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Validation Report of the Human Glucocorticoid Receptor Transactivation (GRTA)

OECD Series on Testing and Assessment

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Foreword

This document contains the Validation report of the human Glucocorticoid Receptor TransActivation (GRTA) assay, developed for the detection of glucocorticoid receptor agonist and antagonist activity of chemicals.

The GRTA assay was proposed by France and Canada. The validation study, conducted in 2022–2023, was coordinated by the public–private platform for the pre-validation of testing methods on endocrine disruptors (PEPPER). The validation report was peer reviewed by an international Peer Review Panel in 2024. The Peer review report and the Validation report of the GRTA assay were circulated for comments to the Working Party of the National Coordinators of the Test Guidelines Programme (WNT) in December 2025. The WNT approved the Validation report at its 38th meeting in April 2026. The WNT also approved the Peer review report of the GRTA assay.

This report is published under the responsibility of the Chemicals and Biotechnology Committee.

Validation report on the Glucocorticoid receptor transactivation method

Revised validation report

11th of July 2025

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Abbreviations

%CV – Percent coefficient of variance
AO – Adverse Outcome
AOP – Adverse Outcome Pathway
AR – Androgen receptor
CV – Coefficient of variance (stdev / mean)
DEX – Dexamethasone
EC_{50} – Effector/activator concentration that induced 50% of full activity
EC_{80} – Effector/activator concentration that induced 80% of full activity (used to measure antagonism)
ECHA – European Chemical Agency (ECHA)
ECVAM – European Centre for the Validation of Alternative Methods
ED or EDC – Endocrine disrupter (chemical)
ER – Estrogen/Oestrogen receptor
GD – Guidance document
GR/hGR – Glucocorticoid receptor/human GR
GR-TA – Glucocorticoid receptor transactivation
GRE – Glucocorticoid Response Element (regulating gene transcription)
IC_{50} – Inhibitor concentration that reduces the signal by 50%
KE – Key event
MIE – Molecular initiation event
MR - Mineralocorticoid receptor
NAM – New approach method
NR3C1 – Glucocorticoid receptor gene
NR – Nuclear receptor
OECD – Organisation for Economic Co-operation and Development
RACI – Responsible, actor/accountable, consulted, informed
RE – Response element
REACH – Registration, Evaluation, Authorisation and Restriction of Chemicals
SOP – Standard operating procedure
SD and Stdev – Standard deviation
TA – Transactivation
TF – Transcription factor

1. Introduction and objectives

1.1 Introduction to Endocrine disrupter biology and guidance

Endocrine disrupters (EDs) are groups of chemicals with the ability to disrupt various endocrine/hormonal systems in humans and animals. The toxicological outcomes are very diverse and ranges from reduced sperm quality and fertility, abnormalities in sex organs, endometriosis, early puberty, and altered nervous system function, to changes in immune function, certain cancers, respiratory problems, metabolic issues, diabetes, obesity, cardiovascular problems, growth, neurological and learning disabilities (as outlined by the Endocrine society: https://www.endocrine.org/patient-engagement/endocrine-library/edcs). Due to the diversity of the affected hormonal systems and the biological outcomes, it is very difficult to test for endocrine disruption per se. Instead, there are efforts to address specific biological systems or hormonal receptors. Several organizations and authorities have formulated guidelines and strategies on EDs, including the 2018 guideline by the European Commission to enable detection of EDs in pesticides and biocides (https://health.ec.europa.eu/endocrine-disruptors/overview_en). The EU agency for chemicals (European Chemical Agency (ECHA)) has also guidelines around testing for EDs under the REACH program (Registration, Evaluation, Authorisation and Restriction of Chemicals; https://echa.europa.eu/hot-topics/endocrine-disruptors). As stated by ECHA: "*Under REACH, endocrine disruptors can be identified as substances of very high concern alongside chemicals known to cause cancer, mutations and toxicity to reproduction. The aim is to reduce their use and ultimately replace them with safer alternatives*". Testing for and removing EDs is included in the general guidelines by OECD on Chemical Safety and Biosafety (https://www.oecd.org/content/dam/oecd/en/topics/policy-issues/chemical-safety-and-biosafety/work-on-chemical-safety-and-biosafety-2021-24.pdf). Furthermore, from 2025 the EU is having a new classification of products under "Classification, labelling and packaging of chemical substances and mixtures" that demands classification as ED or non-ED for substances and mixtures (https://www.europarl.europa.eu/RegData/etudes/BRIE/2023/751425/EPRS_BRI(2023)751425_EN.pdf). The latter is of key importance and the OECD works to develop relevant and validated assays in various safety and toxicology areas including those for EDs. However, there are currently only a limited set of validated assays in the ED space, in particular among the level 2 tests (in vitro assays; OECD GD 150 - https://doi.org/10.1787/9789264304741-en) with only 5 adopted OECD Test Guidelines, though many more areas are in need of coverage (https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/endocrine-disrupters.html).

1.2 EDs and steroid receptors

Many EDs disrupt cellular receptors of various steroid hormones belonging to the nuclear receptor (NR) family and may affect both parents and offspring (Toporova and Balaguer, 2020). Some key receptors are those for estrogen/oestrogen, progesterone, androgen, mineralocorticoids and glucocorticoids. These steroid receptors are generally located in the cytosol of cells, but upon binding and activation by their respective ligands they transition to the nucleus and act as transcription factors (TFs). A common feature is that they bind to their respective specific response elements (RE) that

control gene transcription and activate transcription of target genes, a process termed transactivation (TA).

The Glucocorticoid receptor (GR) is involved in a wealth of biological processes regulating genes that control developmental biology, metabolism, and immune responses. The significance of GR has been established in mice models where gene deletion led to perinatal death, while partial loss resulted in changed corticosteroid levels, neuroendocrine abnormalities, and lung development impairment (Cole et al., 1995; Chourbaji and Gass, 2008).

Of clinical significance is the fact that several mutations in the GR gene (*NR3C1*) underlay the Chrousos syndrome, either from sporadic mutations or inherited in an autosomal recessive or dominant manner (Mendonca et al., 2002). Chrousos syndrome (also known as generalized glucocorticoid resistance) can lead to potentially fatal conditions like hypoglycaemia, alkalosis, or severe hypokalaemia, but may also be asymptomatic (Chrousos et al, 1993; Martins and Castro, 2021). In the description of the outcome of glucocorticoid resistance the authors write: "*In women, hyperandrogenism can result in acne, hirsutism, menstrual irregularities, oligoanovulation, and infertility; in men, it may lead to infertility and in children, to precocious puberty. Different molecular defects, such as point mutations or a microdeletion of the highly conserved glucocorticoid receptor gene, alter the functional characteristics or concentrations of the intracellular receptor and appear to cause glucocorticoid resistance*" (Chrousos et al., 1993).

GR activation is also associated with serious adverse biology. Hypercortisolism/Cushing's syndrome is a serious disease state stemming from prolonged excessive use of corticosteroid medication or over production of Cortisol (can be a tumor). Signs and symptoms can include high blood pressure, abdominal obesity, a round red face due to facial plethora, a fat lump between the shoulders, weak muscles, weak bones, acne, and fragile skin that heals poorly (Batista et al., 2007; Nieman et al., 2005). Women may have more hair and irregular menstruation or loss of menses. Occasionally there may be changes in mood, headaches, and a chronic feeling of tiredness.

There are numerous adverse outcome pathways (AOPs) in the AOP Wiki (www.aopwiki.org) describing adverse outcomes initiated by AR antagonism and activation. Similarly, several AOPs focus on GR disruption (AOPs 14, 64, 66, 71, 318, and 334) where the outcomes relate to fertility, sperm quality, liver steatosis and more. In four of the AOPs the Molecular Initiating Event (MIE) is the activation of GR (AOPs 14, 64, 318 and 334). As mentioned, there are several GR mutations underlying the Chrousos syndrome with many implications as evidence of a diverse set of important biological pathways, again reflecting the diverse sets of genes regulated by GR. In a comprehensive study the GR ligand dexamethasone was inducing 234 different genes involved in numerous pathways and biology (Reddy et al., 2009). This adds to the understating of how disruption of GR can lead to the numerous outcomes seen in Chrousos and Cushing's syndromes, the mentioned mouse models and the AOPs. Further information on the AOPs is gathered in Annex 3.

The clinical and mouse model data points to the severity of interfering with the GR function and gene regulation. A recent review highlight GR in endocrine disruption biology (Zhang et al., 2019). The review also covers the mineralocorticoid receptor (MR) and biology. The review highlights the many

potential ways EDs can interfere with the biology of the receptors and alter gene expression downstream. Chemicals with this potential include metals, metalloids, pesticides, bisphenols, flame retardants, and pharmaceuticals of the steroid family. The literature around chemicals influencing the GR receptor and biology is quite divers and covers a range of in vitro and in vivo models (Zhang et al., 2019). In the in vivo models it was demonstrated that many such chemicals are affecting the glucocorticoid homeostasis (Cole et al., 1995; Chourbaji and Gass, 2008). This warrants the development and use of more simple in vitro models to screen for disrupters of the GR receptor and gene regulation system. It allows for deselection of such chemistry in development or screening for disrupters in for example soil or water samples from the environment. A Dutch team tested surface water samples in several Steroid hormone TA assays (Calux) and found many samples had glucocorticoid-like and other steroid activities (van der Linden et al., 2008).

This brings us to the relevance of such TA assays. Generally, well designed TA assays are robust and show reproducible results with low false positive and negative outcomes. They represent the cellular context of the hormonal system but with added sensitivity. The mentioned Calux assays utilize an artificial gene hosting response element(s) of the relevant receptor controlling the expression of luciferase. This enzyme converts luciferin to a luminescent product (oxyluciferin) which is readily measured by an appropriate luminometer. Thus, activation of the receptor leads to increased luciferase that leads to increased fluorescent light that can be quantified. Typically, the system will also overexpress the receptor itself to increase sensitivity and broaden the utility of the test.

1.3 Introduction to the GR-TA assay, implications and objectives

The current assay (GR-TA) was designed in this manner, based on a human immortalized cell line (HeLa). The cells are transfected with two types of cDNA constructs, MMTV-Luciferase-SV-Neomycin (a GR responsive gene), and pSG5-hGR-puromycin (a glucocorticoid receptor alpha (GRα) expressing plasmid) to obtain the GR and luciferase over-expressing HMLN-hGR cells (Grimaldi et al., 2019; Toso et al. 2023). This allows for culture of the cells over long periods (months), testing of chemicals and assessment of activation or inhibition of GR measured by the induction or suppression of luciferase. A more thorough description of the assay is found in a following chapter. The full Standard Operating Procedure (SOP) is found in Annex 2.

A reliable TA assay for GR disrupting activity would allow for detection of chemicals during screening/counter screening of new compounds in chemical design and pharmaceutical discovery, or for detection in environmental samples as in the surface water samples mentioned. The significance of GR disrupting chemicals is highlighted by the biological evidence and toxicological outcomes. All the steroid receptors are involved in key hormonally controlled biological processes and are core targets of EDs (Toporova and Balaguer, 2020). The 5 mentioned OECD TGs all relate to steroid biology and two are for TA assays (estrogen and androgen).

1.4 Approval and use of a GR-TA assay

There is currently no OECD adopted TG on a GR focused assay that can be used in regulatory submissions. The industry relies on broader in vivo models to evaluate chemicals for steroid-like

properties and ED-like toxicities. As mentioned, the few in vitro assays that have an adopted OECD TG focus on other steroid receptors of significance. The scope of these assays is broader than covering new chemistry that needs regulatory approval but will also allow for reliable detection of steroid-like activities in water, soil or other environmental samples.

All this is principally the basis for the calls and efforts by EU, OECD and others to populate the regulatory and environmental detection space with relevant and reliable approved assays for ED detection. The GR-TA assay was developed to enable detection of chemicals that disrupt GR. It is based on well-established in vitro/cell techniques, and has already shown good results (Grimaldi et al., 2019, Toso et al., 2023). The goal was to validate this assay using several labs and a diverse set of chemicals. This would allow for submission for approval by the OECD to increase the level 2 space of OECD adopted ED relevant assays.

The objectives of this validation study were:

- to evaluate the transferability and reliability of the GR-TA assay (reproducibility within and between laboratories).
- to evaluate the relevance of the test method through comparison of the classifications of the compounds by this test method to reported/expected classifications from other GR relevant test methods.

2. Validation of test method GR-TA

2.1 Test system and assay

As introduced above the GR-TA assay is based on the HMLN-hGR cells (Grimaldi et al., 2019) that stably express the alpha-receptor of GR and a luciferase reporter gene controlled by GR through GR responsive elements (GREs) in the promotor region. The cells are based on the human immortalized HeLa cells that express several steroid hormone and related receptors (Grimaldi et al., 2019). However, the measurable response to endogenous receptors is limited, so usually in TA assays the receptor is also over-expressed to increase the window of response and sensitivity, as was the case here.

The cells were generated by co-transfecting with a glucocorticoid responsive gene, MMTV-Luciferase-SV-Neomycin (this ensures that only GR will regulate the luciferase), and a GRα expressing plasmid (pSG5-hGR-puromycin) and cultured using both neomycin and puromycin to select for cells expressing both plasmids. The selected cell line (HMLN-hGR) can be cultured for up to 50 passages without loss of response (see the SOP). Initially the authors used the cells in a study of bisphenols, but the cells have also been tested using other compounds. To validate the cell line as responsive to the expected chemistry, well known activators of GR were tested and shown to induce a robust and reproducible increase in luciferase (Toso et al., 2023), including dexamethasone, cortisol, bimedrazol, medrol, cortivazol and fluticasone. Dexamethasone (DEX) was used as the control agonist in the current validation study. The cells are available from the developing lab at INSERM (see below).

To simplify the grounds of the assay here is a description of the principles:

Cells are grown and treated with reference or test compounds, these will, to varying degrees, bind and activate GR that in turn induced the luciferase gene to produce luciferase in the cells. After compound treatment the produced luciferase is measured using luciferin that is turned into oxyluciferin producing light. If the test chemical is an activator of GR, it will produce a concentration-dependent induction of light measurable with a luminometer. Also, antagonism can be measured by inducing GR (and luciferase) using DEX and then testing compounds for the ability to reduce the signal induced by DEX. The most common application in antagonist mode is to choose the concentration that induces 80% of the maximal response and then inhibit this response by test compounds. In addition, the assay can be used in an interference mode using higher induction (100 %) by DEX, allowing to discriminate between pure antagonists and interference substances (see below; and Figure 1). A true antagonist will then be right-shifted in dose-response since more agonist must be competed (Figure 1, grey). An interfering compound often have effect without shifting the curve (Figure 1, orange). Often chemicals that reduce the signal from an agonist are classified as antagonists, but only true antagonist behave competitive, while interfering chemicals have other indirect effects that reduce the agonistic signal.

The data are interpreted according to data interpretation criteria for agonism, antagonism or interference (explained elsewhere) and should be consistent between runs and between labs. This support classification in the ED space.

Potency concentration-response data are fitted using the Hill equation for biological samples (https://en.wikipedia.org/wiki/Hill_equation_(biochemistry)). The sigmoidal curve allows for potency assessment by determining the concentration at which the activity is 50% of full response (effector concentration (EC_{50})). A key parameter is to determine the EC_{50} of activating and IC_{50} of inhibiting compounds and compare the potencies between runs, but also between determinations from the different labs involved. The experiments produce a light signal that is quantified by a measuring instrument (luminometer). Each concentration of compound leads to a light measurement and when these are plotted against each other they make for a sigmoidal curve in log-concentration plots. However, the dose-response curves are only generated afterwards by fitting a curve to the measurements according to the Hill equation. If the plotted dose-response measurements are strong agonists or antagonist, the equation will fit the sigmoidal version when using a Hill coefficient of 4. However, compounds with lower affinity or indirect actions may only produce smaller changes where the Hill fit uses lower coefficients to fit the curve.

2.2 Validation overview

The method was developed in the INSERM lab in Montpellier of Professor Patrick Balaguer (Grimaldi et al., 2019). This lab is the test method expert lab with responsibility for the SOP and transfer of knowledge and experience to the other participating labs. The other labs were INERIS under Dr. Selim Ait-Aissa, Toxem headed by Dr. Jérôme Couteau and Tame-Water under Dr. Erwan Michelin.

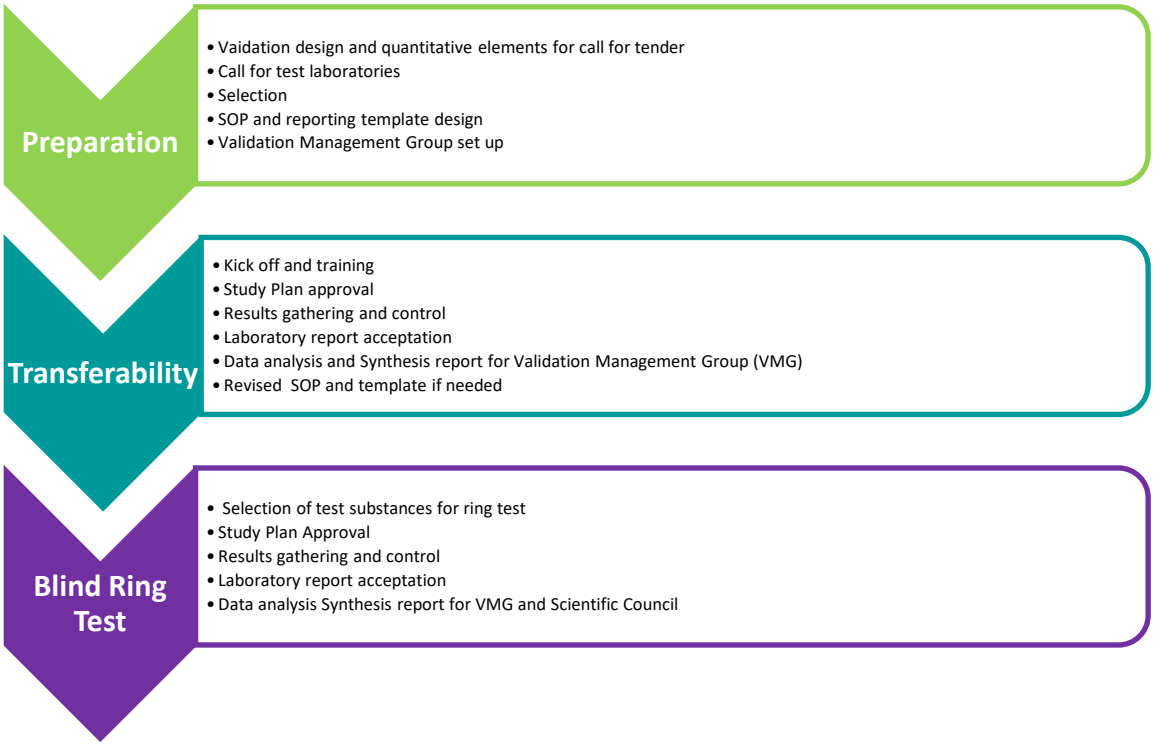
The validation study started with meetings to agree on terms, compounds, materials and other details required for the actual lab work. The SOP and practicals were explained, and the naïve lab had access to the expertise from the parent lab. A visit to the INSERM lab was not possible due to Covid.

The validation study had several steps. First, the transferability phase, where labs setup and tested the method with a small number of compounds to compare results and evaluate the transfer phase. This led to revision of the SOP for clarification. Then followed the second phase where each lab tested a larger number of compounds in a blinded fashion to avoid bias (ring trial). This was the major test phase of the study. Following the testing and reporting of results, the compounds were unblinded, and the data subjected to statistical analyses to evaluate the intra- and inter-laboratory reproducibility. The experience from this part also led to adjustments of the SOP and a supplementary test phase was added to better characterize the limits of the method with higher concentrations of some compounds and one additional substance. The following sections outline details and results, data analyses and conclusions of the study.

2.3 Management of study

Pepper is the central organiser of the assay validation. Pepper organized a call for relevant ED assays and had the initial discussions with Professor Patrick Balaguer, from which lab the assay originates. Then Pepper organised a call for tests labs to join the ring trial and onboarded the chosen labs mentioned above (INERIS, Toxem and Tame-Water). Pepper was also helping to finance the trial.

Validation management outline.



There were four levels of governance: 1) the labs themselves supported by Pepper with the INSERM lab having a head experts role, as compared to the naïve labs that learned the method, 2) Pepper as key coordinator and contracting support from data manger (François Sermier), biostatistician (Sebastian Hoffman), agreement with Charles River Laboratories (Netherlands) for anonymisation and dispatching of test substances, and investigation of literature on compounds (supported by CEHTRA consulting), 3) a Validation Management Group of experts in assays, EDs, toxicology and regulatory,

and 4) the Scientific council of Pepper from which Pepper would seek advice on assay, substances, interpretations and validation questions.

It should be stressed that Pepper is not to be considered as developer and has no particular interest in the method per see. The function of Pepper is, as stated, to facilitate that validation takes place and, thus, have a role as project manager and in financial support of the project.
In Annex 4 is the Project Chart as outlined at the start of the project for documentation of the project management, plans, and deliverables. Here is found further details of the stages, actors, roles and flow outlined. This is largely a RACI document where specific tasks are shown with responsible, actor/accountable, consulted and informed parties, together with some further details on contracts, and organization of the parties' involvement throughout the process.

The VMG would constitute an independent governing body where all open questions, different issues, recommendations and disputes could be formally solved. Pepper organized the meetings also inviting statistician and data manager when relevant. All important decisions about the validation design, study protocols, timelines, data analysis and acceptance were governed by the VMG. In practical terms the main role of the VMG was to advise on chemicals, data interpretation and outlier identification, the criteria to distinguish antagonist from interference substances, decide on validation phases and when to proceed. In particular the choice of chemicals was a difficult task since only few had convincing data attached (see Annex 1) that would allow for assessment of predictability. Therefore, it was decided to include several substances that had been tested in related assays or models, though often with varying results, to expand the chemical space in the ED area. Most of these were only supported by one or two publications while the well-known agonists and antagonist have been assessed in numerous papers (Details for this in Table 37). A key argument for the choice of substances was also to understand if the assay would behave promiscuous and give rise to false positives, a key concern in the ED space.

Overview of the VMG.

Name	Affiliation	Expertise
Ella Atlas	Health Canada	Assays, regulatory, validation, EDs
Katharina Koch	Leibniz research Institute for environmental medical, Germany	EDs, NAMs, AOPs
Laurent Sachs	Museum of Natural History, France	EDs, assays
Marc Audebert	French National Institute for Agricultural Research	EDs, regulatory, assays
Kristina Fant	Research Institutes of Sweden, later Key2Compliance	Chemistry, toxicology

3. Transferability phase

3.1 Setup, controls and modes of study

The transferability work started in December 2021 and was finalised in April 2022.

The HMLN-GR cell line was obtained from INSERM U1194, IRCM laboratory, upon signature of a Material Transfer Agreement.

The four labs implemented the method using the agonist positive control Dexamethasone (DEX, CAS# 50-02-2), the antagonist positive control Mifepristone (RU-486, CAS# 84371-65-3), as well as three test items (Cortisol (CAS# 50-23-7), DEHP (Bis(2-ethylhexyl) phthalate, CAS# 117-81-7) and Drospirenone (CAS# 67392-87-4)).
The purity of all chemicals was ≥ 98%.

Cortisol was expected to have an agonist activity, DEHP to have no effect, and Drospirenone to decrease the receptor activity.

All test items were tested in three modes: agonist, antagonist, and interference. In the antagonist mode, test items are co-exposed with the concentration of the reference agonist (DEX) yielding 80% of the maximum activity (EC_{80}). In the interference mode, they are co-exposed with a concentration of Dexamethasone yielding 100% of response (often 100-fold higher concentration). The increased concentration of the reference agonist should induce a shift in the test item response.

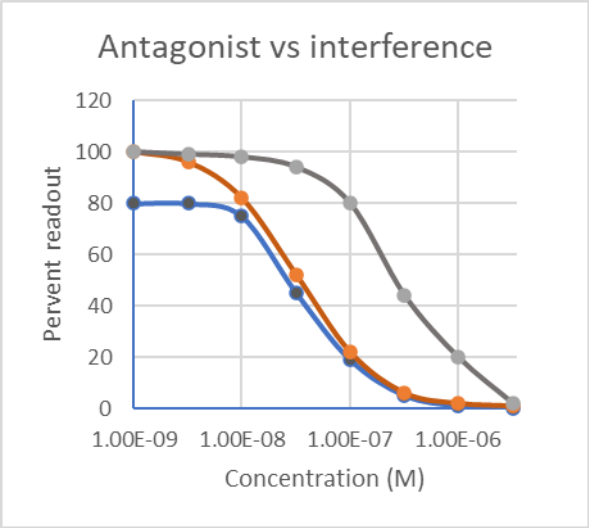


Figure 1: Example of responses of an antagonist chemical (blue curve). In interference mode, this chemical will either be confirmed as a true antagonist (grey curve) or as having an interfering effect (orange curve).

Every plate was tested for absence of cytotoxicity.

3.2 Results from the transferability phase

The acceptance criteria for this method, as defined at the beginning of the transferability phase, are:

- The cells must be mycoplasma-free.
- Induction factor: in each run, the average luciferase activity of the positive control (DEX 10^{-7} M) must be at least 10 times that of the solvent control.
- Chemicals must be tested in the concentration ranges not inducing cytotoxicity.
- Response of the cells to positive controls must be consistent throughout the runs qualitatively (expected effect of reference substances (i.e. sigmoid aspect of the curves)/no effect of solvent control) and quantitatively (EC_{50} must be in the range of values expected for the effect or reference substance, *i.e.* DEX tested from 10^{-10} to 10^{-7} M results in an EC_{50} between log -9.9

and -7.9 ; and Mifepristone tested from 10^{-9} to 10^{-6} M results in an EC_{50} between log -9.2 and -7.2).

- For active chemicals, the concentration-response curve should cover no-effect concentrations (at least two concentrations with no effect), intermediate effect concentrations, and maximum effect concentration of the chemical (excluding toxicity).
- Goodness of fit: when fitting a curve with the Hill model, the calculated R^2 should be above 0.95.

The range of concentrations (in M) tested are presented below.

Table 1: Controls and test items concentrations used (in M).

	Agonism				Antagonism/interference			
	INERIS	INSERM	Toxem	Tame-Water	INERIS	INSERM	Toxem	Tame-Water*
Dexamethasone	$1,0E^{-10} – 1,0E^{-07}$				Not tested			
Mifepristone	Not tested	$1,0E^{-09} – 1,0E^{-06}$	Not tested	$1,0E^{-09} – 1,0E^{-06}$	$1,0E^{-09} – 1,0E^{-06}$			
Cortisol	$1,0E^{-08} – 1,0E^{-05}$ or $1,0E^{-09} – 1,0E^{-06}$		$1,0E^{-08} – 1,0E^{-05}$ or $3,2E^{-09} – 3,2E^{-06}$		$1,0E^{-09} – 1,0E^{-06}$		$1,0E^{-08} – 1,0E^{-05}$	
DEHP	$1,0E^{-08} – 1,0E^{-05}$				$1,0E^{-08} – 1,0E^{-05}$			
Drospirenone	$1,0E^{-08} – 1,0E^{-05}$				$1,0E^{-08} – 1,0E^{-05}$			

* Tame-Water did not test Mifepristone for interference.

Agonism testing

For classification, according to the SOP used for this phase, a chemical is considered to have an agonist effect when it increases luciferase activity above 10% of maximum effect of positive control (100%) for at least two consecutive concentrations.

Also, an agonistic EC_{50} is calculated from the dose-response curve.

Dexamethasone (DEX)

Dexamethasone being the positive control for agonism, the labs were asked to run a full dose-response for each run. INERIS and INSERM did four runs of Dexamethasone, whereas Tame-Water did 5, and Toxem 7. One run from INERIS was invalid due to incorrect concentrations used. The two invalid runs of Tame-Water were due to too high basal level response of the cells (should be < 4% of the maximal activity induced by dexamethasone 100 nM)). All runs from Toxem were valid, regarding the acceptance criteria, however, two were excluded due to a sub-optimal setting of the luminometer and an EC_{50} value within the acceptable range but quite different from the other runs.

Table 2: Dexamethasone EC_{50} values.

	INERIS	INSERM	Toxem	Tame-Water
EC_{50}(±SD) in M	$4,70E^{-09} ± 2,43E^{-10}$	$3,05E^{-09} ± 1,20E^{-10}$	$7,79E^{-09} ± 6,03E^{-10}$	$4,98E^{-09} ± 6,88E^{-10}$
	$8,28E^{-09} ± 8,55E^{-10}$	$3,56E^{-09} ± 2,45E^{-10}$	$8,19E^{-09} ± 7,79E^{-10}$	$6,86E^{-09} ± 7,06E^{-10}$
	$6,61E^{-09} ± 4,631.E^{-10}$	$2,50E^{-09} ± 1,39E^{-10}$	$7,45E^{-09} ± 7,88E^{-10}$	$1,25E^{-08} ± 1,91E^{-09}$
		$2,86E^{-09} ± 2,57E^{-10}$	$8,23E^{-09} ± 6,87E^{-10}$	
			$6.26\ E^{-09} ± 5.5.E^{-10}$	
Mean	$6,53E^{-09} ± 1,79E^{-09}$	$2,99E^{-09} ± 4,42E^{-10}$	$7,58E^{-09} ± 8,06E^{-10}$	$8,11E^{-09} ± 3,91E^{-09}$

The dose-response curves and EC_{50} values of the valid runs are presented below.

All EC_{50} values meet the acceptance criterion of an EC_{50} between $1.26E^{-10}$ and $1.26E^{-8}$ M for DEX tested from 10^{-10} to 10^{-7} M.

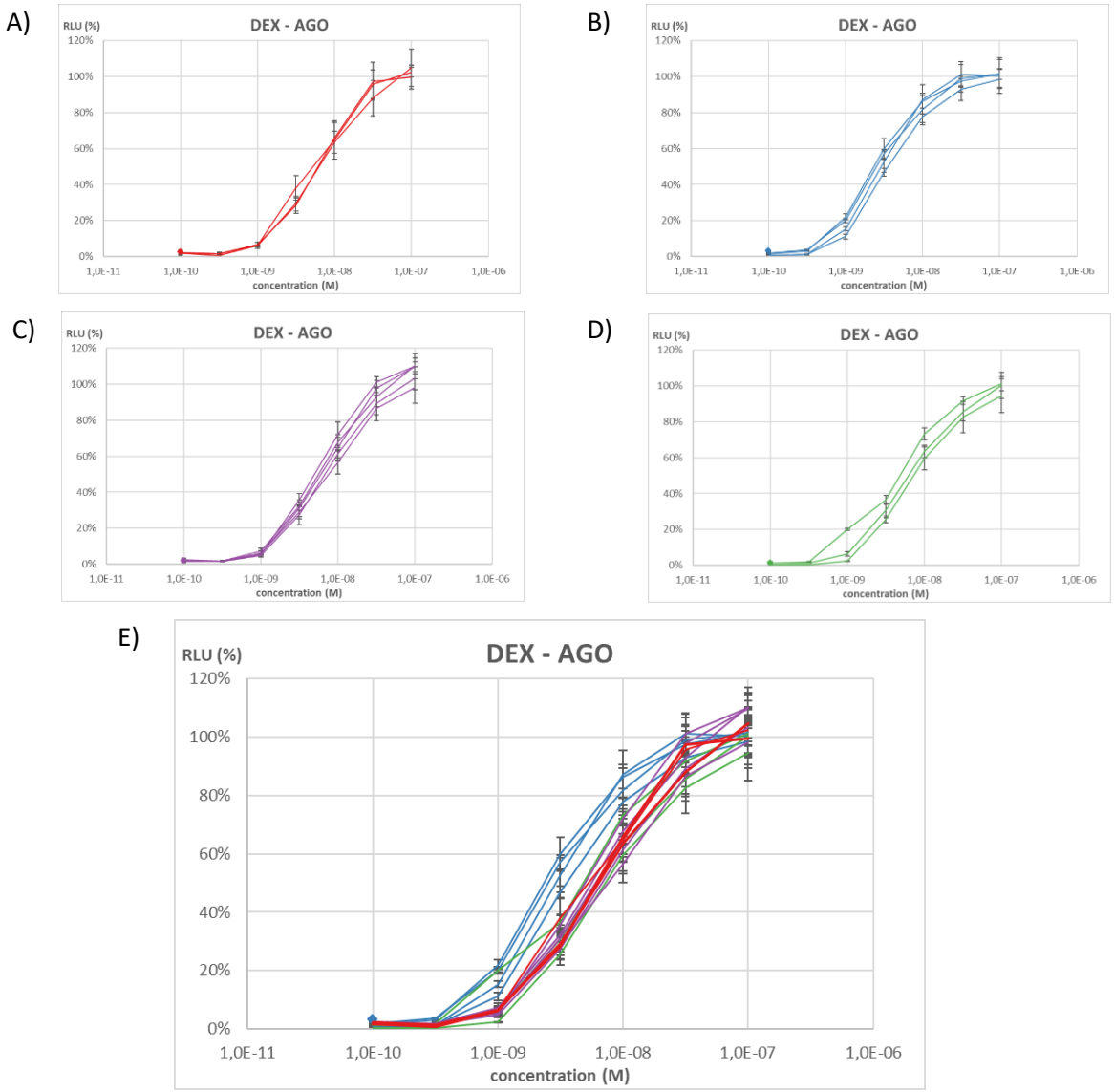


Figure 2: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to dexamethasone in agonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that Dexamethasone is an agonist, increasing luciferase activity above 10% of maximum effect of positive control for at least two consecutive concentrations. The EC_{50} values obtained show a good intra- and inter-laboratory reproducibility.

While testing for agonism activity, the labs established the EC_{80} to be used in presence of Mifepristone for antagonism testing.

Cortisol

All the labs observed that using the highest soluble non-cytotoxic concentration led to a dose-response curve not including two concentrations having no effect. Shifting the highest concentration by half-log or one log allowed them to meet this acceptance criterion.

The dose-response curves and EC_{50} values of the valid runs are presented below.

Table 3: Cortisol EC_{50} values.

	INERIS	INSERM	Toxem	Tame-Water
EC50(±SD) in M	$3{,}74E^{-08} \pm 5{,}59E^{-10}$	$4{,}01E^{-08} \pm 3{,}61E^{-09}$	$1{,}14E^{-07} \pm 6{,}39E^{-09}$	$1{,}20E^{-07} \pm 9{,}43E^{-09}$
	$5{,}05E^{-08} \pm 3{,}13E^{-09}$	$4{,}95E^{-08} \pm 2{,}37E^{-09}$	$8{,}91E^{-08} \pm 2{,}42E^{-09}$	$1{,}27E^{-07} \pm 7{,}06E^{-09}$
	$8{,}24E^{-08} \pm 5{,}24E^{-09}$	$4{,}44E^{-08} \pm 2{,}54E^{-09}$	$7{,}47E^{-08} \pm 5{,}28E^{-09}$	$1{,}29E^{-07} \pm 1{,}29E^{-08}$
Mean	$5{,}68E^{-08} \pm 2{,}31E^{-08}$	$4{,}47E^{-08} \pm 4{,}71E^{-09}$	$9{,}26E^{-08} \pm 1{,}99E^{-08}$	$1{,}25E^{-07} \pm 4{,}73E^{-09}$

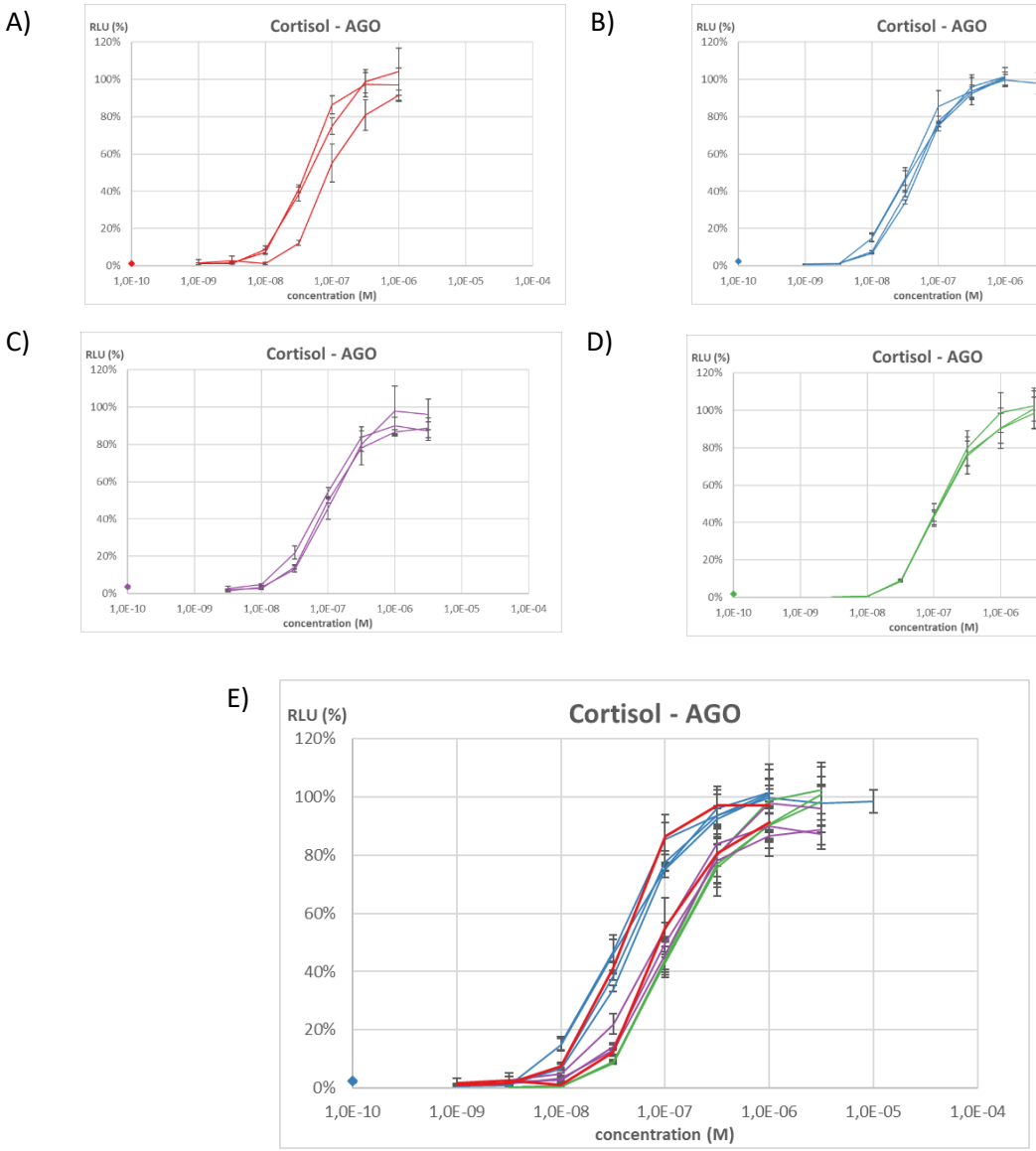


Figure 3: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Cortisol in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

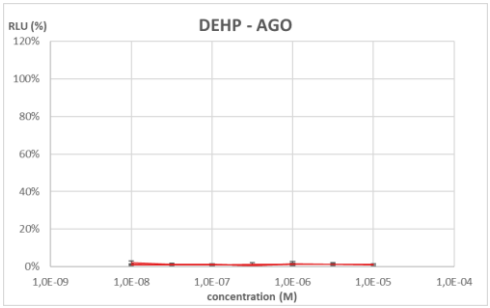
All labs concluded that Cortisol is an agonist, increasing luciferase activity above 10% of maximum effect of positive control for at least two consecutive concentrations. The EC_{50} values obtained show a good reproducibility intra-laboratory, as well as inter-laboratories, even if "two groups" could be seen.

Bis(2-ethylhexyl) phthalate (DEHP)

All the labs used the same concentrations. They did not report any cytotoxicity, and all concluded that the substance had no agonist effect on GR, as expected.

The dose-response curves of the valid runs are presented below.

A)



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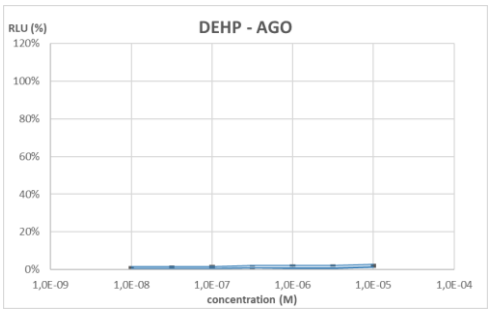
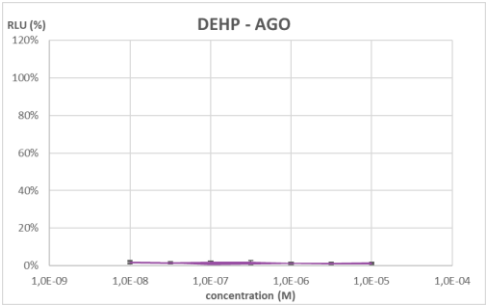
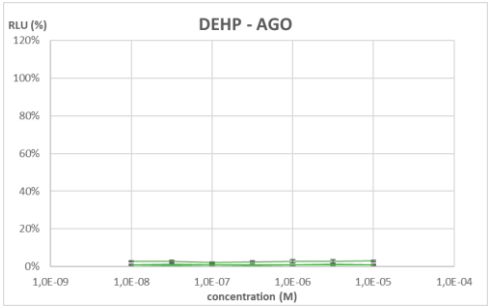


C)



D)



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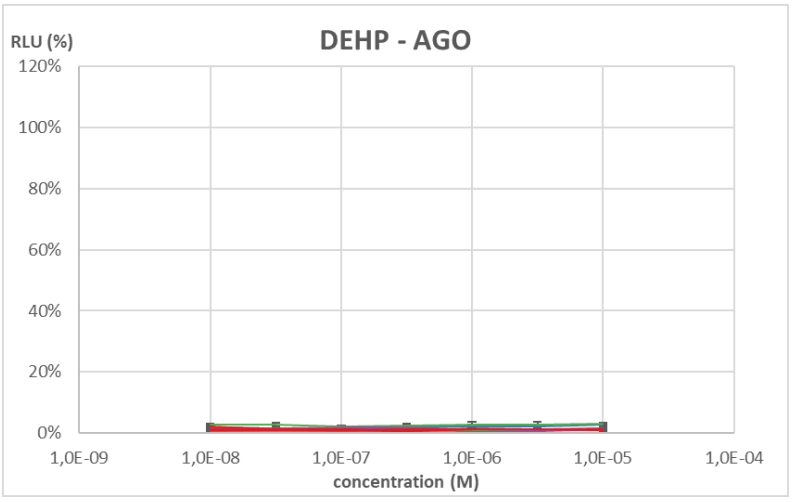


Figure 4: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to DEHP in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Drospirenone

All the labs used the same concentrations. Ineris and Tame-Water did report some cytotoxicity at the highest tested concentrations, but this was not observed on all runs.

The dose-response curves of the valid runs are presented below.

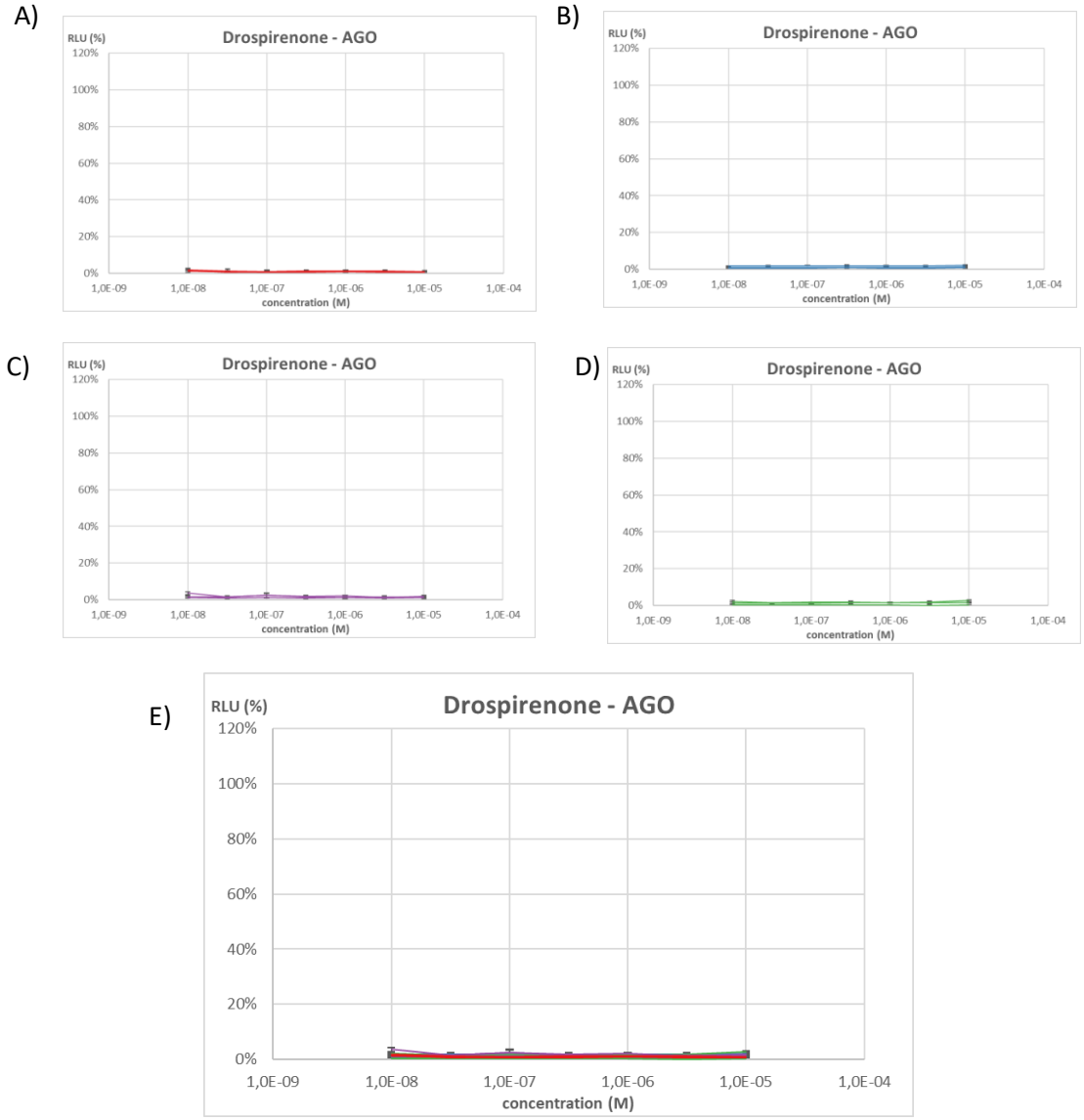


Figure 5: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to dexamethasone in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

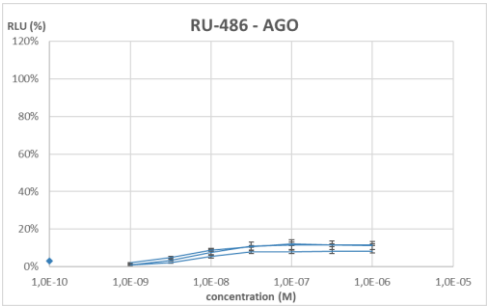
All labs concluded that DEHP is not an agonist.

Mifepristone (RU-486)

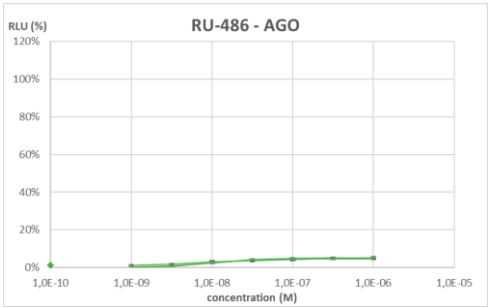
Mifepristone is the reference antagonist. Only two labs (INSERM and Tame-Water) tested it in the agonist mode. INSERM did three runs. Tame-Water did four runs, one of which was invalid due to high basal response of the cells (> 4%).

The dose-response curves of the valid runs are presented below.

A)



B)



C)

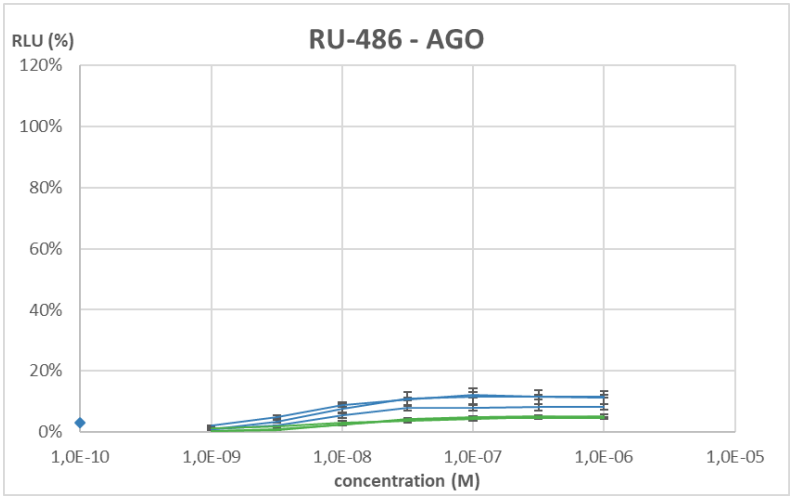


Figure 6: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Mifepristone (RU-486) in agonist mode, in INSERM A), Tame-Water B), or in all labs C).

The labs concluded that, whether Mifepristone induces an increase in luciferase production, it is not enough to be classified as an agonist according to the prediction model. This conclusion agrees with the partial agonist activity of the substance.

Antagonism and interference testing

For antagonism classification, the test item is considered an antagonist if it reduces the DEX induced signal by more than 30%. Here DEX is added at the concentration inducing 80% induction, while for interference the DEX induction was 100% (see above).

The labs were asked to calculate the EC_{80} value of DEX based on the results of their agonist runs. The concentrations of Dexamethasone used in antagonism (EC_{80}) and interference testing are:

Table 4: Dexamethasone concentrations used in antagonism and interference testing.

	INERIS	INSERM	Toxem	Tame-Water
Antagonism testing	$1.0E^{-08}$M	$8.0E^{-09}$M	$1.0E^{-08}$M	$3.1E^{-08}$M
Interference testing	$3.0E^{-06}$M	$1.0E^{-06}$M	$1.0E^{-06}$M	$1.0E^{-06}$M
Fold between modes	300	125	100	32

According to the SOP used for this phase, for antagonist assays, the 80% DEX activity should be between 70% and 80% of the maximum luciferase activity. Moreover, a chemical is considered to have an antagonist effect when it decreases luciferase activity of more than 30% for at least two consecutive concentrations, in the presence of the reference chemical at the concentration inducing 80% of the maximal response.

In case a chemical inhibits luminescence in an antagonist assay, and is tested negative in the cytotoxicity assay, it must be tested in an interference assay, *i.e*. in co-exposure with the reference agonist chemical at a concentration yielding 100% of the response. In the interference assay, the IC_{50} should be shifted to a higher concentration with a factor in the 300-fold range. The concentration of Dexamethasone to be used, and the subsequent shift in IC_{50} were redefined during the transferability.

Mifepristone

Mifepristone is the positive control for antagonism. According to the SOP, it is to be tested from 10^{-9} to 10^{-6} M, resulting in an IC_{50}. between $6{,}31E^{-10}$ and $6{,}31E^{-08}$M, in presence of the EC_{80} of DEX. As positive control, Mifepristone was to be tested (full dose-response) on each run.

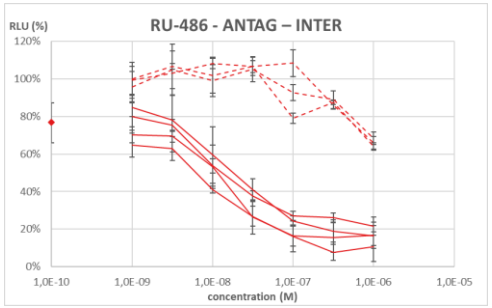
Toxem did 5 runs in antagonist mode, 2 being invalid due to a wrong concentration of EC_{80} of DEX used.

The dose-response curves and IC_{50} values of the valid runs are presented below.

Table 5: Mifepristone IC_{50} values in antagonism and interference modes.

	INERIS		INSERM		Toxem		Tame-Water	
	Antagonism	Interference	Antagonism	Interference	Antagonism	Interference	Antagonism	Interference
IC_{50}(±SD) in M	$1{,}41E^{-08} \pm 2{,}81E^{-09}$	$1{,}11E^{-06} \pm 3{,}97E^{-06}$	$5{,}47E^{-09} \pm 1{,}60E^{-10}$	$3{,}10E^{-07} \pm 1{,}17E^{-08}$	$8{,}50E^{-09} \pm 7{,}13E^{-10}$	$3{,}97E^{-07} \pm 2{,}99E^{-07}$	$1{,}59E^{-08} \pm 3{,}36E^{-10}$	
	$1{,}39E^{-08} \pm 1{,}30E^{-09}$	$3{,}26E^{-06} \pm 4{,}22E^{-07}$	$9{,}69E^{-09} \pm 8{,}68E^{-10}$	$6{,}36E^{-07} \pm 2{,}49E^{-07}$	$7{,}96E^{-09} \pm 5{,}02E^{-10}$	$4{,}27E^{-07} \pm 1{,}54E^{-07}$	$1{,}82E^{-08} \pm 9{,}27E^{-10}$	
	$1{,}13E^{-07} \pm 1{,}31E^{-08}$	$1{,}12E^{-06} \pm 7{,}28E^{-06}$	$1{,}02E^{-08} \pm 8{,}57E^{-10}$	$2{,}86E^{-07} \pm 9{,}10E^{-09}$	$8{,}35E^{-09} \pm 6{,}06E^{-10}$	$5{,}11E^{-07} \pm 2{,}44E^{-07}$	$1{,}66E^{-08} \pm 1{,}62E^{-09}$	
	$1{,}69E^{-08} \pm 1{,}32E^{-09}$							
Mean	$3{,}95E^{-08} \pm 4{,}90E^{-08}$	$1{,}83E^{-06} \pm 1{,}24E^{-06}$	$8{,}45E^{-09} \pm 2{,}60E^{-09}$	$4{,}11E^{-07} \pm 1{,}96E^{-07}$	$8{,}27E^{-09} \pm 2{,}79E^{-10}$	$4{,}45E^{-07} \pm 5{,}91E^{-08}$	$1{,}69E^{-08} \pm 1{,}18E^{-09}$	
IC_{50} shift	46		48		53			

A)



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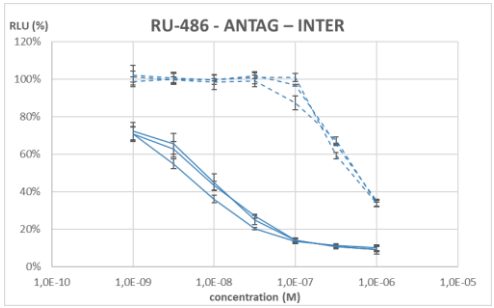

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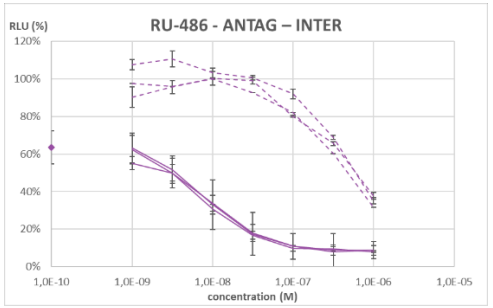
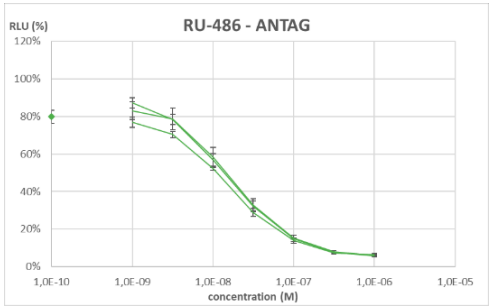


D)



E)

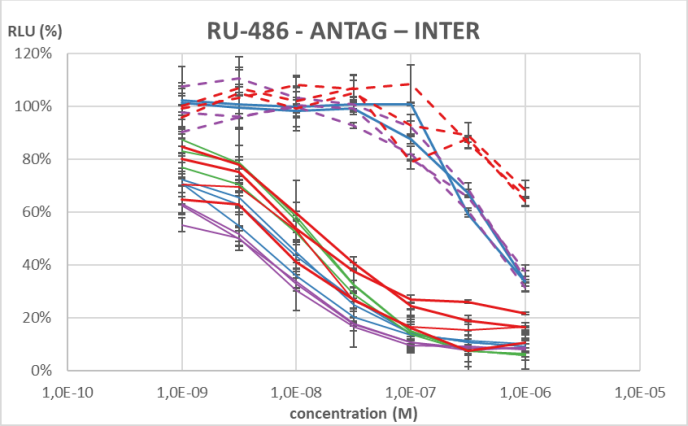


Figure 7: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Mifepristone (RU-486) in antagonist and interference modes, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Solid lines: antagonist mode; dotted lines: interference mode.

The labs concluded that Mifepristone is a true antagonist, decreasing luciferase activity of more than 30% for at least two consecutive concentrations, in the presence of the reference chemical at the concentration inducing 80% of the maximal response, and a shift in IC_{50} around 50 between the two modes.
The criterion regarding the shift in IC_{50} to differentiate between a true antagonist and a substance acting through interference was revised based on the data obtained. Indeed, the initial criteria of 300 was not reached by any of the labs (it cannot be expected that a 300-fold increase in concentration of the agonists leads to 300-fold shift in antagonist potency. Thus, this criterion was later abandoned for a mathematical model on parallel shift of dose-response curves (ECVAM model).

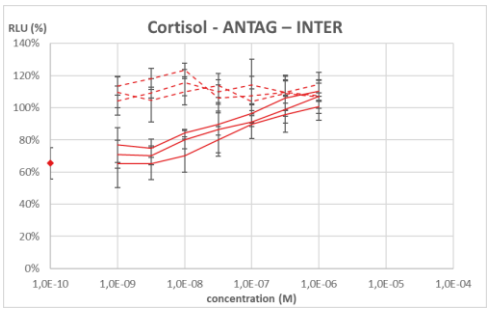
The IC_{50} obtained by Ineris and INSERM show some variability in antagonism mode but are still in the acceptable range. It is to be noted that, due to the higher concentration of agonist used by Ineris in the interference mode, the decrease in signal is lower than for the other labs.

Cortisol

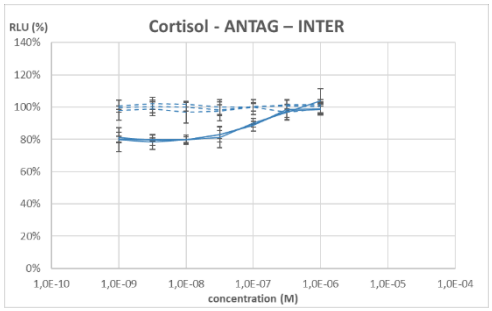
The labs tested slightly different concentrations, however, they all concluded that Cortisol is not an antagonist in this test. The slight increase of activity in antagonist mode can be explained by the fact that Cortisol is a full GR agonist (*i.e*. increasing signal from 80% to 100%).
Toxem and Tame-Water did 4 runs in antagonist mode, 1 being invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

The dose-response curves of the valid runs are presented below.

A)



B)



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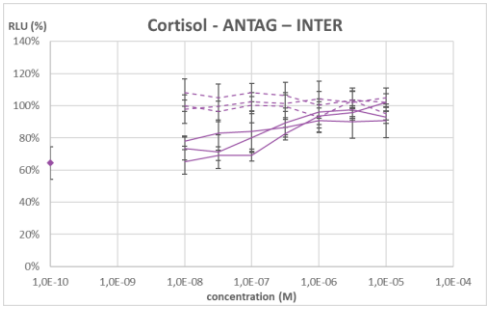

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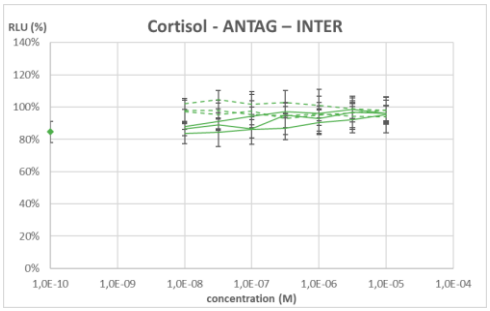


E)

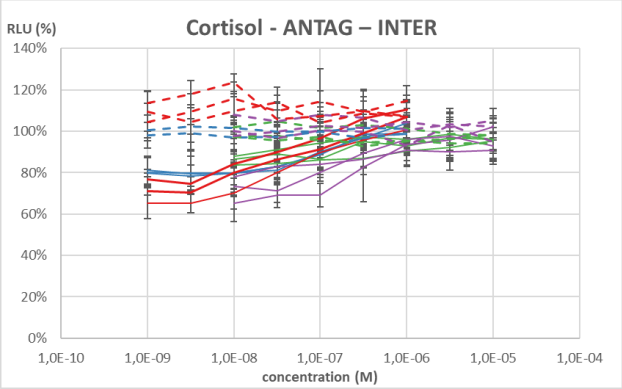


Figure 8: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Mifepristone (RU-486) in antagonist and interference modes, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

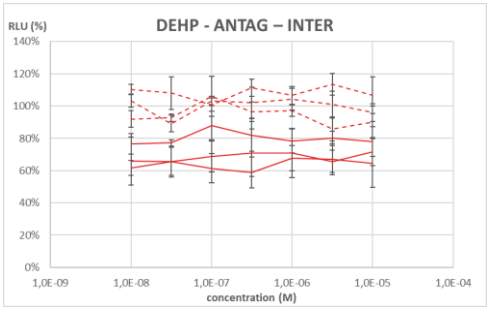
Solid lines: antagonist mode; dotted lines: interference mode.

Bis(2-ethylhexyl) phthalate (DEHP)

All labs concluded that DEHP is not an antagonist in this test.
Toxem did 4 runs in antagonist mode, one being excluded due to high variability compared to other runs. Tame-Water did 5 runs in antagonist mode, 1 being invalid due to the acceptance criterion linked to EC_{80} (inducing activity between 65% and 95%) not being met, one showing some cytotoxicity.

The dose-response curves of the valid runs are presented below.

A)



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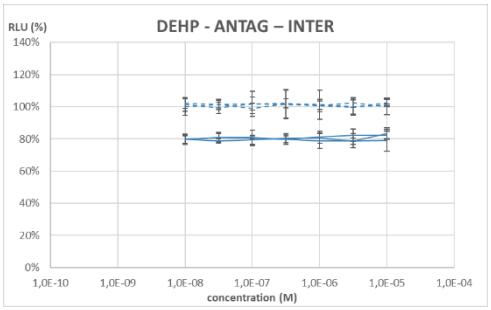

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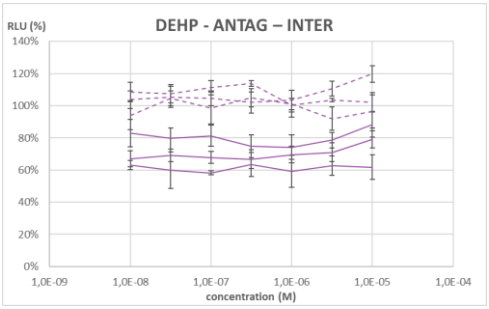

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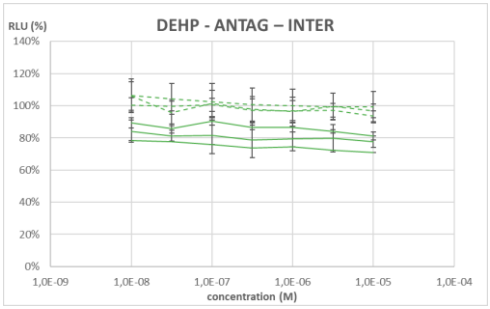

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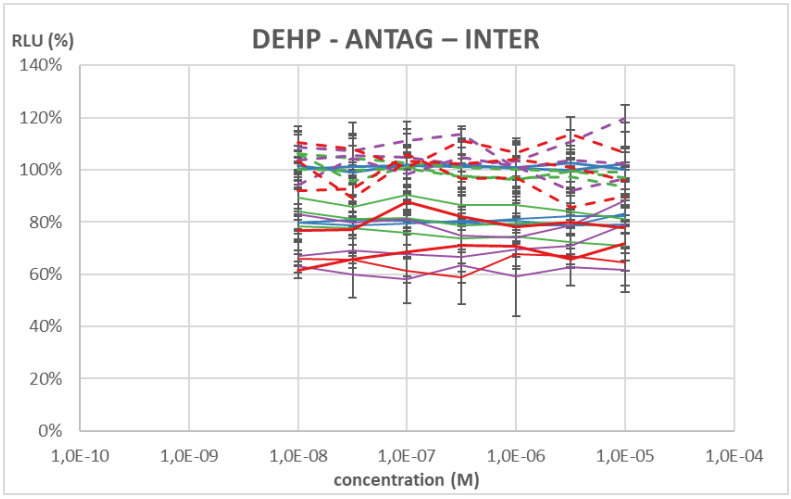


Figure 9: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to DEHP in antagonist and interference modes, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Solid lines: antagonist mode; dotted lines: interference mode.

Drospirenone

All labs observed a decrease in luciferase activity in antagonist testing. The interference testing allowed them to conclude that this decreased signal was due to some interference (and not to true antagonism), with a very low shift in IC_{50} between the two modes.

Toxem did 5 runs in antagonist mode, 2 being invalid due to a wrong concentration of EC_{80} of DEX used. Ineris did 5 runs, 2 being invalid for showing some toxicity.

The dose-response curves and IC_{50} values of the valid runs are presented below.

Table 6: Drospirenone IC_{50} values in antagonism and interference modes.

	INERIS		INSERM		Toxem		Tame-Water	
	Antagonism	Interference	Antagonism	Interference	Antagonism	Interference	Antagonism	Interference
IC_{50}(±SD) in M	$1,83E^{-06}$ ± $5,53E^{-07}$	$2,71E^{-06}$ ± $2,29E^{-07}$	$1,78E^{-06}$ ± $6,54E^{-08}$	$3,66E^{-06}$ ± $4,53E^{-07}$	$1,39E^{-06}$ ± $1,81E^{-07}$	$1,91E^{-06}$ ± $1,60E^{-07}$	$3,04E^{-06}$ ± $2,60E^{-07}$	$6,49E^{-06}$ ± $7,91E^{-06}$
	$1,84E^{-06}$ ± $3,44E^{-07}$	$2,71E^{-06}$ ± $1,57E^{-07}$	$1,87E^{-06}$ ± $9,44E^{-08}$	$3,16E^{-06}$ ± $3,50E^{-08}$	$1,39E^{-06}$ ± $1,94E^{-07}$	$2,22E^{-06}$ ± $3,72E^{-07}$	$3,62E^{-06}$ ± $6,30E^{-07}$	$8,07E^{-06}$ ± $5,41E^{-06}$
	$2,76E^{-06}$ ± $1,16E^{-06}$	$2,67E^{-06}$ ± $5,56E^{-07}$	$1,57E^{-06}$ ± $1,59E^{-07}$	$2,56E^{-06}$ ± $6,73E^{-08}$	$1,56E^{-06}$ ± $2,23E^{-07}$	$2,31E^{-06}$ ± $1,89E^{-07}$	$3,40E^{-06}$ ± $4,33E^{-07}$	$5,74E^{-06}$ ± $5,26E^{-06}$
Mean	$2,15E^{-06}$ ± $5,31E^{-07}$	$2.70E^{-06}$ ± $2.31E^{-08}$	$1,74E^{-06}$ ± $1,58E^{-07}$	$3,13E^{-06}$ ± $5,52E^{-07}$	$1,44E^{-06}$ ± $9,61E^{-08}$	$2,15E^{-06}$ ± $2,13E^{-07}$	$3,35E^{-06}$ ± $2,91E^{-07}$	$6,77E^{-06}$ ± $1,19E^{-06}$
IC_{50} shift	0.87		1.80		1.49		1.13	

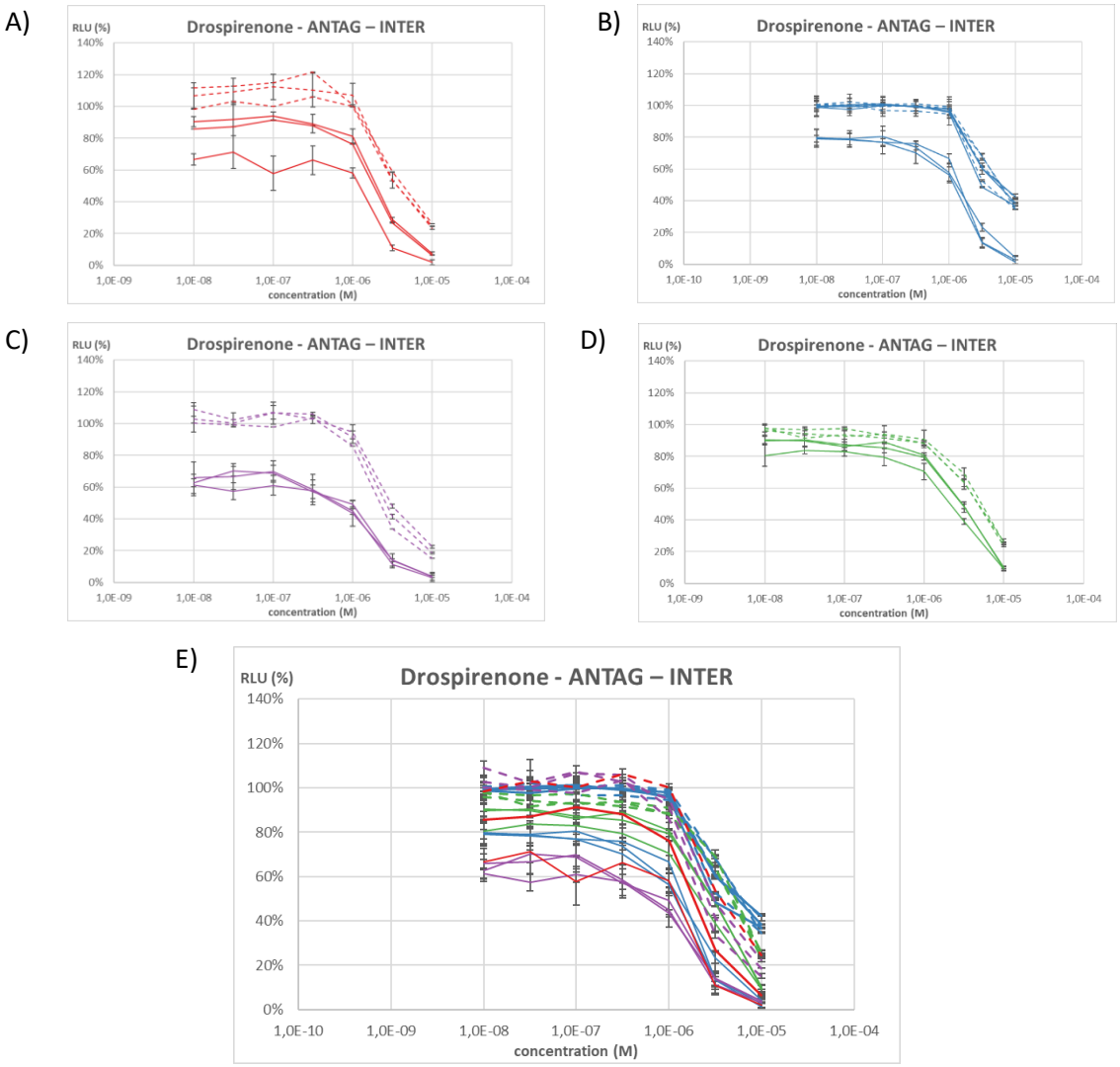


Figure 10: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Drospirenone in antagonist and interference modes, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

Based on the low shift in IC_{50}, all labs concluded that Drospirenone interferes with the luminescence of the cells. The IC_{50} values obtained show a good intra- and inter-laboratory reproducibility.

3.3 Issues encountered

Ineris experienced slight variability in the cytotoxicity data that could possibly be due to early cytotoxic events when using $3E10^{-6}$ M DEX in the interference assay. They decided to reduce the concentration for phase 2.

The main issue experienced by the labs was the presence of a luminescence gradient between the upper half and the lower half of the plate (the luminescence values being always higher at the top than at the bottom). This was seen in all labs and was dealt with by analysing the data by half-plates (normalisation by the solvent control values of the corresponding half-plate). It should be highlighted

that the test items were randomly placed on the top or bottom part of the plate, and that the conclusion regarding the activity of the test item was not impacted by the position on the plate.

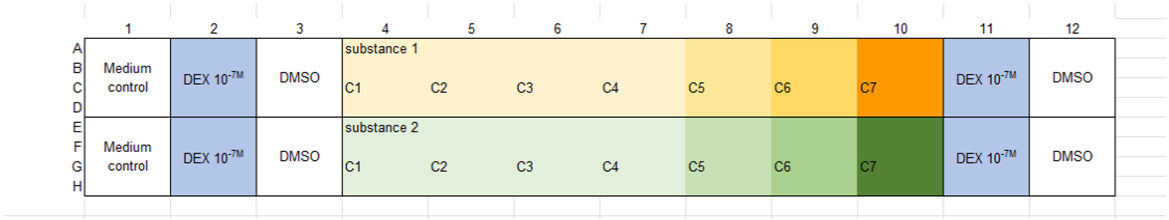


Figure 11: plate design showing the presence of solvent control (DMSO) allowing a normalisation by half-plate.

The cell density was also increased from "at least 50,000 cells per well" to "100,000 cells per well", as the developer mentioned that a higher confluence limited this gradient.

3.4 Changes to the SOP at the end of the transferability phase

Following the transferability phase, some changes were implemented in the Standard Operating Procedure. The main changes are:

Cell culture

Cell seeding in the plates was modified from "50 000 cells per well" to "at least 100,000 cells per well", to decrease variability in measurements (mainly the luminescence gradient observed from the top to the bottom of the plate).

Test item solubility / concentration selection

- The maximum concentration to be used was expressed in M (10^{-3} M stock solution in DMSO). An estimation was made to express it in mg/mL (0.4 mg/mL stock solution in DMSO) to accommodate the fact that the test items being blind-coded for phase 2, the labs would not know the molecular weight.
- Some text was added to clarify how to adjust the concentrations of the test items: "Another possibility to better characterize the concentration-response curve consists in making adjusted dilution curves, e.g., 10-fold dilutions for the first and last concentrations and a narrower range around EC or IC_{50}".

Acceptance criteria

- The identification of outliers was modified from "*A replicate is considered an outlier if its value differs by more than 20% from the mean of the four replicates and if the standard deviation of the four replicates is greater than 10%*", to "*A replicate is considered an outlier if the coefficient of variation (CV) between replicates is above 30% for the wells above basal activity (4%)*". Indeed, the coefficient of variations of values below the basal activity was often high, because it was in the limits of quantification of the plate readers.
- The level of the basal activity of the cells was modified from "around 1-2% of the maximal activity" to "around 4% of maximum luminescence" based on the data from the labs.

- The acceptable range of EC/IC_{50} of the positive controls was narrowed down from "log-9.9 to log-7.9 for DEX tested from 10^{-10} to 10^{-7} M" to "log-8.9 to log-7.9" and log-9.2 to log-7.2 for RU 486 tested from 10^{-9} to 10^{-6} M" to "log-8.5 to log-7.5". The range was also adjusted for RU-486 to account for data from phase 1.
- For antagonist assays, the acceptable range for 80% DEX activity was modified "from 70% to 80%" to "65% to 90%", based on the labs data.

Prediction Model

- The criterion to define a test item as "interferent" was modified from "*In the interference assay, the IC_{50} should be shifted to a higher concentration with a factor in the 300-fold range. If there is no such shift, the chemical is considered to have an interference effect*" to "*To quantify the shift between the antagonism response and the interference response, we use the R2 criterion*". The criterion was also applied in the validation of the AR-CALUX TA assay (Milcamps et al., 2021) and is the root square of the Pearson correlation coefficient applied to two data sets, as follows: This criterion is based on the assumption that the decrease of the test chemicals response which is not due to competitive antagonism is proportionally the same as the decrease of the specificity control response at all (non-cytotoxic) concentration. The criterion R2 is the square of the correlation coefficient between the Relative Induction of the test chemical at concentration C and the Relative Induction of its specificity control at concentration C.
  The following criteria are applied:
  - Competitive antagonist: $R^2 \leq 0.9$
  - False positive antagonist: $R^2 > 0.9$

Indeed, the data from phase 1 showed that the initial criterion was not applicable.

- The data analysis was made more precise, by specifying that a "four-parameter Hill equation" was to be used.

3.5 Conclusions on transfer phase

In conclusion, the labs did not report major issues, except for the luminescence gradient.

All the substances tested were classified as expected about their activity towards the human Glucocorticoid Receptor. A good intra- and inter-laboratory reproducibility was observed during this phase.

Some modifications were made to the SOP based on the data obtained in phase 1 and feedback from the labs.

The data of phase 1 were presented to the Validation Management Group who concluded that the method had been successfully transferred to the naïve labs.

4. Phase 2 (compound blinded test)

4.1 Agreed test criteria

The second phase of the validation started in Summer 2022 and was finalised at the end of 2022. It was run on thirty-five blind-coded test items.

The acceptance criteria for this method, as defined at the beginning of phase 2, are:

- The cells must have normal morphology.
- The cells must have 100% confluence when exposed.
- The cells must be mycoplasma-free (tested every time a vial is thawed).
- Induction factor: in each run, the average luciferase activity of the positive control (DEX 10^{-7} M) must be at least 10 times that of the solvent control.
- The basal activity should be around 4% of maximum luminescence.
- The coefficient of variation (CV) between replicated wells must be under 30% for the points above basal activity (4%).
- Chemicals must be tested in the concentration ranges not inducing cytotoxicity.
- Response of the cells to the reference chemical must be consistent throughout the runs qualitatively (expected effect of reference substances (*i.e.* sigmoid aspect of the curves)/no effect of solvent control) and quantitatively (EC_{50} must be in the range of values expected for the effect or reference substance, *i.e.* DEX tested from 10^{-10} to 10^{-7} M results in an EC_{50} between $1.26E^{-9}$ and $1.26E^{-8}$ M; and RU 486 tested from 10^{-9} to 10^{-6} M results in an IC_{50} between $3.16E^{-9}$ and $3.16E^{-8}$ M).
- For active chemicals, the concentration-response curve should cover no-effect concentrations (at least two concentrations with no effect), intermediate effect concentrations, and maximum effect concentration of the chemical (excluding toxicity). If this condition is not met, the stock solution should be diluted with half-log-serial dilutions to adjust the concentration range and runs should be repeated three times.
- For antagonist assays, the 80% DEX activity should be between 65% and 90% of the maximum luciferase activity.
- Goodness of fit: when fitting a curve with the Hill model, the calculated R^2 should be above 0.95.

4.2 Test items

Thirty-five test items were selected by the Validation Management Group, following a literature search. The criteria for test items selection were:

- *in vitro*, *in vivo* or human relevant data,
- diversity of chemical families and product classes,
- structural diversity,
- about 20% of the chemicals having no effect
- for chemicals having an effect, half to be agonists, half to be antagonists/interferent

A scrutiny of the literature implied that only few compounds could be surely assigned to be true GR agonists or antagonists. However, this would clearly limit the scope and application of the assay and make for a very short compound list, and those would all belong to the steroid family. By searching for other possible chemistry of relevance the chemical and functional domain of compounds would increase and allow for a proper assessment of these in the assay. Some compounds were suspected to have both agonistic and antagonistic properties as listed below. Except for the well described steroids, the assigned properties of agonist and/or antagonist on GR is only based on a first screen of literature which is scarce outside of the few well-known compounds. Eventually the list includes besides steroids, phthalates, salts, pyrethroids, parabens and other chemical families, but are primarily chosen from an

ED viewpoint covering polymers, pesticides, pharmaceuticals, and preservatives of different products as listed.

The selected chemicals are listed below.

Table 7: Test items used in phase 2.

Name	Cas number	Product class	Suggested effect
Bisphenol A	80-05-7	Plasticizer	hypothetic agonist
Diethylhexyl phthalate (DEHP)	117-81-7	Phthalate	hypothetic
Phthalic acid mono-2-ethylhexyl ester (MEHP)	4376-20-9	Phthalate	hypothetic antagonist
Tetrabromobisphenol A	79-94-7	Polymer	hypothetic antagonist
Butylated hydroxyanisole	121-00-6	Polymerisation product	hypothetic antagonist
L-limonene	5989-54-8	monoterpene	hypothetic agonist
Oleic acid	112-80-1	Fatty acid	negative
Ginsenoside Rg1	22427-39-0	Isoprenoid	hypothetic agonist
Triclosan	3380-34-5	Antibacterial, conservative	hypothetic antagonist
Tolylfluanid	731-27-1	Fungicide, wood preserving	hypothetic agonist / antagonist
Butyl paraben	94-26-8	antimicrobial preservative, flavouring additive	hypothetic agonist
Propyl paraben	94-13-3	antimicrobial preservative, flavouring additive	hypothetic agonist
λ-Cyhalothrin	91465-08-6	Pesticide	hypothetic antagonist
Cypermethrin	52315-07-8	Pesticide	hypothetic antagonist
Methoxychlor	72-43-5	Pesticide, Veterinary Agent	hypothetic antagonist
Arsenic	7440-38-2	wood preservative, pesticide, feed additive	hypothetic antagonist
Monocrotophos	6923-22-4	Pesticide	hypothetic agonist
Benzo[a]pyrene	50-32-8	Product of combustion	hypothetic agonist
2-Methylresorcinol	608-25-3	Cosmetic ingredient	hypothetic agonist / antagonist
Perfluorooctane sulfonic acid (PFOS)	1763-23-1	Industrial chemical	hypothetic agonist / antagonist
Cortisol	50-23-7	Pharmaceutical, Veterinary Agent	Steroid agonist
Budesonide	51333-22-3	Pharmaceutical	Steroid agonist
Fluticasone propionate	80474-14-2	Pharmaceutical	Steroid agonist
Aldosterone	52-39-1	Hormone	Steroid agonist
Progesterone	57-83-0	Pharmaceutical, Veterinary Agent	Steroid agonist / antagonist

21-Hydroxyprogesterone	64-85-7	Pharmaceutical	Steroid agonist / antagonist
Mifepristone	84371-65-3	Pharmaceutical	Steroid agonist / antagonist
Ketoconazole	65277-42-1	Pharmaceutical	antagonist
Spironolactone	52-01-7	Pharmaceutical	Steroid antagonist
17β-estradiol	50-28-2	Pharmaceutical	hypothetic steroid antagonist
Fulvestrant	129453-61-8	Pharmaceutical	hypothetic steroid antagonist
Lithium Chloride	7447-41-8	Brazing, desiccant	hypothetic antagonist
Sucrose	57-50-1	Natural sugar	negative
Sodium nitrite	7632-00-0	Food preservative	negative
Sodium azide	26628-22-8	Chemical Intermediate, Fungicide, Herbicide	negative

The chemicals were blind-coded by Charles River (The Netherlands), and aliquots were sent (as powder or liquid) to the labs, with a modified Safety Data Sheet. The full Data Sheet were sent in a sealed envelope to be open in case of emergency.
The identity of the test items was lifted after the statistical analysis of the data.

4.3 Results from phase 2

Solubility and cytotoxicity testing

The first step of this phase consisted in solubilising the test items and testing their potential cytotoxicity.
All test items were dissolved in DMSO, at 10 mg/mL for Ineris, and 3 mg/mL for the other labs. Ineris deviated from the SOP which suggested a maximal stock concentration of 3 mg/mL.
Arsenic was not soluble in DMSO, ethanol or water.

No cytotoxicity was observed for any of the test items at a concentration up to 3 mg/mL.

It is to be noted that beyond this initial cytotoxicity testing, cytotoxicity is systematically checked on each run (in parallel to luminescence).

Agonism testing

The labs tested the test items at concentration from $3.0E^{-6}$ to $3.0E^{-3}$ mg/mL, except for those listed in the table below.

Table 8: Concentrations used in mg/mL.

	Ineris	INSERM	Tame-Water	Toxem
Cortisol	$3.0E^{-7} – 3.0E^{-4}$	$3.0E^{-7} – 3.0E^{-4}$	$9.5E^{-7} – 9.5E^{-4}$	$9.5E^{-7} – 9.5E^{-4}$
Budesonide	$1.0E^{-8} – 1.0E^{-5}$	$1.0E^{-8} – 1.0E^{-5}$	$3.0E^{-8} – 3.0E^{-5}$	$9.5E^{-9} – 9.5E^{-6}$
Fluticasone propionate	$1.0E^{-9} – 1.0E^{-6}$	$3.0E^{-9} – 3.0E^{-6}$	$9.5E^{-9} – 9.5E^{-6}$	$9.5E^{-10} – 9.5E^{-7}$
Mifepristone	$1.0E^{-8} – 1.0E^{-4}$	$3.1E^{-7}$ - $3.1E^{-4}$	$9.5E^{-7} – 9.5E^{-4}$	

Dexamethasone (Positive control)

Dexamethasone (DEX) was tested by all labs from $1.0E^{-10}$ to $1.0E^{-7}$M, as specified in the SOP. At these concentrations, the EC_{50} should range from $1.26E^{-9}$ to $1.26E^{-8}$.

At Tame-Water, three runs of DEX were considered not valid due to an EC_{50} outside of the acceptable range. All test items tested in the same runs were considered as not valid and had to be repeated.

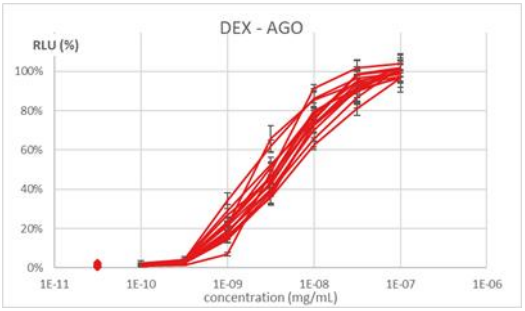
The concentration-response curves and EC_{50} obtained for the valid runs are presented below.

Table 9: Dexamethasone EC_{50} values in agonism mode.

	Ineris	INSERM	Tame-Water	Toxem
EC_{50}(±SD) in M	$4,9E^{-09} \pm 5,1E^{-10}$	$3,3E^{-09} \pm 1,7E^{-10}$	$4,6E^{-09} \pm 4,6E^{-10}$	$8,1E^{-09} \pm 1,4E^{-09}$
	$4,2E^{-09} \pm 4,2E^{-10}$	$4,6E^{-09} \pm 4,4E^{-10}$	$1,0E^{-08} \pm 1,5E^{-09}$	$3,9E^{-09} \pm 3,5E^{-10}$
	$3,3E^{-09} \pm 3,4E^{-10}$	$3,2E^{-09} \pm 9,2E^{-11}$	$2,2E^{-09} \pm 1,9E^{-10}$	$7,2E^{-09} \pm 5,9E^{-10}$
	$3,4E^{-09} \pm 2,4E^{-10}$	$3,7E^{-09} \pm 1,8E^{-10}$	$4,1E^{-09} \pm 4,1E^{-10}$	$7,3E^{-09} \pm 8,0E^{-10}$
	$3,0E^{-09} \pm 3,3E^{-10}$	$5,8E^{-09} \pm 6,1E^{-10}$	$2,1E^{-09} \pm 1,5E^{-10}$	$6,5E^{-09} \pm 7,4E^{-10}$
	$2,0E^{-09} \pm 1,5E^{-10}$	$4,4E^{-09} \pm 2,2E^{-10}$	$4,7E^{-09} \pm 3,6E^{-10}$	$7,0E^{-09} \pm 7,8E^{-10}$
	$3,0E^{-09} \pm 2,6E^{-10}$	$4,4E^{-09} \pm 2,7E^{-10}$	$2,3E^{-09} \pm 2,7E^{-10}$	$6,2E^{-09} \pm 6,7E^{-10}$
	$6,2E^{-09} \pm 6,3E^{-10}$	$3,9E^{-09} \pm 2,1E^{-10}$	$5,9E^{-09} \pm 1,1E^{-09}$	$5,0E^{-09} \pm 4,3E^{-10}$
	$1,8E^{-09} \pm 1,6E^{-10}$	$5,2E^{-09} \pm 4,6E^{-10}$	$8,2E^{-09} \pm 1,2E^{-09}$	$5,7E^{-09} \pm 3,4E^{-10}$
	$5,0E^{-09} \pm 4,0E^{-10}$	$4,5E^{-09} \pm 2,1E^{-10}$	$8,0E^{-09} \pm 1,8E^{-09}$	$7,1E^{-09} \pm 4,9E^{-10}$
	$3,8E^{-09} \pm 1,4E^{-10}$	$3,5E^{-09} \pm 1,5E^{-10}$	$4,3E^{-09} \pm 4,8E^{-10}$	
	$3,4E^{-09} \pm 9,3E^{-11}$		$2,3E^{-09} \pm 3,3E^{-10}$	
	$3,8E^{-09} \pm 4,1E^{-10}$			
	$6,3E^{-09} \pm 7,4E^{-10}$			
	$4,4E^{-09} \pm 4,0E^{-10}$			
Mean (±SD)	$3,9E^{-09} \pm 1,30E^{-09}$	$4,2E^{-09} \pm 8,11E^{-10}$	$4,9E^{-09} \pm 2,67E^{-09}$	$6,3E^{-9} \pm 1,2E^{-9}$

All labs show a good reproducibility in their EC_{50} values, with the lowest variability in INSERM (developer lab).

A)



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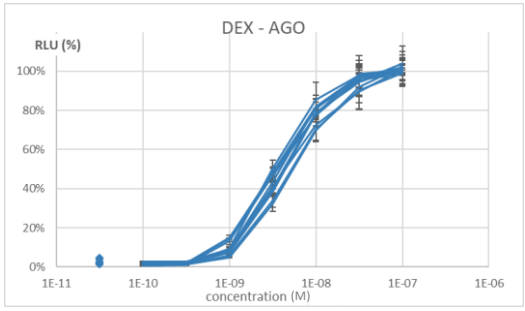

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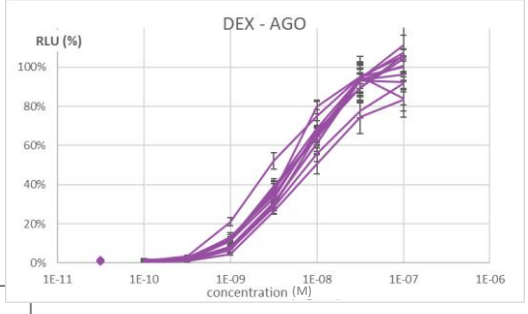

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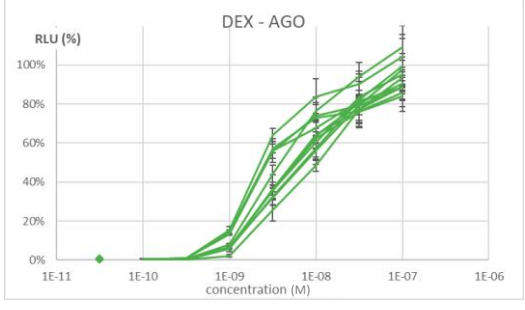


E)

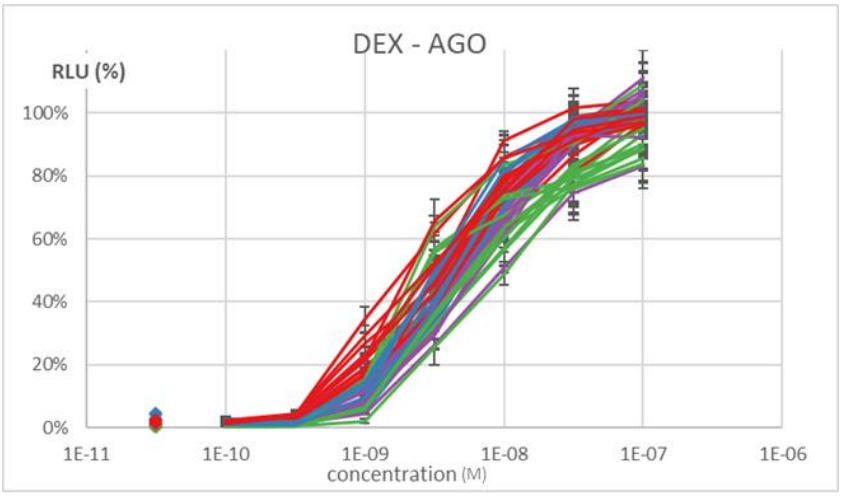


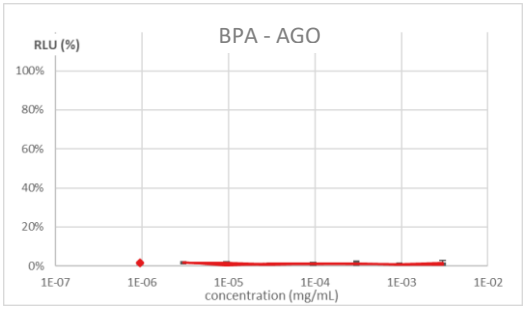
Figure 12: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Dexamethasone in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs demonstrated that Dexamethasone is an agonist in this method.

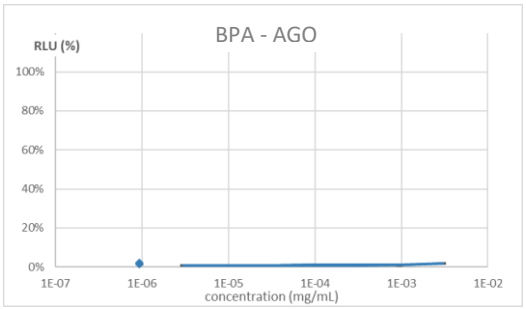
Bisphenol A

All labs tested Bisphenol A from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

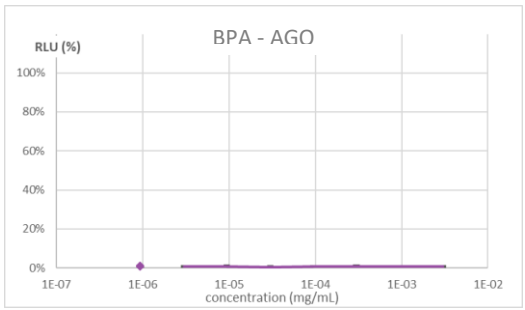
A)



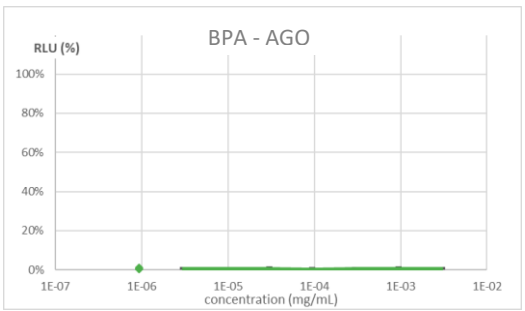
B)



C)



D)



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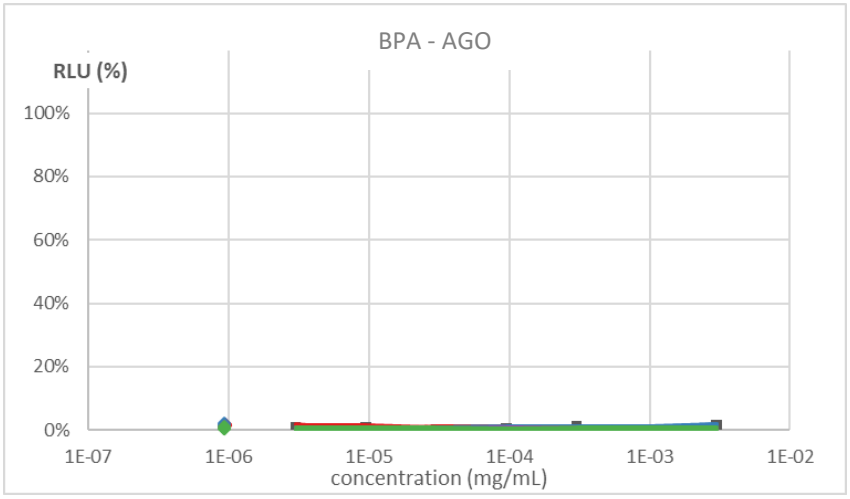


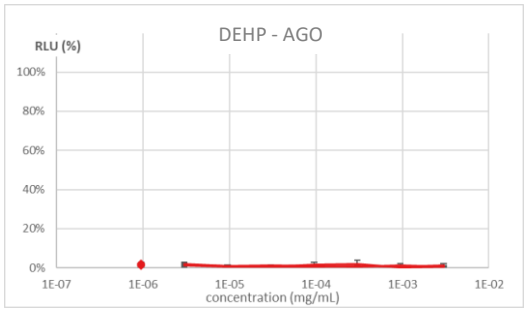
Figure 13: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Bisphenol A in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Bisphenol A is not an agonist in this method.

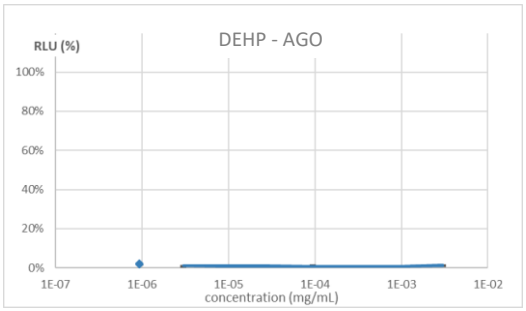
Diethylhexyl phthalate (DEHP)

All labs tested DEHP from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

A)



B)



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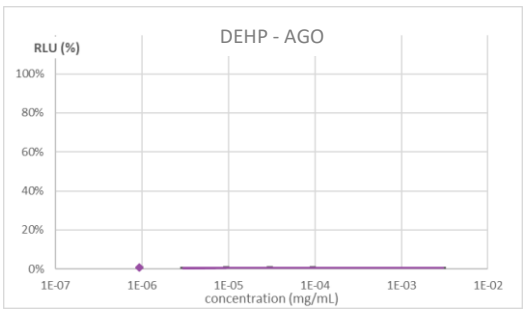
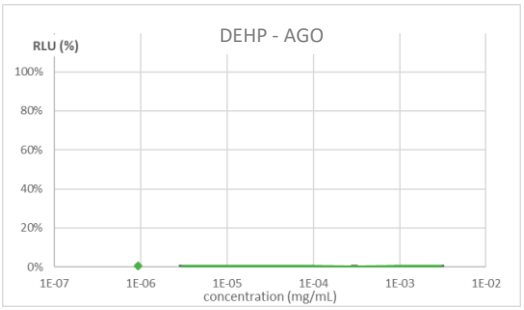


D)



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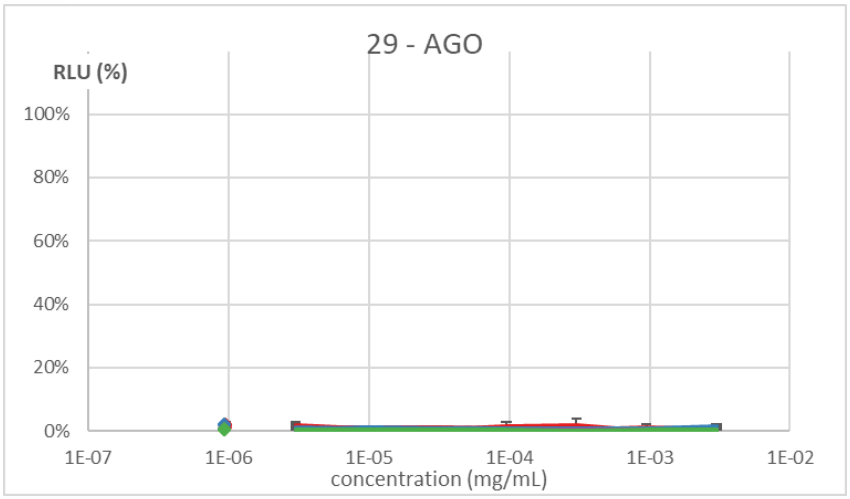


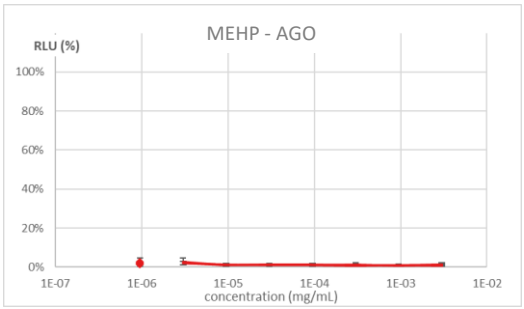
Figure 14: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to DEHP in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, DEHP is not an agonist in this method.

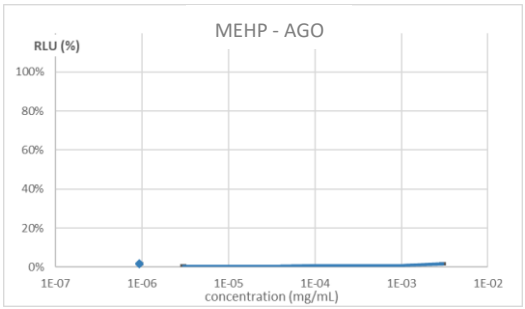
Phthalic acid mono-2-ethylhexyl ester (MEHP)

All labs tested MEHP from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

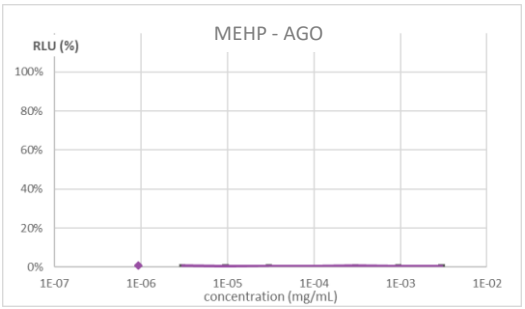
A)



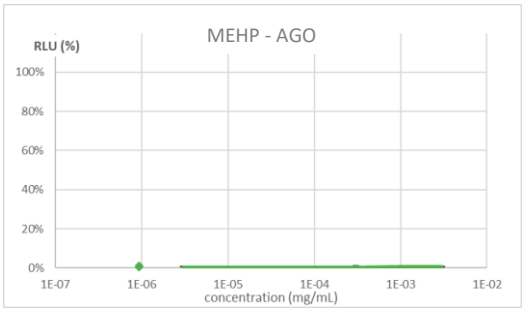
B)



C)



D)



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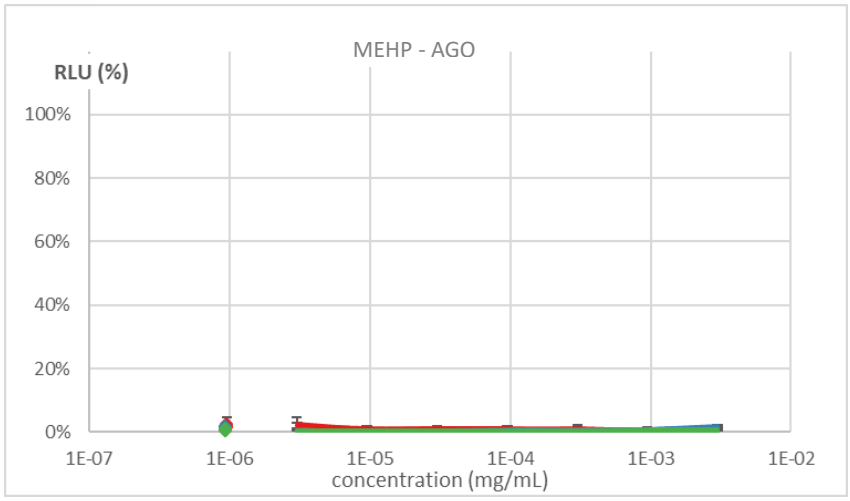


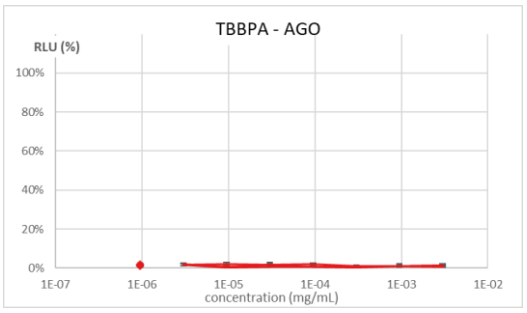
Figure 15: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to MEHP in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, MEHP is not an agonist in this method.

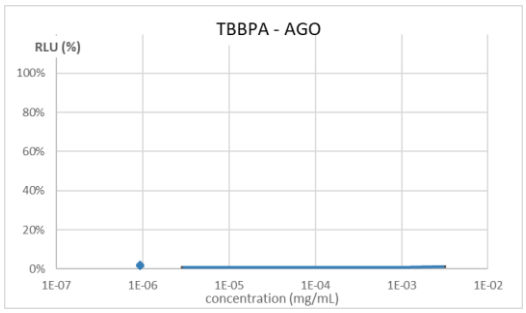
Tetrabromobisphenol A (TBBPA)

All labs tested TBBPA from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

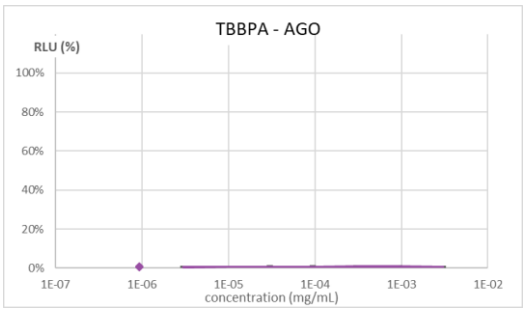
A)



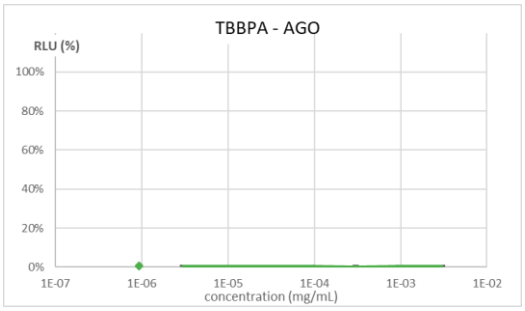
B)



C)



D)



E)

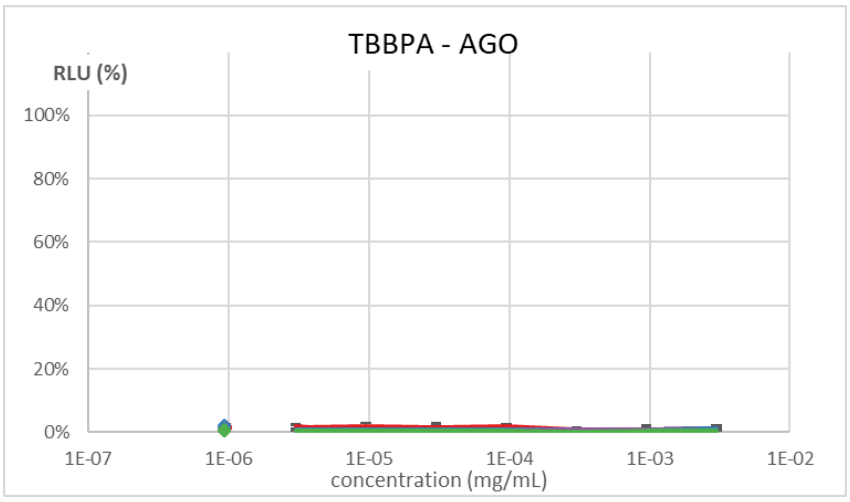


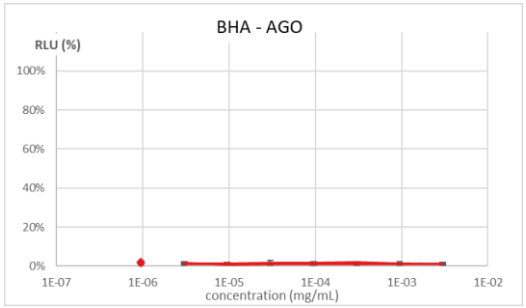
Figure 16: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to TBBPA in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, TBBPA is not an agonist in this method.

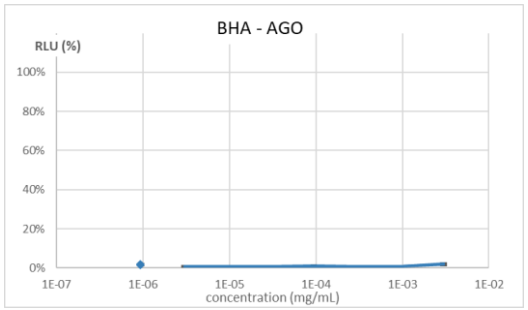
Butylated hydroxyanisole (BHA)

All labs tested BHA from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

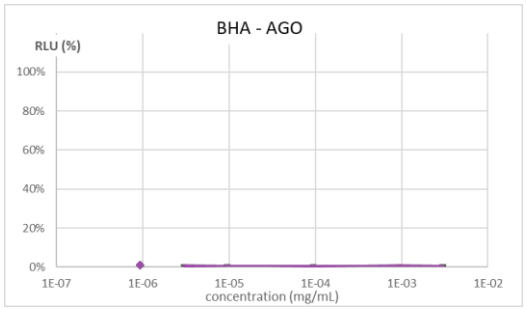
L)



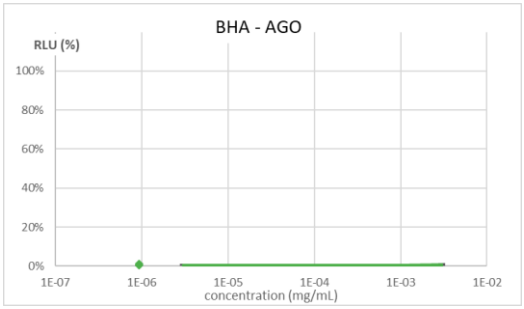
B)



C)



D)



E)

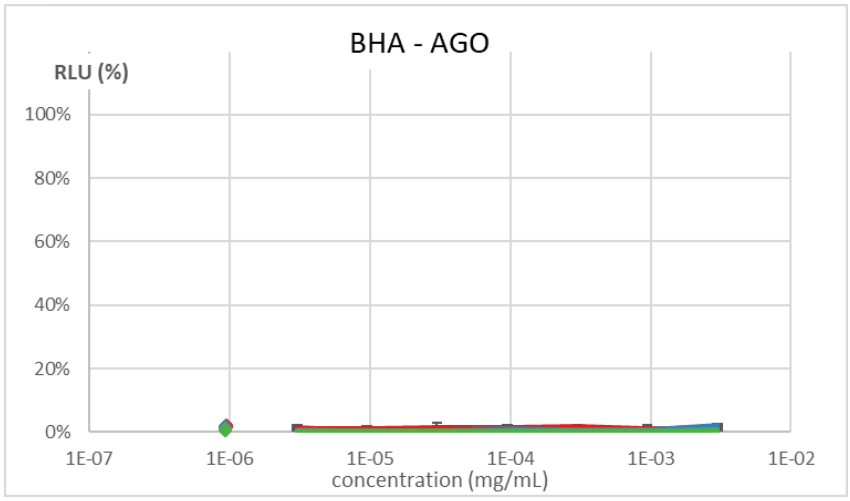


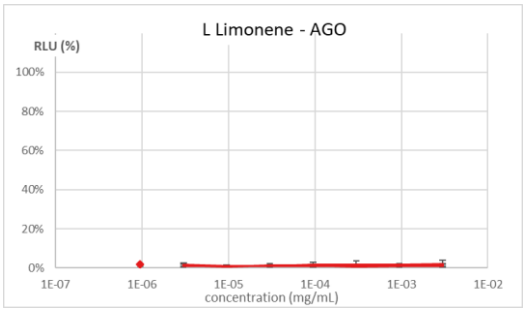
Figure 17: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to BHA in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, BHA is not an agonist in this method.

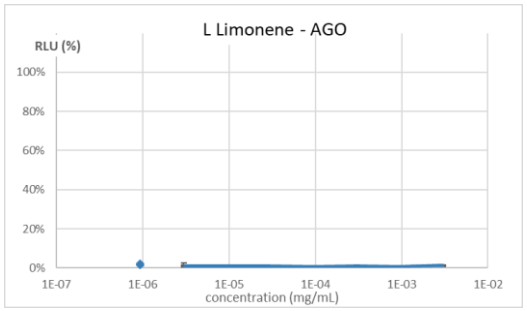
L-limonene

All labs tested L-limonene from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

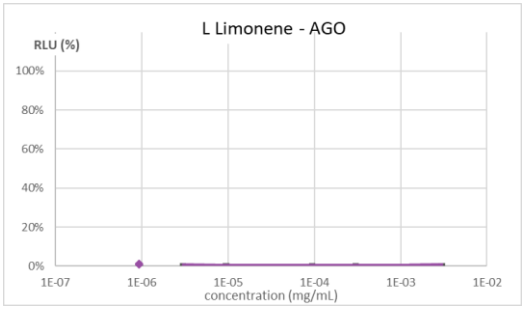
A)



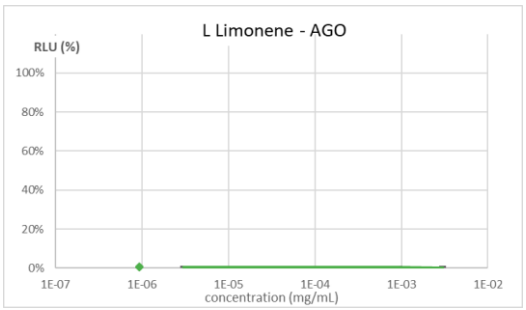
B)



C)



D)



E)

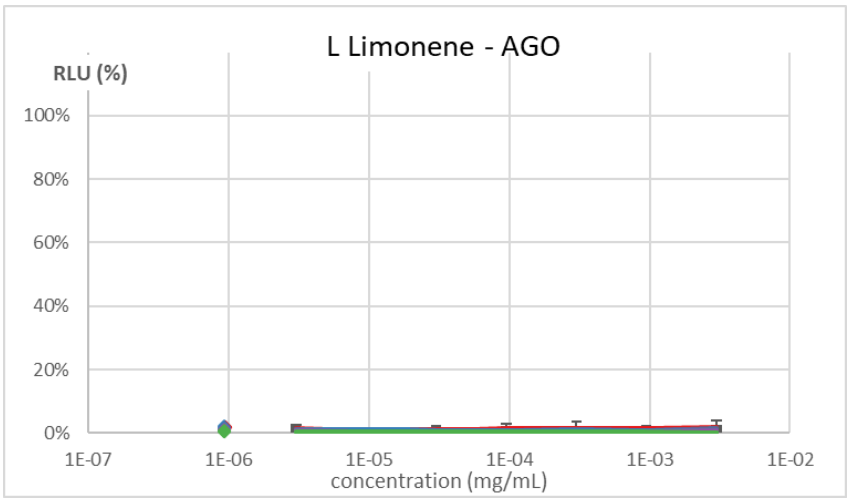


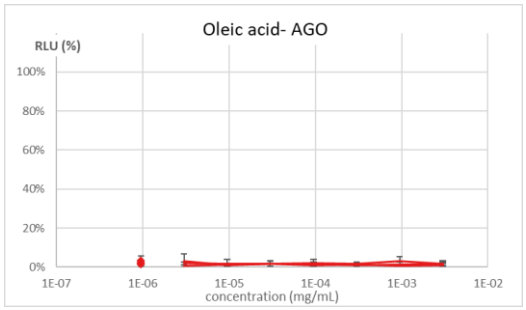
Figure 18: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to L-Limonene in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, L-limonene is not an agonist in this method.

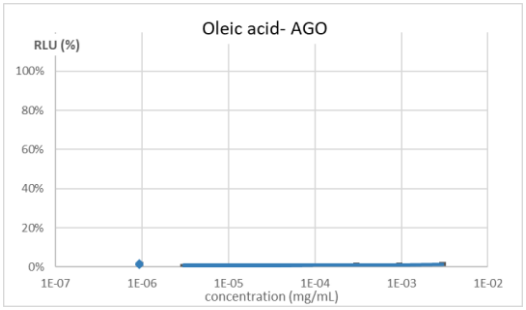
Oleic acid

All labs tested oleic acid from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

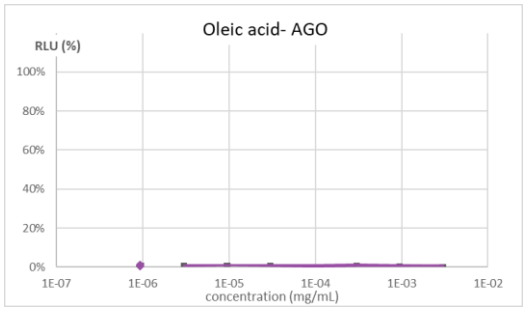
A)



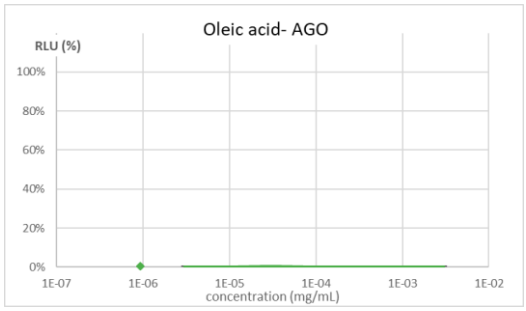
B)



C)



D)



E)

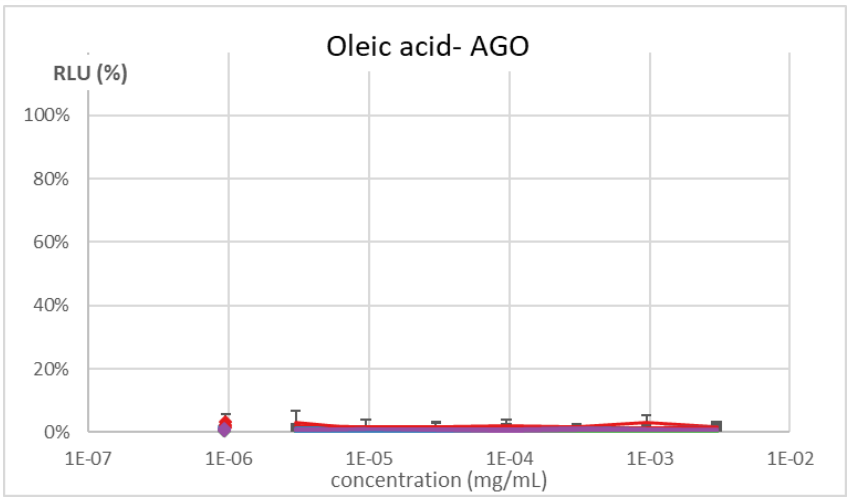


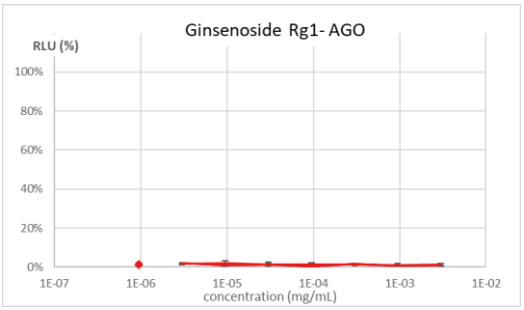
Figure 19: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Oleic acid in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Oleic acid is not an agonist in this method.

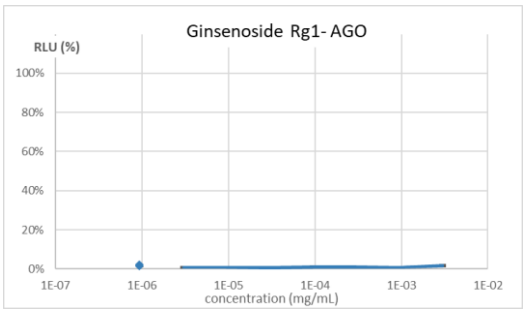
Ginsenoside Rg1

All labs tested Ginsenoside Rg1 from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

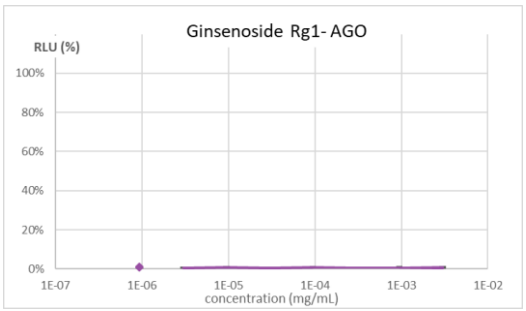
A)



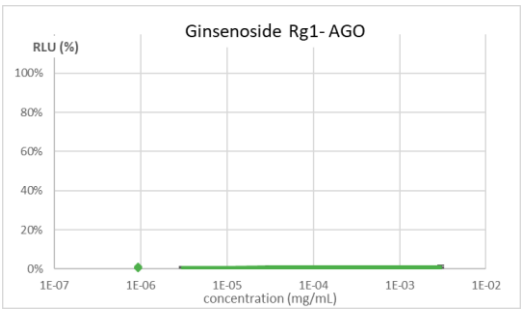
B)



C)



D)



E)

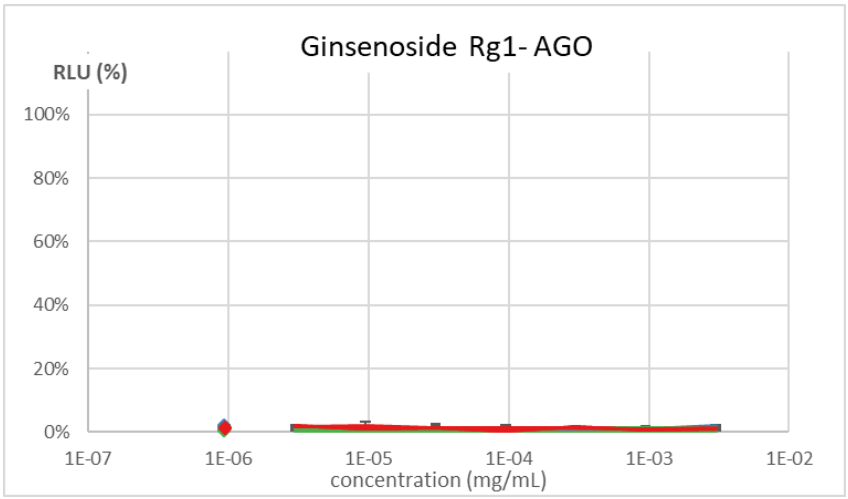


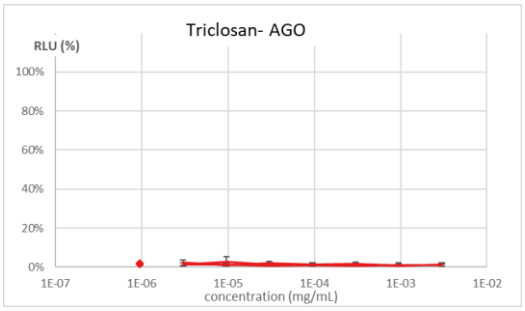
Figure 20: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Ginsenoside Rg1 in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Ginsenoside Rg1 is not an agonist in this method.

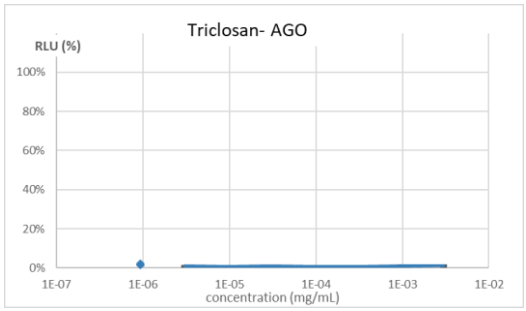
Triclosan

All labs tested Triclosan from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

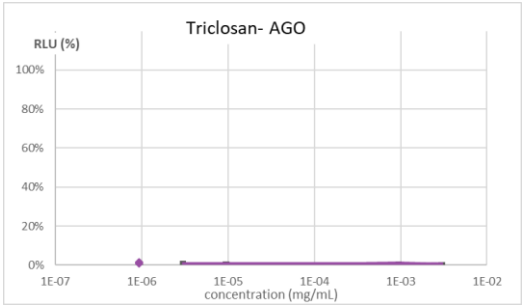
A)



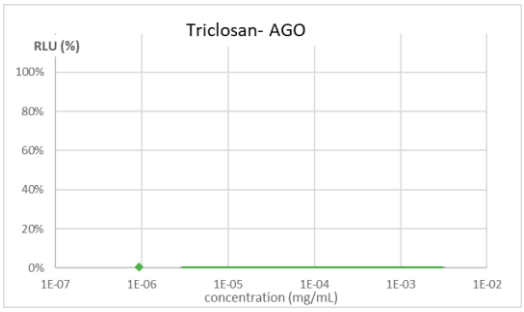
B)



C)



D)



E)

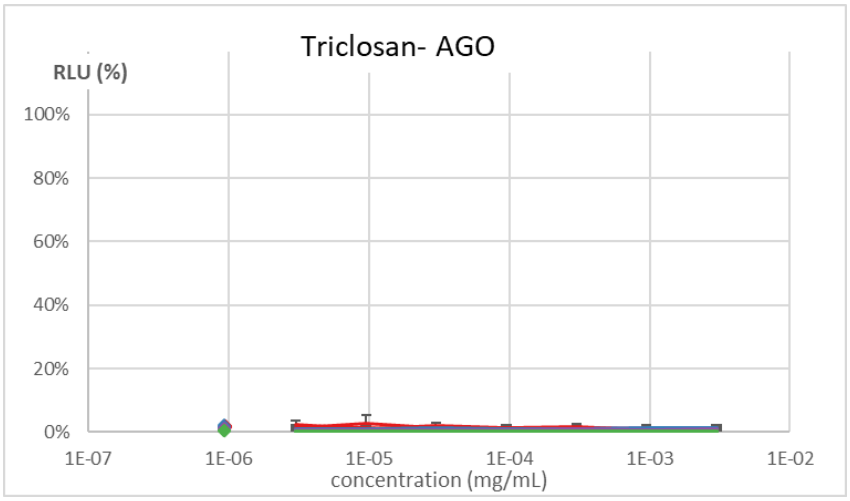


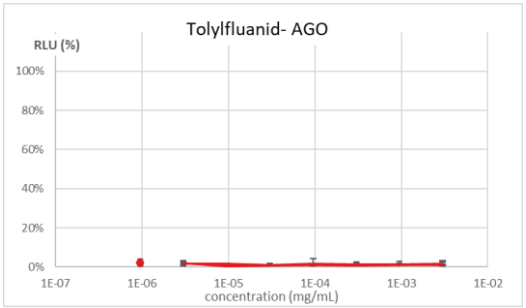
Figure 21: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Triclosan in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Triclosan is not an agonist in this method.

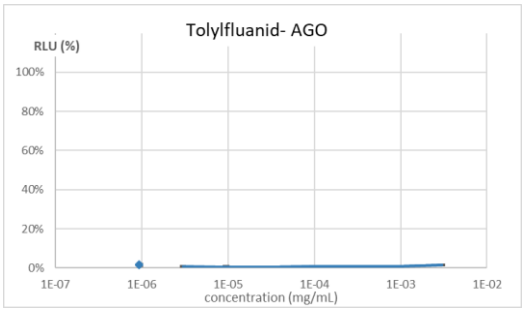
Tolylfluanid

All labs tested Tolylfluanid from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

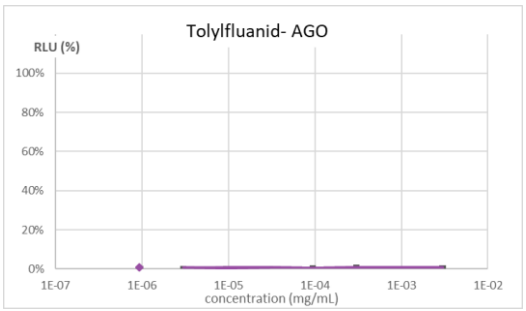
A)



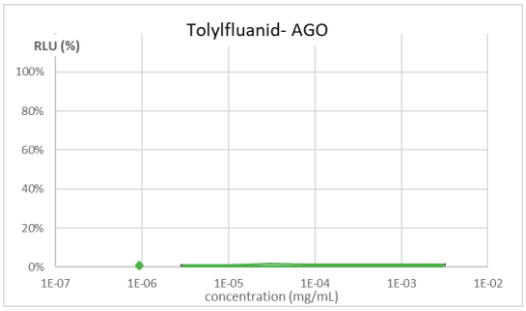
B)



C)



D)



E)

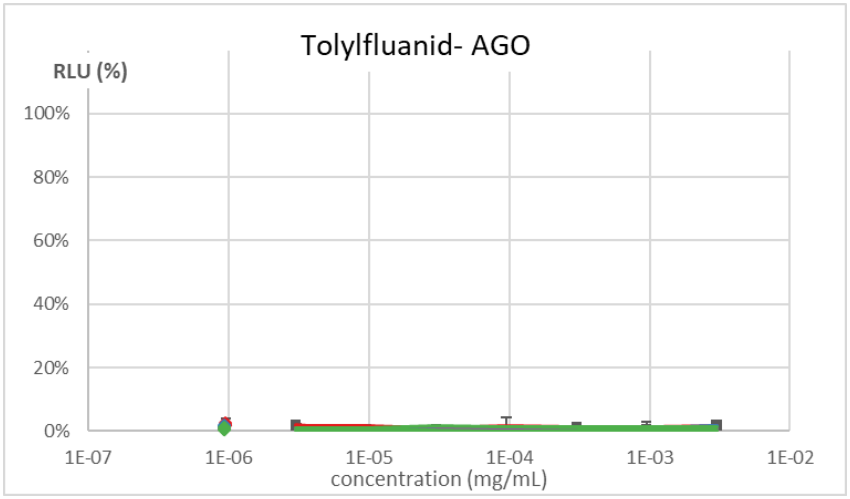


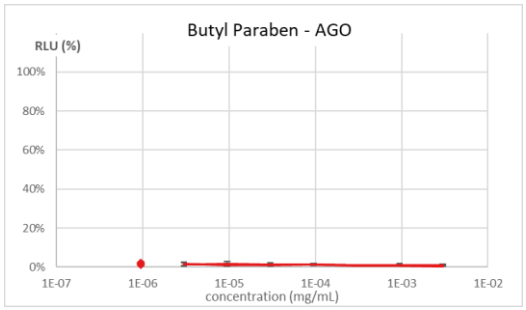
Figure 22: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Tolylfluanid in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Tolylfluanid is not an agonist in this method.

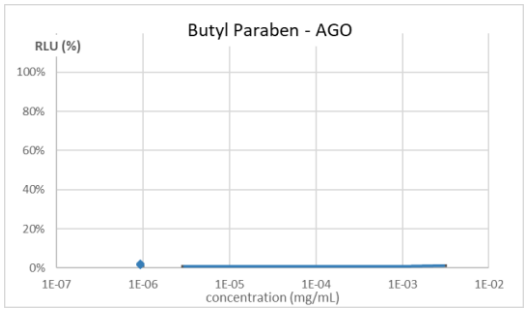
Butyl paraben

All labs tested Butyl paraben from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

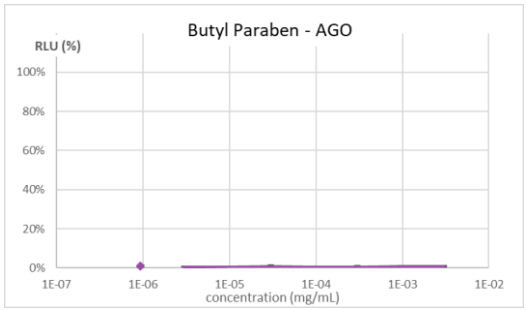
A)



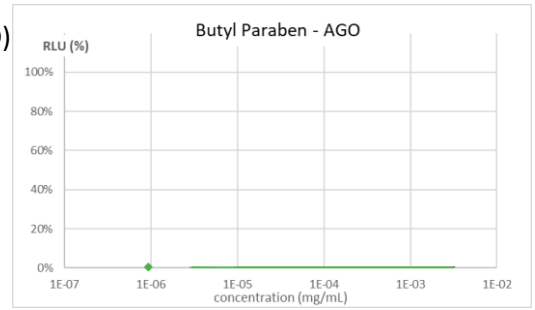
B)



C)



D)



E)

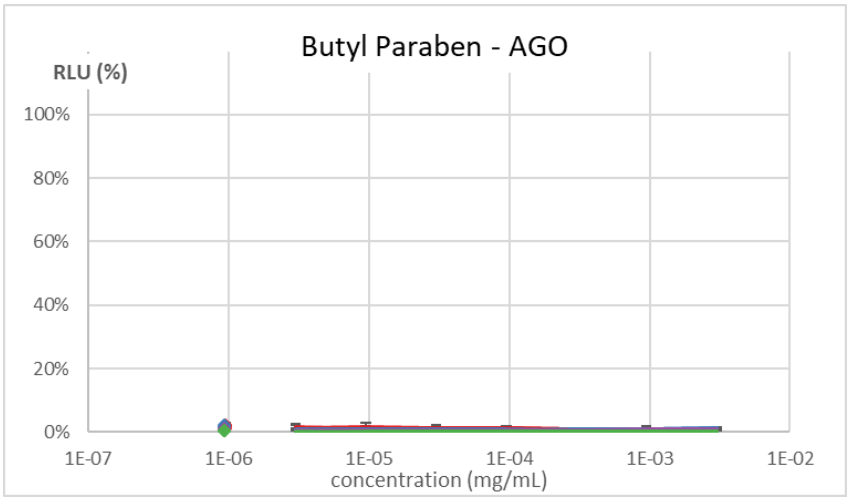


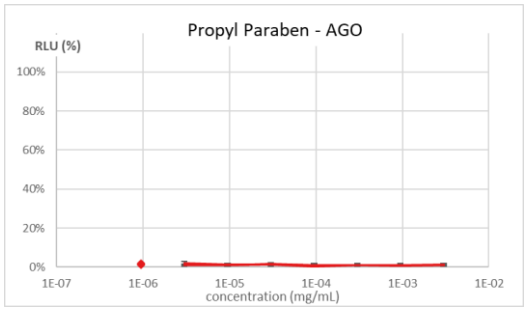
Figure 23: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Butyl paraben in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Butyl paraben is not an agonist in this method.

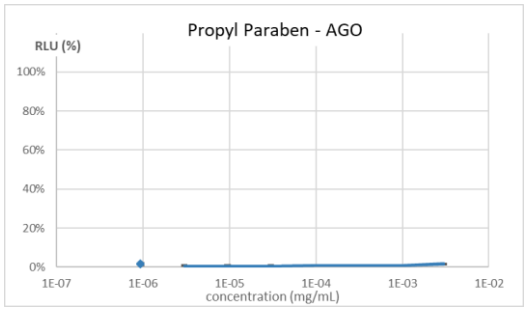
Propyl paraben

All labs tested Propyl paraben from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

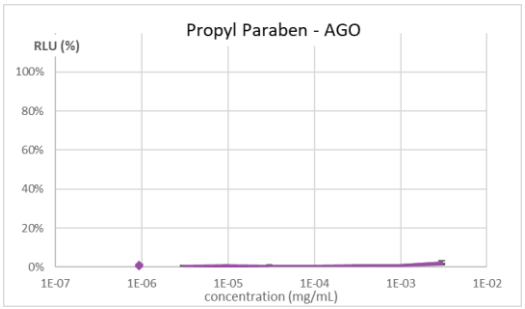
A)



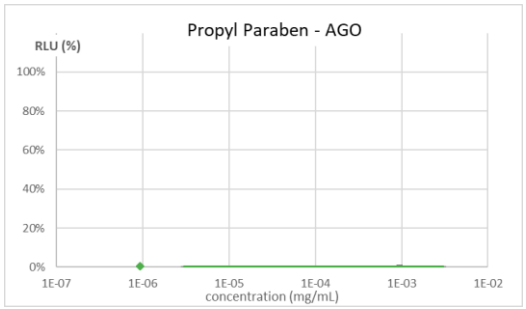
B)



C)



D)



E)

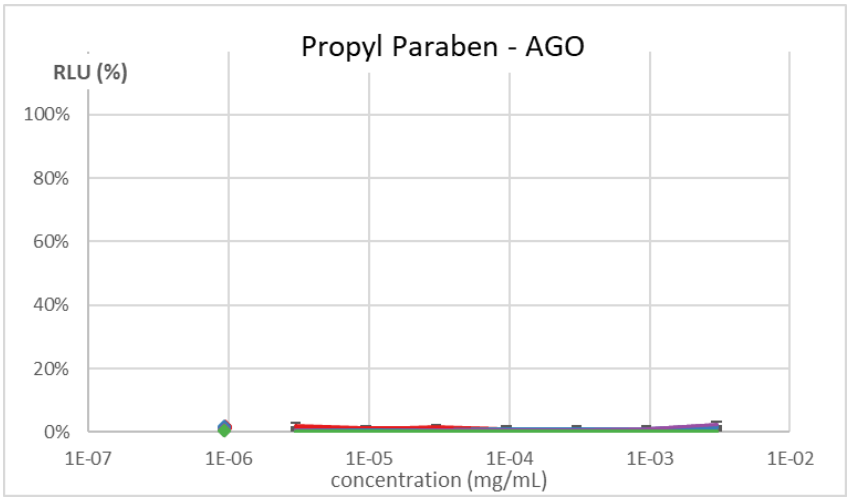


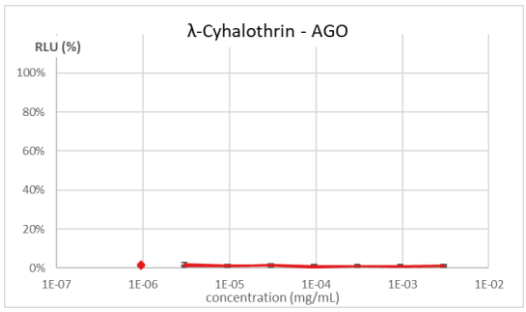
Figure 24: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Propyl paraben in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Propyl paraben is not an agonist in this method.

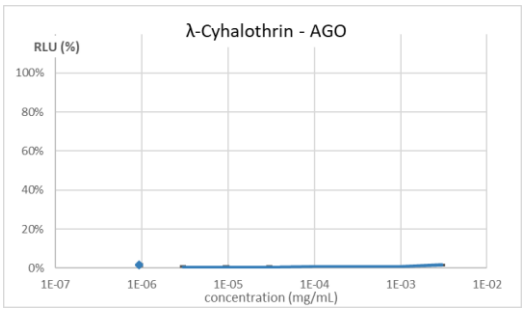
<u>λ-Cyhalothrin</u>

All labs tested λ-Cyhalothrin from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

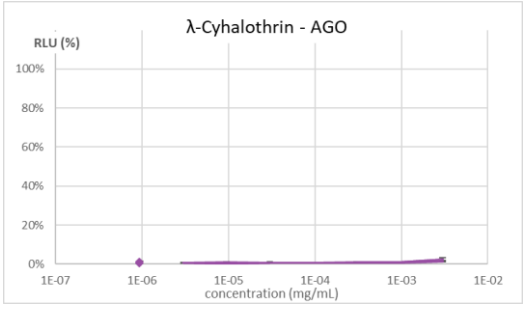
A)



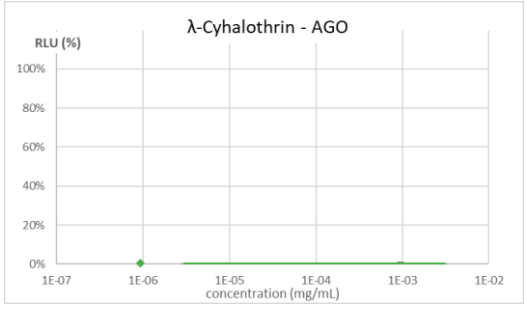
B)



C)



D)



E)

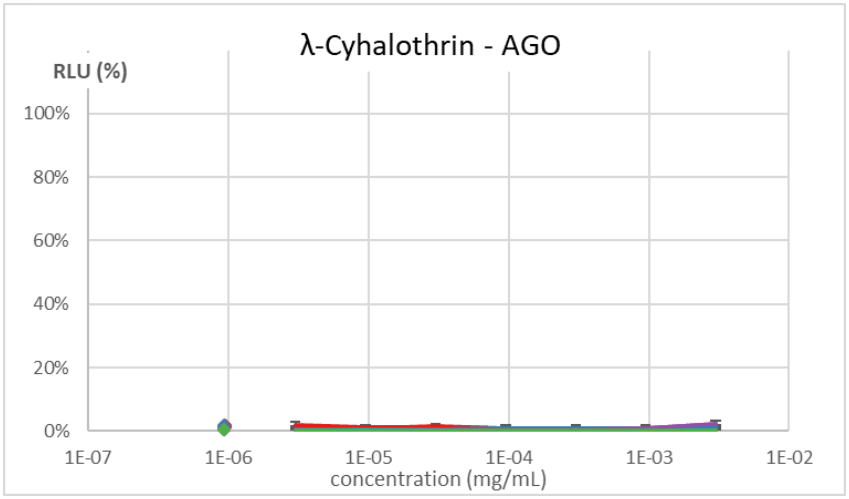


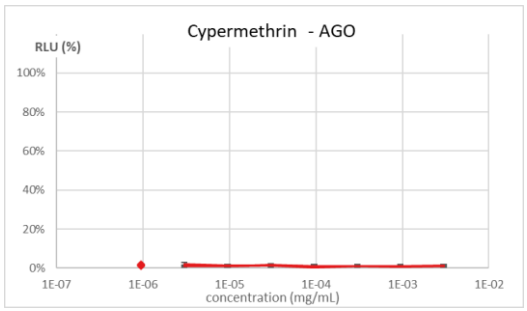
Figure 25: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to λ-Cyhalothrin in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, λ-Cyhalothrin is not an agonist in this method.

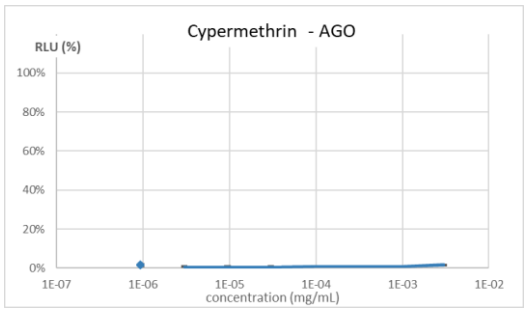
Cypermethrin

All labs tested Cypermethrin from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

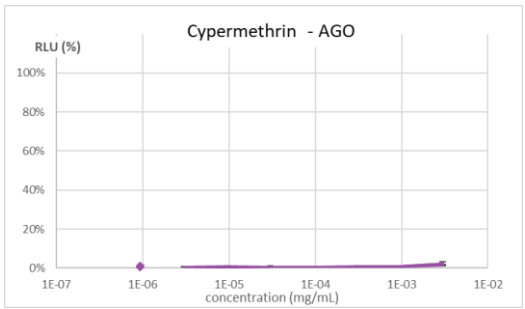
A)



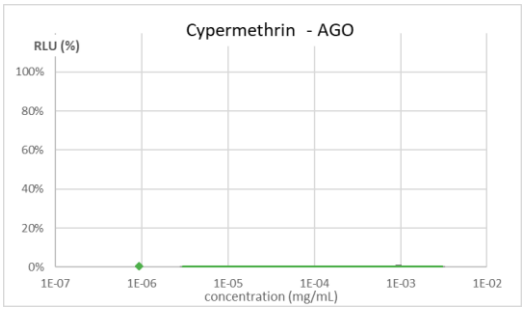
B)



C)



D)



E)

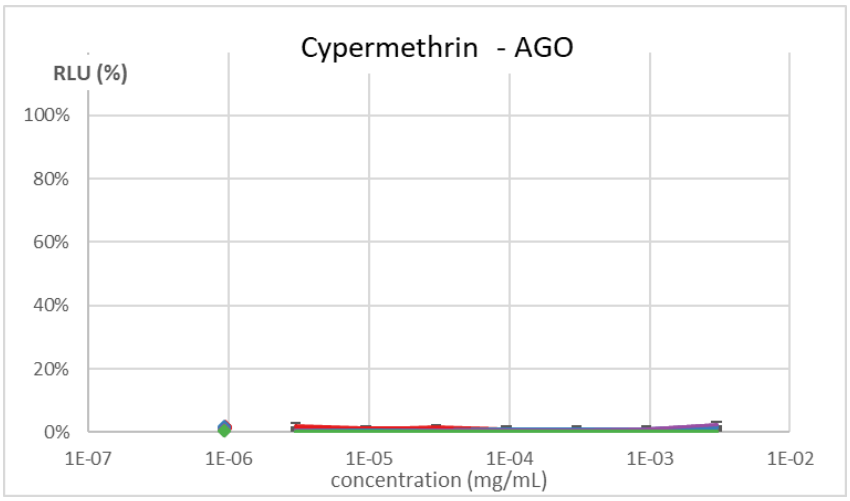


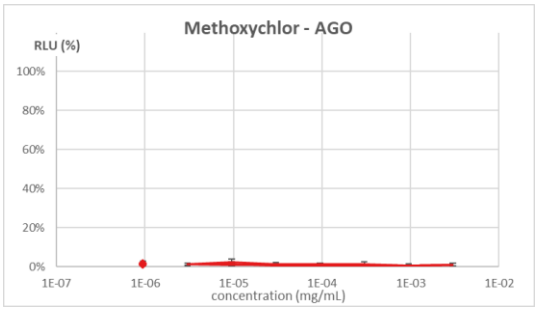
Figure 26: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Cypermethrin in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E)

All labs concluded that at the concentrations tested, Cypermethrin is not an agonist in this method.

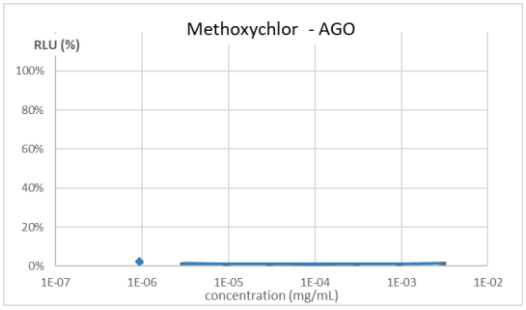
Methoxychlor

All labs tested Methoxychlor from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

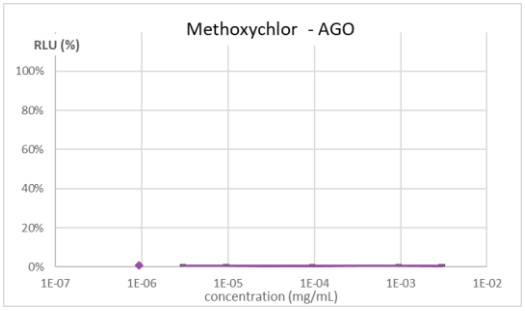
A)



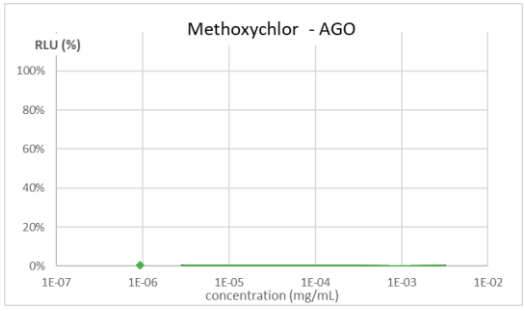
B)



C)



D)



E)

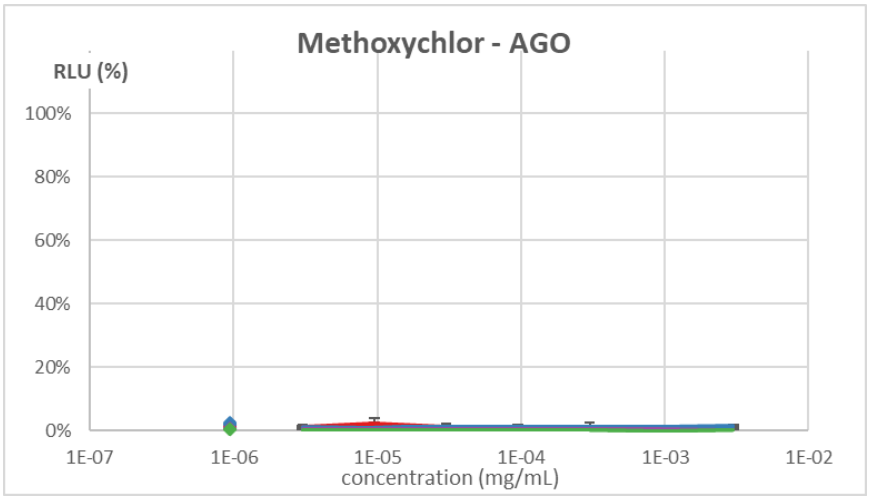


Figure 27: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Methoxychlor in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Methoxychlor is not an agonist in this method.

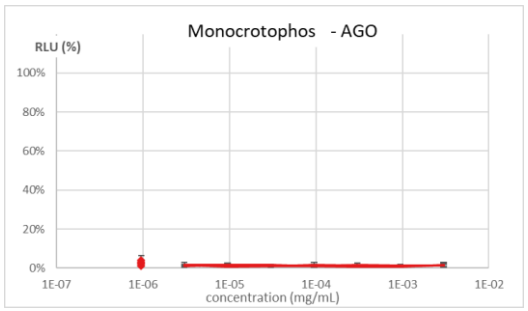
Arsenic

Arsenic was not tested as it was found insoluble.

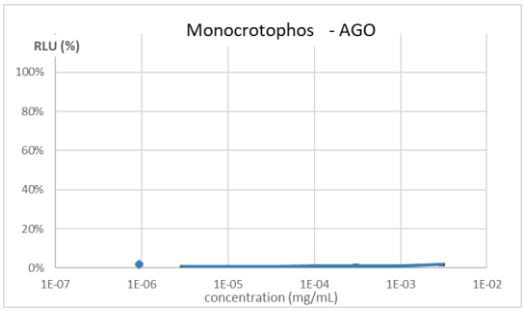
Monocrotophos

All labs tested Monocrotophos from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

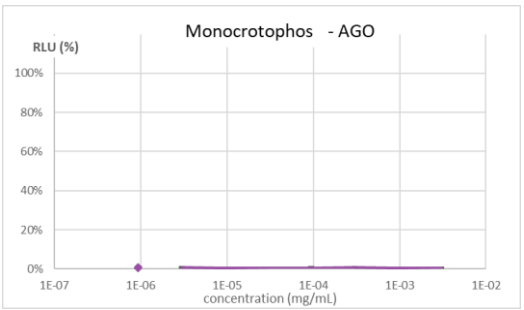
A)



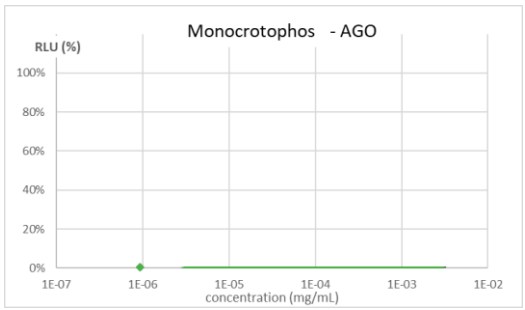
B)



C)



D)



E)

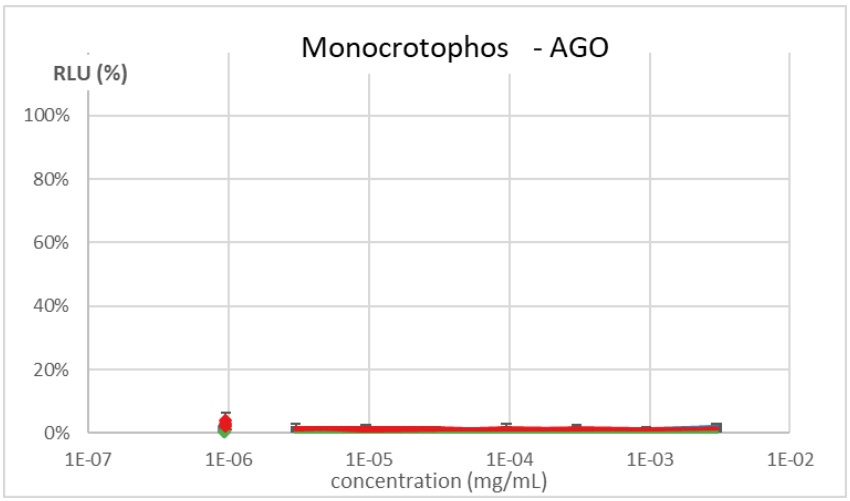


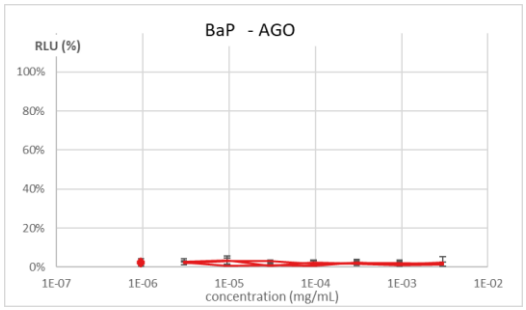
Figure 28: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Monocrotophos in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E)

All labs concluded that at the concentrations tested, Monocrotophos is not an agonist in this method.

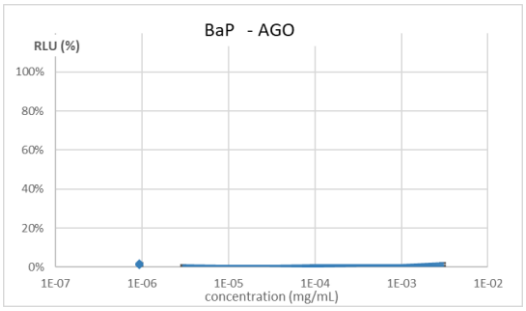
Benzo[a]pyrene

All labs tested Benzo[a]pyrene from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

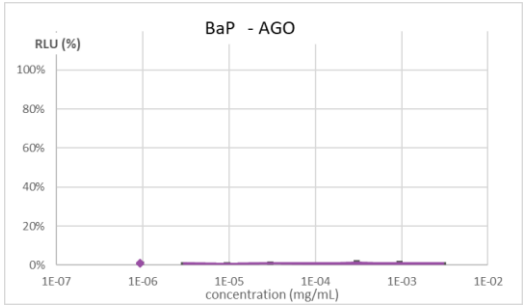
A)



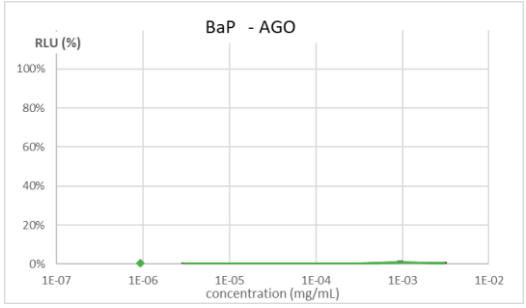
B)



C)



D)



E)

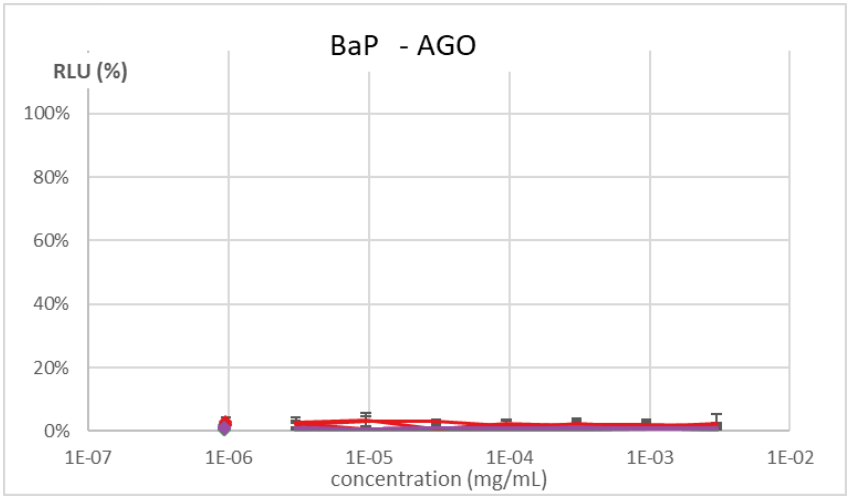


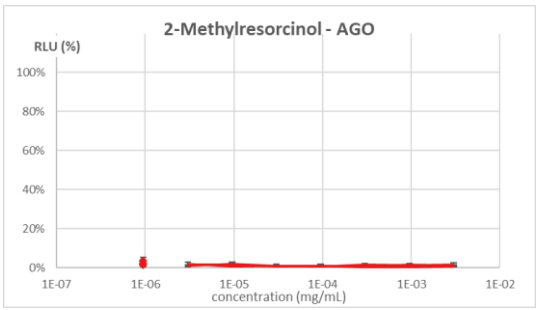
Figure 29: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to BaP in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Benzo[a]pyrene is not an agonist in this method.

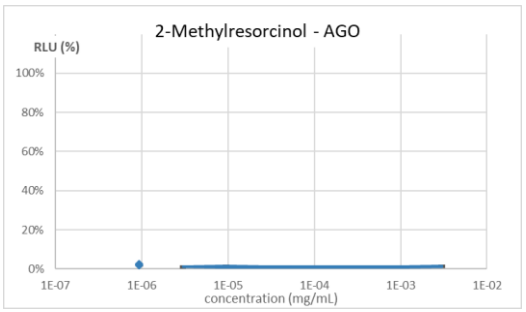
2-Methylresorcinol

All labs tested 2-Methylresorcinol from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

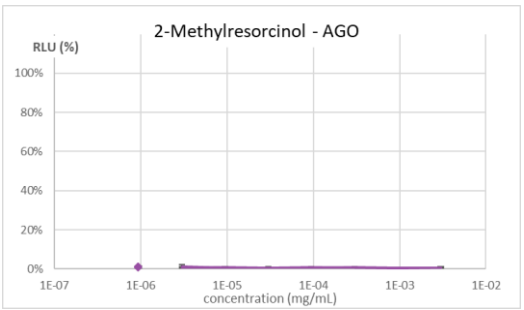
A)



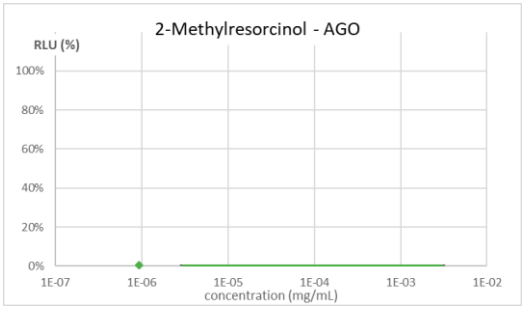
B)



C)



D)



E)

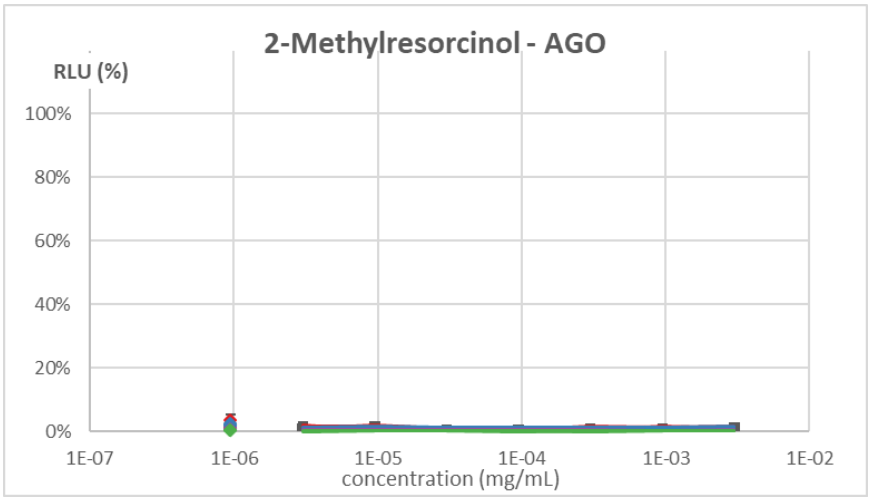


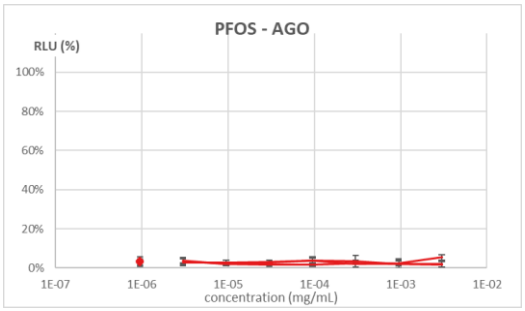
Figure 30: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to 2-Methylresorcinol in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, 2-Methylresorcinol is not an agonist in this method.

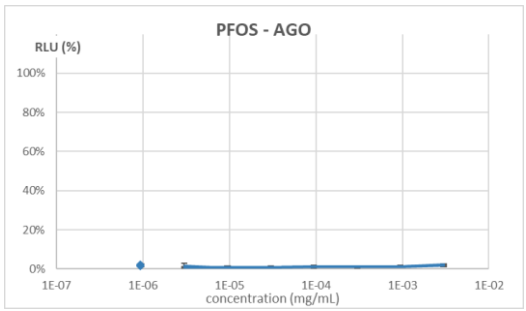
Perfluorooctane sulfonic acid (PFOS)

All labs tested PFOS from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

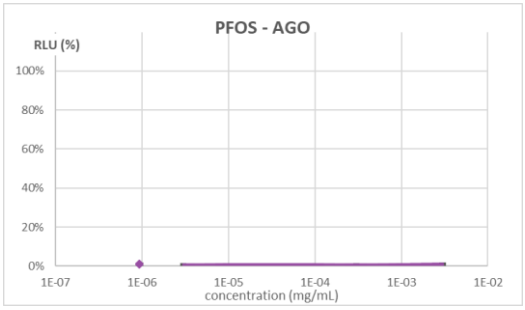
A)



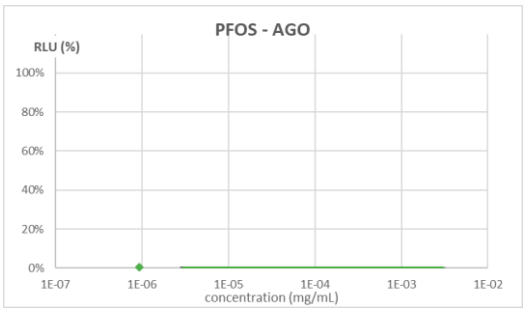
B)



C)



D)



E)

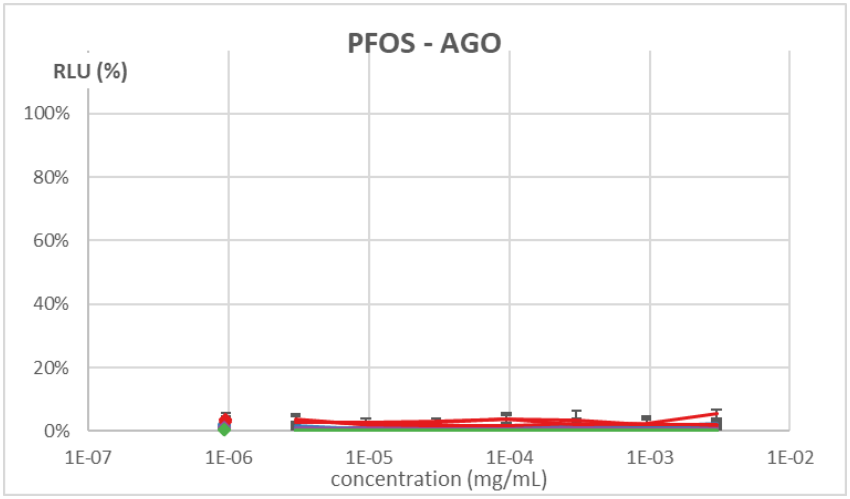


Figure 31: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to PFOS in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, PFOS is not an agonist in this method.

Cortisol

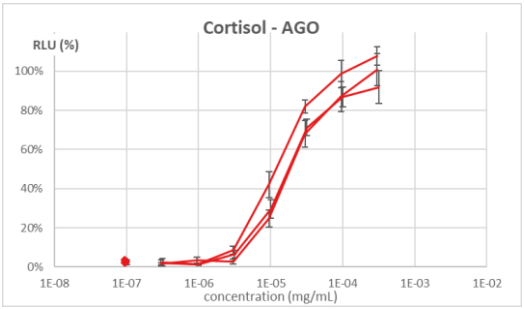
In agonism mode, all labs found that Cortisol had a strong activity, which led them to test at lower concentrations in order to obtain a full dose-response.
The concentration-response curves and EC_{50} obtained are presented below.

Table 10 : Cortisol EC_{50} values in agonism mode.

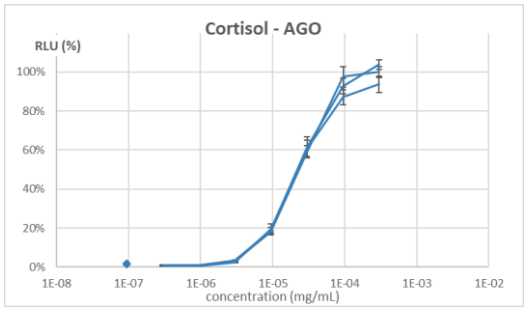
	Ineris	INSERM	Tame-Water	Toxem
EC_{50} (±SD) in mg/mL	$1{,}6E^{-05} \pm 1{,}0E^{-06}$	$2{,}1E^{-05} \pm 8{,}5E^{-07}$	$9{,}9E^{-05} \pm 1{,}3E^{-05}$	$3{,}5E^{-05} \pm 3{,}5E^{-06}$
	$1{,}9E^{-05} \pm 1{,}7E^{-06}$	$2{,}5E^{-05} \pm 9{,}6E^{-07}$	$4{,}4E^{-05} \pm 2{,}3E^{-06}$	$3{,}3E^{-05} \pm 1{,}4E^{-06}$
	$1{,}3E^{-05} \pm 9{,}1E^{-07}$	$2{,}5E^{-05} \pm 9{,}4E^{-07}$	$5{,}8E^{-05} \pm 4{,}8E^{-06}$	$3{,}3E^{-05} \pm 1{,}8E^{-06}$
Mean (±SD)	$1{,}6E^{-05} \pm 3{,}1E^{-06}$	$2.4E^{-05} \pm 2.3E^{-06}$	$6{,}7E^{-05} \pm 2{,}9E^{-05}$	$3.4e^{-05} \pm 1.2E^{-06}$

The IC_{50} show a good intra-laboratory reproducibility, with a higher variability in Tame-Water. Some variability can be observed inter-laboratories, which may be explained by the various concentration tested.

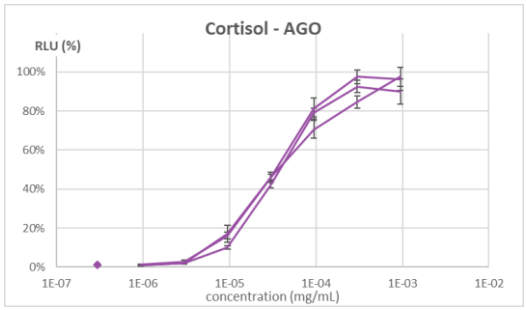
A)



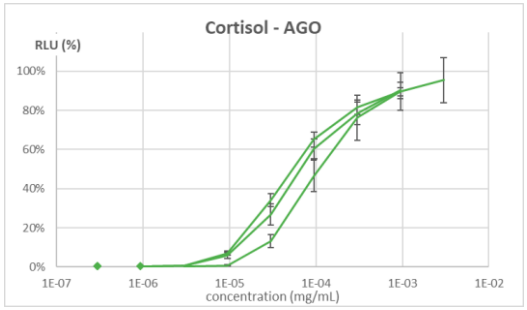
B)



C)



D)



E)

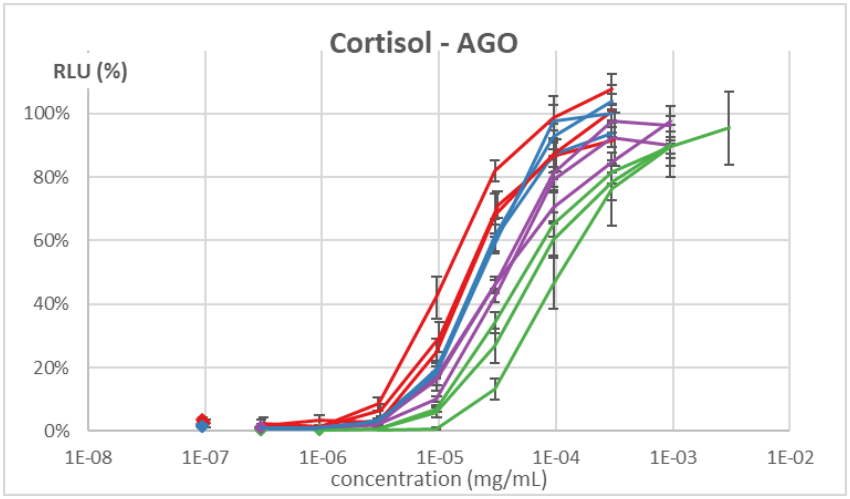


Figure 32: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Cortisol in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Cortisol is an agonist in this method, increasing luciferase activity above 10% of maximum effect of positive control the basal activity (which is maximum 4%) for at least two consecutive concentrations or the highest concentration.

Budesonide

In agonism mode, all labs found that Budesonide had a strong activity, which led them to test at lower concentrations in order to obtain a full dose-response.
The concentration-response curves and EC_{50} obtained are presented below.

Table 11: Budesonide EC_{50} values in agonism mode

	Ineris	INSERM	Tame-Water	Toxem
EC_{50} (±SD) in mg/mL	$4{,}1E^{-07} \pm 3{,}6E^{-08}$	$5{,}0E^{-07} \pm 2{,}6E^{-08}$	$1{,}2E^{-06} \pm 9{,}1E^{-08}$	$4{,}6E^{-07} \pm 4{,}8E^{-08}$
	$3{,}2E^{-07} \pm 3{,}0E^{-08}$	$5{,}7E^{-07} \pm 1{,}7E^{-08}$	$1{,}2E^{-06} \pm 1{,}7E^{-07}$	$3{,}0E^{-07} \pm 2{,}2E^{-08}$
	$2{,}9E^{-07} \pm 2{,}9E^{-08}$	$5{,}6E^{-07} \pm 2{,}8E^{-08}$	$1{,}2E^{-06} \pm 1{,}3E^{-07}$	$4{,}4E^{-07} \pm 3{,}0E^{-08}$
		$6{,}2E^{-07} \pm 1{,}6E^{-08}$		
Mean (±SD)	$3{,}4E^{-07} \pm 6{,}2E^{-08}$	$5{,}6E^{-07} \pm 4{,}9E^{-08}$	$1{,}2E^{-06}$	$4{,}0E^{-07} \pm 8{,}7E^{-08}$

The EC_{50} values show a very good intra-laboratory reproducibility. Tame-Water has an EC_{50} slightly different from the other labs.

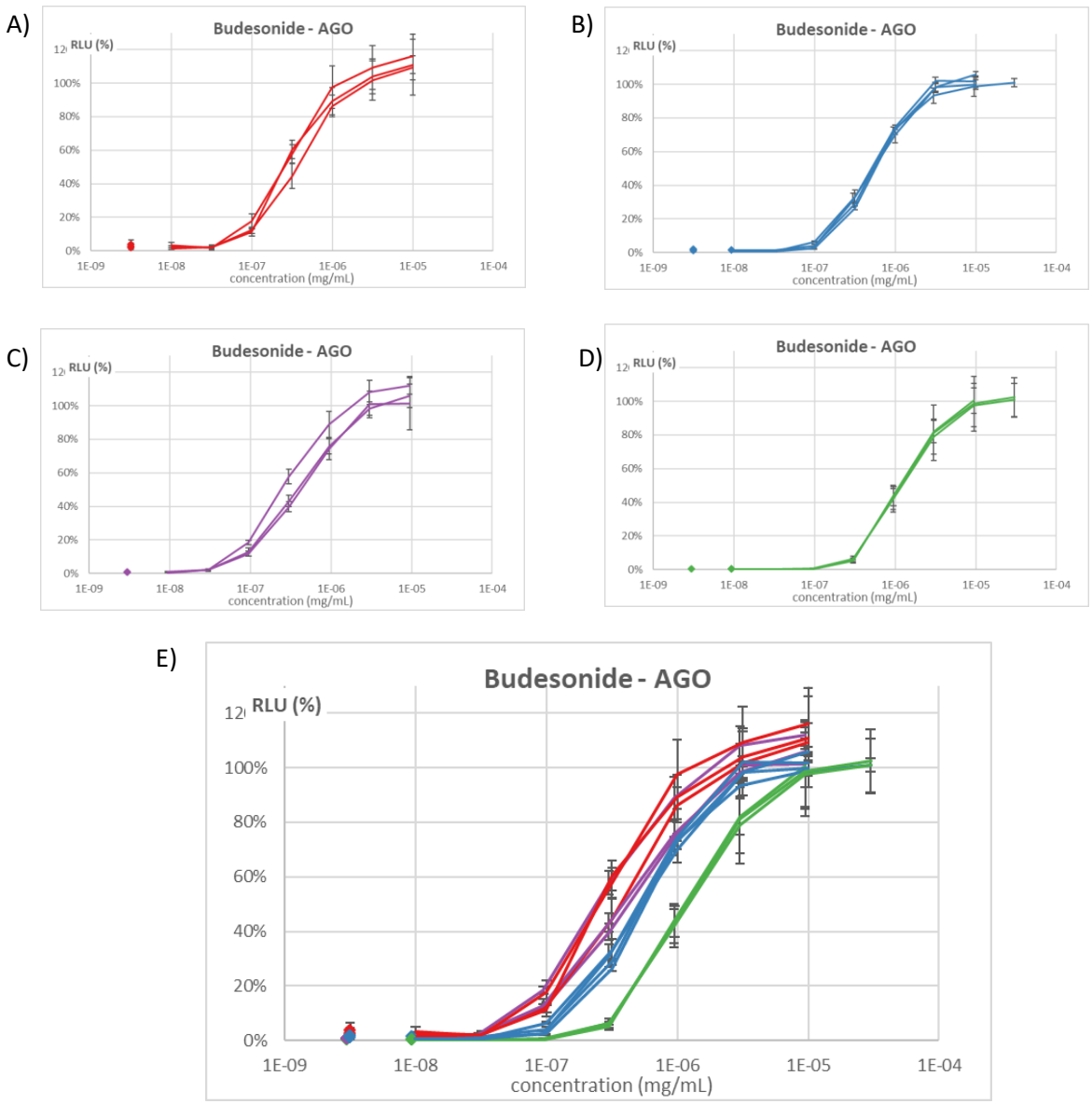


Figure 33: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Budesonide in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Budesonide is an agonist in this method, increasing luciferase activity above 10% of maximum effect of positive control the basal activity (which is maximum 4%) for at least two consecutive concentrations or the highest concentration.

Fluticasone propionate

In agonism mode, all labs found that Fluticasone propionate had a strong activity, which led them to test at lower concentrations in order to obtain a full dose-response.
The concentration-response curves and EC_{50} obtained are presented below.

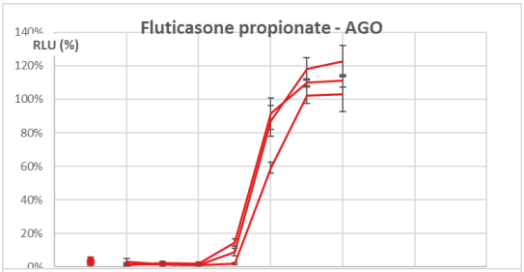
INSERM had one invalid run due to too high basal signal of the cells.

Table 12: Fluticasone propionate EC_{50} values in agonism mode.

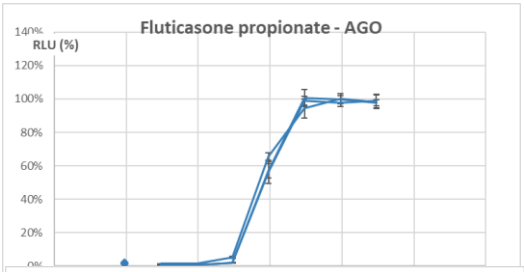
	Ineris	INSERM	Tame-Water	Toxem
EC_{50} (±SD) in mg/mL	$6{,}1E^{-08} \pm 2{,}0E^{-09}$	$9{,}0E^{-08} \pm 3{,}5E^{-09}$	$1{,}1E^{-07} \pm 7{,}8E^{-09}$	$7{,}0E^{-08} \pm 2{,}6E^{-09}$
	$7{,}5E^{-08} \pm 3{,}6E^{-09}$	$9{,}0E^{-08} \pm 1{,}0E^{-08}$	$1{,}5E^{-07} \pm 1{,}1E^{-08}$	$4{,}3E^{-08}$ $2{,}1E^{-09}$
	$9{,}5E^{-08} \pm 3{,}8E^{-09}$	$7{,}7E^{-08} \pm 2{,}5E^{-09}$	$1{,}6E^{-07} \pm 4{,}3E^{-09}$	$7{,}3E^{-08} \pm 2{,}9E^{-09}$
Mean (±SD)	$7{,}7E^{-08} \pm 1{,}7E^{-08}$	$8{,}6E^{-08} \pm 7{,}5E^{-09}$	$1{,}4E^{-07} \pm 2{,}6E^{-08}$	$6{,}2E^{-08} \pm 1{,}7E^{-08}$

The EC_{50} values show a very good intra-laboratory reproducibility. Tame-Water has an EC_{50} slightly different from the other labs.

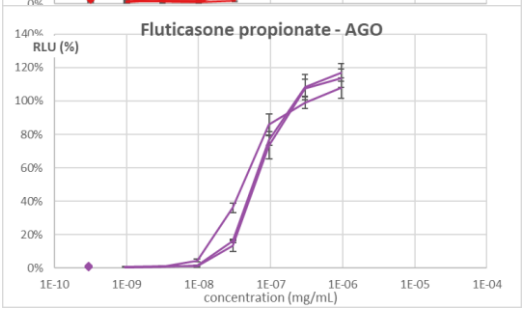
A)



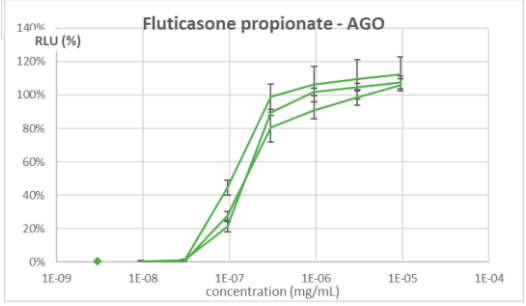
B)



C)



D)



E)

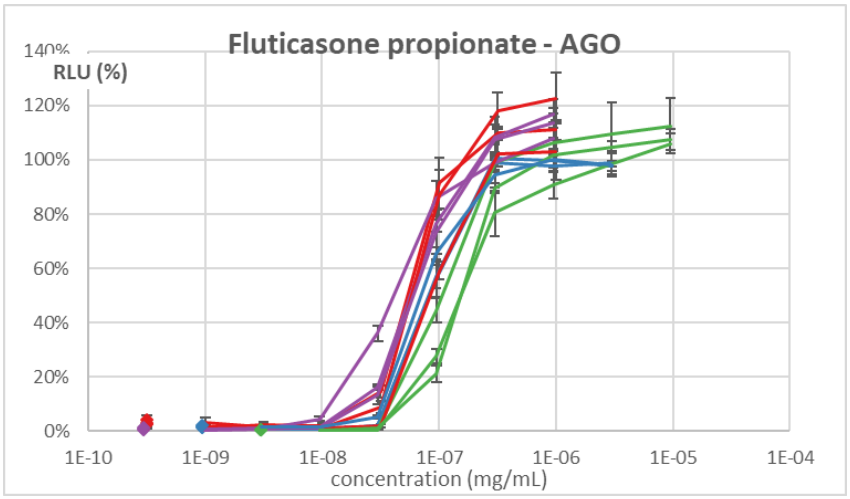


Figure 34: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Fluticasone propionate in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Fluticasone propionate is an agonist in this method, increasing luciferase activity above 10% of maximum effect of positive control the basal activity (which is maximum 4%) for at least two consecutive concentrations or the highest concentration.

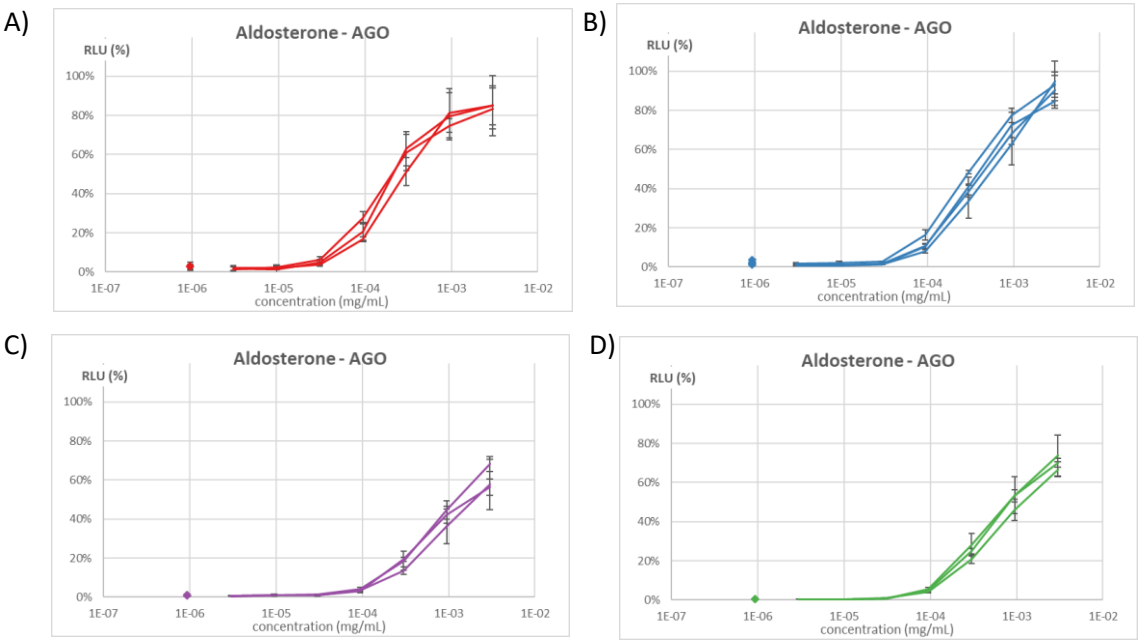
Aldosterone

In agonism mode, all labs found that Aldosterone was active, using the "by default" concentrations suggested in the SOP.
The concentration-response curves and EC_{50} obtained are presented below.

Table 13: Aldosterone EC_{50} values in agonism mode.

	Ineris	INSERM	Tame-Water	Toxem
EC_{50} (±SD) in mg/mL	$2{,}5E^{-04} \pm 2{,}5E^{-05}$	$7{,}8E^{-04} \pm 2{,}3E^{-04}$	$5{,}0E^{-04} \pm 2{,}4E^{-05}$	$8{,}8E^{-04} \pm 2{,}9E^{-04}$
	$1{,}7E^{-04} \pm 1{,}9E^{-05}$	$4{,}6E^{-04} \pm 5{,}0E^{-05}$	$5{,}3E^{-04} \pm 1{,}1E^{-04}$	$7{,}8E^{-04} \pm 9{,}6E^{-05}$
	$1{,}5E^{-04} \pm 1{,}7E^{-05}$	$3{,}1E^{-04} \pm 2{,}0E^{-05}$	$6{,}6E^{-04} \pm 6{,}8E^{-05}$	$5{,}2E^{-04} \pm 4{,}4E^{-05}$
		$3{,}3E^{-04} \pm 2{,}0E^{-05}$		
Mean (±SD)	$1{,}9E^{-04} \pm 5{,}3E^{-05}$	$4{,}7E^{-04} \pm 2{,}2E^{-04}$	$5{,}6E^{-04} \pm 8{,}5E^{-05}$	$7{,}4^{-04} \pm 1{,}9E^{-04}$

The EC_{50} values show a very good intra-laboratory reproducibility, with some inter-laboratory variability, which may be due to the fact that the dose-response curves are not complete, creating some uncertainty on the fitting and thus on the EC_{50} determination.



E)

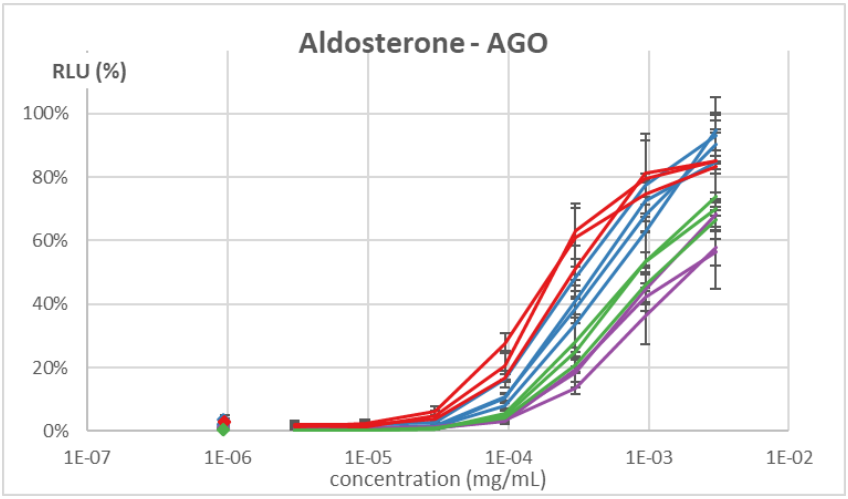


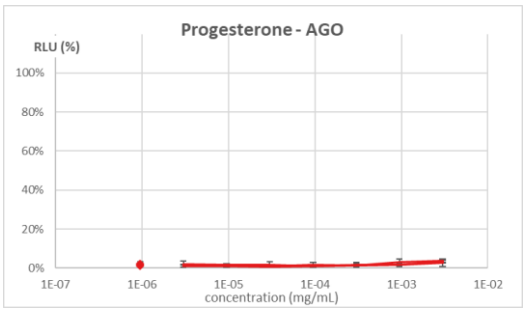
Figure 35: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Aldosterone in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Aldosterone is an agonist in this method, increasing luciferase activity above 10% of maximum effect of positive control the basal activity (which is maximum 4%) for at least two consecutive concentrations or the highest concentration.

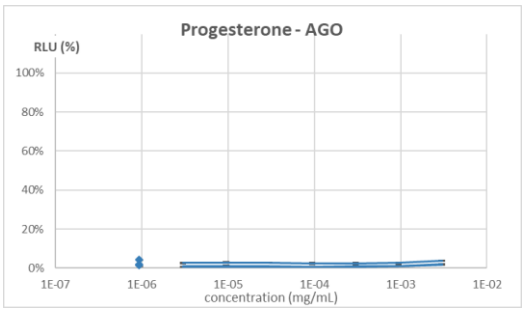
Progesterone

All labs tested Progesterone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

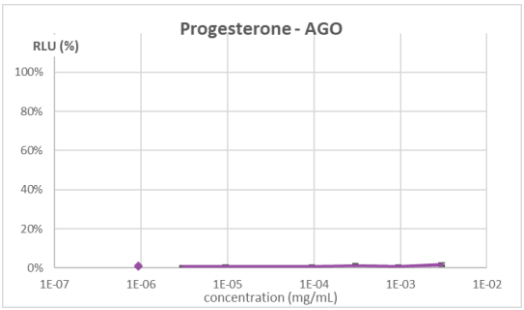
A)



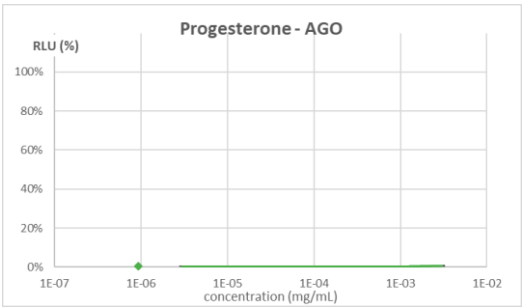
B)



C)



D)



E)

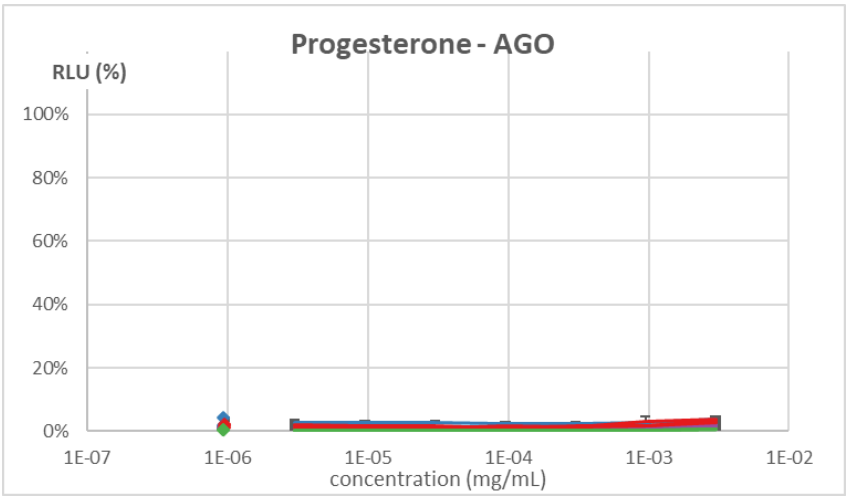


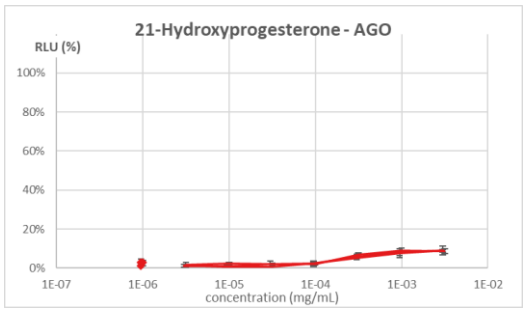
Figure 36: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Progesterone in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Progesterone is not an agonist in this method.

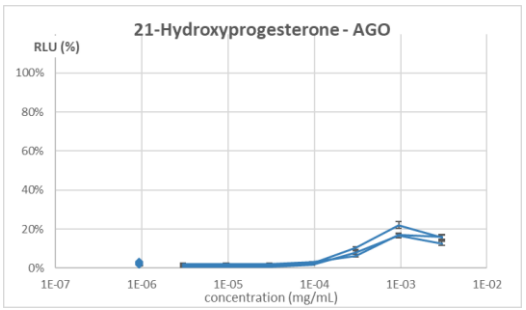
21-Hydroxyprogesterone

All labs tested 21-Hydroxyprogesterone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL and observed a slight increase of signal at the highest concentrations. The concentration-response curves obtained are presented below.

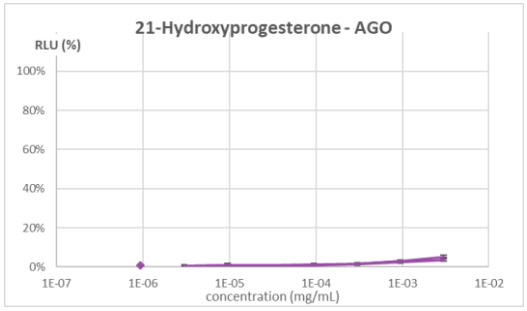
A)



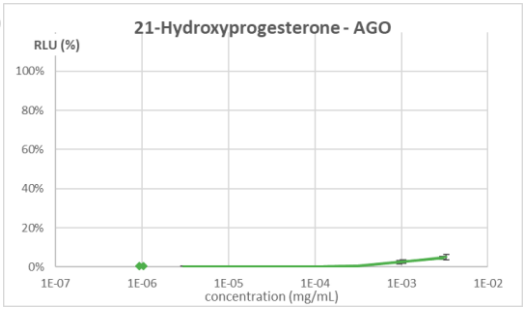
B)



C)



D)



E)

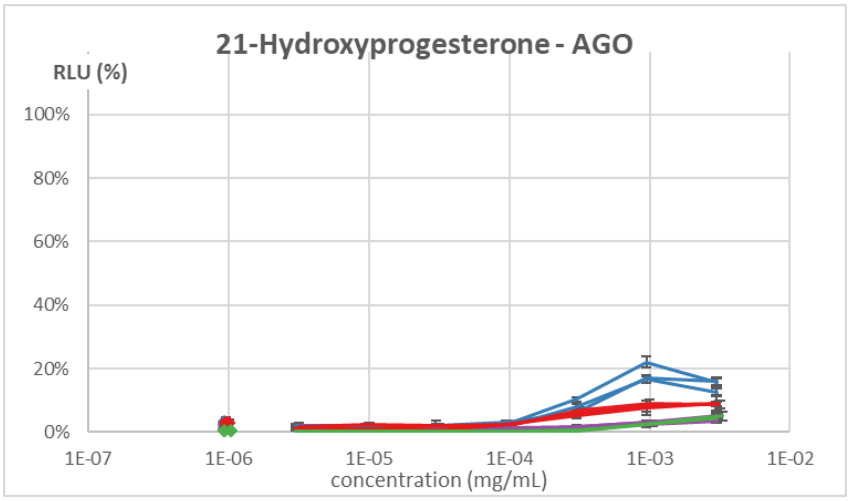


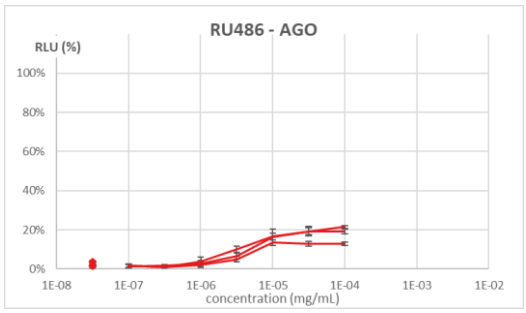
Figure 37: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to 21-Hydroxyprogesterone in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Despite the slight increase of activity, no curve could be modelled to derive an EC_{50}. Only INSERM concluded that 21-Hydroxyprogesterone is an agonist at the tested concentrations, the other labs did not, as the increase in activity did not reach 10% of maximum effect of positive control the basal activity (which is maximum 4%) for at least two consecutive concentrations or the highest concentration.

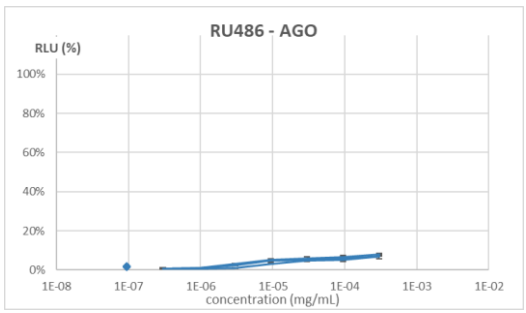
Mifepristone (RU-486)

In agonism mode, all labs found that Mifepristone had some activity, which led three of them to test at lower concentrations in order to obtain a better dose-response. The concentration-response curves obtained are presented below.

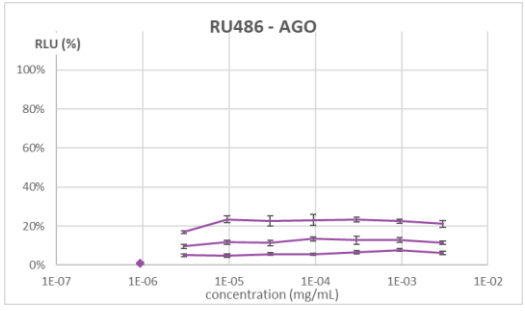
A)



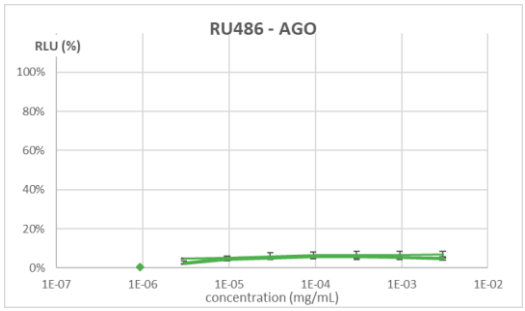
B)



C)



D)



E)

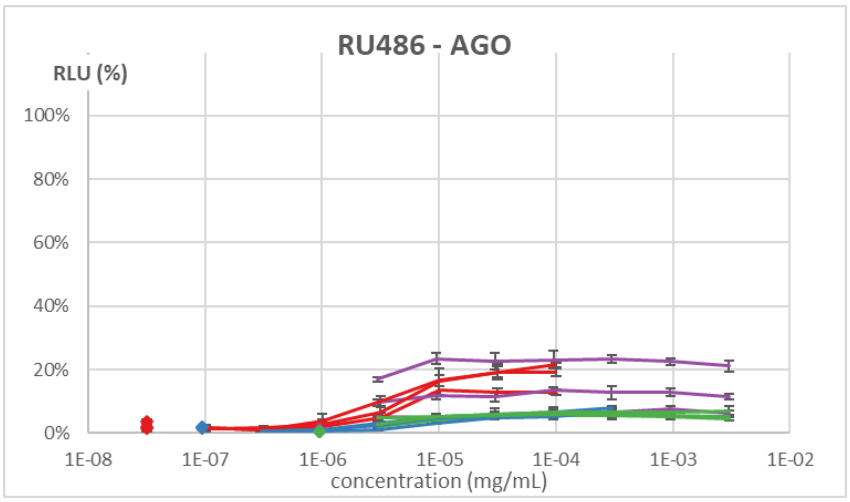


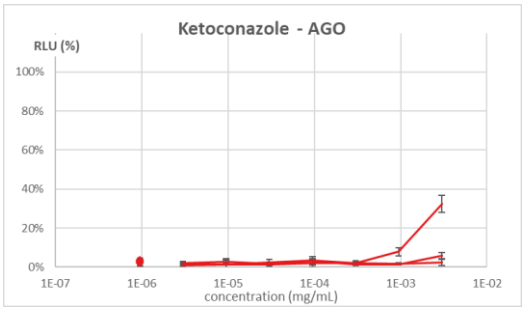
Figure 38: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Mifepristone (RU-486) in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

The conclusions of the labs on Mifepristone activity varies. Tame-Water, who tested using the concentrations by default found Mifepristone not active. According to Toxem, Ineris and INSERM Mifepristone shows an increased activity, but not enough to qualify as "agonist" according to the prediction model.
These data are consistent with the testing of Mifepristone in agonism mode during the transferability.

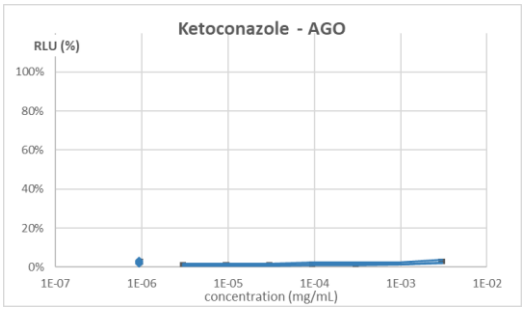
Ketoconazole

All labs tested Ketoconazole from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

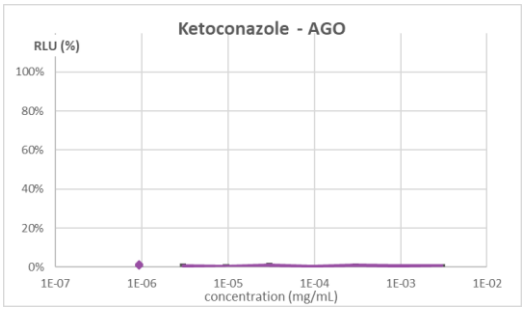
A)



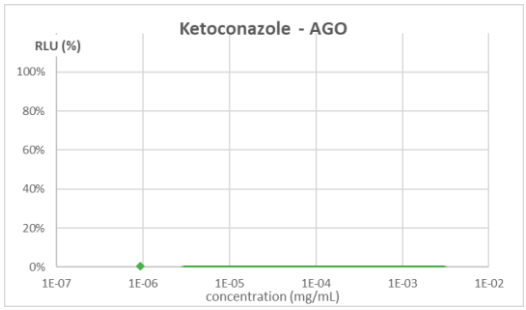
B)



C)



D)



E)

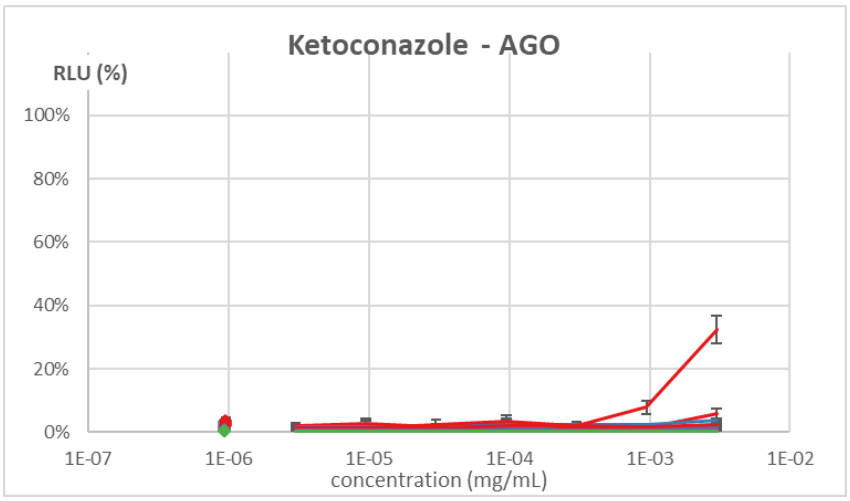


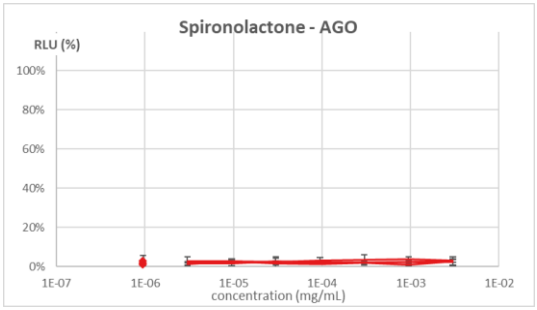
Figure 39: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Ketoconazole in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Ketoconazole is not an agonist in this method. The increase of activity observed for the last concentration of one run at Ineris was considered as not relevant, as not repeated in the other runs.

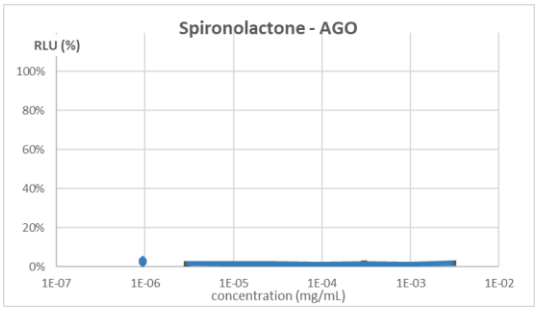
Spironolactone

All labs tested Spironolactone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

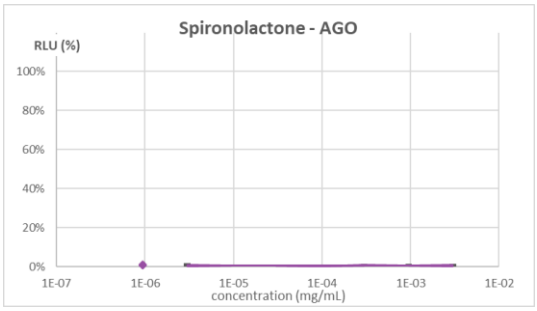
A)



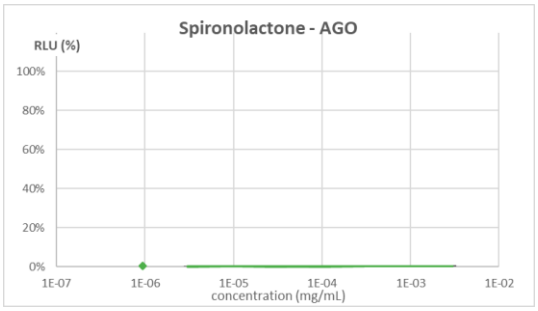
B)



C)



D)



E)

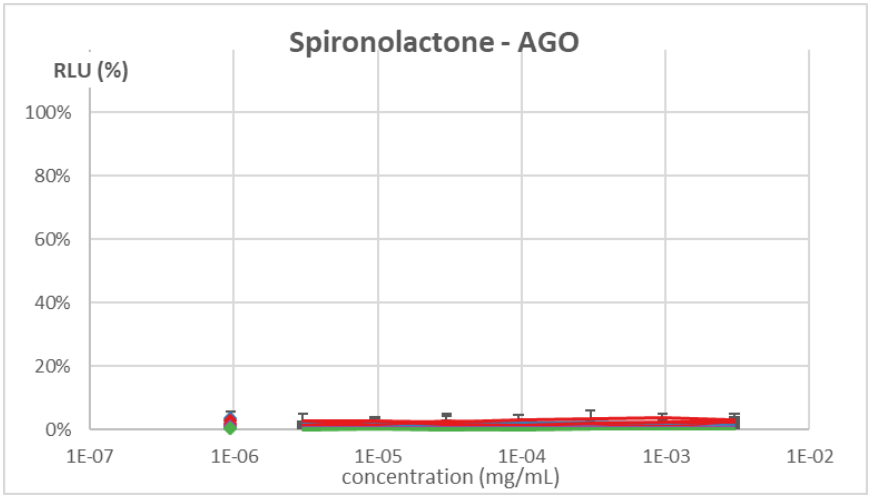


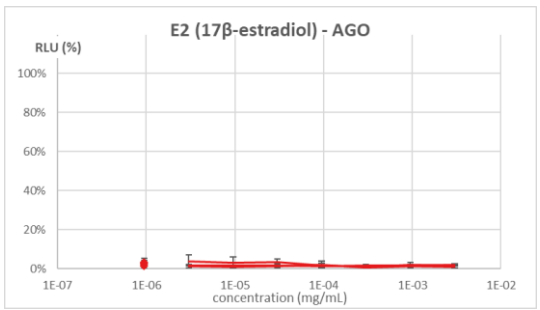
Figure 40: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Spironolactone in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Spironolactone is not an agonist in this method.

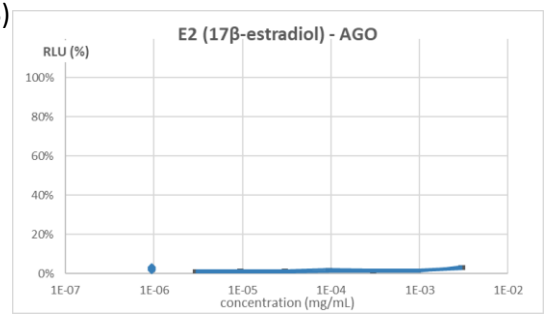
<u>17ß-estradiol</u>

All labs tested 17β-estradiol from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

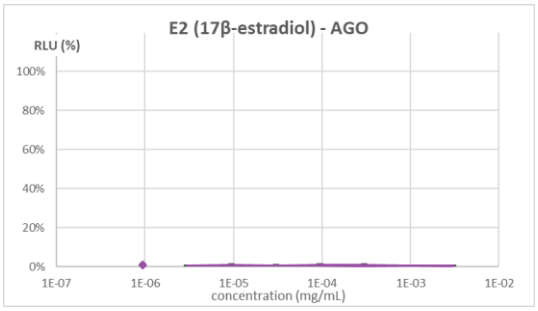
A)



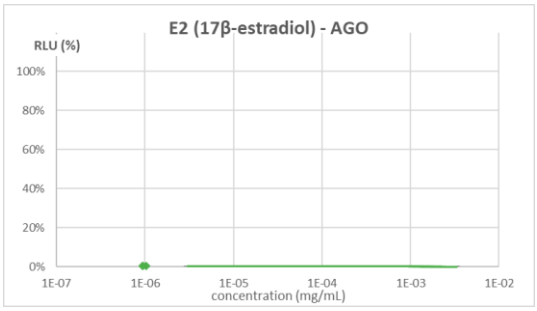
B)



C)



D)



E)

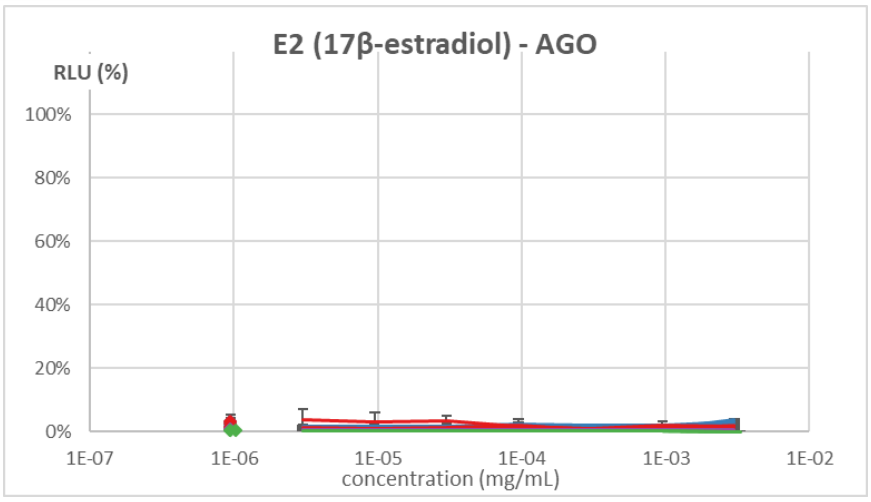


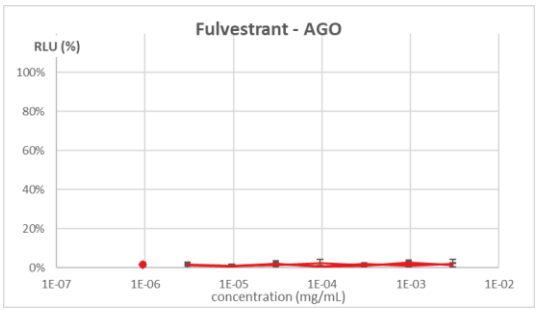
Figure 41: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to 17β-estradiol in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, 17β-estradiol is not an agonist in this method.

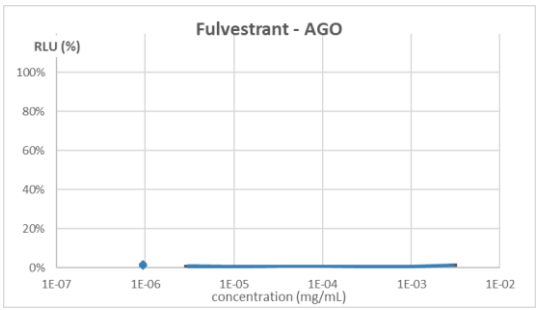
Fulvestrant

All labs tested Fulvestrant from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

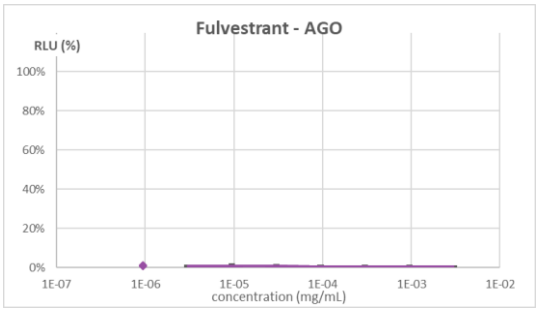
A)



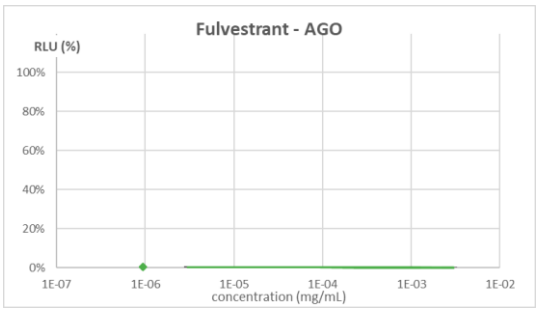
B)



C)



D)



E)

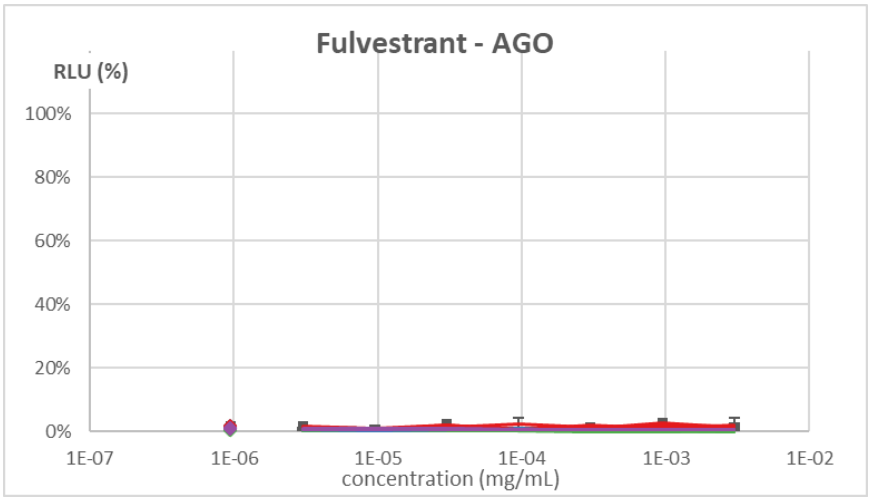


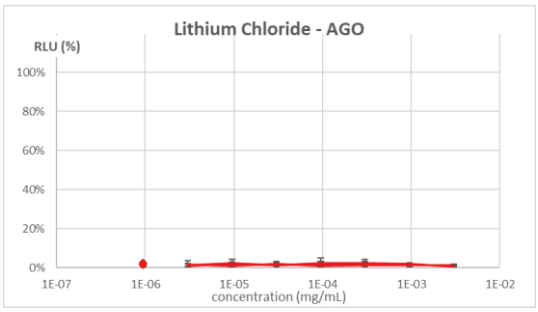
Figure 42: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Fulvestrant in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Fulvestrant is not an agonist in this method.

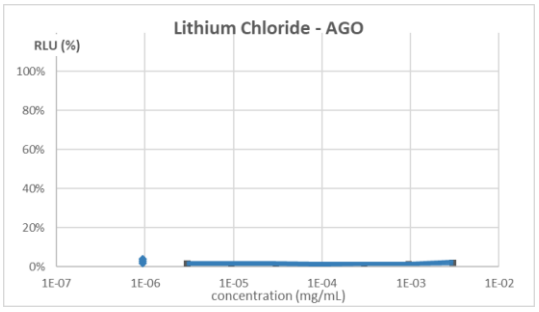
Lithium Chloride

All labs tested Lithium chloride from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

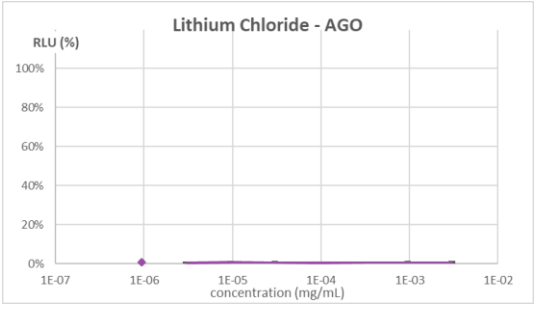
A)



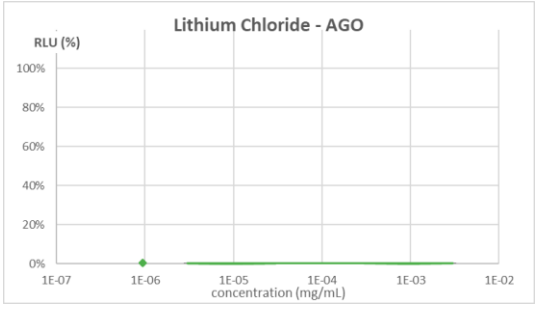
B)



C)



D)



E)

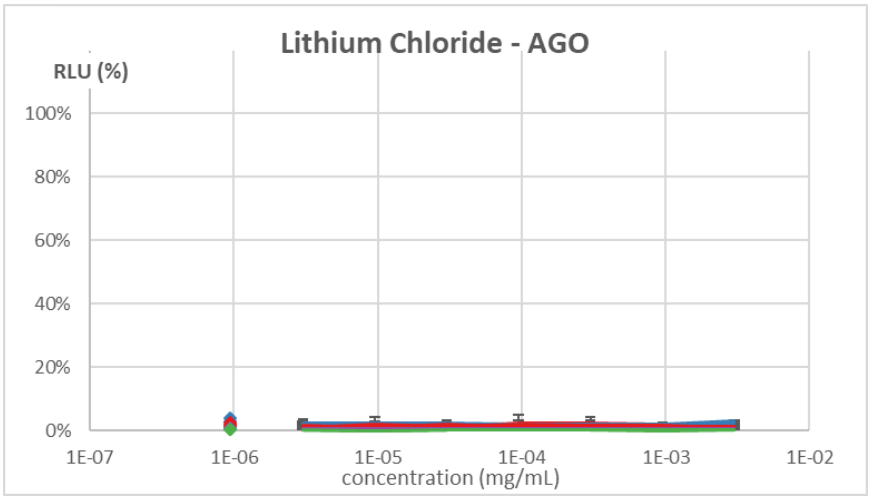


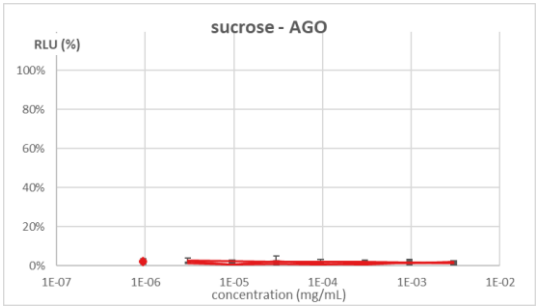
Figure 43: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Lithium chloride in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Lithium chloride is not an agonist in this method.

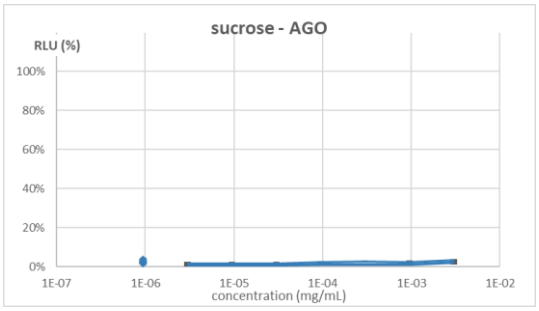
Sucrose

All labs tested Sucrose from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

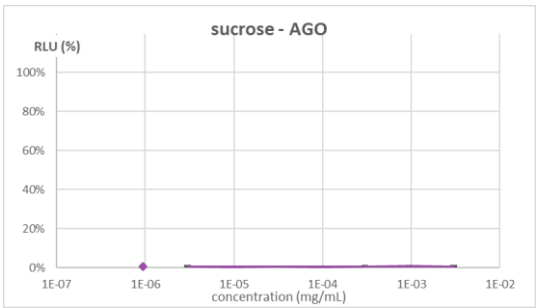
A)



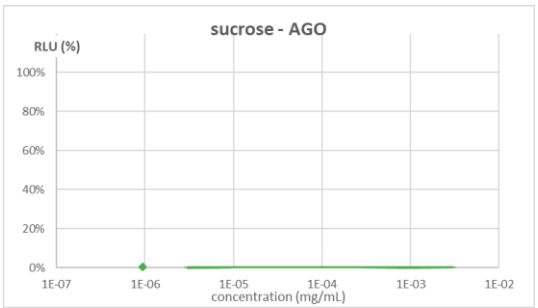
B)



C)



D)



E)

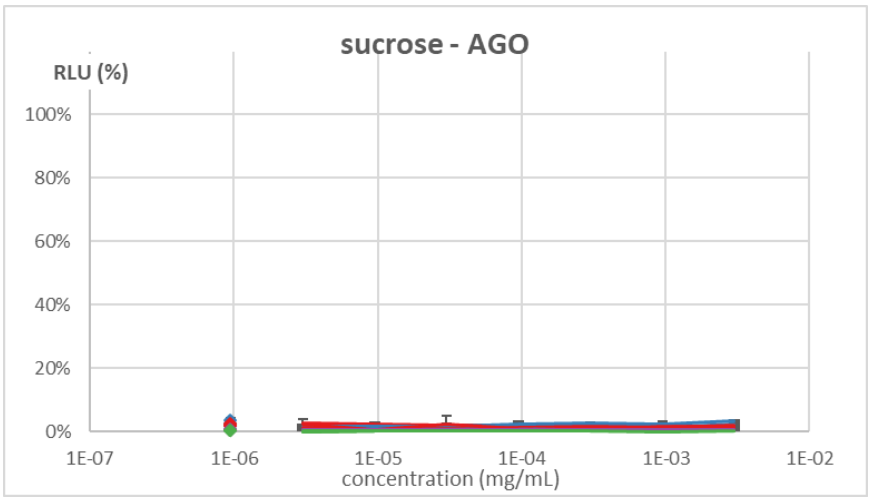


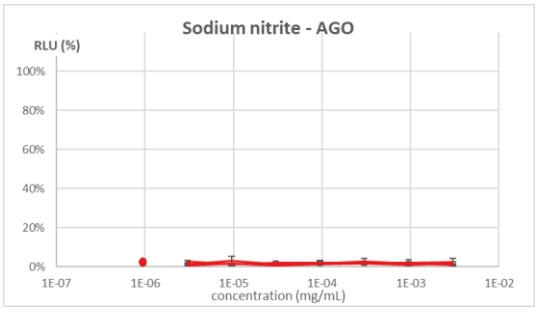
Figure 44: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sucrose in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Sucrose is not an agonist in this method.

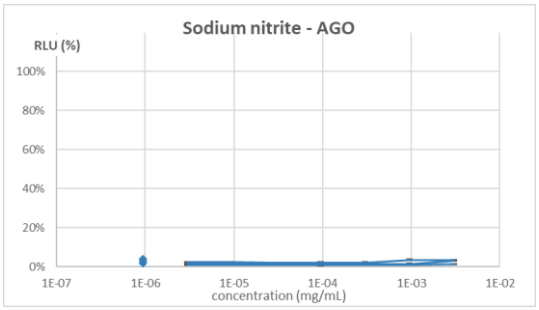
Sodium nitrite

All labs tested Sodium nitrite from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

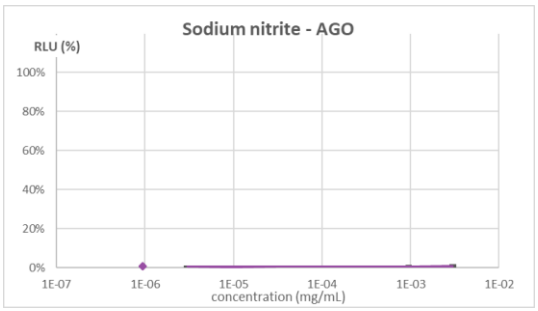
A)



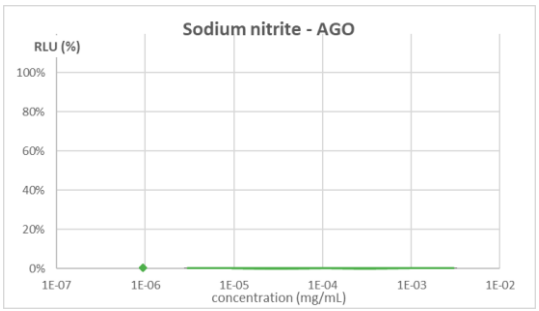
B)



C)



D)



E)

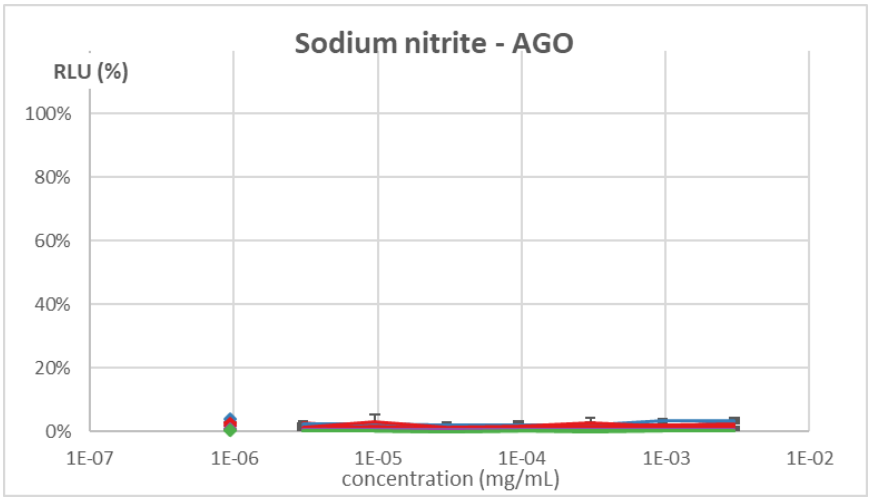


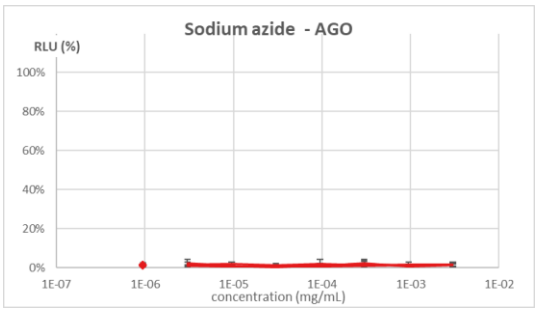
Figure 45: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sodium nitrite in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Sodium nitrite is not an agonist in this method.

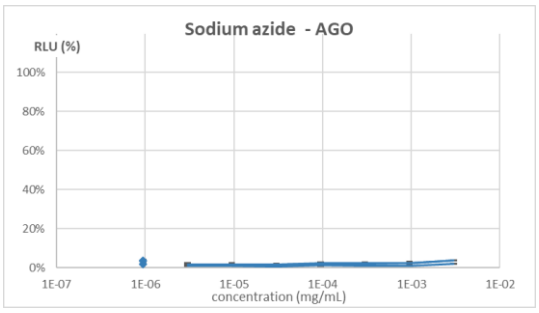
Sodium azide

All labs tested Sodium azide from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

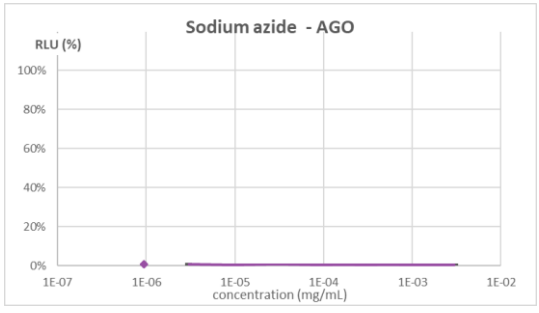
A)



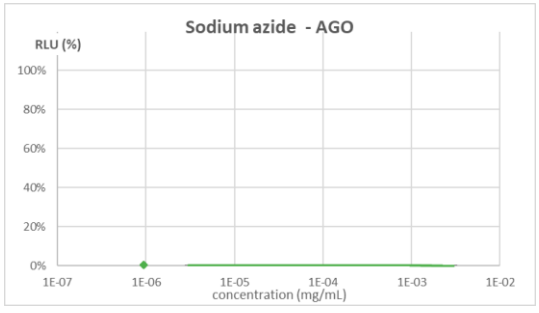
B)



C)



D)



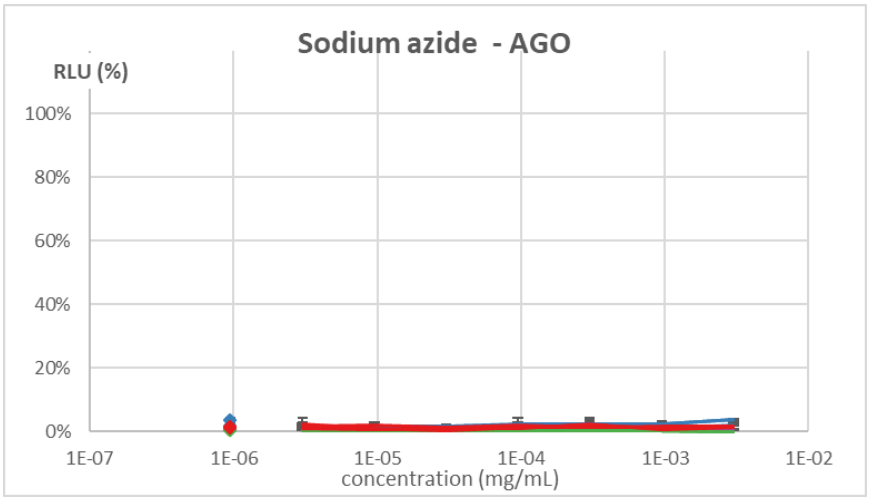


Figure 46: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sodium azide in agonist mode, in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Toxem excluded one of its runs which was valid (according to the acceptance criteria) but was different from the other ones.
All labs concluded that at the concentrations tested, Sodium azide is not an agonist in this method.

Antagonism and interference testing

The labs tested the test items at concentration from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL, except for those listed in the table below.

Table 14: Concentrations used in mg/mL.

	Ineris	INSERM	Tame-Water	Toxem
Budesonide	$1.0E^{-8} – 1.0E^{-5}$	$1.0E^{-7} – 1.0E^{-4}$		
Fluticasone propionate	$1.0E^{-9} – 1.0E^{-6}$	$3.0E^{-9} – 3.0E^{-6}$		
Mifepristone	$1.0E^{-7} – 1.0E^{-4}$	$3.0E^{-7} – 3.0E^{-4}$	$9.5E^{-7} – 9.5E^{-4}$	

In the antagonist mode, the concentration of Dexamethasone used by the labs to reach 80% of the maximum signal was $1,0E^{-8}$M for Ineris, $8,0E^{-9}$M for INSERM, $8,0E^{-9}$M for Tame-Water and $2,5E^{-8}$M for Toxem. All labs used Dexamethasone at $1,0E^{-6}$M for co-exposure in the 65interference mode.

Only test items demonstrating a decrease of luciferase signal were also tested for interference, using the same concentrations.

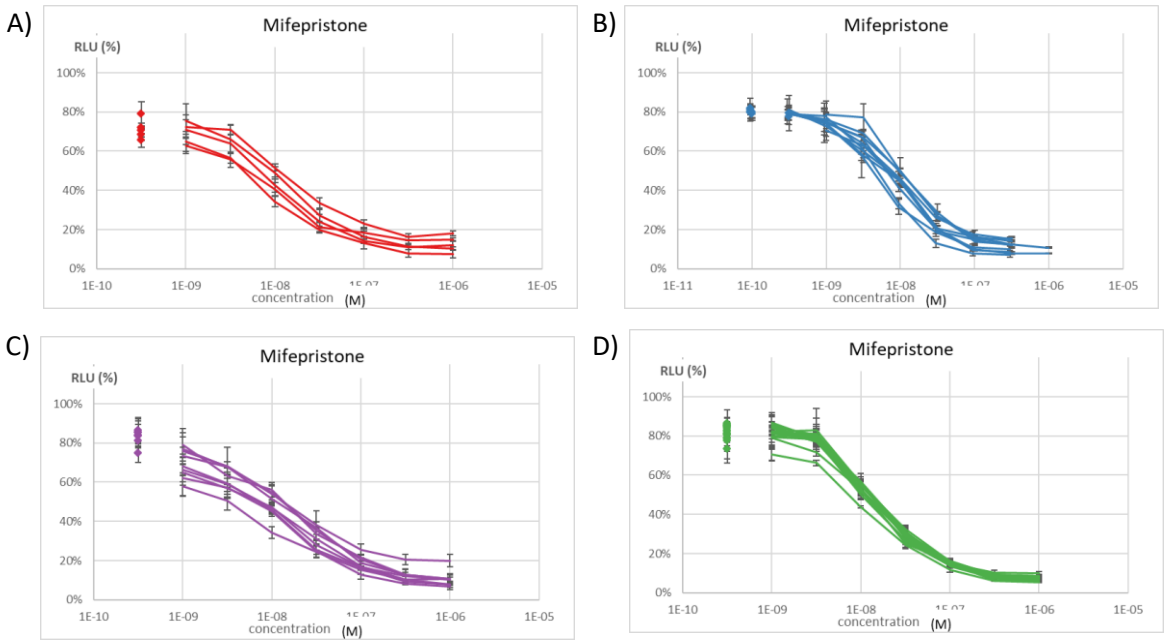
Mifepristone (Positive control)

Mifepristone was tested as positive control from $1.0E^{-9}$ to $1.0E^{-6}$ M, inducing IC_{50} between $3.16E^{-9}$ and $3.16E^{-8}$ M.
One run of Toxem was invalid due to an IC_{50} outside of the acceptable range.
It should be noted that INSERM did two runs using the concentrations defined in the SOP, and then deviate from it, using concentrations between $3,0E^{-10}$ and $3,0E^{-7}$ M.

Table 15: Mifepristone IC_{50} values in antagonism mode.

	Ineris	INSERM	Tame-Water	Toxem
IC_{50} (± SD) in M	$1{,}1E^{-08} \pm 1{,}0E^{-09}$	$1{,}2E^{-08} \pm 4{,}8E^{-10}$	$1{,}3E^{-08} \pm 9{,}9E^{-10}$	$1{,}4E^{-08} \pm 3{,}1E^{-09}$
	$1{,}2E^{-08} \pm 1{,}9E^{-09}$	$1{,}2E^{-08} \pm 1{,}5E^{-09}$	$1{,}5E^{-08} \pm 1{,}4E^{-09}$	$1{,}3E^{-08} \pm 3{,}4E^{-09}$
	$8{,}1E^{-09} \pm 1{,}3E^{-09}$	$6{,}4E^{-09} \pm 5{,}8E^{-10}$	$1{,}6E^{-08} \pm 8{,}7E^{-10}$	$1{,}6E^{-08} \pm 2{,}2E^{-09}$
	$1{,}0E^{-08} \pm 1{,}0E^{-09}$	$9{,}1E^{-09} \pm 6{,}2E^{-10}$	$1{,}5E^{-08} \pm 1{,}0E^{-09}$	$1{,}7E^{-08} \pm 1{,}8E^{-09}$
	$1{,}5E^{-08} \pm 1{,}9E^{-09}$	$5{,}0E^{-09} \pm 1{,}1E^{-09}$	$1{,}5E^{-08} \pm 1{,}1E^{-09}$	$1{,}4E^{-08} \pm 3{,}0E^{-09}$
		$1{,}3E^{-08} \pm 1{,}2E^{-09}$	$1{,}4E^{-08} \pm 1{,}6E^{-09}$	$1{,}9E^{-08} \pm 1{,}6E^{-09}$
		$1{,}2E^{-08} \pm 9{,}0E^{-10}$	$1{,}3E^{-08} \pm 1{,}3E^{-09}$	$1{,}8E^{-08} \pm 1{,}7E^{-09}$
		$7{,}0E^{-09} \pm 9{,}8E^{-10}$	$1{,}5E^{-08} \pm 1{,}2E^{-09}$	$2{,}0E^{-08} \pm 3{,}1E^{-09}$
		$7{,}5E^{-09} \pm 1{,}1E^{-09}$	$1{,}3E^{-08} \pm 1{,}1E^{-09}$	$8{,}9E^{-09} \pm 1{,}9E^{-09}$
		$9{,}0E^{-09} \pm 8{,}0E^{-10}$	$1{,}5E^{-08} \pm 7{,}5E^{-10}$	
			$1{,}2E^{-08} \pm 7{,}3E^{-10}$	
			$1{,}5E^{-08} \pm 7{,}8E^{-10}$	
			$1{,}2E^{-08} \pm 7{,}7E^{-10}$	
			$1{,}3E^{-08} \pm 5{,}3E^{-10}$	
			$1{,}3E^{-08} \pm 8{,}2E^{-10}$	
			$1{,}6E^{-08} \pm 7{,}3E^{-10}$	
Mean (± SD)	$1{,}1E^{-08} \pm 2{,}5E^{-09}$	$9{,}3E^{-09} \pm 2{,}8E^{-09}$	$1{,}4E^{-08} \pm 1{,}3E^{-09}$	$1{,}5E^{-08} \pm 3{,}5E^{-09}$

All labs respected the acceptance criterion regarding Mifepristone IC_{50} and demonstrated a good intra- and inter-laboratory reproducibility.



E)

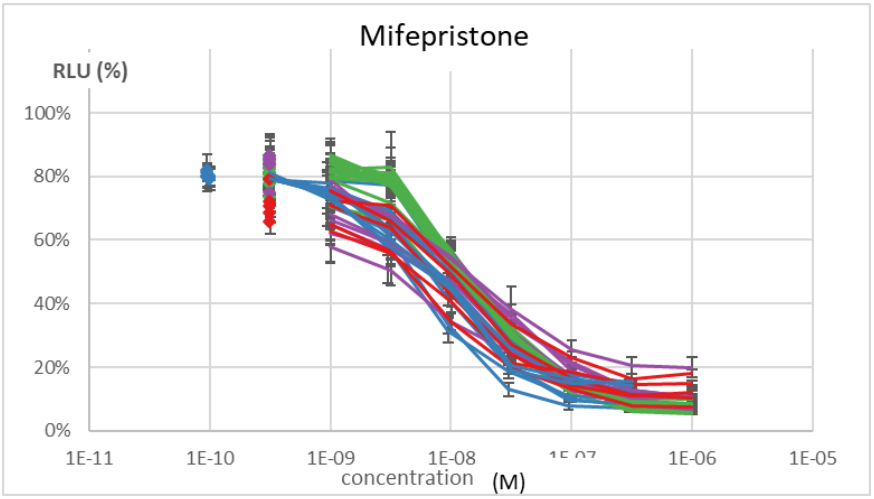


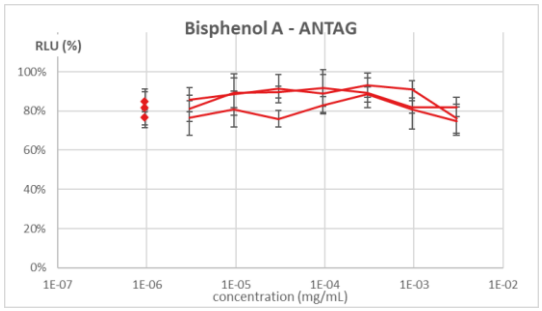
Figure 47: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Mifepristone in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs demonstrated that Mifepristone is an antagonist in this method.

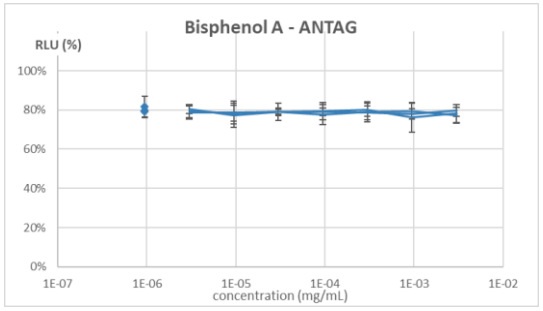
Bisphenol A

All labs tested Bisphenol A from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

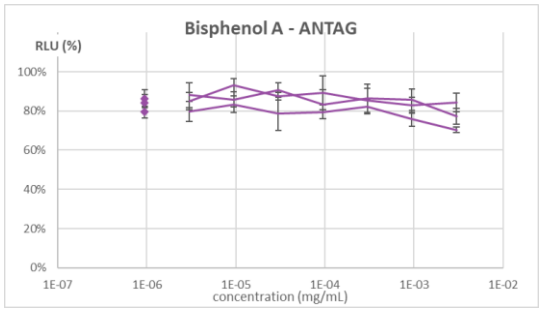
A)



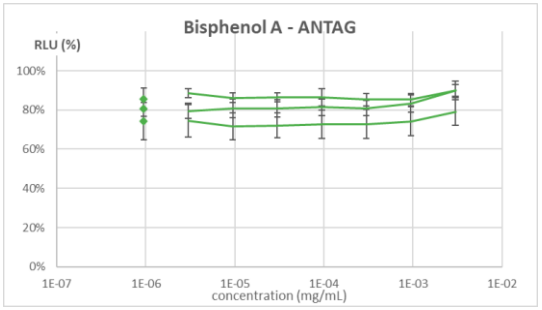
B)



C)



D)



E)

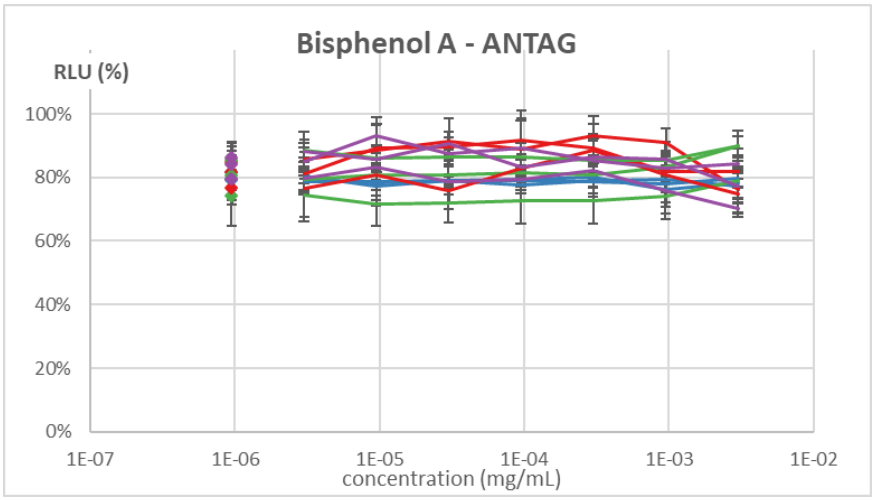


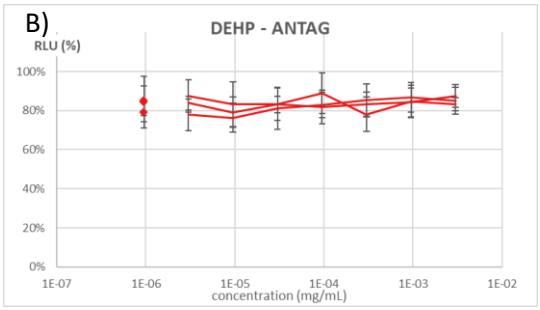
Figure 48: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to BPA in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

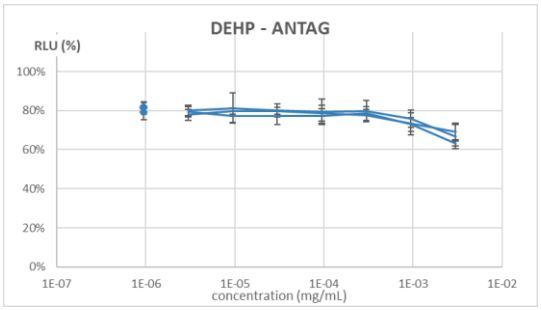
Toxem had one run invalid due to the criterion of the 80% DEX activity not met.
All labs concluded that at the concentrations tested, Bisphenol A is not an antagonist in this method.

Diethylhexyl phthalate (DEHP)

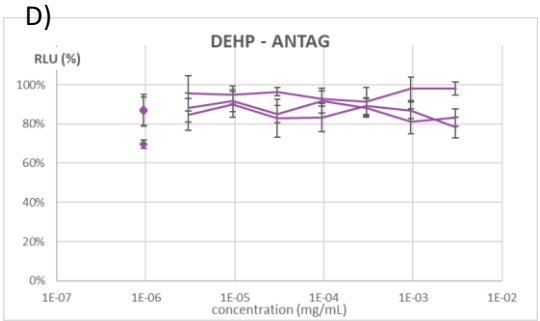
All labs tested DEHP from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

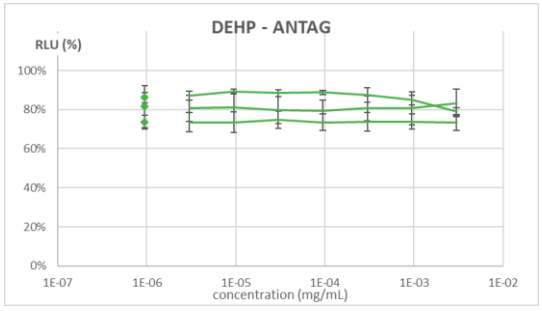
B) B)





C) D)





E)

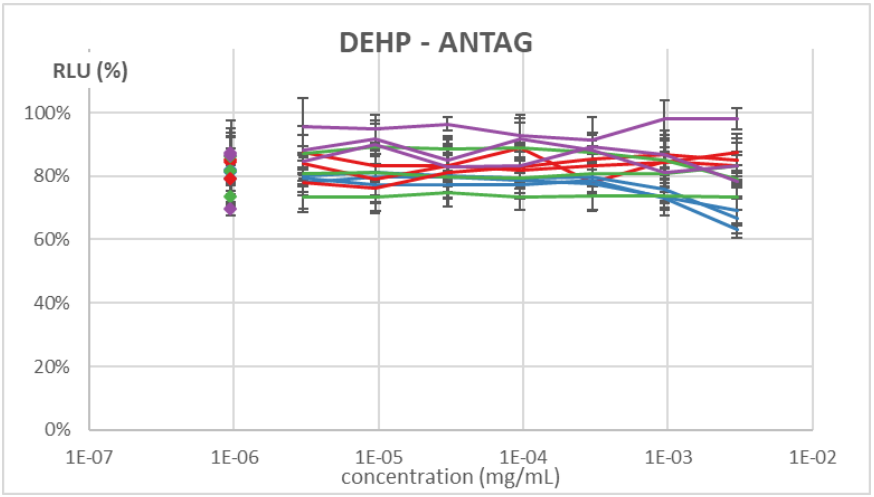


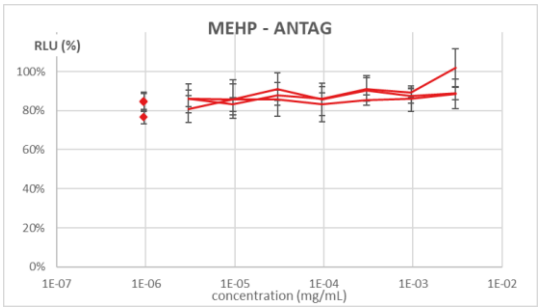
Figure 49: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to DEHP in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, DEHP is not an antagonist in this method.

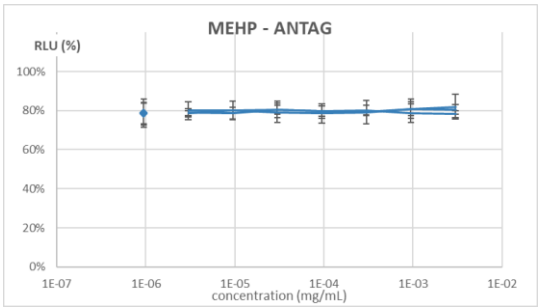
Phthalic acid mono-2-ethylhexyl ester (MEHP)

All labs tested MEHP from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

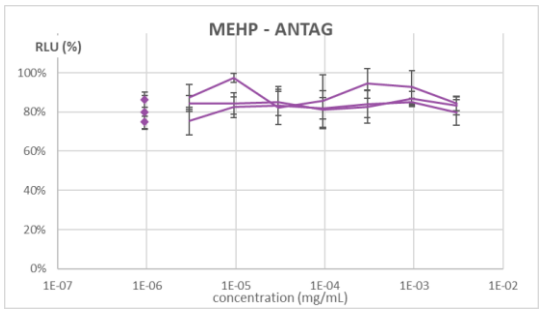
A)



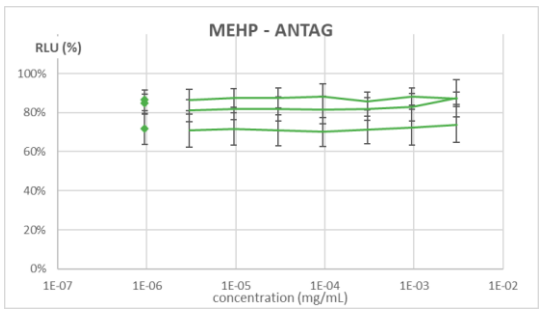
B)



C)



D)



E)

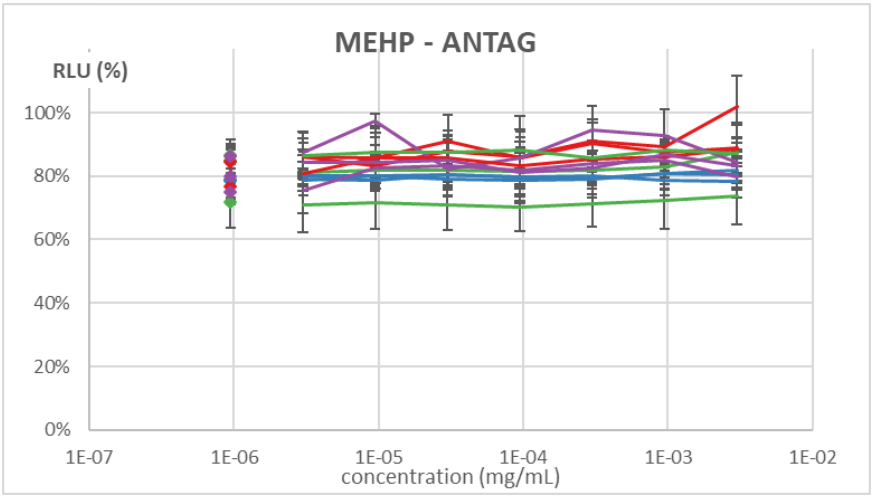


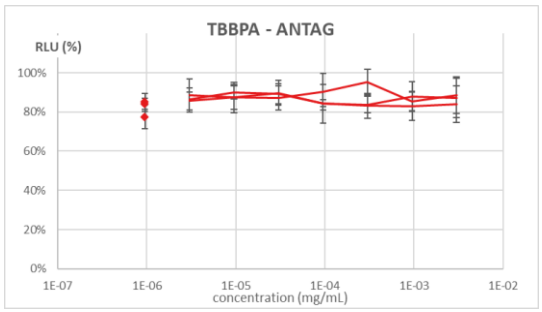
Figure 50: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to MEHP in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, MEHP is not an antagonist in this method.

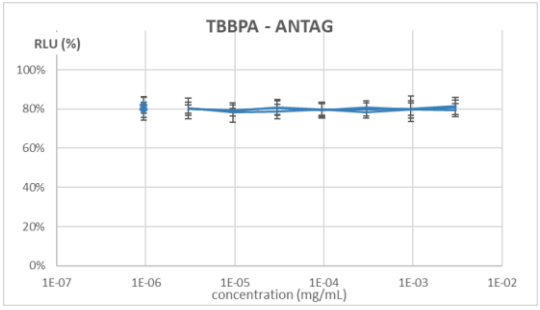
Tetrabromobisphenol A

All labs tested TBBPA from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.

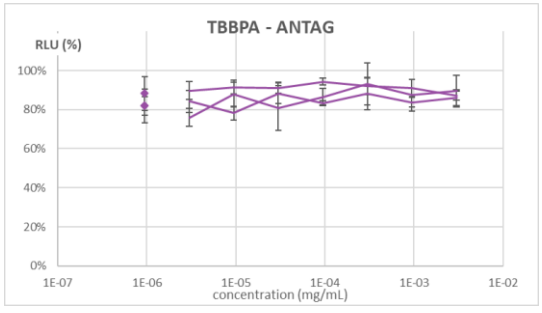
A)



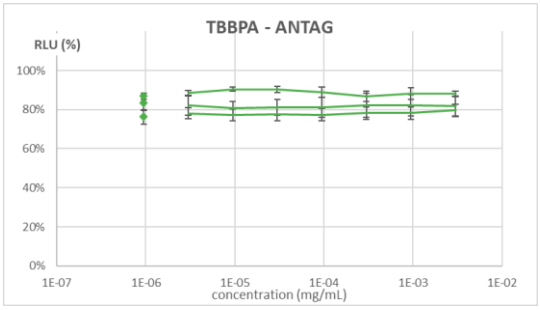
B)



C)



D)



E)

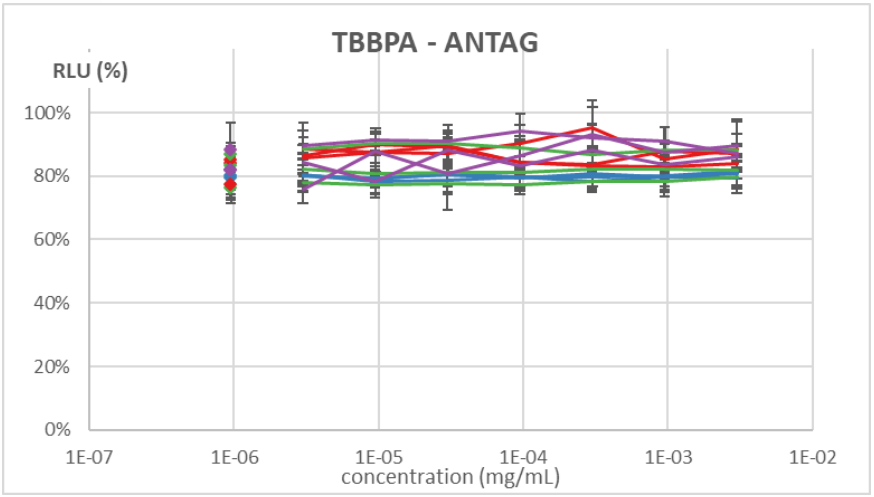


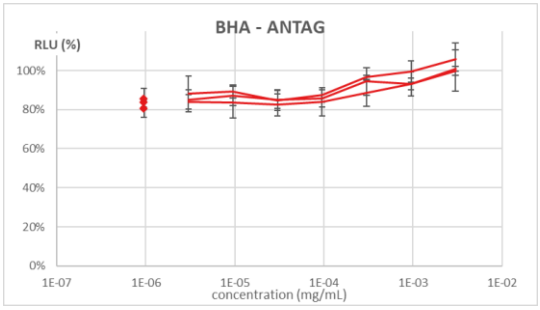
Figure 51: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to TBBPA in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, TBBPA is not an antagonist in this method.

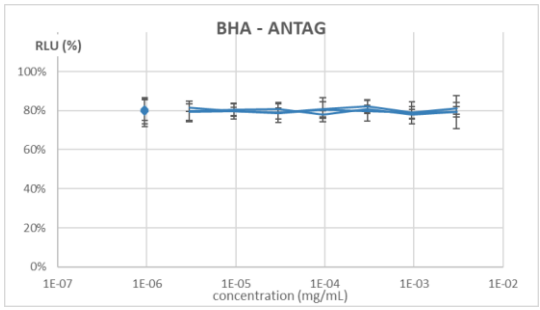
<u>Butylated hydroxyanisole (BHA)</u>

All labs tested BHA from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.

A)



B)



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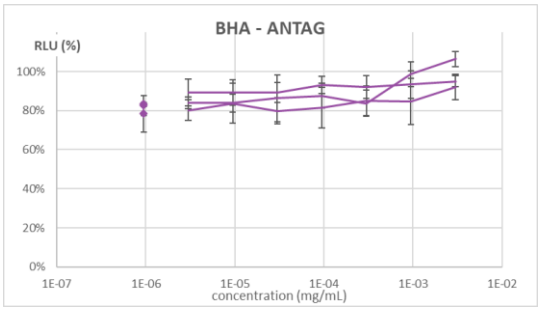
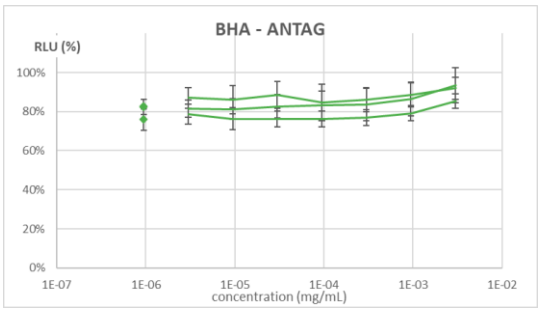


D)



E)

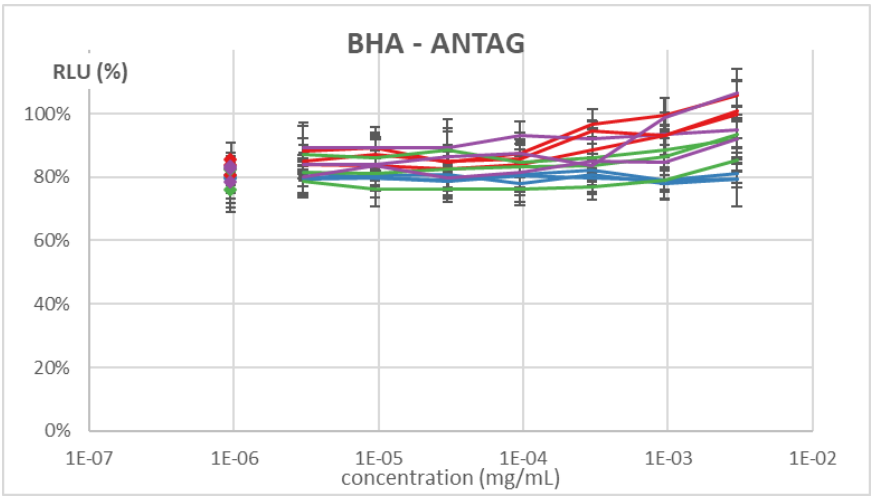


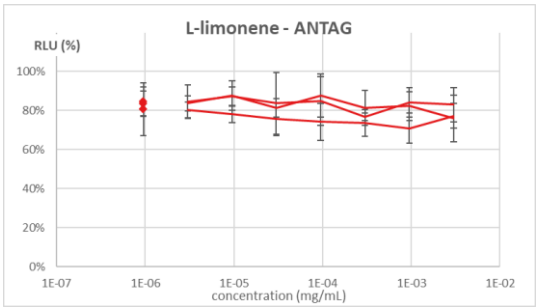
Figure 52: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to BHA in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, BHA is not an antagonist in this method.

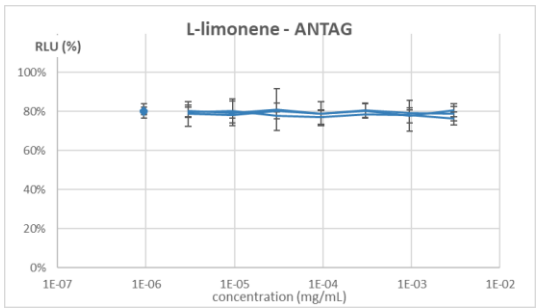
<u>L-Limonene</u>

All labs tested L-Limonene from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.

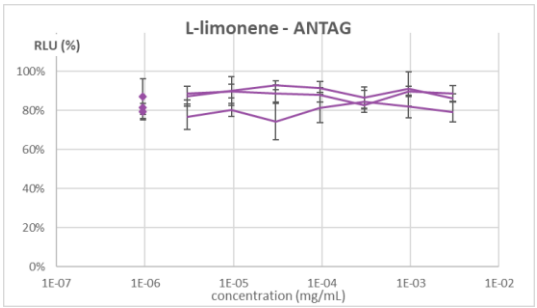
A)



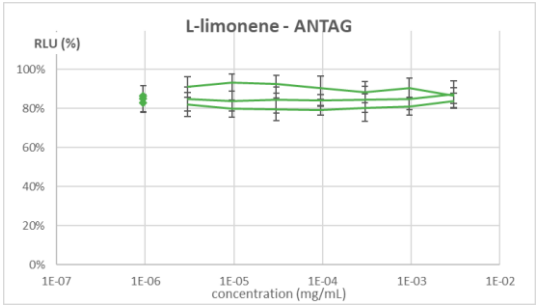
B)



C)



D)



E)

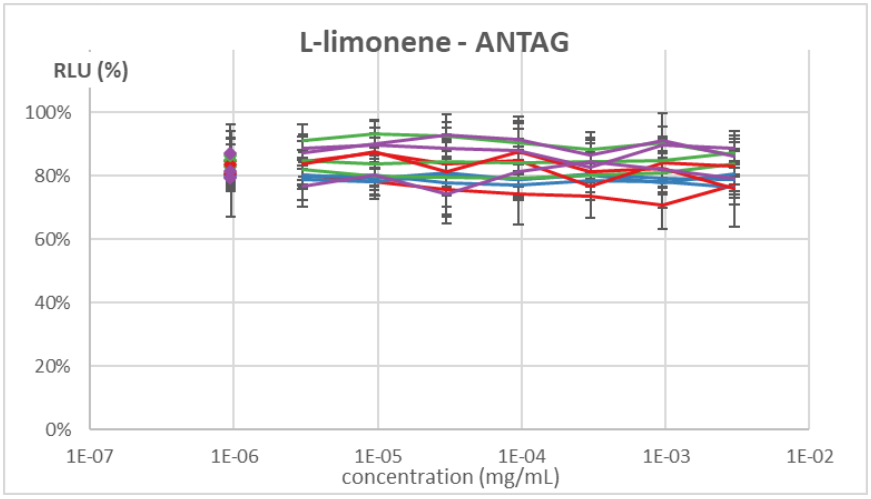


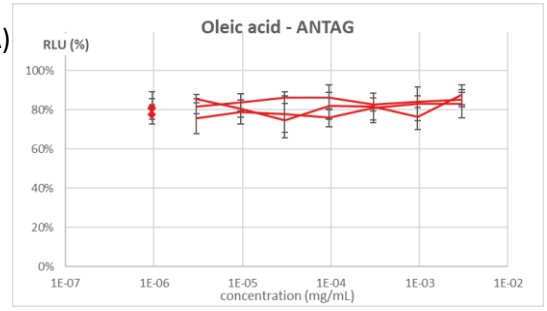
Figure 53: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to L-Limonene in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, L-Limonene is not an antagonist in this method.

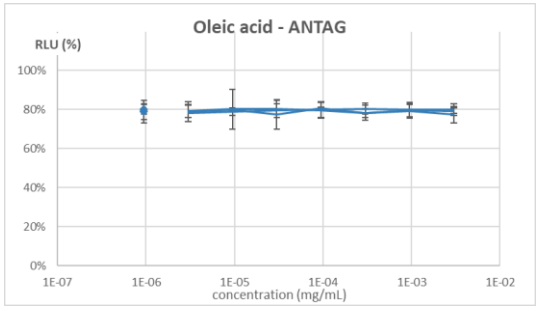
Oleic acid

All labs tested Oleic acid from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.

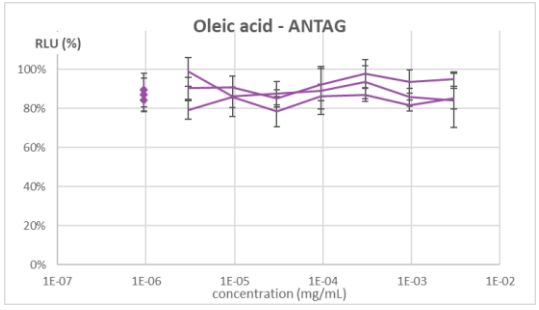
A)



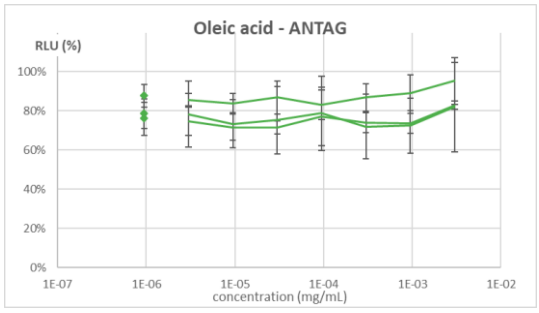
B)



C)



D)



E)

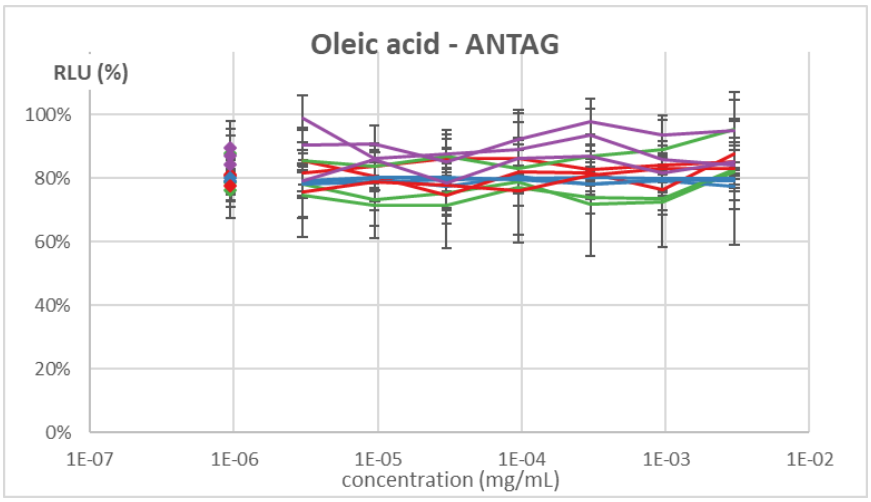


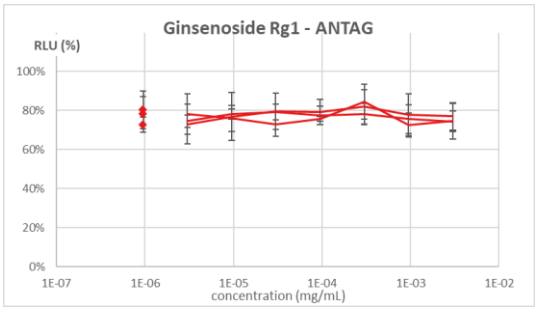
Figure 54: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Oleic acid in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Oleic acid is not an antagonist in this method.

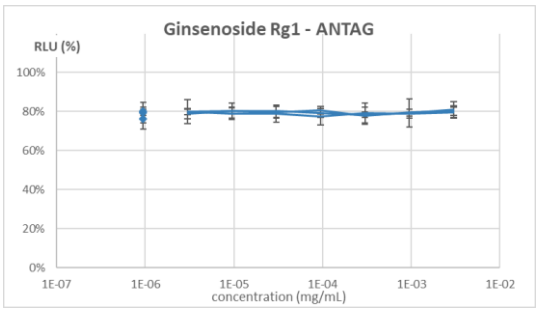
Ginsenoside Rg1

All labs tested Ginsenoside Rg1 from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.

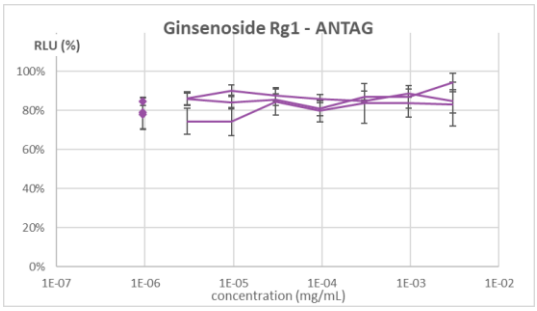
A)



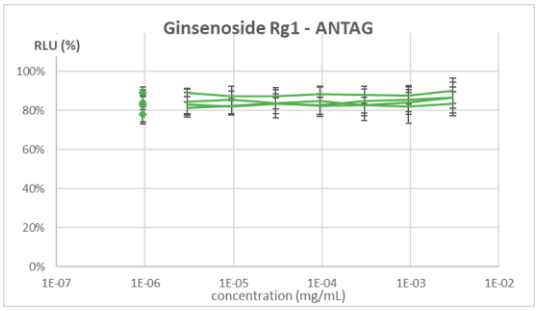
B)



C)



D)



E)

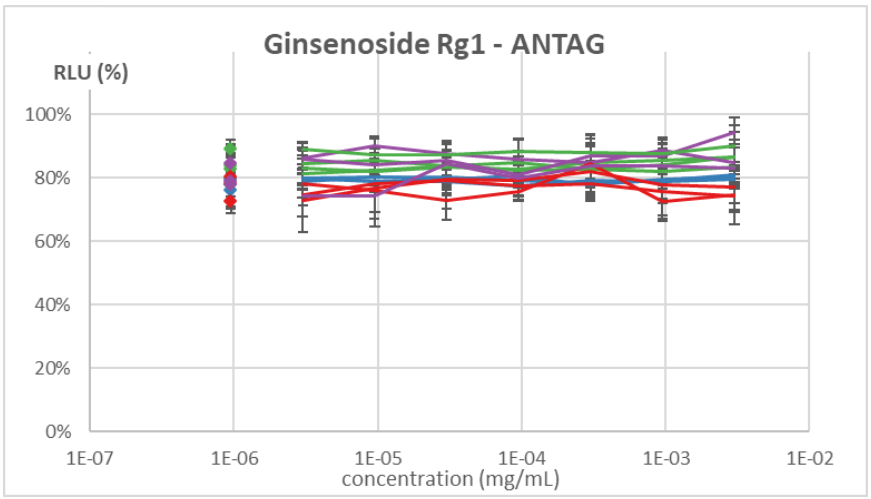


Figure 55: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Ginsenoside Rg1 in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Ginsenoside Rg1 is not an antagonist in this method.

Triclosan

All labs tested Triclosan from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves and IC_{50} obtained are presented below.
Toxem rejected one run as it was different from the others, even though it met the acceptance criteria.

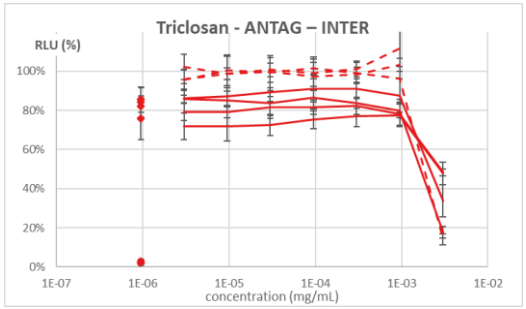
Table 16 : Triclosan IC_{50} values in antagonism mode.

	Ineris		INSERM		Tame-Water		Toxem	
IC_{50} (mg/mL)	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
	$4,6E^{-03}$ *	$2,2E^{-03}$ *	$3,6E^{-03*}$	$3,2E^{-03} \pm 2,4E^{-02}$			$3,6E^{-03} \pm 1,6E^{-02}$	$3,8E^{-03} \pm 3,5E^{-02}$
	$4,2E^{-03}$ *		$3,2E^{-03*}$	$3,4E^{-03*}$			$5,8E^{-03} \pm 2,5E^{-03}$	$3,4E^{-03}$
	$3,7E^{-03} \pm 8,7E^{-03}$		$6,2E^{-03*}$	$4,1E^{-03*}$			$5,1E^{-03}$	$4,0E^{-03} \pm 8,4E^{-03}$
	$4,2E^{-03}$ *						$5,4E^{-03} \pm 1,5E^{-02}$	
mean	$4,2E^{-03} \pm 3,6E^{-04}$		$4,3E^{-03} \pm 1,6E^{-03}$	$3,5E^{-03}$			$5,0E^{-03} \pm 9,6E^{-04}$	$3,8E^{-03} \pm 3,0E^{-04}$
R^2	0,97		0,99				0,95	

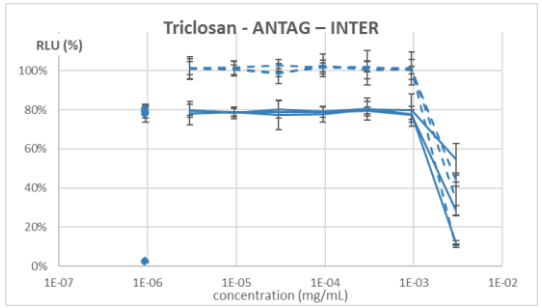
* estimation does not converge

IC_{50} values show a good intra- and inter-lab reproducibility for the three labs observing a decrease of luciferase signal.

A)



B)



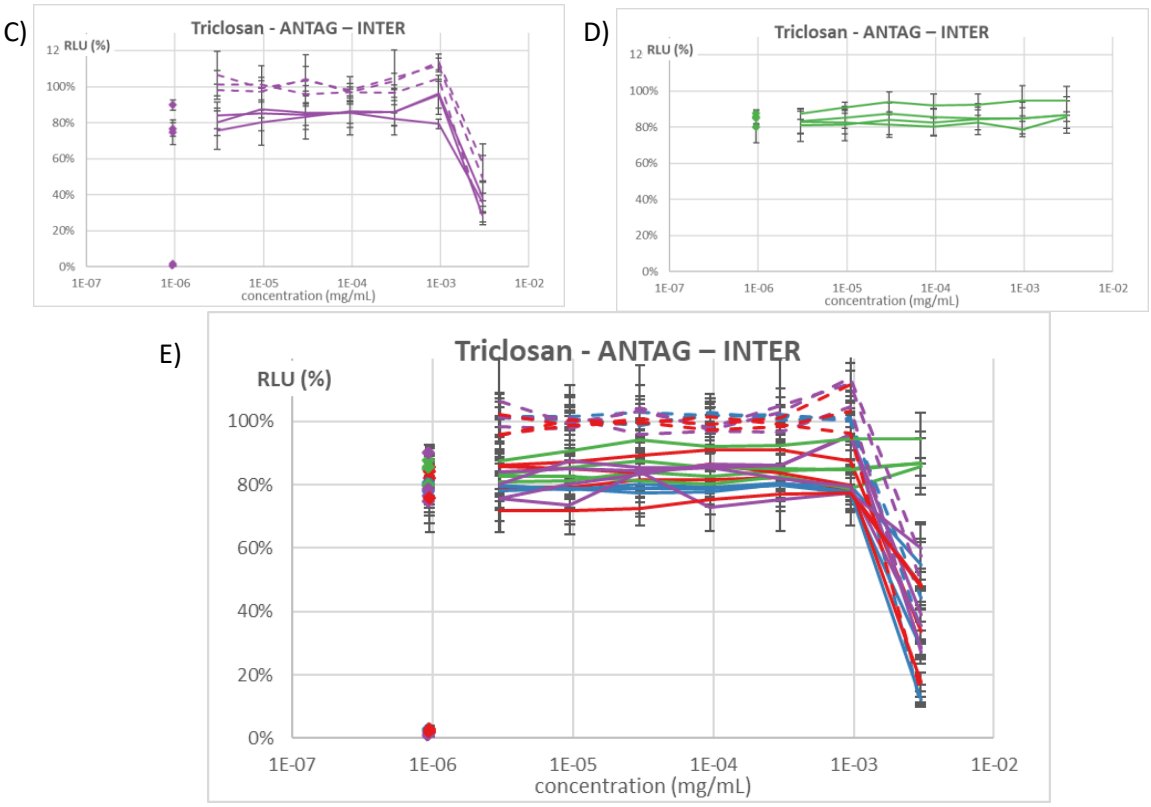
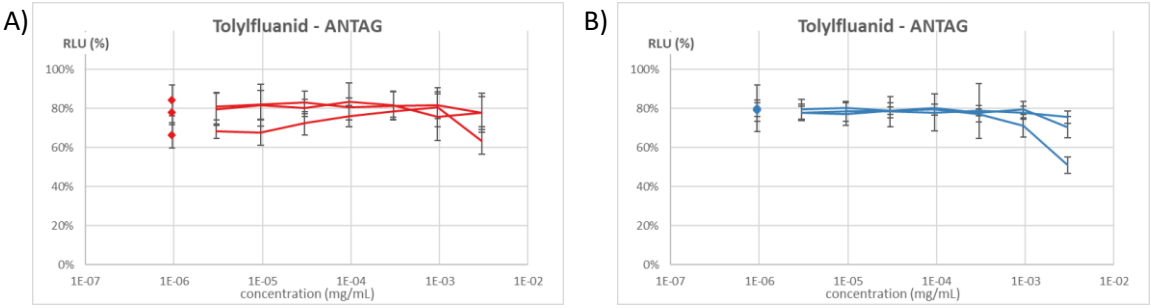


Figure 56: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Triclosan in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Toxem excluded one run on the basis that it was different from others, despite meeting the acceptance criteria.
The conclusions from the labs differ. Tame-Water did not observe any effect of Triclosan at the tested concentrations. Ineris, INSERM and Toxem noted a decreased activity at the highest concentration and tested it in the interference mode. All labs calculated a correlation coefficient between antagonist and interference runs (R^2) above 0.9, indicating that Triclosan is not an antagonist, but interferes with the method. The steep curve seen in many runs indicate that it may have had toxic effect (cell death) at the highest concentration.

Tolylfluanid

All labs tested Tolylfluanid from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.



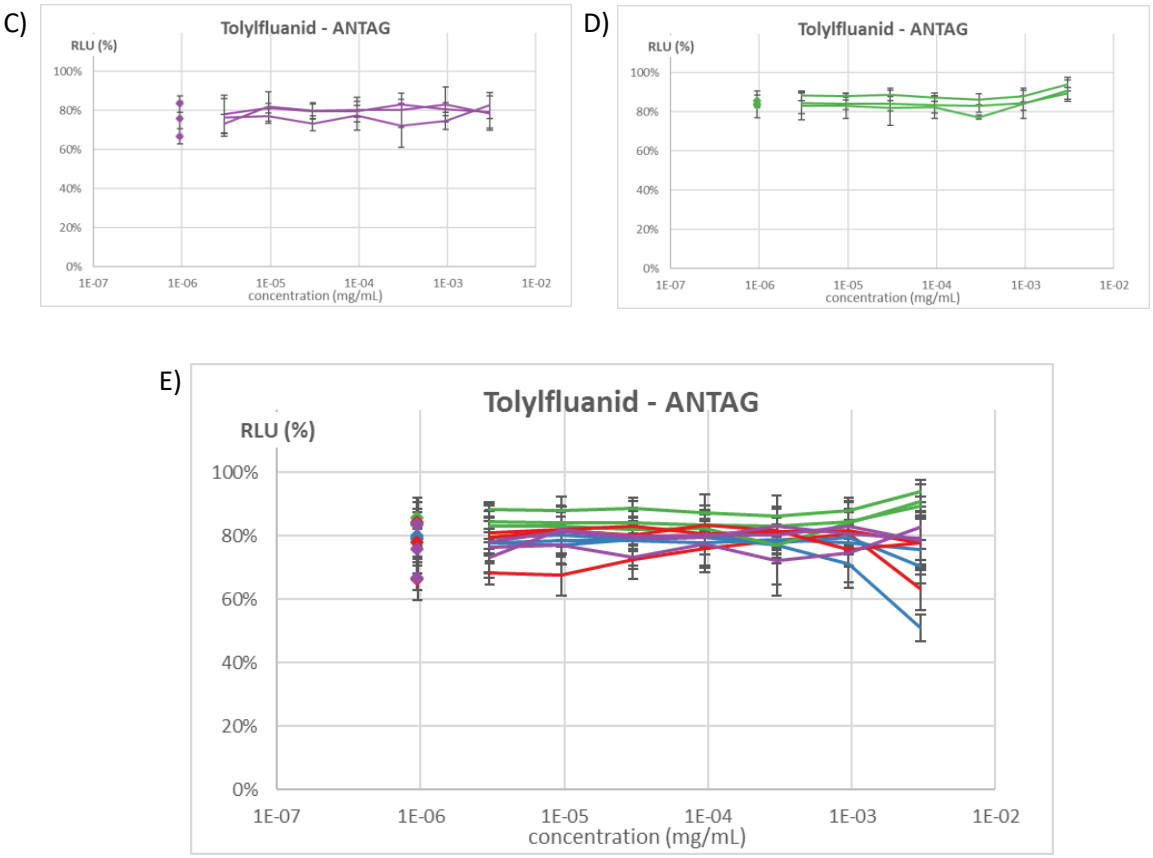
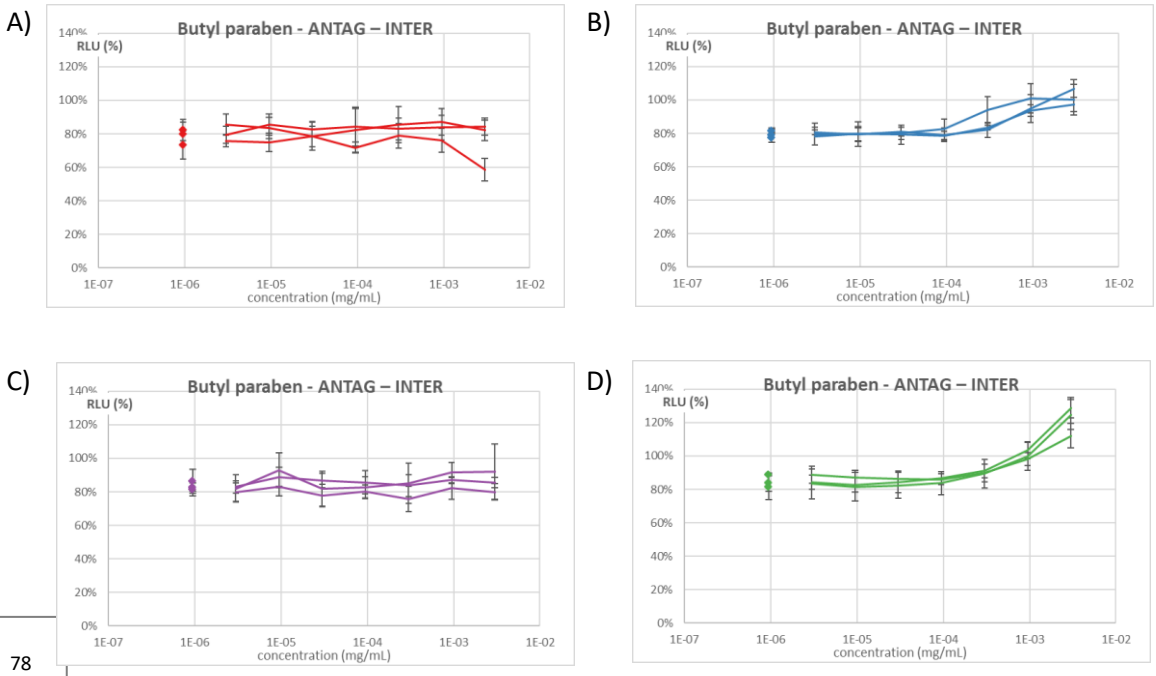


Figure 57: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Tolylfluanid in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Tolylfluanid is not an antagonist in this method.

Butyl paraben

All labs tested Butyl paraben from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.



E)

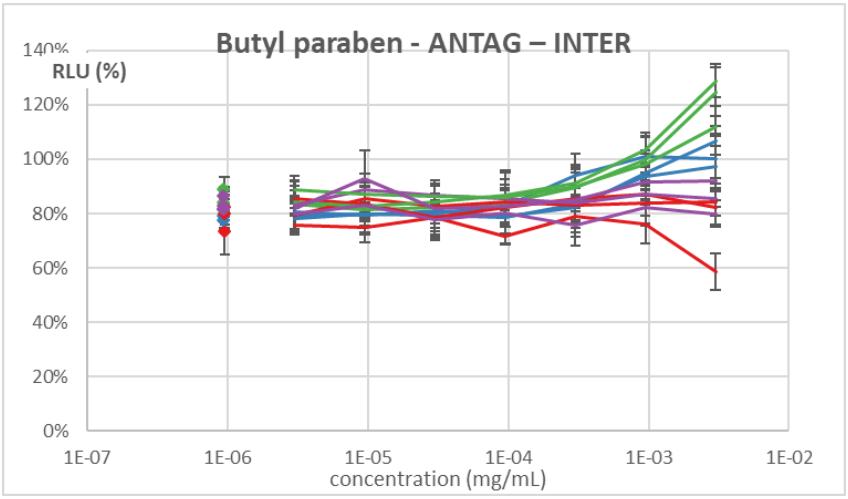


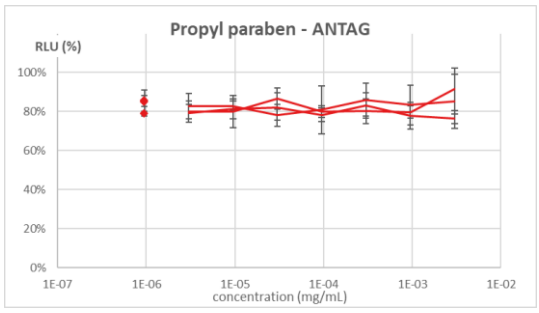
Figure 58: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Butyl paraben in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

INSERM and Tame-Water did observe a slight increase of the signal at the highest concentrations tested.
All labs concluded that at the concentrations tested, Butyl paraben is not an antagonist in this method.

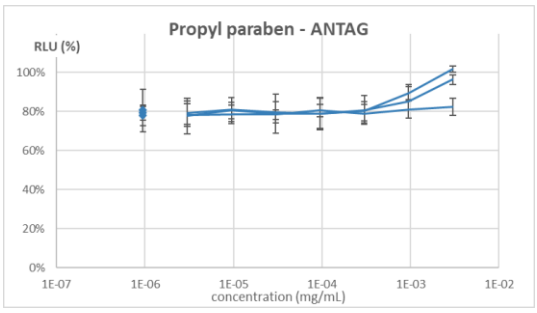
Propyl paraben

All labs tested Propyl paraben from $3.0E^{-6}$ to $3.0E^{-3}$mg /mL. The concentration-response curves obtained are presented below.

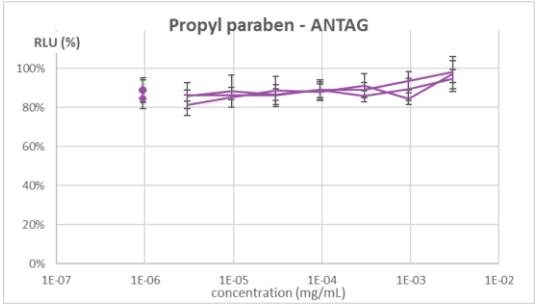
A)



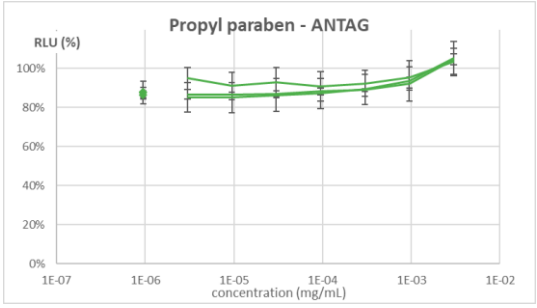
B)



C)



D)



E)

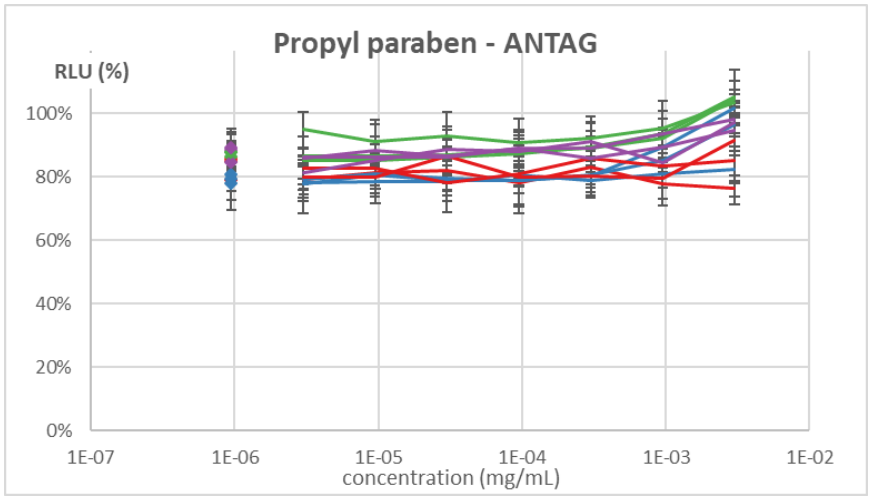


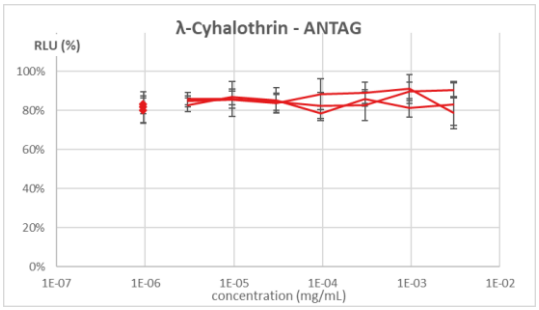
Figure 59: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Propyl paraben in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Propyl paraben is not an antagonist in this method.

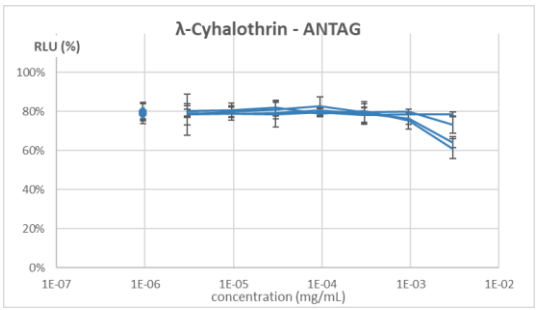
λ-Cyhalothrin

All labs tested λ-Cyhalothrin from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.
INSERM had one invalid run due to some cytotoxicity.

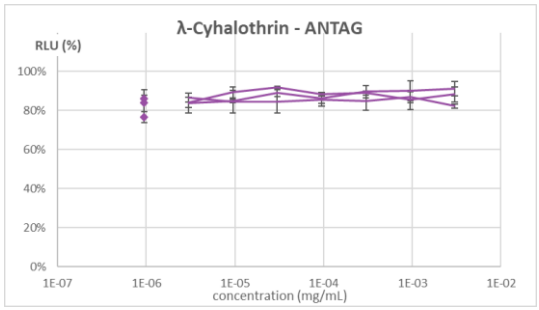
A)



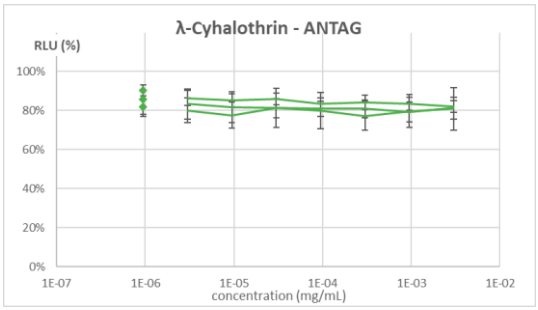
B)



C)



D)



E)

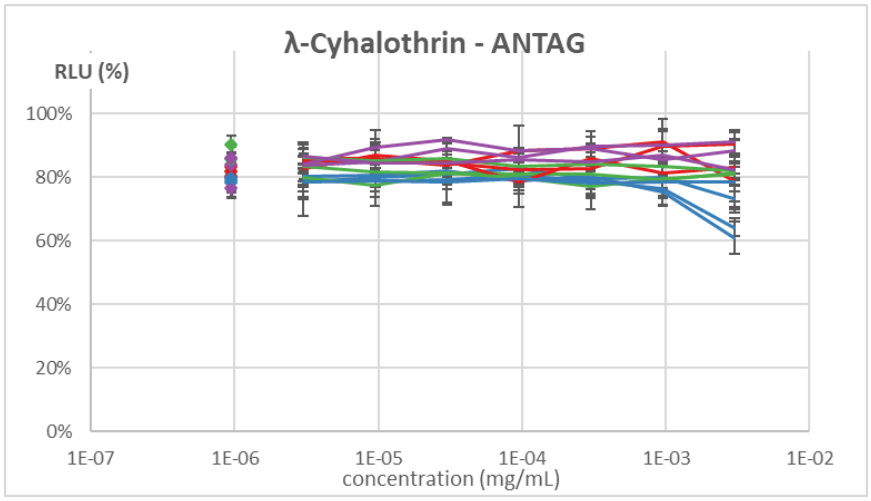


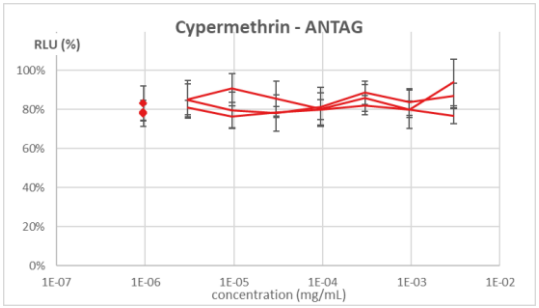
Figure 60: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to λ-Cyhalothrin in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, λ-Cyhalothrin is not an antagonist in this method.

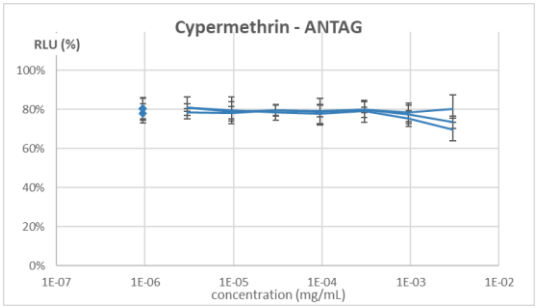
Cypermethrin

All labs tested Cypermethrin from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

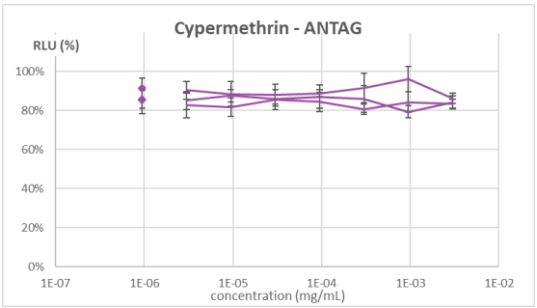
A)



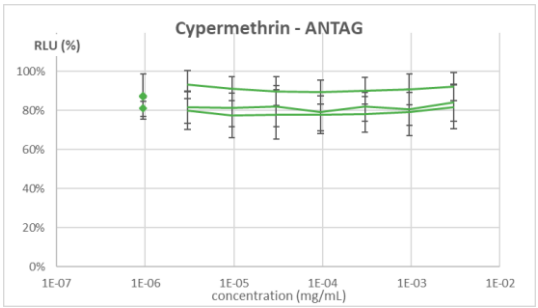
B)



C)



D)



E)

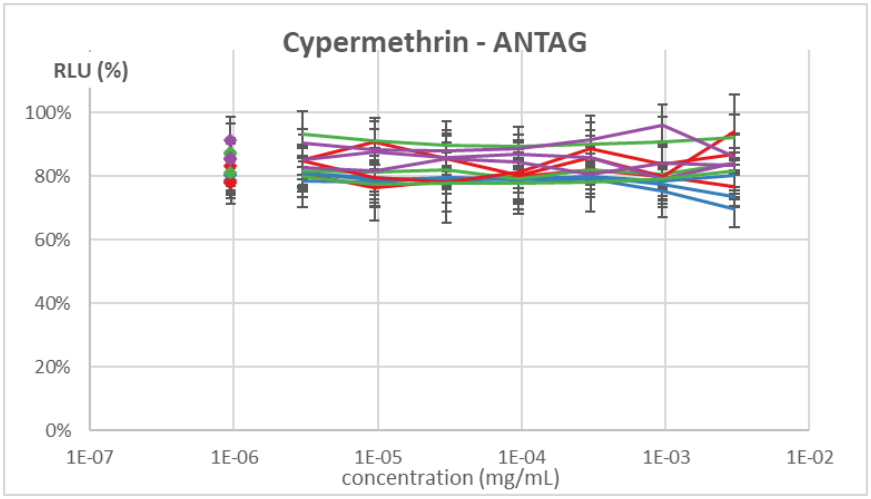


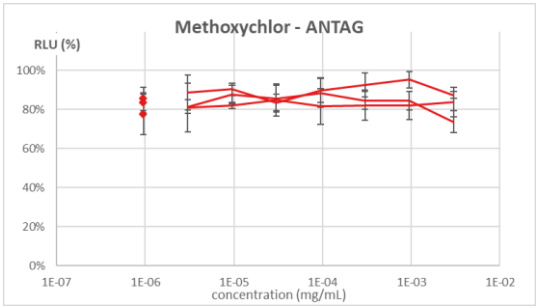
Figure 61: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Cypermethrin in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Cypermethrin is not an antagonist in this method.

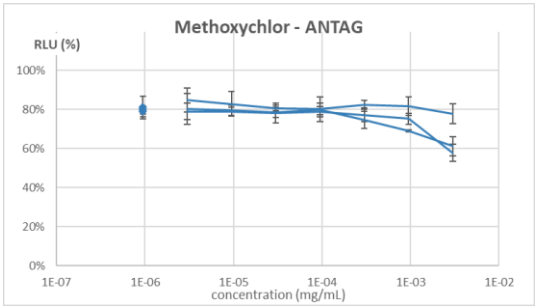
Methoxychlor

All labs tested Methoxychlor from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

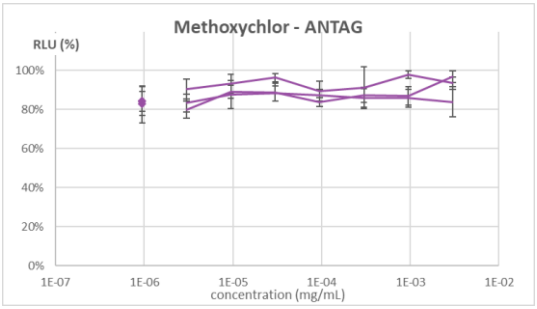
A)



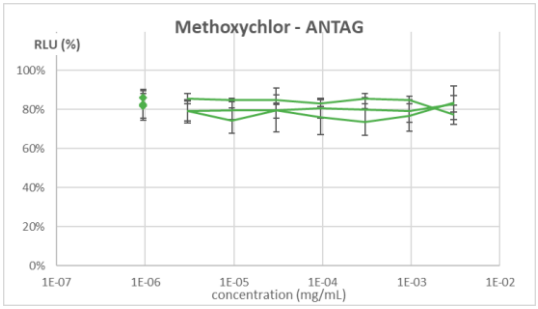
B)



C)



D)



E)

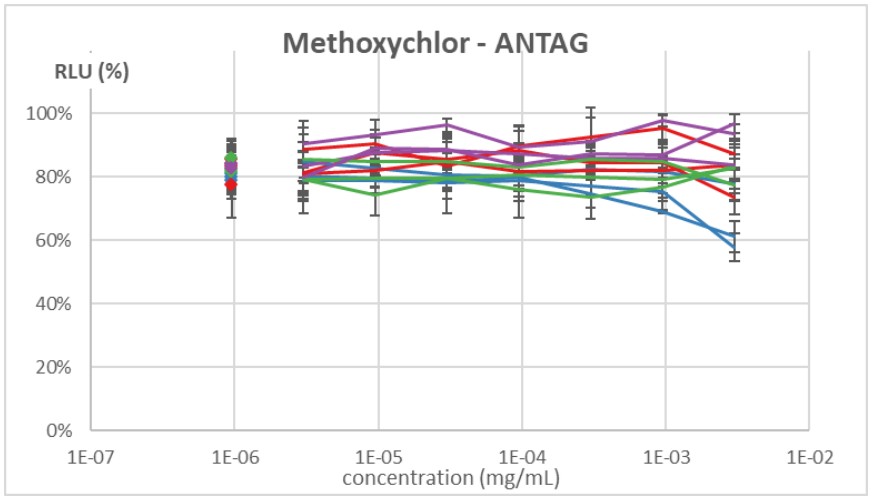


Figure 62: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Methoxychlor in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Methoxychlor is not an antagonist in this method.

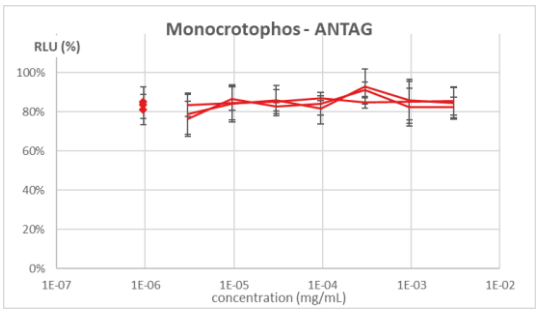
Arsenic

Arsenic was not tested due to solubility issue.

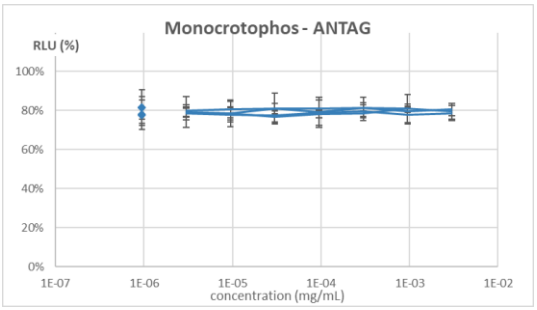
Monocrotophos

All labs tested Monocrotophos from $3.0E^{-6}$ to $3.0e^{-3}$mg/mL. The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

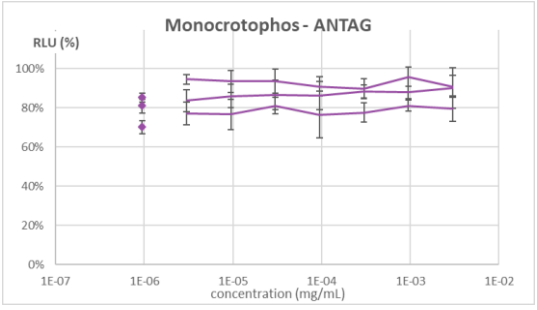
A)



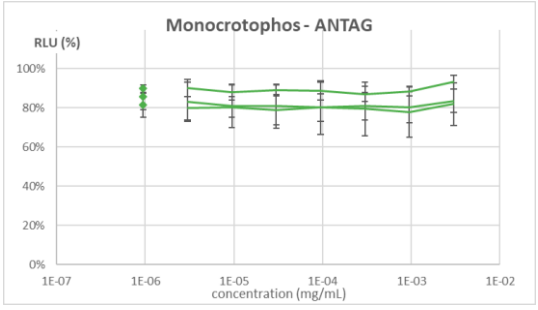
B)



C)



D)



E)

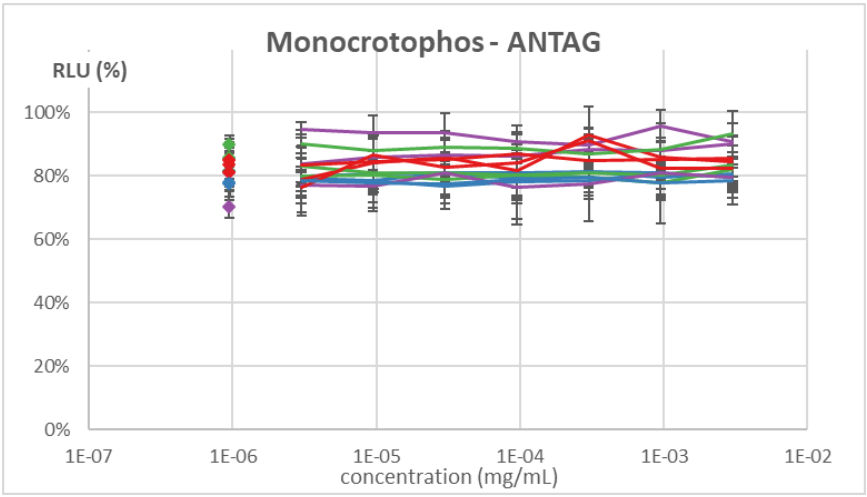


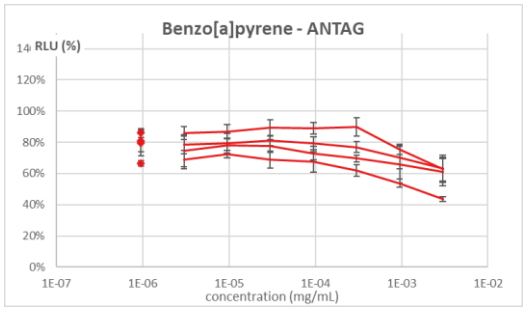
Figure 63: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Monocrotophos in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Monocrotophos is not an antagonist in this method.

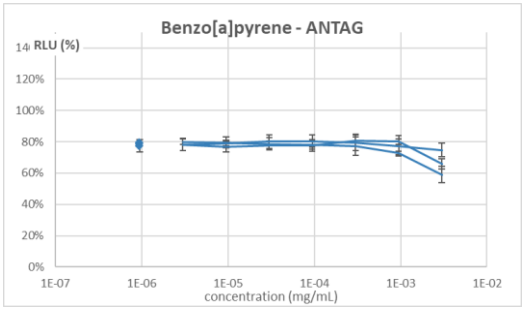
Benzo[a]pyrene (BaP)

All labs tested BaP from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

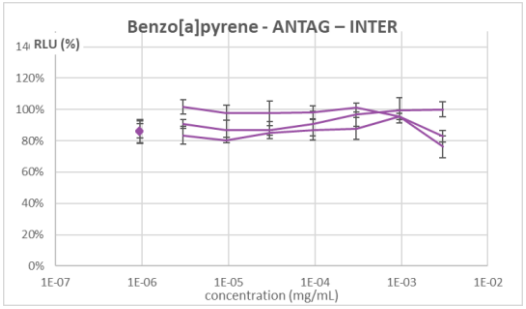
A)



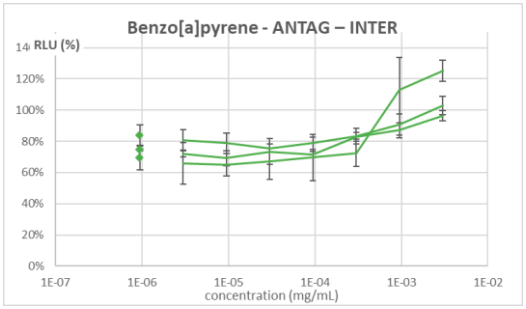
B)



C)



D)



E)

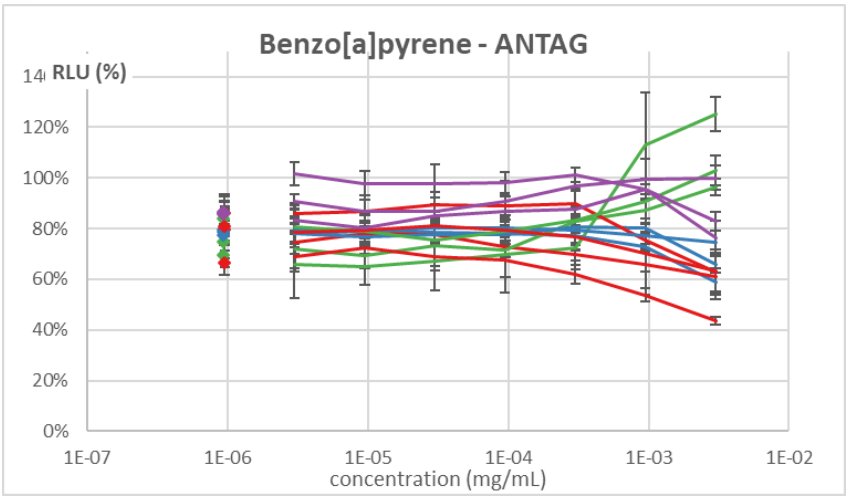


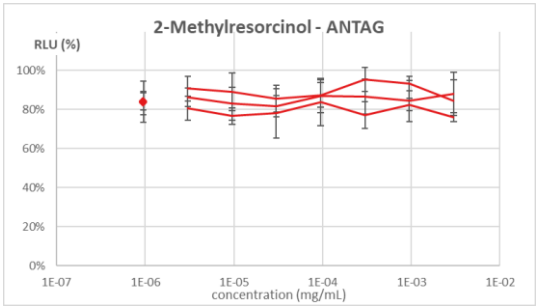
Figure 64: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to BaP in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, BaP is not an antagonist in this method.

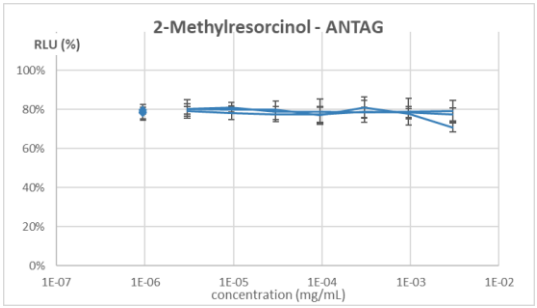
2-Methylresorcinol

All labs tested 2-Methylresorcinol from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

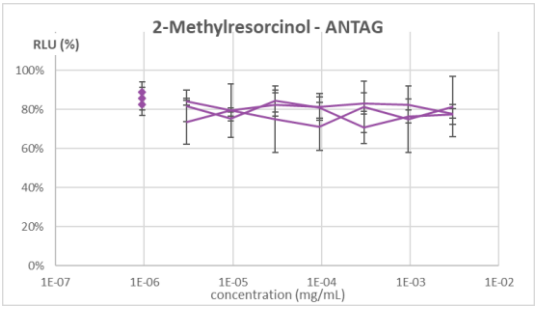
A)



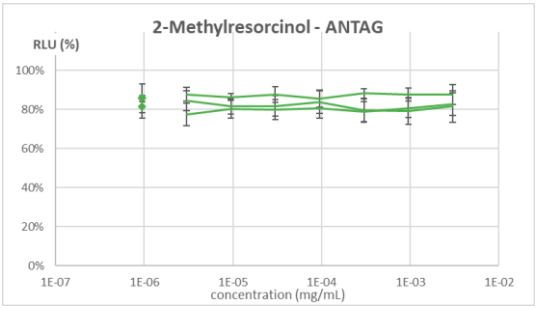
B)



C)



D)



E)

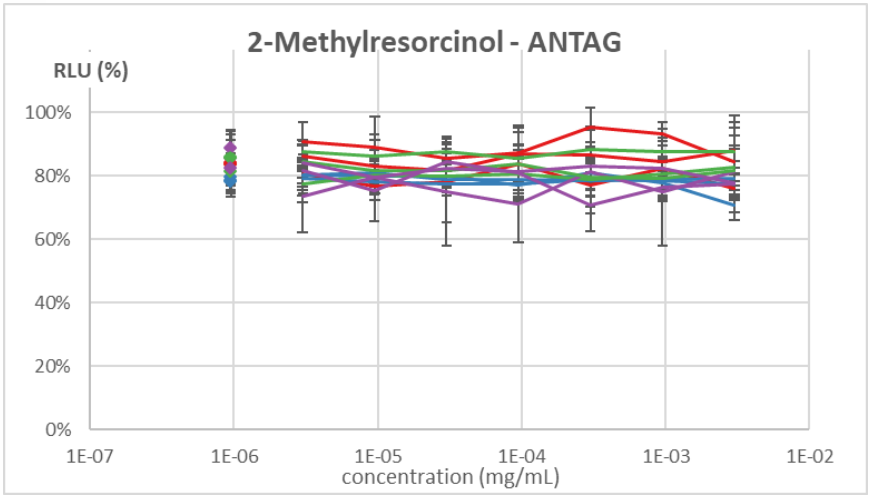


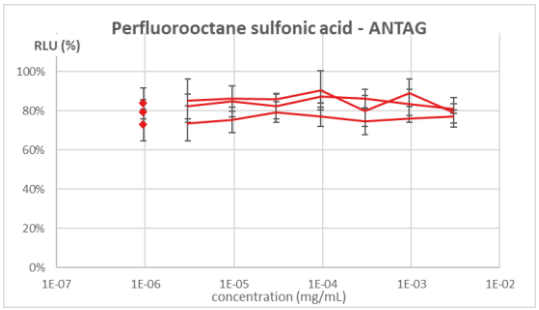
Figure 65: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to 2-Methylresorcinol in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, 2-Methylresorcinol is not an antagonist in this method.

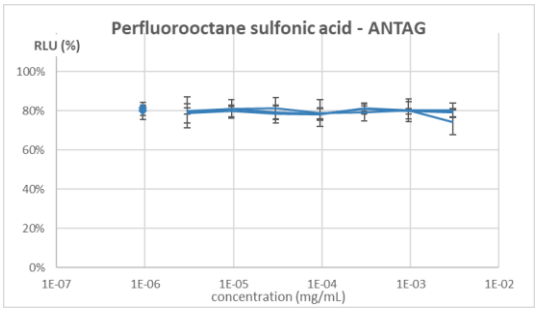
Perfluorooctane sulfonic acid (PFOS)

All labs tested PFOS from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

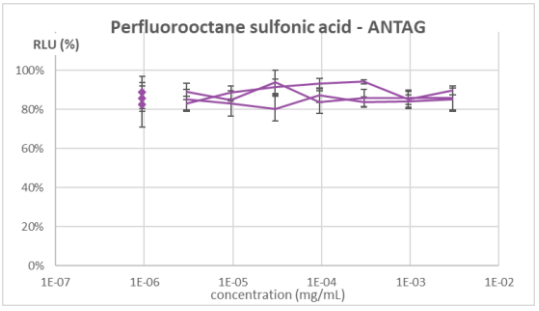
A)



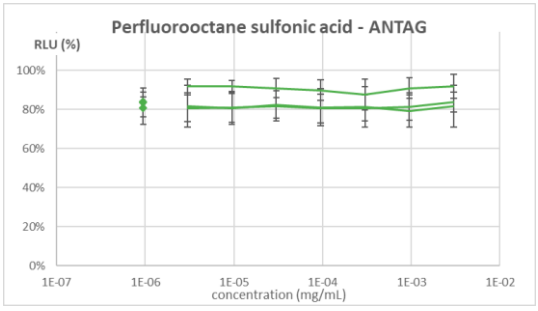
B)



C)



D)



E)

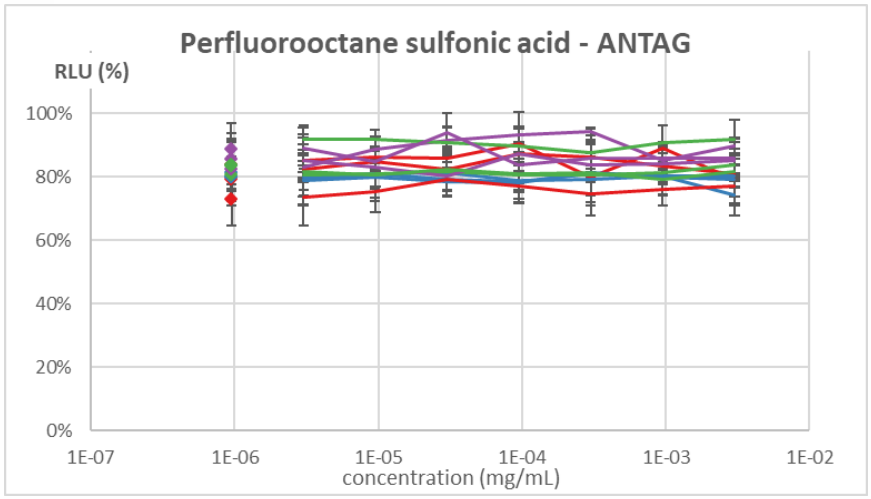


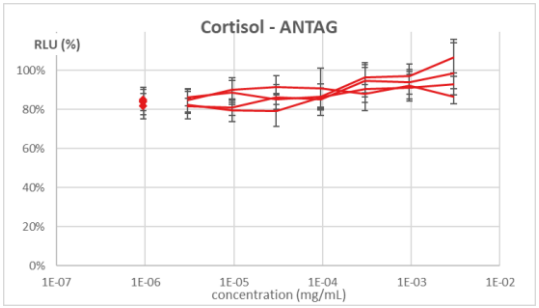
Figure 66: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Perfluorooctane sulfonic acid in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, PFOS is not an antagonist in this method.

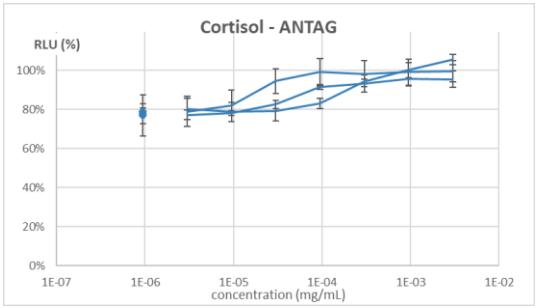
Cortisol

All labs tested Cortisol from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

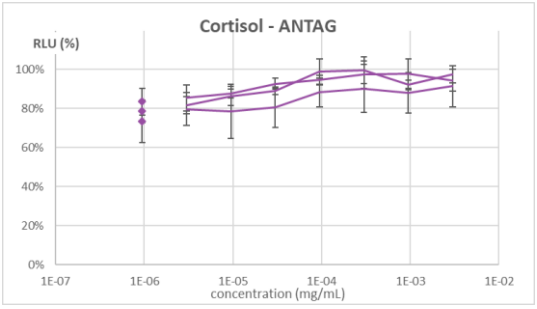
A)



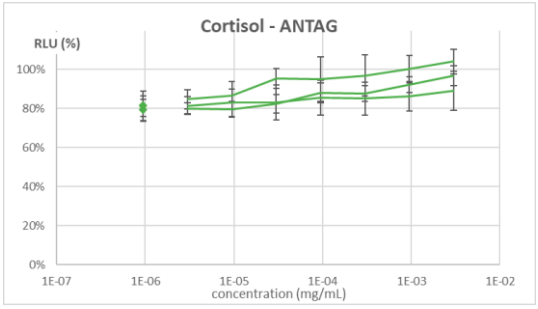
B)



C)



D)



E)

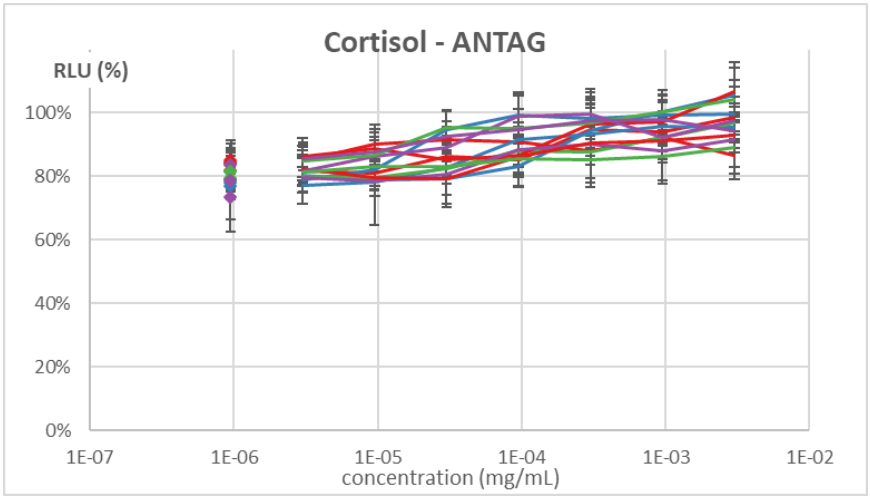


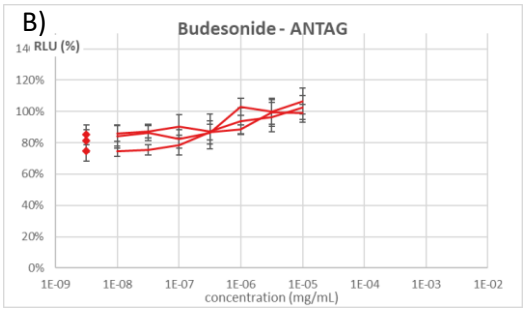
Figure 67: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Cortisol in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

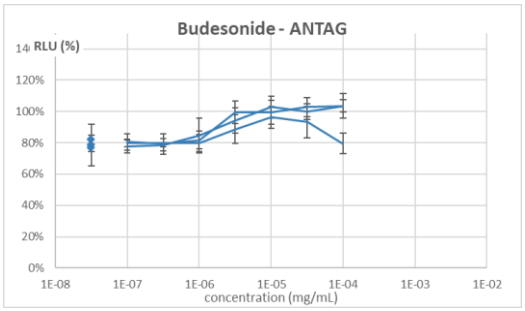
All labs concluded that at the concentrations tested, Cortisol is not an antagonist in this method.

Budesonide

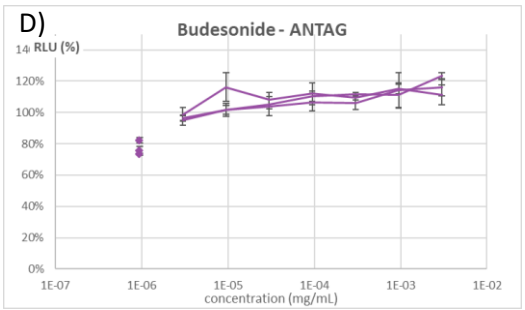
Two labs tested Budesonide from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. Ineris and INSERM used lower concentrations (the same as in the agonist mode), in deviation from the SOP.
The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

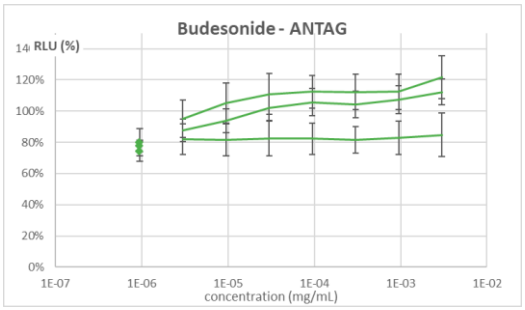
B)





C)





E)

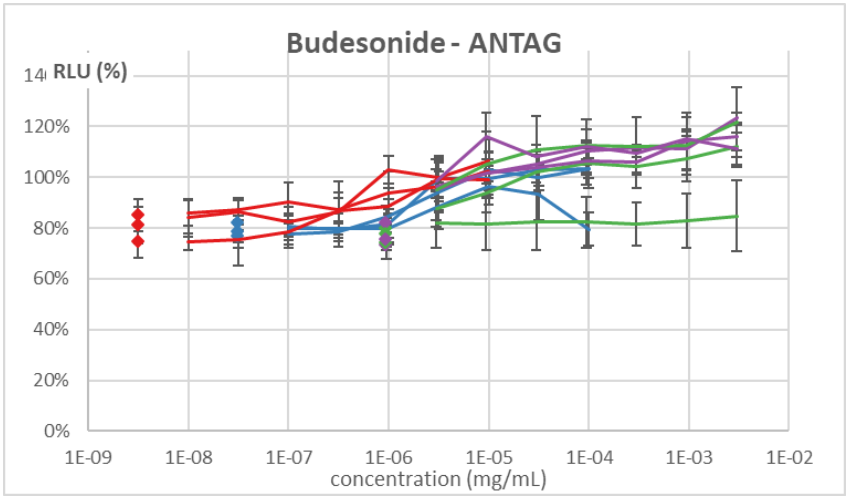


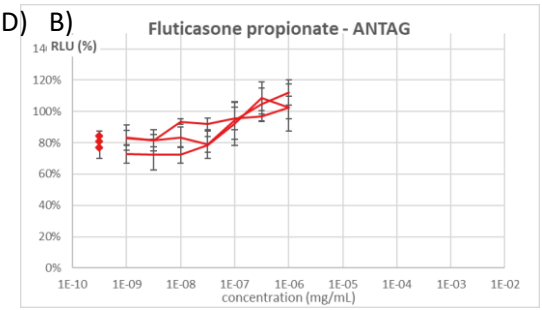
Figure 68: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Cortisol in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

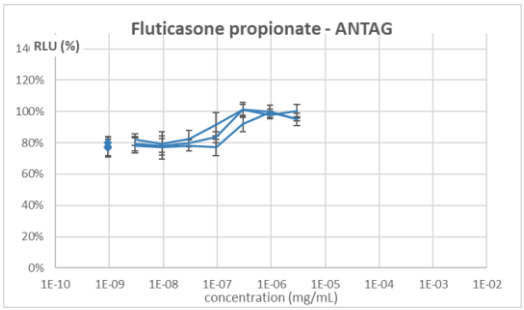
All labs concluded that at the concentrations tested, Budesonide is not an antagonist in this method.

Fluticasone propionate

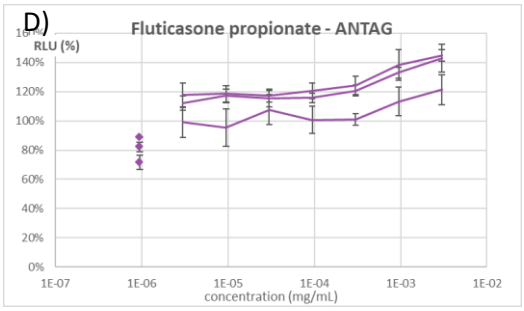
Two labs tested Fluticasone propionate from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. Ineris and INSERM used lower concentrations (the same as in the agonist mode), in deviation from the SOP.
The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

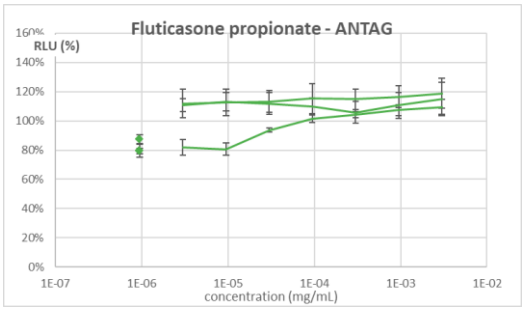
D) B)





E) D)





E)

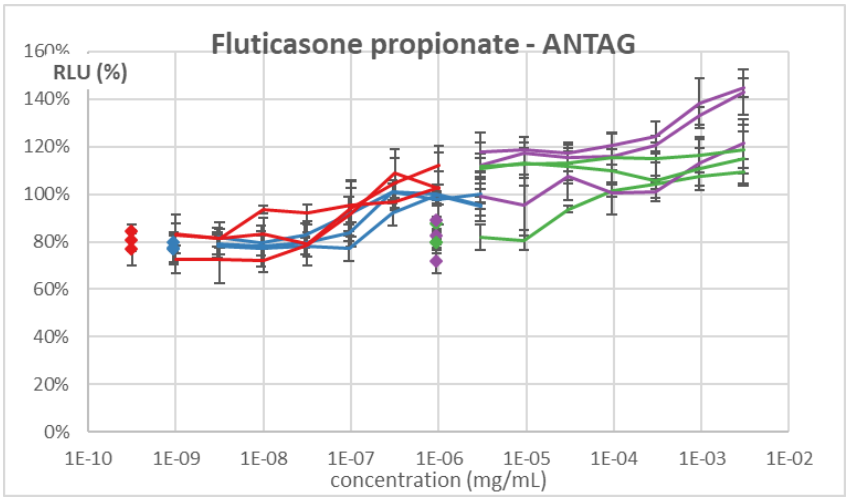


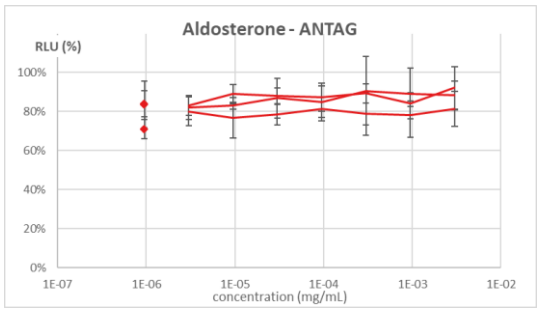
Figure 69: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Fluticasone propionate in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Fluticasone propionate is not an antagonist in this method.

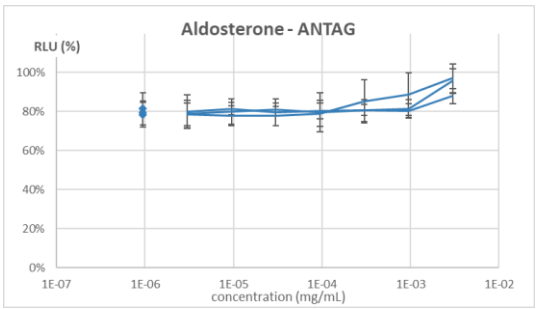
Aldosterone

All labs tested Aldosterone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

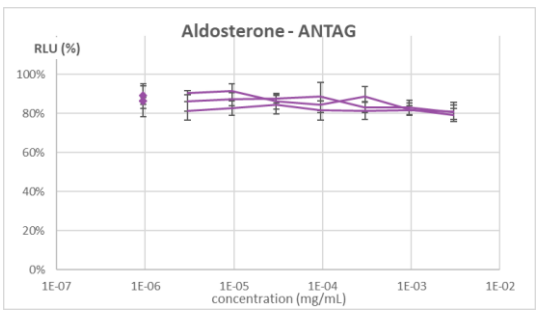
A)



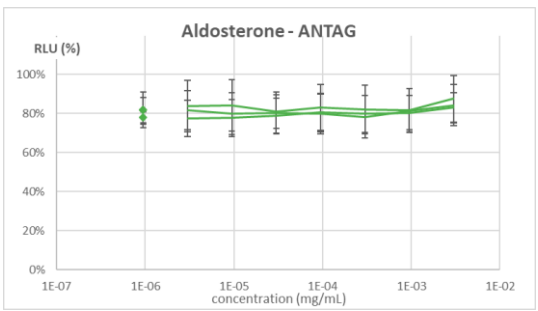
B)



C)



D)



E)

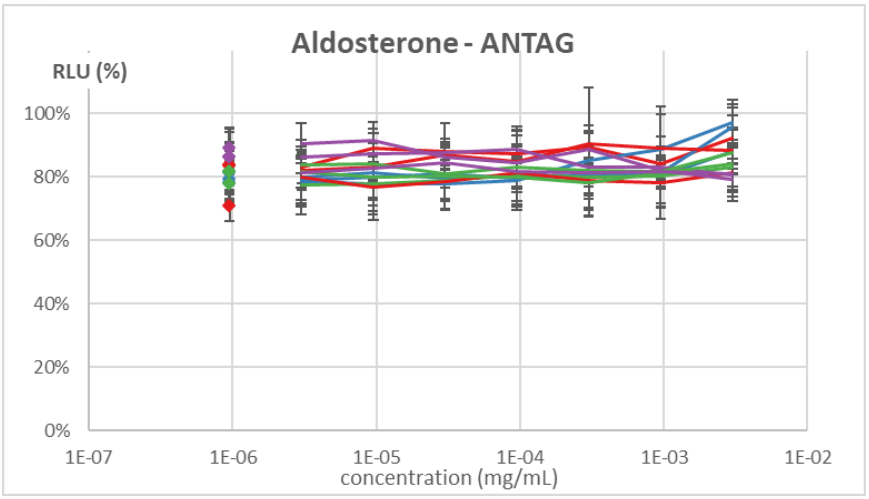


Figure 70: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Aldosterone in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Aldosterone is not an antagonist in this method.

Progesterone

All labs tested Progesterone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL and observed a decrease in signal. They, then, tested it for potential interference. The correlation coefficient between antagonist and interference runs was above 0.9 for three of the labs, indicating that the decreased signal was due to interference. The IC_{50} values concentration-response curves obtained are presented below.
Tame-Water rejected one run showing no effect of Progesterone.

Table 17: Progesterone IC_{50} values in antagonism and interference modes.

	Ineris		INSERM		Tame-Water		Toxem	
	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
IC_{50} (mg/mL)	$8,2E^{-04}$ ± $3,2E^{-04}$	$6,5E^{-04}$ ± $6,1E^{-05}$	$4,2E^{-04}$ ± $3,6E^{-04}$	$7,9E^{-04}$ ± $8,2E^{-05}$		$4,9E^{-07}$ ± $1,9E^{-06}$	$9,3E^{-04}$ ± $4,0E^{-05}$	$9,5E^{-04}$ ± $7,0E^{-05}$
	$7,8E^{-04}$ ± $4,3E^{-04}$	$5,7E^{-04}$ ± $7,2E^{-05}$	$5,3E^{-04}$ ± $4E^{-05}$	$8,9E^{-04}$ ± $3,3E^{-05}$	$3,2E^{-03}$ ± $6,2E^{-03}$	$1,8E^{-03}$ ± $1,6E^{-02}$	$7,2E^{-04}$ ± $6,0E^{-05}$	$9,5E^{-03}$ ± $2,9E^{-02}$
	$8,0E^{-04}$ ± $4,1E^{-04}$	$7,5E^{-04}$ ± $2,3E^{-04}$	$7,3E^{-04}$ ± $3,2E^{-04}$	$7,0E^{-04}$ ± $4,0E^{-05}$	$3,6E^{-03}$*	$4,1E^{-03}$ ± $1,3E^{-02}$	$9,0E^{-04}$ ± $2,0E^{-04}$	$6,7E^{-04}$ ± $1,6E^{-04}$
	$8,3E^{-04}$ ± $2,7E^{-04}$				$3,4E^{-03}$ ± $8,2E^{-03}$			
mean	$8,1E^{-04}$ ± $2,3E^{-05}$	$6,6E^{-04}$ ± $9,1E^{-05}$	$5,6^{E-04}$ ± $1,6^{E-04}$	$7,9E^{-04}$ ± $9,3E^{-05}$	$3,4E^{-03}$ ± $2,1E^{-04}$	$2,0E^{-03}$ ± $2,1E^{-03}$	$8,5E^{-04}$ ± $1,2E^{-04}$	$3,7E^{-03}$ ± $5,0E^{-03}$
R^2	0.95		0.95		0.70		0.95	

*estimation does not convert

The IC_{50} values show a good intra- and inter-laboratory reproducibility, with a slight difference for Tame-Water.

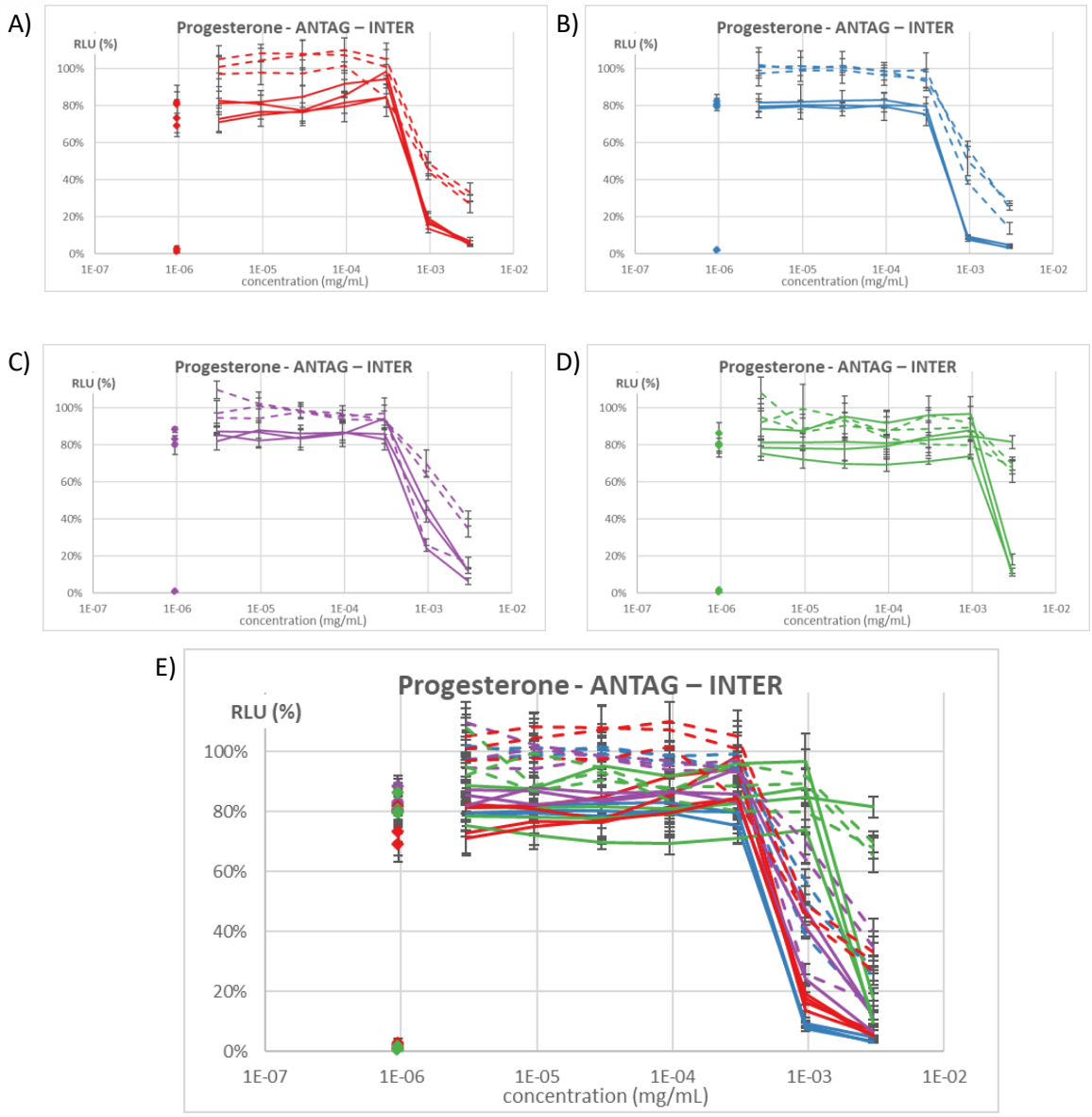


Figure 71: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Progesterone in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

Three labs concluded that at the concentrations tested, Progesterone is not an antagonist in this method, but interferes with the method. Tame-Water concluded that it is an antagonist.

21-Hydroxyprogesterone

All labs tested 21-Hydroxyprogesterone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL and observed a decrease in signal. They, then, tested it for potential interference. The correlation coefficient between antagonist and interference runs was above 0.9 for three of the labs, indicating that the decreased signal was due to interference. INSERM observed an R^2 of 0.87.
The concentration-response curves and IC_{50} obtained are presented below.

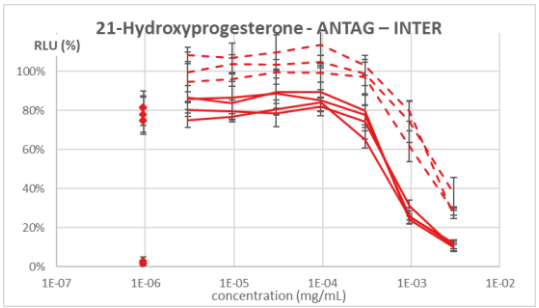
Table 18: 21-Hydroxyprogesterone IC_{50} values in antagonism and interference modes.

	Ineris		INSERM		Tame-Water		Toxem	
IC_{50} (mg/mL)	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
	$7{,}2E^{-04} \pm 5{,}0E^{-05}$	$1{,}6E^{-03} \pm 8{,}2E^{-04}$	$5{,}3E^{-04} \pm 5{,}1E^{-05}$	$2{,}0E^{-03} \pm 1{,}2E^{-03}$	$1{,}1E^{-03} \pm 3{,}1E^{-04}$	$3{,}5E^{-03} \pm 1{,}5E^{-02}$	$7{,}2E^{-04} \pm 5{,}8E^{-04}$	$2{,}7E^{-03} \pm 2{,}9E^{-03}$
	$3{,}6E^{-04} \pm 2{,}1E^{-04}$	$1{,}1E^{-03} \pm 2{,}7E^{-04}$	$8{,}1E^{-04} \pm 8{,}8E^{-05}$	$5{,}7E^{-03} \pm 2{,}5E^{-02}$	$3{,}4E^{-03}$	$2{,}1E^{-03}$	$8{,}2E^{-04} \pm 4{,}6E^{-05}$	$1{,}0E^{-03} \pm 8{,}6E^{-05}$
	$6{,}0E^{-04} \pm 6{,}4E^{-05}$	$9{,}4E^{-04} \pm 5{,}9E^{-05}$	$1{,}1E^{-03} \pm 2{,}6E^{-04}$	$1{,}8E^{-03} \pm 1{,}4E^{-03}$	$3{,}5E^{-03}$*	$2{,}8E^{-03} \pm 1{,}4E^{-02}$	$1{,}0E^{-03} \pm 7{,}5E^{-05}$	$4{,}9E^{-04} \pm 5{,}8E^{-04}$
	$5{,}4E^{-04} \pm 5{,}5E^{-05}$				$4{,}3E^{-03}$*			
mean	$5{,}5E^{-04} \pm 1{,}5E^{-04}$	$1{,}2E^{-03} \pm 3{,}3E^{-04}$	$8{,}1E^{-04} \pm 2{,}7E^{-04}$	$3{,}2E^{-03} \pm 2{,}3E^{-03}$	$3{,}0E^{-03} \pm 1{,}4E^{-03}$	$2{,}8E^{-03} \pm 6{,}8E^{-04}$	$8{,}5E^{-04} \pm 1{,}4E^{-04}$	$1{,}4E^{-03} \pm 1{,}1E^{-03}$
R^2	0.91		0.87		0.96		0.97	

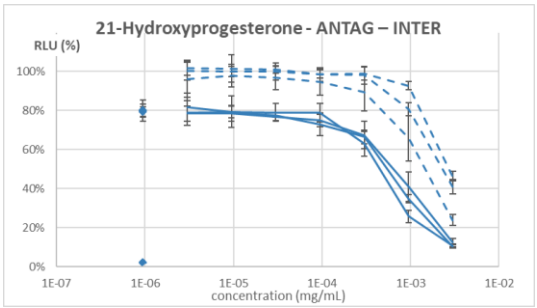
*estimation does not convert.

The IC_{50} values show a good intra- and inter-laboratory reproducibility, INSERM having a higher variability. Tame-Water had slightly higher values.

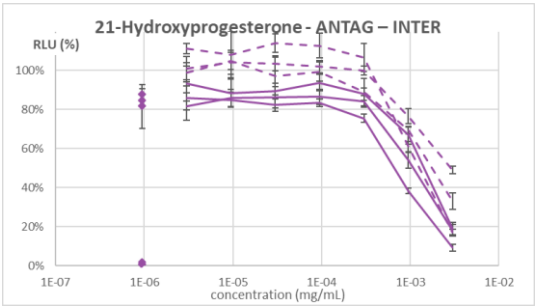
A)



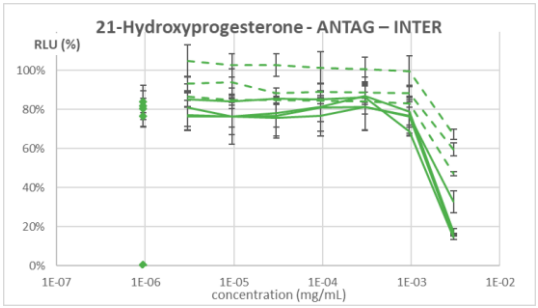
B)



C)



D)



E)

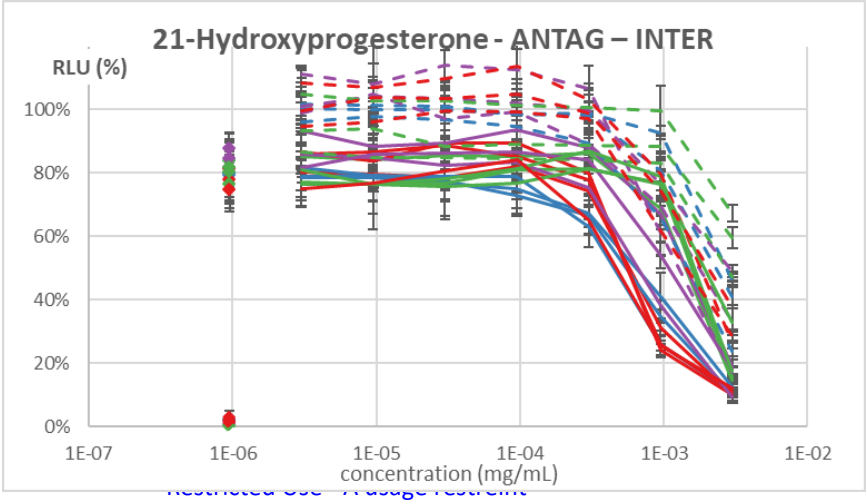


Figure 72: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to 21-Hydroxyprogesterone in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

Three labs concluded that at the concentrations tested, 21-Hydroxyprogesterone is not an antagonist in this method but interferes with the method. INSERM concluded that it is an antagonist. The difference in conclusions may be due to the fact that the dose-response curves are not complete, leading to uncertainties on the determination of IC_{50}.

Mifepristone

INSERM, Tame-Water and Toxem adjusted the concentrations of Mifepristone following the first run, to obtain a dose-response covering the full spectrum of responses. Ineris used the same concentrations as in the agonist mode, in deviation from the SOP. All labs observed a decrease in signal and tested Mifepristone for potential interference. The correlation coefficient between antagonist and interference runs was below 0.9 for all labs, indicating that the decreased signal was not due to interference. The concentration-response curves and IC_{50} obtained are presented below.

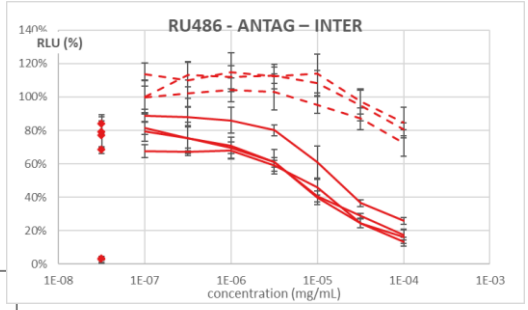
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

Table 19: 21-Hydroxyprogesterone IC_{50} values in antagonism and interference modes.

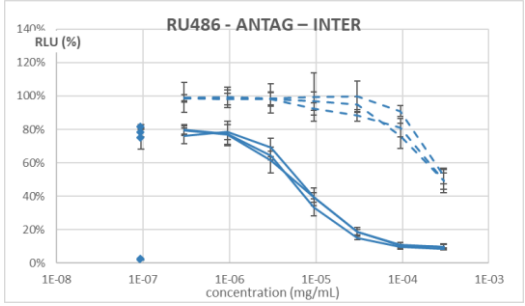
	Ineris		INSERM		Tame-Water		Toxem	
IC_{50} (mg/mL)	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
	$7{,}5E^{-06} \pm 1{,}5E^{-06}$		$7{,}1E^{-06} \pm 8{,}7E^{-07}$	$1{,}2E^{-04} \pm 2{,}9E^{-05}$	$1{,}0E^{-05} \pm 7{,}5E^{-07}$	$5{,}9E^{-04} \pm 2{,}9E^{-04}$	$6{,}1E^{-06} \pm 7{,}0E^{-07}$	$6{,}7E^{-05} \pm 7{,}7E^{-05}$
	$1{,}3E^{-05} \pm 2{,}2E^{-06}$		$6{,}4E^{-06} \pm 4{,}5E^{-07}$	$1{,}9E^{-03} \pm 2{,}8E^{-03}$	$9{,}6E^{-06} \pm 8{,}2E^{-07}$	$9{,}7E^{-04} \pm 1{,}1E^{-03}$	$5{,}1E^{-06} \pm 6{,}4E^{-07}$	$1{,}3E^{-04} \pm 2{,}0E^{-04}$
	$8{,}4E^{-06} \pm 2{,}2E^{-06}$		$8{,}4E^{-06} \pm 7{,}1E^{-07}$	$1{,}3E^{-04} \pm 2{,}2E^{-04}$	$9{,}4E^{-06} \pm 8{,}3E^{-07}$	$2{,}9E^{-04} \pm 1{,}2E^{-04}$	$5{,}3E^{-06} \pm 9{,}0E^{-07}$	$2{,}7E^{-05} \pm 1{,}1E^{-05}$
	$1{,}5E^{-05} \pm 2{,}3E^{-06}$					$3{,}3E^{-04} \pm 1{,}8E^{-04}$		
mean	$1{,}1E^{-05} \pm 3{,}4E^{-06+}$		$7{,}3E^{-06} \pm 1{,}0E^{-06}$	$7{,}3E^{-04} \pm 1{,}1E^{-03}$	$9{,}8E^{-06} \pm 4{,}7E^{-07}$	$5{,}5E^{-04} \pm 3{,}1E^{-04}$	$5{,}5E^{-06} \pm 5{,}1E^{-07}$	$7{,}3E^{-05} \pm 5{,}0E^{-05}$
R^2	0.69		0.44		0.64		0.73	

The IC_{50} values show a good intra- and inter-laboratory reproducibility.
The weak decrease of signal in interference mode for Ineris did not allow the calculation of an IC_{50}.

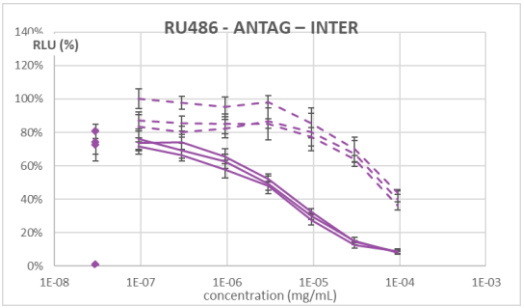
A)



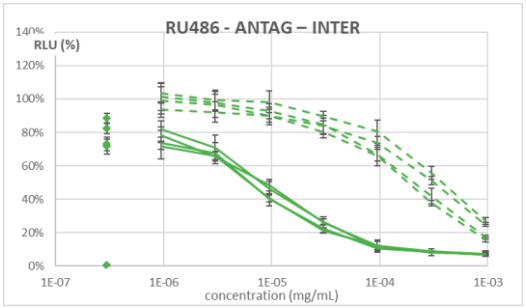
B)



C)



D)



E)

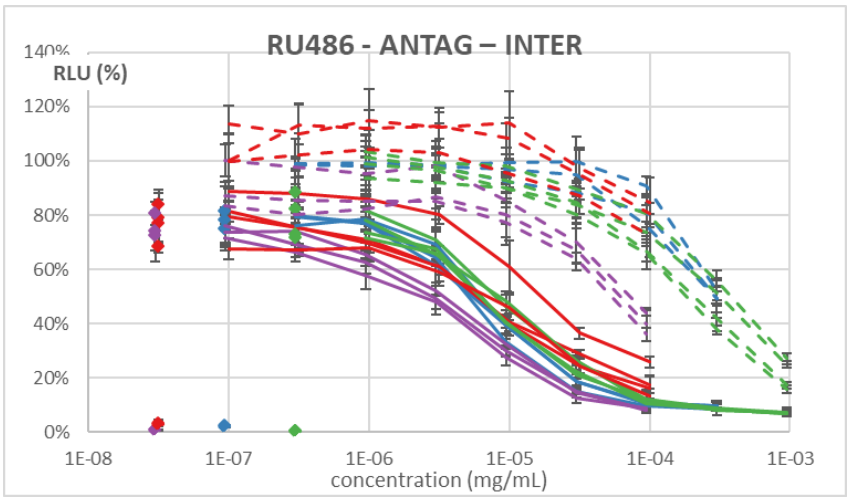


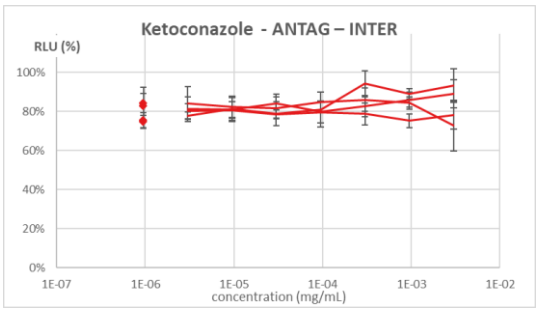
Figure 73: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Mifepristone in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

The labs concluded that at the concentrations tested, Mifepristone is an antagonist in this method.

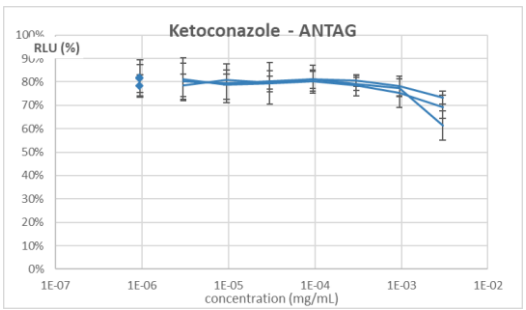
<u>Ketoconazole</u>

All labs tested Ketoconazole from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

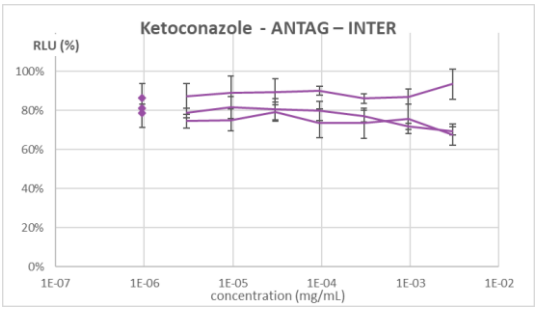
A)



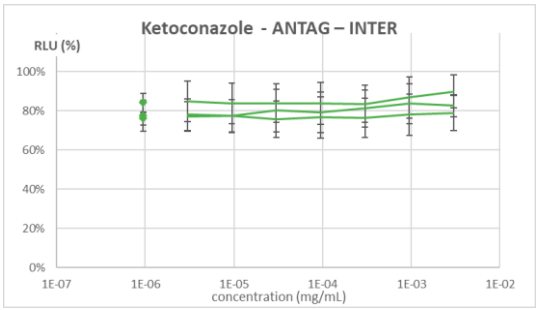
B)



C)



D)



E)

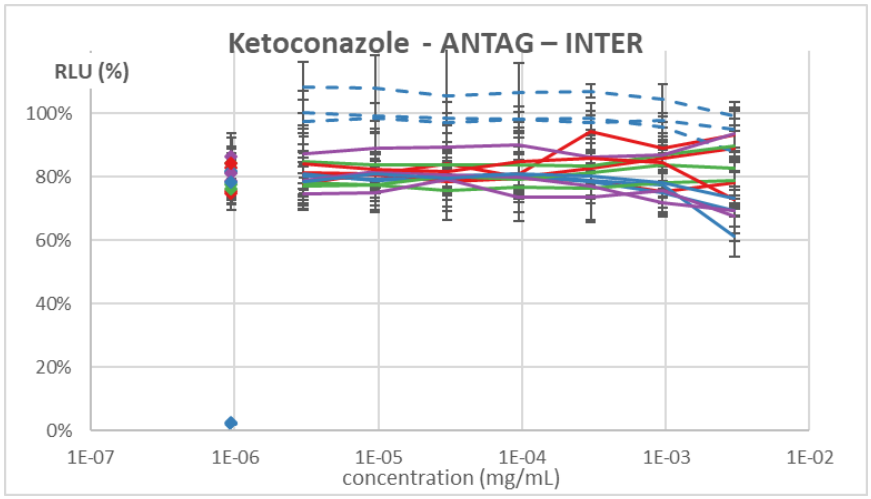


Figure 74: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Ketoconazole in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

All labs concluded that at the concentrations tested, Ketoconazole is not an antagonist in this method.

Spironolactone

All labs tested Spironolactone from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. They all observed a decrease in signal and tested it for potential interference
The correlation coefficient between antagonist and interference runs was above 0.9 for Tame-Water (0.92), INSERM (0.97) and Ineris (0.96), but below for Toxem (0.87).
The concentration-response curves and IC_{50} obtained are presented below.
INSERM had one run invalid due to too high basal response of the cells (>4%).
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.

Table 20: Spironolactone IC_{50} values in antagonism and interference modes.

	Ineris		INSERM		Tame-Water		Toxem	
IC_{50} (mg/mL)	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
	$3,7E^{-03} \pm 2,3E^{-02}$		$1,9E^{-03} \pm 3,1E^{-03}$		$3,9E^{-03} \pm 8,8E^{-03}$		$4,8E^{-03*}$	
	$8,2E^{-03} \pm 2,4E^{-02}$		$1,8E^{-03} \pm 1,5E^{-03}$		$1,2E^{-03} \pm 5,5E^{-04}$		$8,0E^{-03} \pm 6,3E^{-02}$	
	$1,7E^{-03} \pm 1,5E^{-03}$		$4,6E^{-03} \pm 2,0E^{-02}$		$4,2E^{-03} \pm 1,8E^{-02}$		$3,3E^{-03} \pm 7,6E^{-03}$	
	$7,9E^{-03} \pm 2,2E^{-02}$							
mean	$5,4E^{-03} \pm 3,2E^{-03}$		$2,8E^{-03} \pm 1,6E^{-03}$		$3,1E^{-03} \pm 1,7E^{-03}$		$5,4E^{-03} \pm 2,4E^{-03}$	
R^2	0.96		0.97		0.92		0.87	

*estimation does not converge

IC_{50} values show a good intra- and inter-laboratory reproducibility.
IC_{50} values could not be determined for the interference mode as the decrease of signal was too weak.

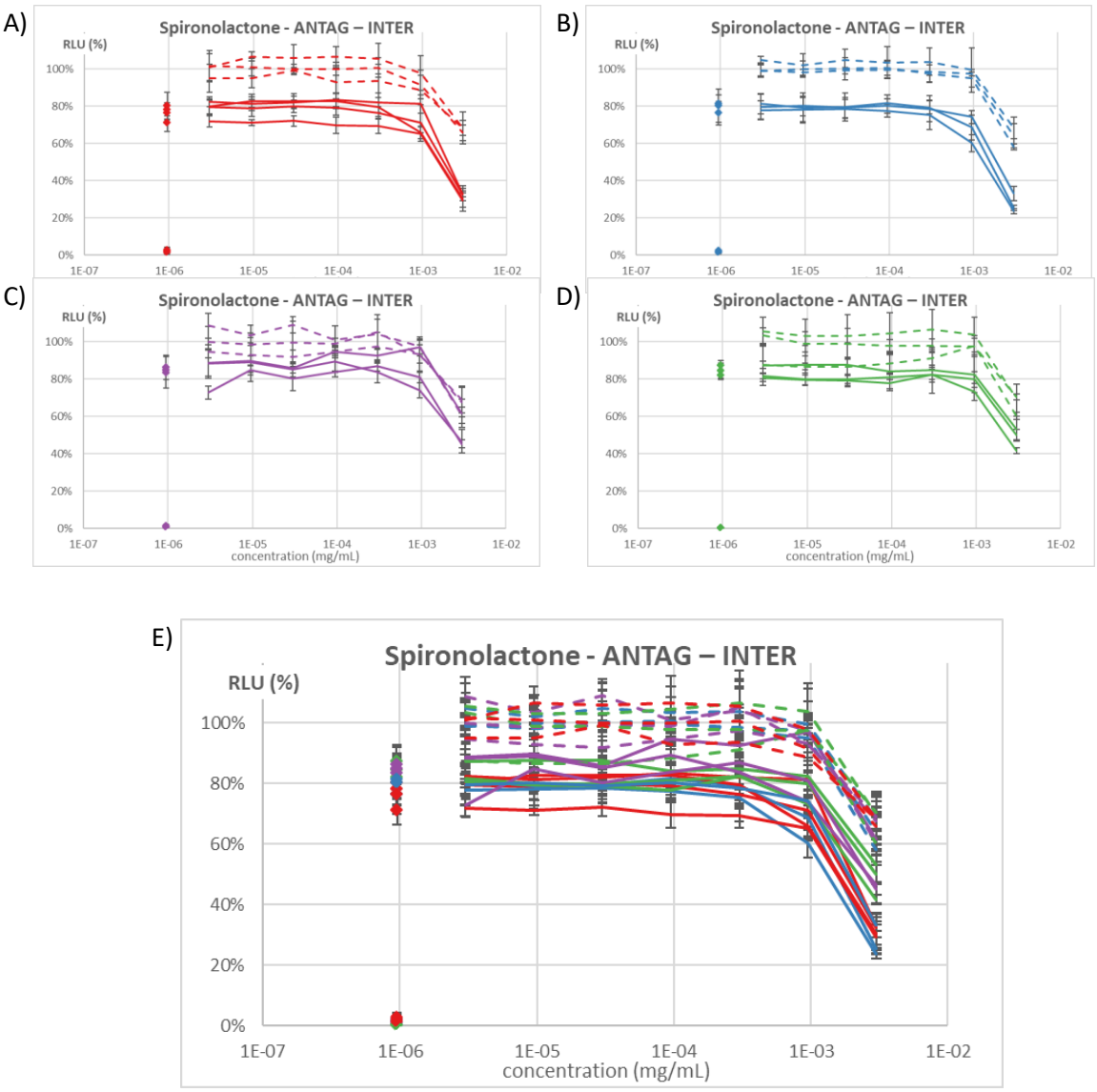


Figure 75: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Spironolactone in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode

The labs had diverging conclusions regarding Spironolactone. INSERM, Ineris and Tame-Water classified it as interferent, Toxem antagonist, at the concentrations tested. These differences may be due to the fact that only the last concentration tested showed a decrease in the signal, making the determination of IC_{50} and R^2 not fully reliable.

17ß-estradiol

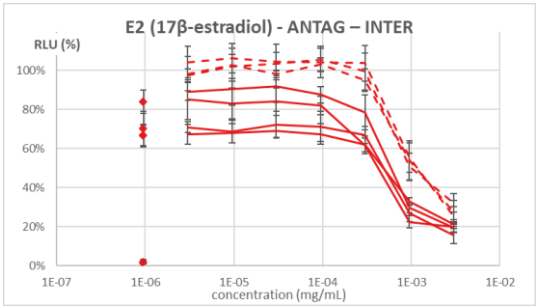
All labs tested 17β-estradiol from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL and observed a decrease in signal. They, then, tested it for potential interference mode. The correlation coefficient between antagonist and interference runs was above 0.9 for all labs, indicating that the decreased signal was due to interference.
The concentration-response curves and IC_{50} obtained are presented below.
INSERM had one run invalid due to too high basal response of the cells (>4%).
Toxem rejected two runs on the basis that they were different from others, even though they met the acceptance criteria.

Table 21: 17ß-estradiol IC_{50} values in antagonism and interference modes.

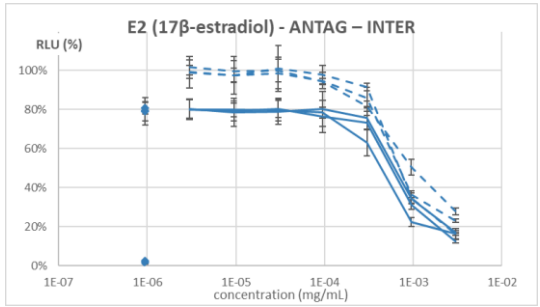
	Ineris		INSERM		Tame-Water		Toxem	
IC_{50} (mg/mL)	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
	$5,3E^{-04} \pm 4,8E^{-05}$	$8,4E^{-04} \pm 8,7E^{-05}$	$6,9E^{-04} \pm 4,1E^{-05}$	$7,0E^{-04} \pm 9,4E^{-05}$	$1,6E^{-02} \pm 3,1E^{-02}$	$1,1E^{-03} \pm 1,4E^{-04}$	$1,1E^{-03} \pm 3,1E^{-04}$	$9,3E^{-04} \pm 6,6E^{-05}$
	$6,4E^{-04} \pm 7,3E^{-05}$	$7,2E^{-04} \pm 8,0E^{-05}$	$4,4E^{-04} \pm 3,9E^{-05}$	$5,6E^{-04} \pm 4,3E^{-05}$	$7,9E^{-04} \pm 1,1E^{-04}$	$1,0E^{-02} \pm 1,8E^{-02}$	$7,0E^{-04} \pm 6,1E^{-05}$	$8,6E^{-04} \pm 1,5E^{-04}$
	$3,7E^{-04} \pm 3,4E^{-05}$	$7,9E^{-04} \pm 8,0E^{-05}$	$7,1E^{-04} \pm 7,1E^{-05}$	$6,4E^{-04} \pm 7,0E^{-05}$	$1,2E^{-03} \pm 2,6E^{-04}$	$8,2E^{-03} \pm 2,1E^{-02}$	$2,9E^{-03} \pm 5,1E^{-03}$	$5,0E^{-04} \pm 4,1E^{-05}$
	$6,4E^{-04} \pm 4,5E^{-05}$						$8,1E^{-04} \pm 7,3E^{-05}$	
							$7,9E^{-04} \pm 7,6E^{-05}$	
mean	$5,5E^{-04} \pm 1,3E^{-04}$	$7,9E^{-04} \pm 6,1E^{-05}$	$6,1E^{-04} \pm 1,5E^{-04}$	$6,3E^{-04} \pm 7,3E^{-05}$	$6,1E^{-03} \pm 8,8E^{-03}$	$6,5E^{-03} \pm 4,8E^{-03}$	$1,3E^{-03} \pm 9,2E^{-04}$	$7,6E^{-04} \pm 2,3E^{-04}$
R^2	0.96		0.98		0.95		0.93	

Despite some variability in IC_{50} within and between labs (mainly Tame-Water and Toxem), the conclusion regarding the effect of the substance is consistent, with R^2 above 0.9.

A)



B)



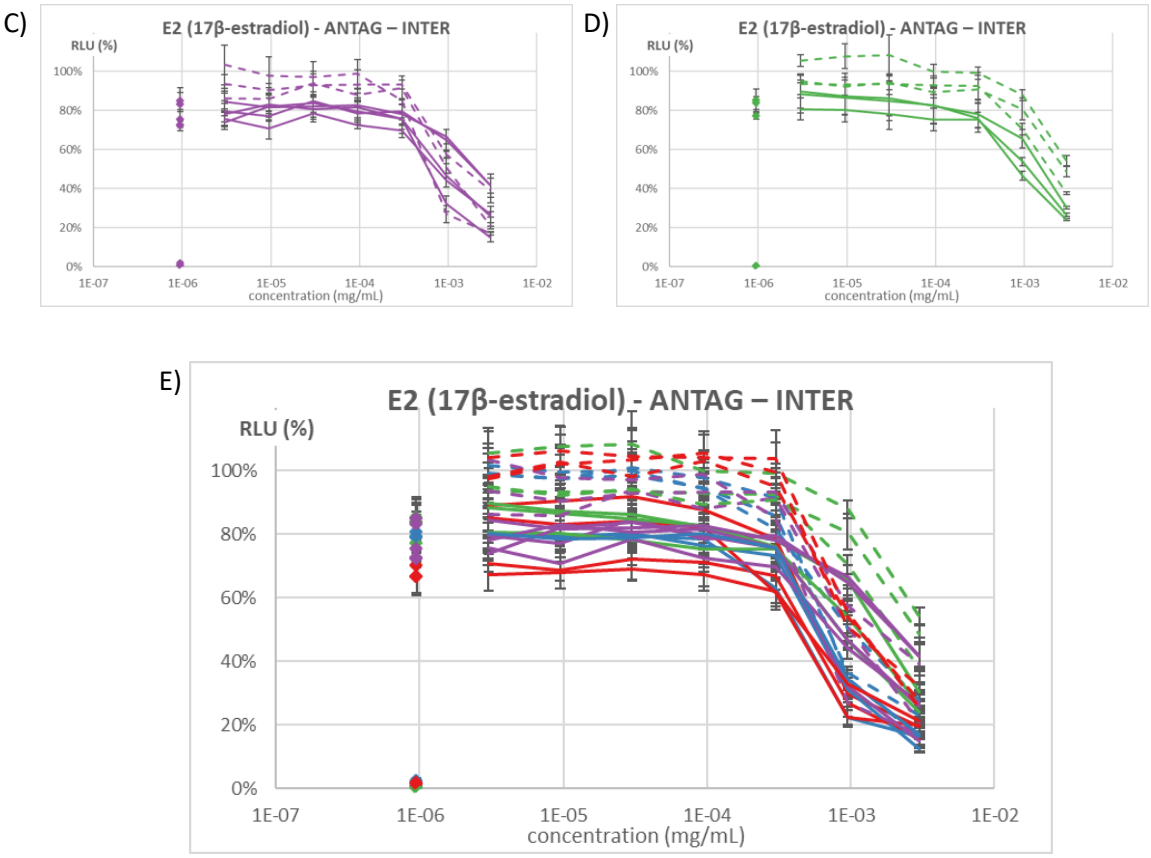


Figure 76: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to 17β-estradiol in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

The labs concluded that at the concentrations tested, 17β-estradiol is not an antagonist in this method but interferes with the method.

Fulvestrant

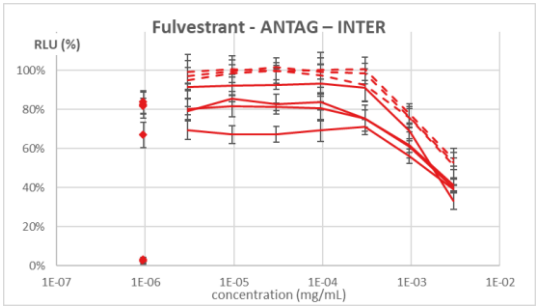
All labs tested Fulvestrant from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL and observed a decrease in signal. They, then, tested it for potential interference mode. The correlation coefficient between antagonist and interference runs was above 0.9 for three labs, indicating that the decreased signal was due to interference. Toxem found an R^2 of 0.88.
The concentration-response curves and IC_{50} obtained are presented below.

Table 22: Fulvestrant IC_{50} values in antagonism and interference modes.

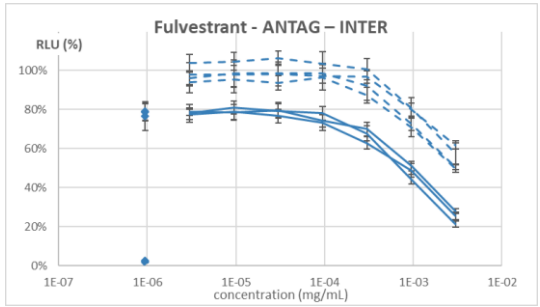
	Ineris		INSERM		Tame-Water		Toxem	
IC_{50} (mg/mL)	antagonism	interference	antagonism	interference	antagonism	interference	antagonism	interference
	$1,1E^{-03} \pm 2,6E^{-04}$	$1,3E^{-03} \pm 8,5E^{-04}$	$2,8E^{-03} \pm 3,2E^{-03}$	$1,0E^{-03} \pm 2,0E^{-04}$	$1,7E^{-03} \pm 2,2E^{-03}$	$1,1E^{-02} \pm 2,3E^{-02}$	$5,7E^{-04} \pm 1,9E^{-04}$	$7,6E^{-04} \pm 3,7E^{-04}$
	$1,5E^{-03} \pm 9,0E^{-04}$	$9,5E^{-04} \pm 5,8E^{-05}$	$8,9E^{-04} \pm 1,6E^{-04}$	$1,1E^{-03} \pm 2,6E^{-04}$	$5,5E^{-04} \pm 9,7E^{-05}$	$2,4E^{-03} \pm 5,2E^{-03}$	$1,3E^{-03} \pm 1,7E^{-03}$	$1,2E^{-03} \pm 6,0E^{-04}$
	$1,2E^{-03} \pm 3,8E^{-04}$	$9,6E^{-04} \pm 7,8E^{-05}$	$1,4E^{-03} \pm 6,7E^{-04}$	$3,8E^{-04} \pm 2,9E^{-04}$	$7,7E^{-04} \pm 8,2E^{-05}$	$4,7E^{-03} \pm 1,6E^{-02}$	$1,0E^{-03} \pm 3,0E^{-04}$	
	$9,8E^{-04} \pm 1,2E^{-04}$			$9,6E^{-04} \pm 9,1E^{-05}$				
mean	$1,2E^{-03} \pm 2,3E^{-04}$	$1,1E^{-03} \pm 1,8E^{-04}$	$1,7E^{-03} \pm 1,0E^{-03}$	$8,7E^{-04} \pm 3,4E^{-04}$	$1,0E^{-03} \pm 6,1E^{-04}$	$6,0E^{-03} \pm 4,3E^{-03}$	$9,4E^{-04} \pm 3,4E^{-04}$	$9,8E^{-04}$
R^2	0.98		0.97		0.92		0.88	

Some variability is observed in the IC_{50}, which could be due to the fact that the latest point of the curves is around the IC_{50}, leading also to variability in the R^2 and the discrepancy of conclusion between the labs.

A)



B)



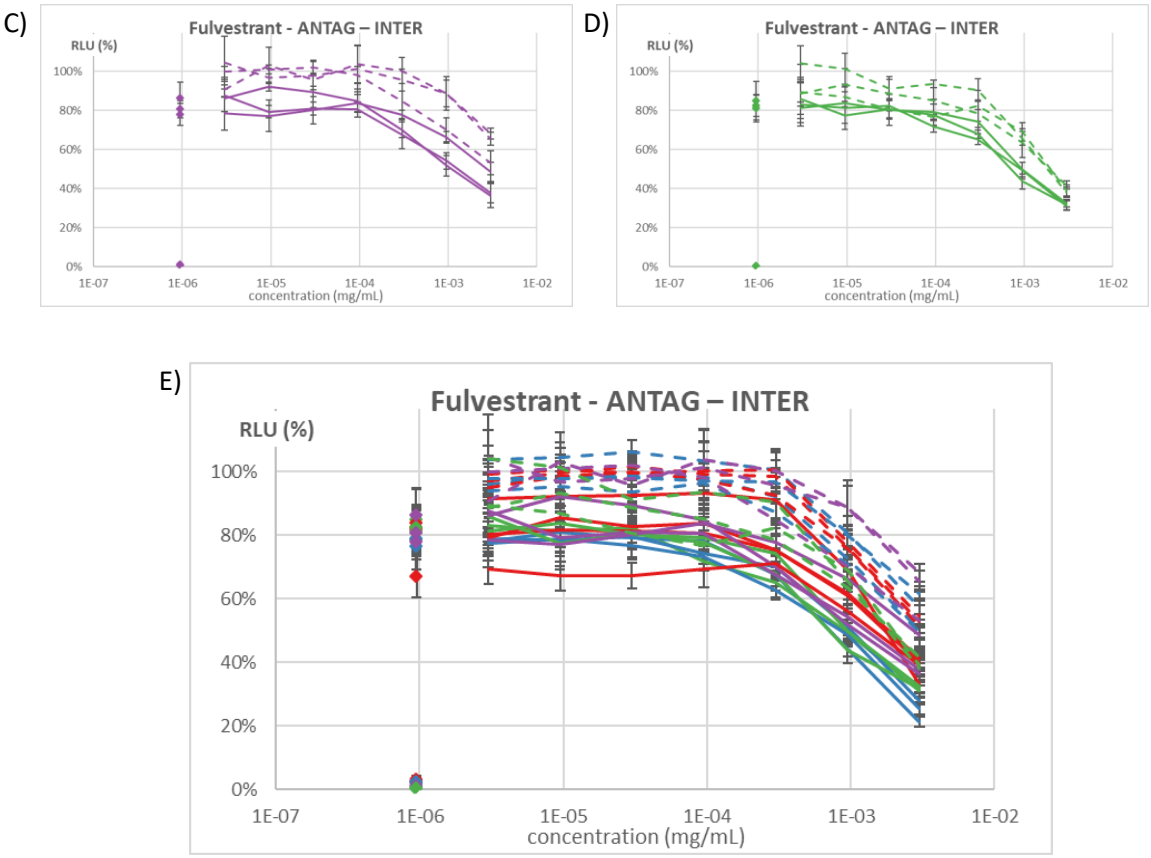
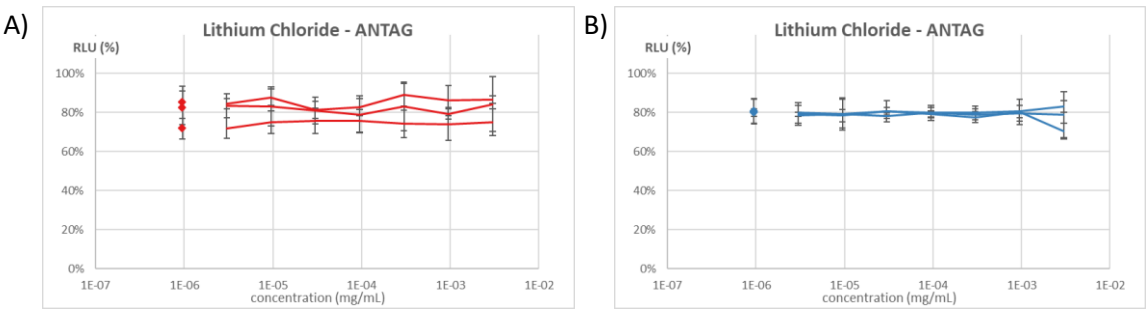


Figure 77: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Fulvestrant in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E). Solid lines: antagonist mode; dotted lines: interference mode.

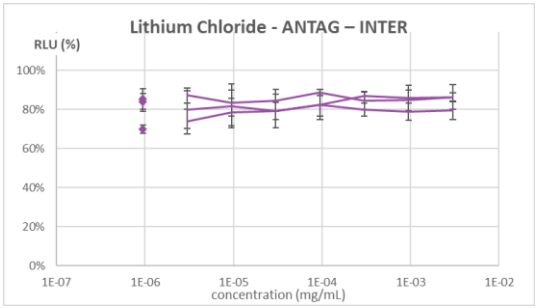
Three labs concluded that at the concentrations tested, Fulvestrant is not an antagonist in this method, but interferes with the method. One lab concluded, that Fulvestrant is antagonist. This discrepancy may be due to the fact that for some labs, the last point of the curve is around the IC_{50}.

Lithium Chloride

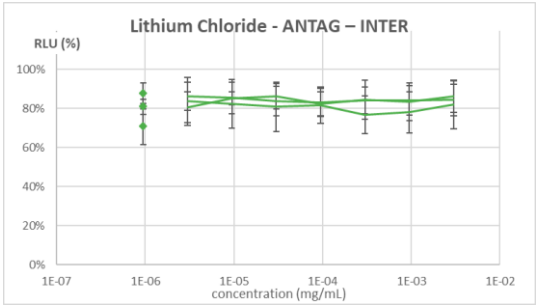
All labs tested Lithium chloride from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.



C)



D)



E)

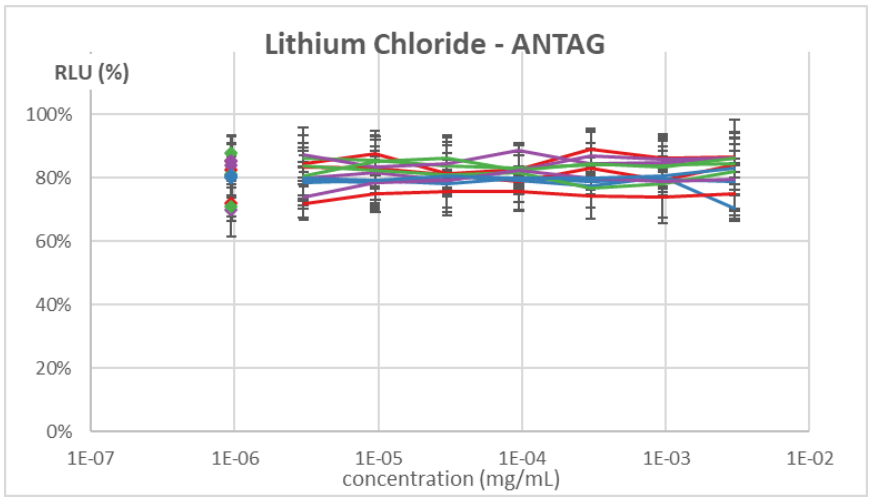


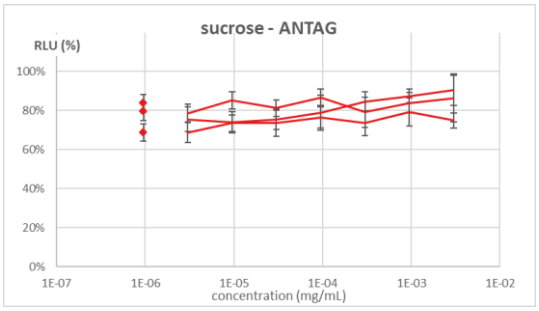
Figure 78: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Lithium Chloride in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Lithium chloride is not an antagonist in this method.

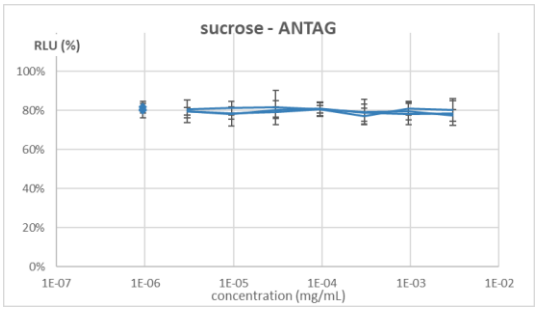
Sucrose

All labs tested Sucrose from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.

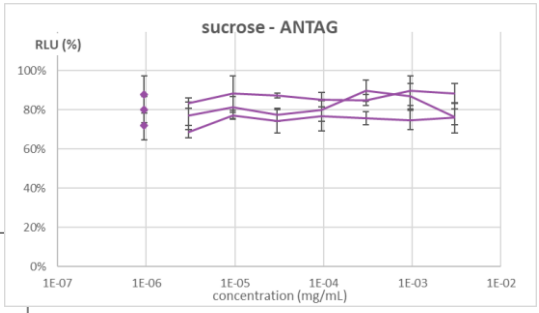
A)



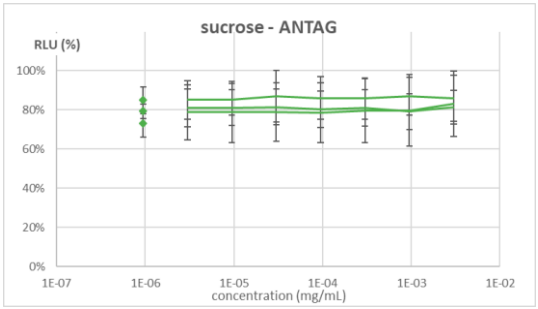
B)



C)



D)



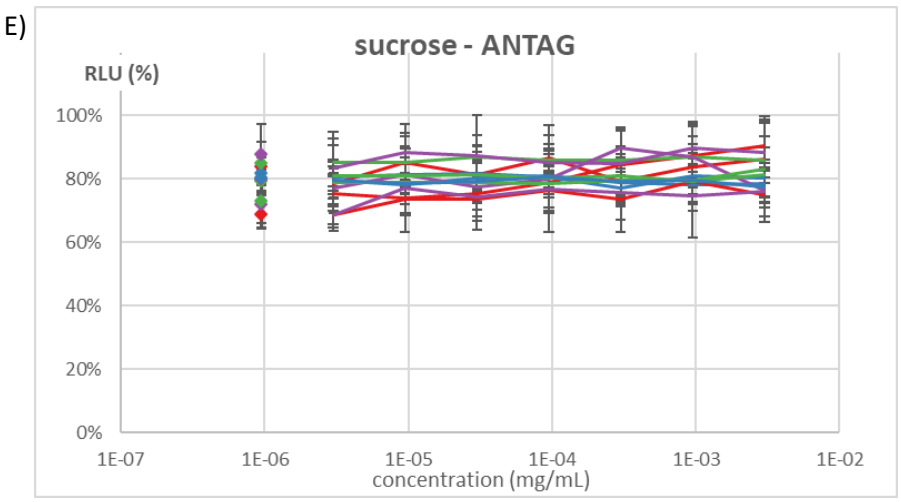
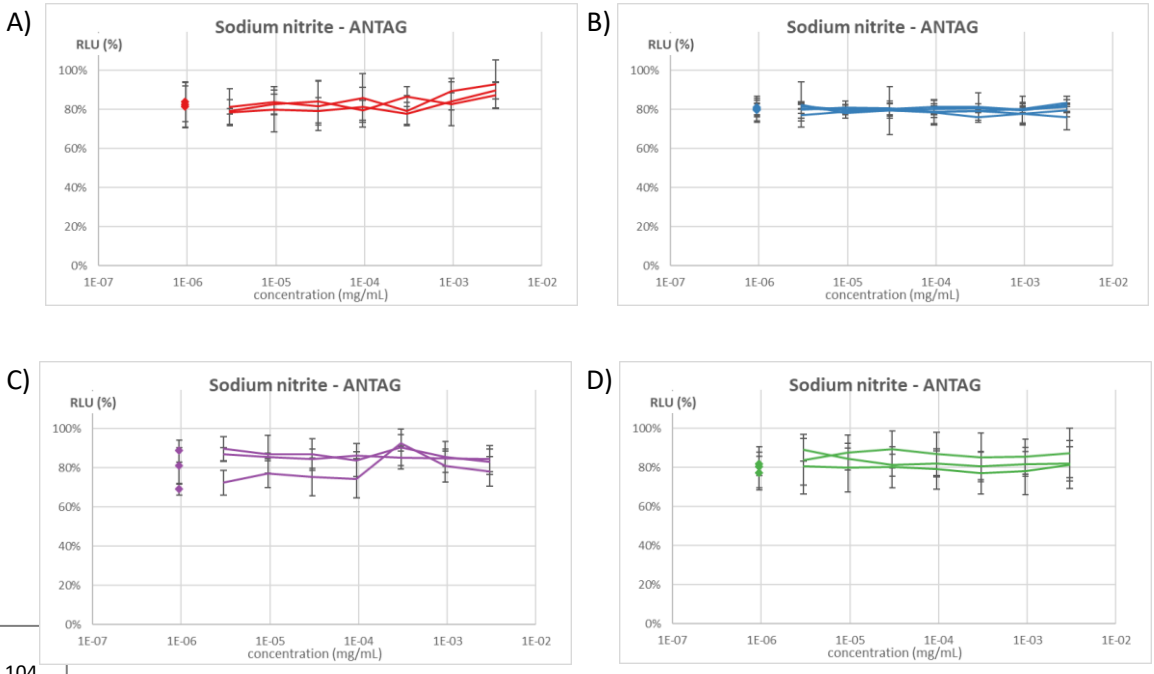


Figure 79: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sucrose in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Sucrose is not an antagonist in this method.

Sodium nitrite

All labs tested Sodium nitrite from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. The concentration-response curves obtained are presented below.
INSERM had one run invalid due to too high basal response of the cells (>4%), and one due to some cytotoxicity.
Toxem had one run invalid due to the acceptance criterion linked to EC_{80} (inducing between 65%-95% of activity) not being met.



E)

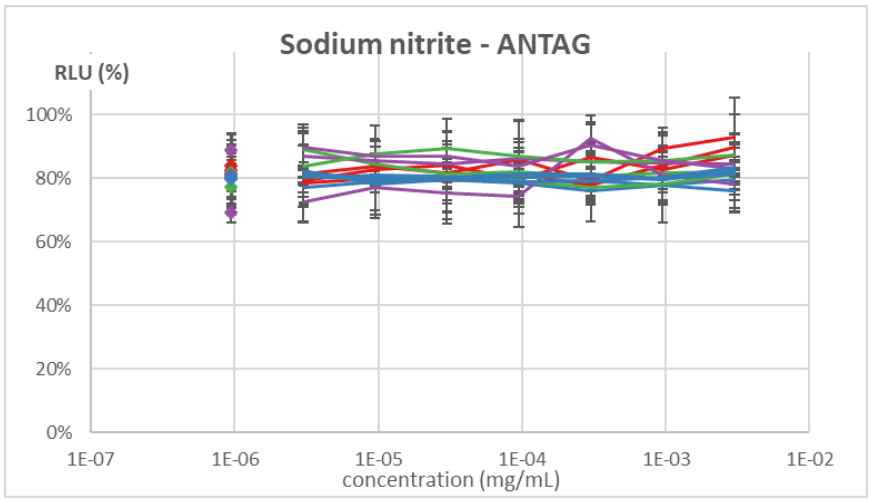


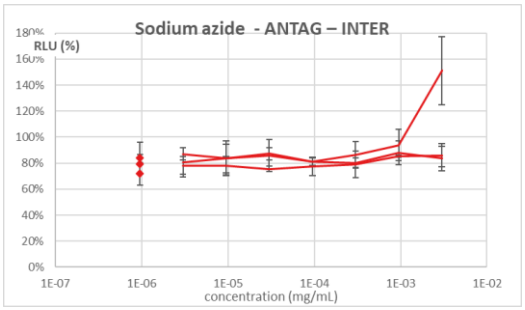
Figure 80: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sodium nitrite in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

All labs concluded that at the concentrations tested, Sodium nitrite is not an antagonist in this method.

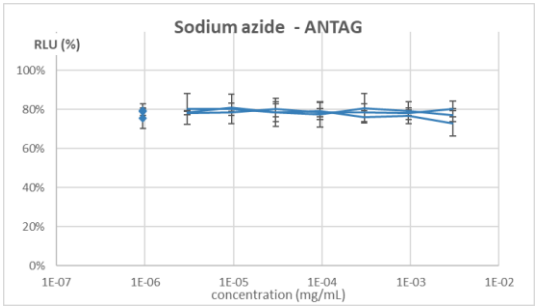
Sodium azide

All labs tested Sodium azide from $3.0E^{-6}$ to $3.0E^{-3}$mg/mL. Tame-Water observed a decrease in the signal and tested it in the interference mode.
The concentration-response curves obtained are presented below.

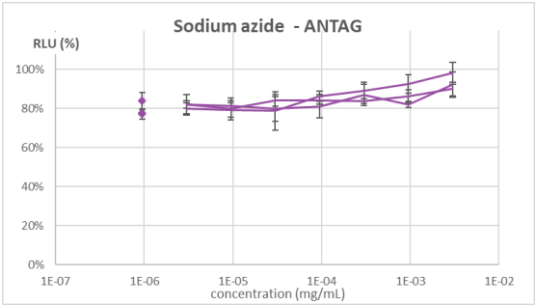
A)



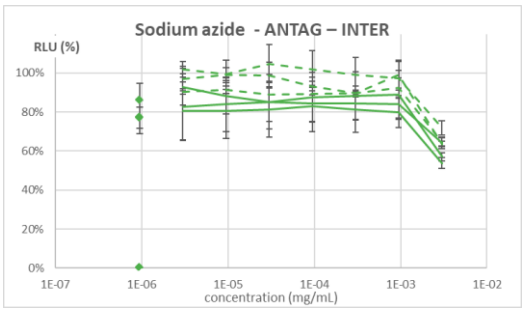
B)



C)



D)



E)

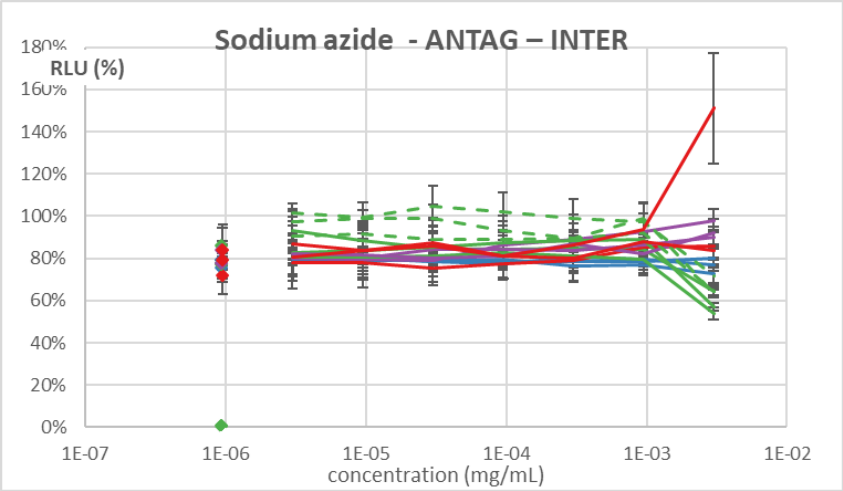


Figure 81: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sodium azide in antagonist mode in Ineris A), INSERM B), Toxem C), Tame-Water D), or in all labs E).

Three labs concluded that at the concentrations tested, Sodium azide is not an antagonist in this method. One lab obtained a correlation coefficient between antagonist and interference runs of 0.89, thus concluded that Sodium azide should be considered as an antagonist according to the criteria defined in the SOP.

4.4 Within laboratory reproducibility

The classifications (agonist/not agonist) for each valid run of the four laboratories are presented below.

Table 23: Laboratories classification per run for agonist mode of each run..

Name	Ineris			INSERM			Tame-Water			Toxem		
Bisphenol A	N	N	N	N	N	N	N	N	N	N	N	N
DEHP	N	N	N	N	N	N	N	N	N	N	N	N
MEHP	N	N	N	N	N	N	N	N	N	N	N	N
TBBPA	N	N	N	N	N	N	N	N	N	N	N	N
BHA	N	N	N	N	N	N	N	N	N	N	N	N
L-limonene	N	N	N	N	N	N	N	N	N	N	N	N
Oleic acid	N	N	N	N	N	N	N	N	N	N	N	N
Ginsenoside Rg1	N	N	N	N	N	N	N	N	N	N	N	N
Triclosan	N	N	N	N	N	N	N	N	N	N	N	N
Tolylfluanid	N	N	N	N	N	N	N	N	N	N	N	N
Butyl paraben	N	N	N	N	N	N	N	N	N	N	N	N
Propyl paraben	N	N	N	N	N	N	N	N	N	N	N	N
λ-Cyhalothrin	N	N	N	N	N	N	N	N	N	N	N	N
Cypermethrin	N	N	N	N	N	N	N	N	N	N	N	N
Methoxychlor	N	N	N	N	N	N	N	N	N	N	N	N
Arsenic	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Monocrotophos	N	N	N	N	N	N	N	N	N	N	N	N
Benzo[a]pyrene	N	N	N	N	N	N	N	N	N	N	N	N
2-Methylresorcinol	N	N	N	N	N	N	N	N	N	N	N	N
PFOS	N	N	N	N	N	N	N	N	N	N	N	N
Cortisol	P	P	P	P	P	P	P	P	P	P	P	P

Budesonide	P	P	P	P	P	P	P	P	P	P	P	P
Fluticasone propionate	P	P	P	P	P	P	P	P	P	P	P	P
Aldosterone	P	P	P	P	P	P	P	P	P	P	P	P
Progesterone	N	N	N	N	N	N	N	N	N	N	N	N
21-Hydroxyprogesterone	N	N	N	P	P	P	N	N	N	N	N	N
Mifepristone	N	N	N	N	N	N	N	N	N	N	N	N
Ketoconazole	N	N	P	N	N	N	N	N	N	N	N	N
Spironolactone	N	N	N	N	N	N	N	N	N	N	N	N
17β-estradiol	N	N	N	N	N	N	N	N	N	N	N	N
Fulvestrant	N	N	N	N	N	N	N	N	N	N	N	N
Lithium Chloride	N	N	N	N	N	N	N	N	N	N	N	N
sucrose	N	N	N	N	N	N	N	N	N	N	N	N
Sodium nitrite	N	N	N	N	N	N	N	N	N	N	N	N
Sodium azide	N	N	N	N	N	N	N	N	N	N	N	N
WLR	99%			100%			100%			100%		

N: negative agonist, P: positive agonist, In red the in-lab deviation.

The within laboratory reproducibility (WLR) for agonist testing is between 99% and 100% for each laboratory. The only discrepancy marked in red.

The classifications (antagonist/not antagonist) for each valid run of the four laboratories are presented below.

Table 24: Laboratories classification per run for antagonist/interference mode for each run.

Name	Ineris			INSERM			Tame-Water			Toxem		
Bisphenol A	N	N	N	N	N	N	N	N	N	N	N	N
DEHP	N	N	N	N	N	N	N	N	N	N	N	N
MEHP	N	N	N	N	N	N	N	N	N	N	N	N
TBBPA	N	N	N	N	N	N	N	N	N	N	N	N
BHA	N	N	N	N	N	N	N	N	N	N	N	N
L-limonene	N	N	N	N	N	N	N	N	N	N	N	N
Oleic acid	N	N	N	N	N	N	N	N	N	N	N	N
Ginsenoside Rg1	N	N	N	N	N	N	N	N	N	N	N	N
Triclosan	P	P	P	P	P	P	N	N	N	P	P	P
Tolylfluanid	N	N	N	N	N	N	N	N	N	N	N	N
Butyl paraben	N	N	N	N	N	N	N	N	N	N	N	N
Propyl paraben	N	N	N	N	N	N	N	N	N	N	N	N
λ-Cyhalothrin	N	N	N	N	N	N	N	N	N	N	N	N
Cypermethrin	N	N	N	N	N	N	N	N	N	N	N	N
Methoxychlor	N	N	N	N	N	N	N	N	N	N	N	N
Arsenic	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Monocrotophos	N	N	N	N	N	N	N	N	N	N	N	N
Benzo[a]pyrene	N	N	N	N	N	N	N	N	N	N	N	N
2-Methylresorcinol	N	N	N	N	N	N	N	N	N	N	N	N
PFOS	N	N	N	N	N	N	N	N	N	N	N	N
Cortisol	N	N	N	N	N	N	N	N	N	N	N	N
Budesonide	N	N	N	N	N	N	N	N	N	N	N	N
Fluticasone propionate	N	N	N	N	N	N	N	N	N	N	N	N
Aldosterone	N	N	N	N	N	N	N	N	N	N	N	N
Progesterone	P	P	P	P	P	P	P	P	P	P	P	P

21-Hydroxyprogesterone	P	P	P	P	P	P	P	P	P	P	P	P
Mifepristone	P	P	P	P	P	P	P	P	P	P	P	P
Ketoconazole	N	N	N	N	N	N	N	N	N	N	N	N
Spironolactone	P	P	P	P	P	P	P	P	P	P	P	P
17β-estradiol	P	P	P	P	P	P	P	P	P	P	P	P
Fulvestrant	P	P	P	P	P	P	P	P	P	P	P	P
Lithium Chloride	N	N	N	N	N	N	N	N	N	N	N	N
sucrose	N	N	N	N	N	N	N	N	N	N	N	N
Sodium nitrite	N	N	N	N	N	N	N	N	N	N	N	N
Sodium azide	N	N	N	N	N	N	N	N	N	N	N	N
	100%			100%			100%			100%		

N: negative antagonist, P: positive antagonist or interferent

The within laboratory reproducibility for antagonist testing is 100% for each laboratory.

4.5 Between laboratory reproducibility

The between laboratory reproducibility for agonist testing is presented below.

Table 25: Laboratory reproducibility for agonist mode.

Name	Ineris	INSERM	Tame-Water	Toxem	BLR
Bisphenol A	N	N	N	N	100%
DEHP	N	N	N	N	100%
MEHP	N	N	N	N	100%
TBBPA	N	N	N	N	100%
BHA	N	N	N	N	100%
L-limonene	N	N	N	N	100%
Oleic acid	N	N	N	N	100%
Ginsenoside Rg1	N	N	N	N	100%
Triclosan	N	N	N	N	100%
Tolylfluanid	N	N	N	N	100%
Butyl paraben	N	N	N	N	100%
Propyl paraben	N	N	N	N	100%
λ-Cyhalothrin	N	N	N	N	100%
Cypermethrin	N	N	N	N	100%
Methoxychlor	N	N	N	N	100%
Arsenic	nt	nt	nt	nt	nt
Monocrotophos	N	N	N	N	100%
Benzo[a]pyrene	N	N	N	N	100%
2-Methylresorcinol	N	N	N	N	100%
PFOS	N	N	N	N	100%
Cortisol	P	P	P	P	100%
Budesonide	P	P	P	P	100%
Fluticasone propionate	P	P	P	P	100%
Aldosterone	P	P	P	P	100%
Progesterone	N	N	N	N	100%
21-Hydroxyprogesterone	N	P	N	N	75%
Mifepristone	N	N	N	N	100%
Ketoconazole	N	N	N	N	100%
Spironolactone	N	N	N	N	100%

17β-estradiol	N	N	N	N	100%
Fulvestrant	N	N	N	N	100%
Lithium Chloride	N	N	N	N	100%
sucrose	N	N	N	N	100%
Sodium nitrite	N	N	N	N	100%
Sodium azide	N	N	N	N	100%

N: negative agonist, P: positive agonist, in red the between-lab deviation.

The between laboratory reproducibility is exceptionally high (97%), with only 1 test item (red) out of 34 showing discrepancy in classification.
Indeed, for 21-Hydroxyprogesterone, all labs observed an increase of luciferase activity, but only INSERM noted an increase above 10% of maximum effect of positive control for at least two consecutive concentrations, qualifying the test item as "agonist".

The between laboratory reproducibility for antagonist testing is presented below.
Here was also only one discrepancy, the negative for triclosan from one lab.

Table 26: Laboratory reproducibility for antagonist/interference mode.

Name	Ineris	INSERM	Tame-Water	Toxem	BLR
Bisphenol A	N	N	N	N	100%
DEHP	N	N	N	N	100%
MEHP	N	N	N	N	100%
TBBPA	N	N	N	N	100%
BHA	N	N	N	N	100%
L-limonene	N	N	N	N	100%
Oleic acid	N	N	N	N	100%
Ginsenoside Rg1	N	N	N	N	100%
Triclosan	P	P	N	P	75%
Tolylfluanid	N	N	N	N	100%
Butyl paraben	N	N	N	N	100%
Propyl paraben	N	N	N	N	100%
λ-Cyhalothrin	N	N	N	N	100%
Cypermethrin	N	N	N	N	100%
Methoxychlor	N	N	N	N	100%
Arsenic	nt	nt	nt	nt	nt
Monocrotophos	N	N	N	N	100%
Benzo[a]pyrene	N	N	N	N	100%
2-Methylresorcinol	N	N	N	N	100%
PFOS	N	N	N	N	100%
Cortisol	N	N	N	N	100%
Budesonide	N	N	N	N	100%
Fluticasone propionate	N	N	N	N	100%
Aldosterone	N	N	N	N	100%
Progesterone	P	P	P	P	100%
21-Hydroxyprogesterone	P	P	P	P	100%
Mifepristone	P	P	P	P	100%
Ketoconazole	N	N	N	N	100%
Spironolactone	P	P	P	P	100%
17β-estradiol	P	P	P	P	100%

Fulvestrant	P	P	P	P	100%
Lithium Chloride	N	N	N	N	100%
sucrose	N	N	N	N	100%
Sodium nitrite	N	N	N	N	100%
Sodium azide	N	N	N	N	100%

N: negative antagonist, P: positive antagonist or interferent, in red the between-lab deviation.

4.6 Conclusions on reproducibility

The results on inter- and intra-laboratory reproducibility are exceptionally high (tables 25 and 26). This points to a very robust and reproducible assay that can be transferred to other labs with confidence and that it will produce similar results as in other labs. It means that cells can be grown/maintained very similarly in different labs, the SOP is well written and can be followed with ease, the treatment and the results are highly similar in and between labs. If one applies the criteria that if more than 3 labs agree on the call for each compound there is consensus, there would be 100 % consensus in this validation study.

When looking further at the reproducibility, the assessment of potency for DEX (Table 7) is a good example where all labs have made numerous determinations. Here the labs determined the EC_{50} a total of 48 times and in each case the standard deviation was also calculated. Generally, the %CV (percentage deviation from average; STDEV/EC*100) was calculated to be close to 15% or below (often well below), with three exceptions above. The outlier criterion of 30% ensures that the %CV would be below this, but in far the most cases the %CV is much lower. The generally low %CV speaks to the robustness and strong reproducibility of the assay.

Table 27. Calculated %CV of the EC_{50} and Stdev determinations in Table 9.

	Ineris	INSERM	Tame-Water	Toxem
%CV of EC_{50} values	10,4	5,2	10,0	17,3
	10,0	9,6	15,0	9,0
	10,3	2,9	8,6	8,2
	7,1	4,9	10,0	11,0
	11,0	10,5	7,1	11,4
	7,5	5,0	7,7	11,1
	8,7	6,1	11,7	10,8
	10,2	5,4	18,6	8,6
	8,9	8,8	14,6	6,0
	8,0	4,7	22,5	6,9
	3,7	4,3	11,2	
	2,7		14,3	
	10,8			
	11,7			
	9,1			

4.7 Changes to the SOP at the end of phase 2

Following phase 2, some changes were implemented in the SOP. The main changes were:

- Solubility assay

The description of the determination of solubility using the stepwise (tiered) procedure (from OECD guidance document 129) was added, as well as precision regarding mixing procedure.

The starting point for solubility testing (as per OECD guidance document) is 200 mg/mL.

- Cytotoxicity assay

The starting point to assess the cytotoxicity of test items was clarified: "*Chemicals are tested up to the maximum soluble concentration in test medium*". The dilution factor was also enlarged (from 3.162 half-log serial dilutions to a log dilution factor), to cover a larger concentrations range.

A mention regarding the morphology of the cells was added, as it must be assessed and if considered as not normal, the concentration must be excluded (even if not cytotoxic in the MTT assay).

- Range Finding and concentration selection

Following results obtained with the cytotoxicity assay, a range-finding experiment is performed (note, that it was not done by the labs in phase II), where the concentrations to use in further experiments are selected. In phase II, highest concentration used was the same for all test items; SOP now precises that the highest concentration, in the first range-finding experiment is the maximum soluble non-toxic concentration and that the dilution factor is test item-dependent.

- In vitro transcriptional activation assay

Precisions were added regarding interference mode (for chemicals showing an antagonist activity):

- Interference and antagonist modes are tested on the same plate, for the same test item.

Test items that showed antagonist activity during the range finding should be tested in three independent antagonist/interference runs (which means 4 antagonist runs when test item is positive in antagonist mode of the range-finding assay).

- Data Analysis

Outlier precision/check: Maximum one outlier can be removed (1/4) and an explanation for the discrimination must be provided.

- Prediction Model

Precisions were added regarding classification as agonist or antagonist.

- Agonistic: a test item is positive when at least two consecutive concentrations or when the highest concentration increases the luciferase activity.

- Antagonistic: When the luciferase activity is decreased by more than 20% for at least two consecutive concentrations, in the presence of the reference chemical at the concentration inducing 80% of the maximal response, it is classified as an antagonist. Previously, the threshold percentage was 30%.

- Acceptance criteria

Precision was added regarding the basal activity of the cells: it must be ≤ 4% of maximum luminescence (instead of around 4%).

4.8 New test phase

During the second phase of the validation, the labs did not report any particular issues.
The intra- and inter-laboratory reproducibility's were very good. However, as many compounds were not showing any activities it was discussed to add further compounds and implement the amendments to the SOP. In some cases, the labs would change the concentration of a test item to investigate if that would change the results and test the limits of the assay.

Below is this supplementary phase with two labs (INSERM and Toxem) participating. This phase had 12 test items (Table 28), implementing the changes listed above on the SOP, in particular higher testing concentrations.

5. Supplementary Phase

5.1 Test items

The concentrations used by the labs for this supplementary phase are presented below.
INSERM did some extra runs focused on the concentration range close to cytotoxicity, using a lower dilution factor.
During phase 2, the labs were supposed to test Arsenic (in its metal form). As they were unable to solubilize it, it was decided to test during this supplementary phase Sodium arsenite.

Table 28: Concentrations used for supplementary phase.

Substances	INSERM (mg/mL)	INSERM (re-test) (mg/mL)	Toxem (mg/mL)
Bisphenol A	$2.01E^{-4} – 2.0E^{-1}$	$5.27E^{-3} – 6.0E^{-2}$	$1.28E^{-6} – 2.0E^{-2}$
Ginsenoside	$6.0E^{-9} – 6.0E^{-3}$		$1.28E^{-5} – 2.0E^{-1}$
Triclosan	$2.01E^{-4} – 2.0E^{-1}$	$5.27E^{-3} – 6.0E^{-2}$	$4.03E^{-7} – 6.3E^{-3}$
Butylparaben	$2.01E^{-4} – 2.0E^{-1}$		$4.03E^{-6} – 6.3E^{-2}$
Propylparaben	$2.01E^{-4} – 2.0E^{-1}$		$1.3E^{-6} – 2.0E^{-2}$
λ-Cyhalothrin	$2.0E^{-7} – 2.0E^{-1}$		$1.3E^{-6} – 2.0E^{-2}$
Cypermethrin	$2.0E^{-7} – 2.0E^{-1}$		$4.0E^{-7} – 6.3E^{-3}$
Methoxychlor	$2.01E^{-4} – 2.0E^{-1}$		$4.0E^{-7} – 6.3E^{-3}$
PFOS	$2.0E^{-7} – 2.0E^{-1}$		$4.03E^{-6} – 6.3E^{-2}$
Ketoconazole	$2.0E^{-5} – 2.0E^{-2}$	$1.76E^{-3} – 2.0E^{-2}$	$4.03E^{-7} – 6.3E^{-3}$
Lithium Chloride	$2.0E^{-8} – 2.0E^{-2}$		$3.8E^{-6} – 6.0E^{-2}$
Sodium Arsenite	$2.0E^{-5} – 2.0E^{-3}$	$1.8E^{-3} – 2.0E^{-2}$	$2.03E^{-5} – 2.0E^{-3}$

5.2 Results from supplementary phase

Agonist testing

The tests run in agonist mode did not change the classifications obtained in phase 2 despite the higher concentrations used.

Antagonist/interference testing

Only the test items for which a difference was observed compared to phase 2 are presented here.

Bisphenol A

The two labs tested BPA at different concentrations ($1.28E^{-06} – 2.2E^{-02}$ and $2.0E^{-07} – 6.0E^{-02}$ mg/mL), leading to different conclusions regarding its activity in the method.
INSERM observed a decrease in signal at the highest concentrations and tested it in interference mode.
The IC_{50} values and dose-response curves obtained are presented below.

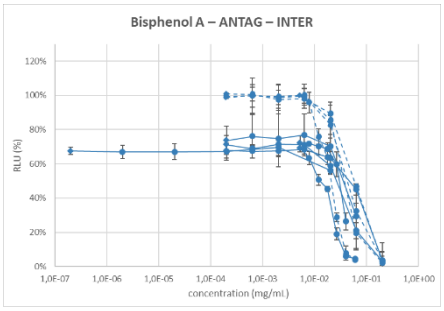
Table 29: IC_{50} values in antagonism and interference modes.

	INSERM	
	Antagonism	Interference
IC_{50}(±SD) in mg/mL	$1,40E^{-02} \pm 1,09E^{-01}$	$6,46E^{-02} \pm 6,04E^{-03}$
	$1,17E^{-01} \pm 3,83E^{-02}$	$4,47E^{-02} \pm 5,90E^{-03}$
	$3,07E^{-02} \pm 5,78E^{-02}$	$3,76E^{-02} \pm 3,17E^{-03}$
	$4,17E^{-02} \pm 1,96E^{-03}$	$3,94E^{-02} \pm 5,37E^{-02}$
	$4,98E^{-02} \pm 4,42E^{-02}$	$2,03E^{-02} \pm 8,80E^{-04}$
	$2,01E^{-02} \pm 1,06E^{-03}$	
Mean	$4,56E^{-02} \pm 3,4E^{-02}$	$4,13E^{-02} \pm 1,4E^{-02}$
R^2	0.99	

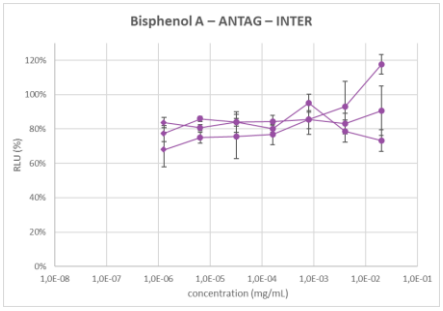
*estimation does not converge

A low variability was observed in the IC_{50}, except for one run.

A)



B)



C)

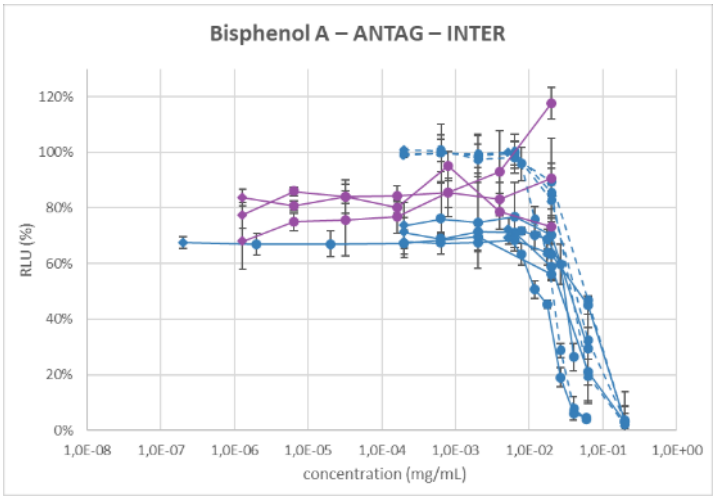


Figure 82: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Bisphenol A in antagonist mode in INSERM A), Toxem B), or in all labs C). Solid lines: antagonist mode; dotted lines: interference mode.

One lab concluded that BPA is interferent, one concluded no effect. The difference in conclusions seems due to the difference in the concentrations tested.

These data are in agreement with the conclusions obtained during the phase 2 where BPA, tested up to $3.0E^{-3}$mg/mL did not show any activity.

Triclosan

Triclosan was tested from $5.2E^{-04}$ to $6.0E^{-01}$ at INSERM, and from $4.0E^{-07}$ to $6.3E^{-03}$ mg/mL at Toxem.

Labs observed a decrease in signal at the highest concentrations and tested Triclosan in interference mode.
The IC_{50} values and dose-response curves obtained are presented below.

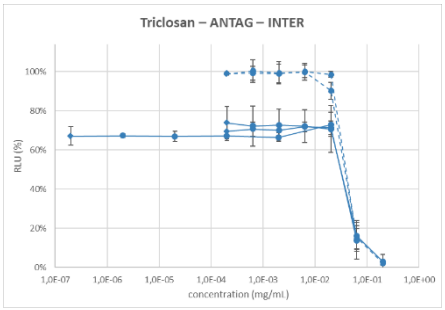
Table 30: IC_{50} values in antagonism and interference modes.

	INSERM		Toxem	
	Antagonism	Interference	Antagonism	Interference
IC_{50}(±SD) in mg/mL		$7,55E^{-02}$± $7,54E^{-01}$	$6,13E^{-03}$± $5,64E^{-02}$	$2,09E^{-03}$± $1,01E^{-04}$
	$7,13E^{-02}$± $1,55E^{-01}$	$3,77E^{-02}$± $2,05E^{-03}$	$3,25E^{-03}$± $2,81E^{-04}$	$2,26E^{-03}$± $6,60E^{-04}$
	$4,51E^{-02}$± $7,41E^{-03}$		$3,50E^{-03}$± $2,16E^{-04}$	$2,88E^{-03}$± $1,72E^{-04}$
			$1,46E^{-02}$± $2,44E^{-02}$	
Mean	$5,82E^{-02}$	$5,66E^{-02}$	$6,88E^{-03}$± $4,6E^{-03}$	$2,41E^{-03}$± $3,4E^{-04}$
R^2	0.99		0.70	

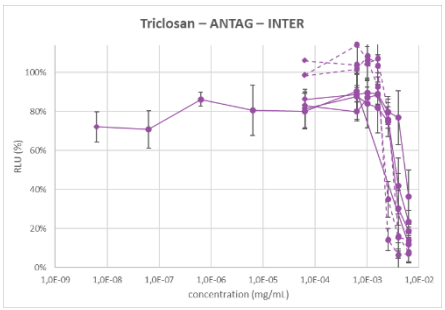
A good intra-laboratory reproducibility in IC_{50} values can be observed.
There is a difference between the IC_{50} on the two laboratories of about one log. This difference can also be found in the data from INSERM between phase 2 and the supplementary phase. The lab could not exclude a mistake in the preparation of the dilutions during the supplementary phase.

Based on the correlation coefficient between antagonist and interference runs, labs conclusion diverges. Triclosan is considered as interferent by INSERM and antagonist by Toxem.

A)



B)



C)

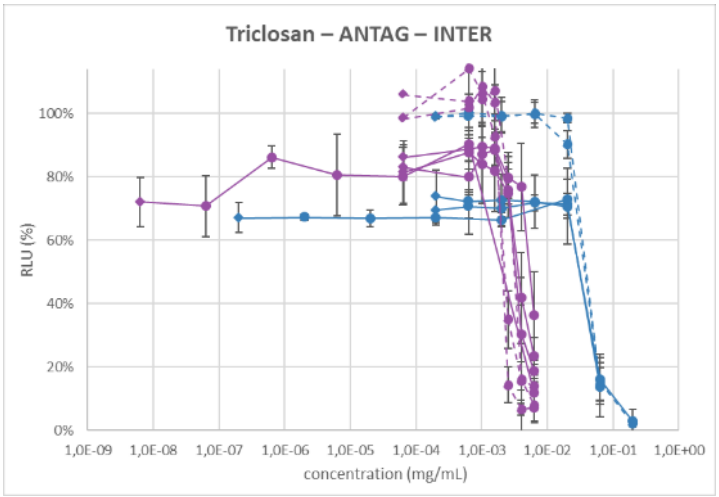


Figure 83: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Triclosan in antagonist and interference modes in INSERM A), Toxem B), or in all labs C). Solid lines: antagonist mode; dotted lines: interference mode.

The conclusion of the labs converges on the reality of an interaction, but they diverge regarding the modality through which Triclosan interacts (antagonist vs interferent). This may be linked to the method criterion, which is based on the correlation coefficient between runs. It is worth noticing that INSERM had very few/no point on the slope of the curve, leading to some uncertainty on the fitting of the curves. This may explain why INSERM concluded that Triclosan is interfering with the assay. Note that in phase 2, the two labs concluded that Triclosan was an interferent in the method.

Butyl paraben

Butyl paraben was tested from $2.0E^{-04}$ to $2.0E^{-01}$ at INSERM and from $6.4E^{-04}$ to $6.3E^{-02}$mg/mL at Toxem.

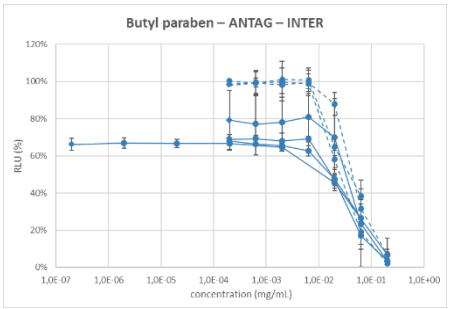
The IC_{50} values and dose-response curves obtained are presented below.

Table 31: IC_{50} values in antagonism and interference modes.

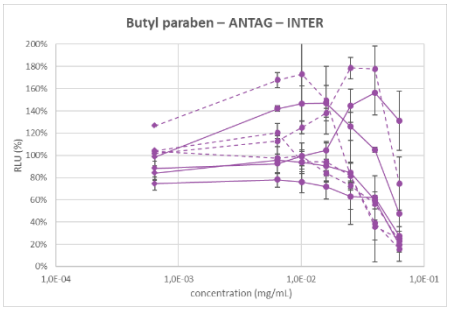
	INSERM		Toxem	
	Antagonism	Interference	Antagonism	Interference
IC_{50}(±SD) in mg/mL	$4,13E^{-02} \pm 1,92E^{-02}$	$1,93E^{-02} \pm 2,80E^{-03}$	$4,88E^{-02} \pm 2,09E^{-02}$	$2,41E^{-02} \pm 1,87E^{-03}$
	$2,73E^{-02} \pm 9,05E^{-03}$	$4,43E^{-02} \pm 2,96E^{-03}$	$4,58E^{-02} \pm 7,81E^{-03}$	$3,50E^{-02} \pm 7,01E^{-03}$
	$4,43E^{-02} \pm 1,47E^{-02}$	$2,45E^{-02} \pm 1,85E^{-03}$	$1,85E^{-01} \pm 3,55E^{-01}$	$2,09E^{-01} \pm 5,37E^{-01}$
	$3,12E^{-02} \pm 2,79E^{-03}$			
Mean	$3,60E^{-02} \pm 8,07E^{-03}$	$2,93E^{-02} \pm 1,32E^{-02}$	$9,31E^{-02} \pm 7,93E^{-02}$	$8,95E^{-02} \pm 1,04E^{-01}$
R^2	0.99		0.81	

The IC_{50} show a very good intra-laboratory reproducibility.
The two labs diverge regarding the classification of Butyl paraben, regarding the correlation coefficient between antagonist and interference runs.

A)



B)



C)

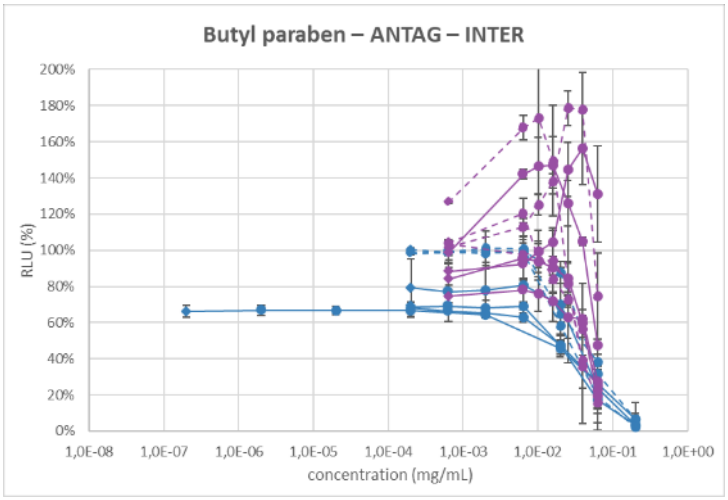


Figure 84: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Butyl paraben in antagonist mode in INSERM A), Toxem B), or in all labs C). Solid lines: antagonist mode; dotted lines: interference mode.

Both labs conclude that Butyl paraben decrease the luciferase activity in the method. They diverge on the classification; Butyl paraben being interferent for INSERM and antagonist for Toxem.

These data are in agreement with the conclusions obtained during the phase 2 where Butyl paraben, tested up to 3.0E-3mg/mL did not show any activity.

Propyl paraben

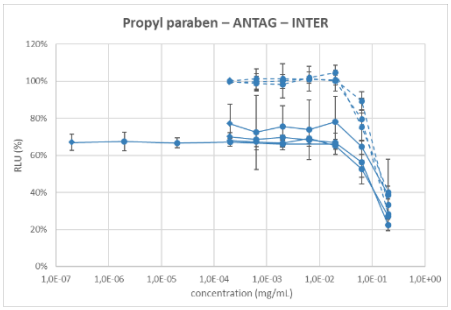
Propyl paraben was tested from $2.0E^{-04}$ to $2.0E^{-01}$ at INSERM and from $1.3E^{-06}$ to $2.0E^{-02}$mg/mL at Toxem. The IC_{50} values and dose-response curves obtained are presented below.

Table 32: IC_{50} values in antagonism and interference modes.

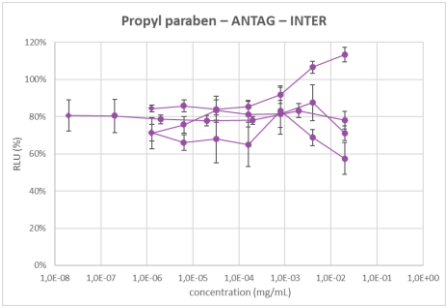
	INSERM	
	Antagonism	Interference
IC_{50}(±SD) in mg/mL	$9,73E^{-02} \pm 3,93E^{-02}$	$6,58E^{-02} \pm 5,96E^{-03}$
	$7,23E^{-02} \pm 6,90E^{-02}$	$7,62E^{-02} \pm 5,67E^{-02}$
	$2,51E^{-01} \pm 3,76E^{-01}$	$1,03E^{-01} \pm 1,05E^{-01}$
Mean	$1,40E^{-01} \pm 9,67E^{-02}$	$8,16E^{-02} \pm 1,90E^{-02}$
R^2	0.98	

The IC_{50} show a good intra-laboratory reproducibility.

A)



B)



C)

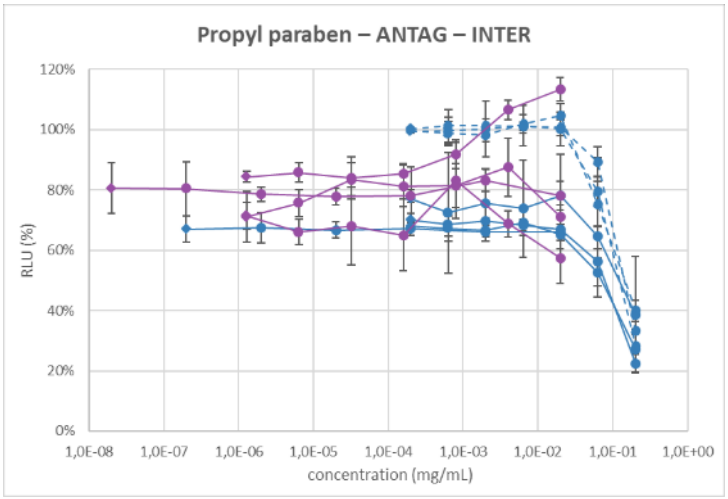


Figure 85: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Propyl paraben in antagonist mode in INSERM A), Toxem B), or in all labs C). Solid lines: antagonist mode; dotted lines: interference mode.

Only INSERM observed some activity for Propyl paraben in the antagonist/interference mode, classifying it as interferent. The difference of classification between the labs may be due to the different concentrations tested.

These data are in agreement with the conclusions obtained during the phase 2 where Propyl paraben, tested up to $3.0E^{-3}$mg/mL did not show any activity.

Methoxychlor

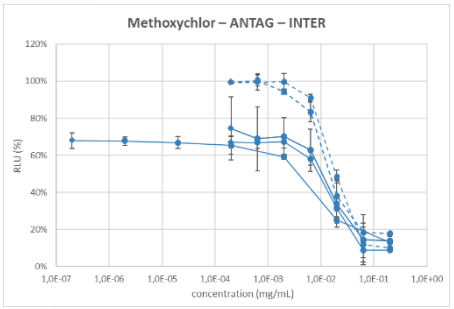
Methoxychlor was tested from $2.0E^{-04}$ to $2.0E^{-01}$ by INSERM, and from $4.0E^{-07}$ to $6.3E^{-03}$mg/mL by Toxem.
The IC_{50} values and dose-response curves obtained are presented below.

Table 33: IC_{50} values in antagonism and interference modes.

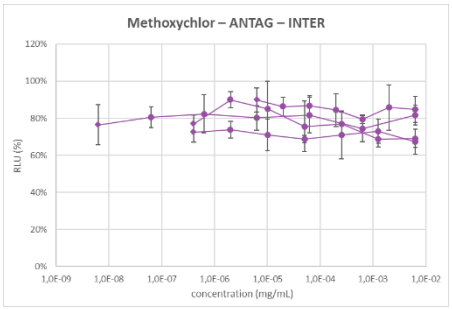
	INSERM	
	Antagonism	Interference
IC_{50}(±SD) in mg/mL	$7,97E^{-03}$± $9,34E^{-04}$	$1,23E^{-02}$± $7,64E^{-04}$
	$1,50E^{-02}$± $3,22E^{-03}$	$1,23E^{-02}$± $7,64E^{-04}$
	$1,50E^{-02}$± $3,22E^{-03}$	$1,79E^{-02}$± $8,64E^{-04}$
	$1,61E^{-02}$±$8,93E^{-04}$	
Mean	$1,35E^{-02}$± $3,2E^{-03}$	$1,41E^{-02}$± $2,6E^{-03}$
R^2	0.99	

INSERM observed a decrease in signal at the highest concentrations and tested Methoxychlor in interference mode.

A)



B)



C)

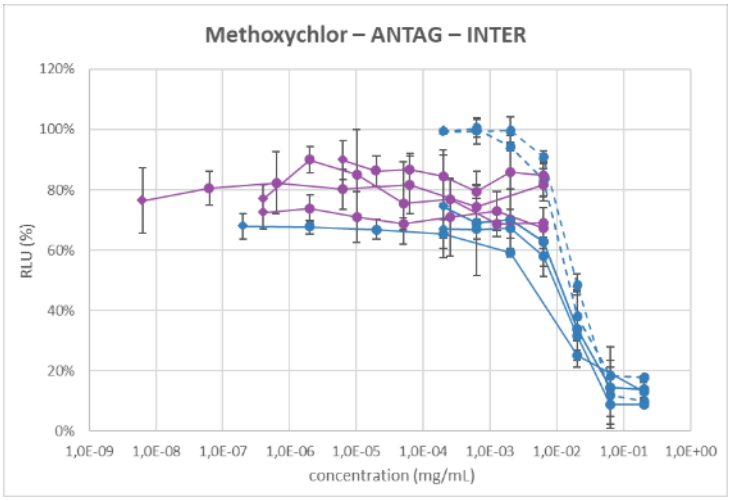


Figure 86: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Methoxyclor in antagonist mode in INSERM A), Toxem B), or in all labs C). Solid lines: antagonist mode; dotted lines: interference mode.

One lab concluded that Methoxychlor is interferent, one concluded no-effect. The difference in conclusions seems due to the difference in the concentrations tested.

These data are in agreement with the conclusions obtained during the phase 2 where Methoxychlor, tested up to 3.0E-3mg/mL did not show any activity.

Ketoconazole

Ketoconazole was tested from $2.0E^{-5}$ to $2.0E^{-2}$ by INSERM, and $4.03E^{-7}$ to $6.3E^{-3}$mg/mL by Toxem.
The IC_{50} values and dose-response curves obtained are presented below.

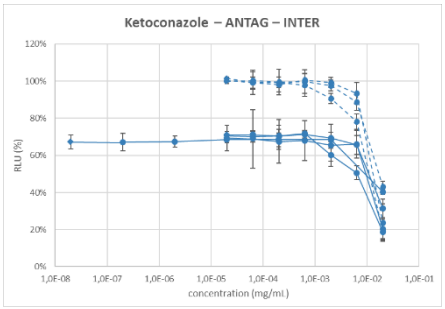
Table 34: IC_{50} values in antagonism and interference modes.

	INSERM	
	Antagonism	Interference
IC_{50}(±SD) in mg/mL	$2,85E^{-02}$± $3,39E^{-01}$	$3,91E^{-02}$± $1,37E^{-01}$
	$3,03E^{-02}$± $6,63E^{-02}$	$2,33E^{-02}$± $1,17E^{-01}$
	$2,08E^{-02}$± $1,69E^{-01}$	$9,55E^{-02}$± $1,69E^{-01}$
	$7,44E^{-02}$±$1,22E^{-01}$	
Mean	$3,85E^{-02}$ ± $2,43E^{-02}$	$5,26E^{-02}$ ± $3,80E^{-02}$
R^2	0.99	

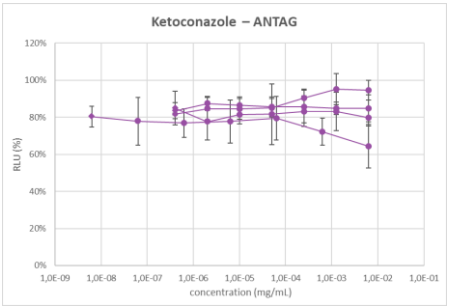
A good reproducibility can be observed for the IC_{50}, in both modes.

INSERM observed a decrease in signal at the highest concentrations and tested it in interference mode.

A)



B)



C)

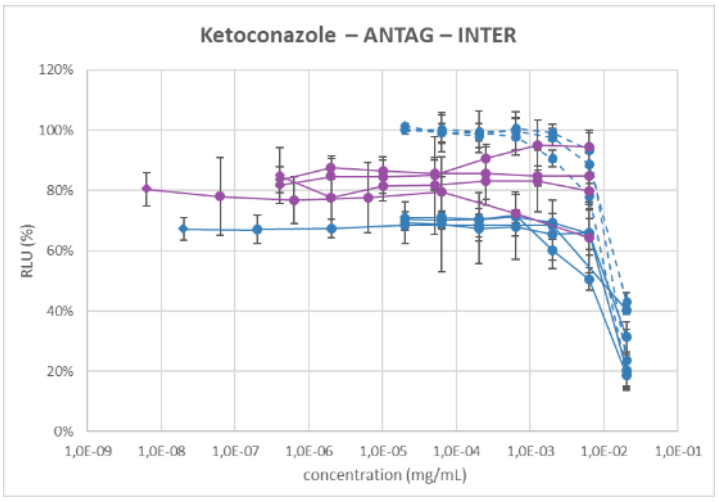


Figure 87: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Ketoconazole in antagonist mode in INSERM A), Toxem B), or in all labs C). Solid lines: antagonist mode; dotted lines: interference mode.

One lab concluded that Ketoconazole is interferent, one concluded no effect. The difference in conclusions seem due to the difference in the concentrations tested. These data are in agreement with the conclusions obtained during the phase 2 where Ketoconazole, tested up to 3.0E-3mg/mL did not show any activity.

Sodium Arsenite

Sodium arsenite was tested in the supplementary phase as "replacement" of Arsenic which could not be tested during phase 2. It was tested between $2.0E^{-05}$ and $2.0E^{-03}$ at INSERM, and $1.3E^{-07}$ and $2.0E^{-3}$ mg/mL at Toxem.

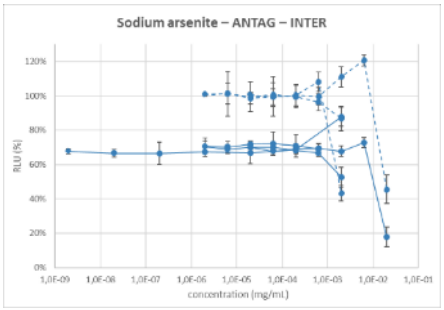
The IC_{50} values and dose-response curves obtained are presented below.

Table 35: IC_{50} values in antagonism and interference modes.

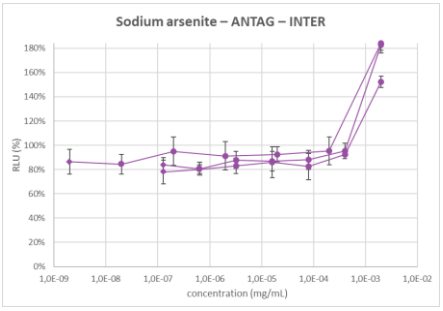
	INSERM	
	Antagonism	Interference
IC_{50}(±SD) in mg/mL	$2,41E^{-02}$± $5,44E^{-02}$	$2,56E^{-02}$± $4,17E^{-02}$
		$2,36E^{-03}$± $1,06E^{-02}$
Mean		$2,46E^{-02}$
R^2	0.91	

INSERM observed a decrease in signal at the highest concentrations and tested it in interference mode.

A)



B)



C)

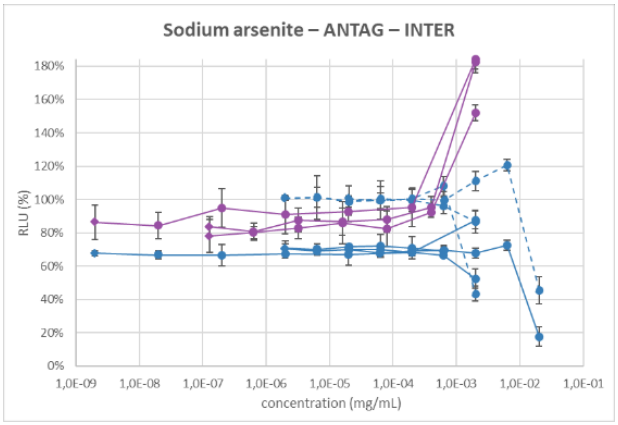


Figure 88: Concentration-response of luciferase activity (including SD) in HMLN-hGR cells exposed to Sodium arsenite in antagonist mode in INSERM A), Toxem B), or in all labs). Solid lines: antagonist mode; dotted lines: interference mode.

The conclusions of the labs differ, INSERM observing a decrease in signal, qualifying Sodium arsenite as interferent, whereas Toxem noted an increase. The labs did not provide any explanation for these results.

5.3 Reproducibility

For agonism testing, both labs found all the test items negative, with 100% of intra-laboratory and inter-laboratory reproducibility. Again, a testament to a very robust and reproducible assay in agonist mode.

Regarding the intra-laboratory reproducibility, it was 100% for both labs in the antagonist/interference testing, while there were some differences between labs.

The result of antagonist/interference testing is summarized in the table below.

Table 36: Concordance between laboratories for antagonist/interference mode.

Name	Ineris	INSERM	Tame-Water	Toxem	INSERM	Toxem
	Phase 2				Supp phase	
Bisphenol A	N	N	N	N	P	N
DEHP	N	N	N	N		
MEHP	N	N	N	N		
TBBPA	N	N	N	N		
BHA	N	N	N	N		

L-limonene	N	N	N	N		
Oleic acid	N	N	N	N		
Ginsenoside Rg1	N	N	N	N	N	N
Triclosan	P	P	N	P	P	P
Tolylfluanid	N	N	N	N		
Butyl paraben	N	N	N	N	P	P
Propyl paraben	N	N	N	N	P	N
λ-Cyhalothrin	N	N	N	N	N	N
Cypermethrin	N	N	N	N	N	N
Methoxychlor	N	N	N	N	P	N
Arsenic	nt	nt	nt	nt		
Monocrotophos	N	N	N	N		
Benzo[a]pyrene	N	N	N	N		
2-Methylresorcinol	N	N	N	N		
PFOS	N	N	N	N	N	N
Cortisol	N	N	N	N		
Budesonide	N	N	N	N		
Fluticasone propionate	N	N	N	N		
Aldosterone	N	N	N	N		
Progesterone	P	P	P	P		
21-Hydroxyprogesterone	P	P	P	P		
Mifepristone (RU-486)	P	P	P	P		
Ketoconazole	N	N	N	N	P	N
Spironolactone	P	P	P	P		
17β-estradiol	P	P	P	P		
Fulvestrant	P	P	P	P		
Lithium Chloride	N	N	N	N	N	N
sucrose	N	N	N	N		
Sodium nitrite	N	N	N	N		
Sodium azide	N	N	N	N		
Sodium arsenite					P	?

N: negative antagonist, P: positive antagonist or interference, ?: is unknown, letter in red indicate deviating interpretation

Yellow background: Different call from phase 2, Orange background: Different call from phase 2 in one lab

The increased concentrations meant that Butyl paraben was now considered positive in the antagonist/interference mode, though the two labs got to different conclusions (yellow background). Triclosan was confirmed as a positive in this assay also, but again the two labs disagreed on antagonism vs. interference. A number of compounds had no effect at Toxem but were positive at INSERM (Bisphenol A, Propyl paraben, Methoxychlor, Ketoconazole, and arsenite (orange background). Toxem saw an increase in signal with arsenite.

One aim of the supplementary phase was to check if testing at higher concentrations than during phase 2 (maximum concentration 3 mg/mL) and applying the range finding procedure from the SOP can result in modifications of test items classifications regarding their activity on GR. Bisphenol A, Butyl paraben, Propyl paraben, Methoxychlor and Ketoconazole were classified as positive in antagonism/ interference mode in at least one lab. Triclosan, Ginsenoside Rg1, λ-Cyhalothrin, Cypermethrin, PFOS, Lithium chloride classification did not change in antagonism/interference mode. No change in classification was observed in agonism testing.

By increasing the concentrations of the test substances (highest for INSERM) it is possible to achieve results that may differ in the antagonist/interference mode, but the conclusion may not be the same (antagonism vs interference). Considering the somewhat sharp decline in signal, these concentrations may in fact trigger pathways relating to cell toxicity or survival, even if cell death was not prominent. This may also explain the differences in the conclusions and the rather big standard deviations for some measurements. To a large degree this has tested the limits of the assay and the cells.

Based on these results we would recommend setting a limit for the maximal test concentration of test items to 100 µM. There is clear evidence that at higher test concentrations there is risk of significant of target effects some of which a related to cytotoxicity. The updated SOP will clarify this point.

6. Result analyses on compounds

6.1 Literature and in silico comparison

Initially compounds were selected based on known activities like the well described agonists and antagonist (all steroids). In addition, various families of ED chemistry previously tested in other assays, relating to GR activity or to corticosteroid biology were added (e.g. Ginsenoside Rg1, parabens, Bisphenol A, and more). Some further unrelated substances were also added like salts, a fatty acid, and a carbohydrate.

With the help of CEHTRA Consulting this selection was then analysed by Toxcast (https://www.epa.gov/comptox-tools/toxicity-forecasting-toxcast) on the following assays: TOX21_GR_BLA_Antagonist_ratio, TOX21_GR_BLA_Agonist_ratio, NVS_NR_hGR, ATG_GRE_CIS, ATG_GR_TRANS, CEETOX_H295R_DOC, CEETOX_H295R_CORTIC, CEETOX_H295R_11DCORT, CEETOX_H295R_CORTISOL and QSAR (Endocrine Disroptome (http://endocrinedisruptome.ki.si/receptor/4/ and Nuclear receptor-mediated Endocrine Activity (NRMEA) Model 1.1.1 (https://www.vegahub.eu)) prediction tools. At the same time a thorough literature search and analysis was also performed to further understand the expected outcome from the assay and in order to compare to the lab results. This covered 20 of the ED compounds while the most well-known agonists and antagonists were covered by the labs and Pepper. Table 37 summarizes the expected (in silico and literature) results and compare to the results obtained by the labs using the GR-TA assay. Numerous compounds had different and not very convincing predictions attached to them, thus there are many question marks in the table due to inconsistencies, partial or indirect results in relation to GR. In Annex 1 the literature assessment can be found with further details.

Table 37: Predictions and results of the compound classification. Antag is antagonist, Ago is agonist, Int is interference, ´-´ means no effect, ´?´ means there is doubt (discrepancies, low quality data, partial data), x means no prediction. Antag? may refer to interference that is not confirmed. Metabolites refers to that effects may be due to conversion of the compound to active chemistry. Unclear is based on contradictory data. Confirmed compounds have coloured background (green for agonists and orange for antagonists). In yellow are compounds found to be antagonistic (dark) and interfering (light) in the GRTA assay. All substances are labelled with a number indicating the number or articles that covers the compound in relation to GR regulation.

Name - number of articles	Toxcast	QSAR	Literature	GR-TA consensus
Bisphenol A – 6	Antag?	Ago?	Ago? Antag?	-
DEHP – 2	-	-	Ago?	-
MEHP – 1	x	x	-	-

TBBPA – 3	x	Ago?	Antag?	-
BHA – 2	-	-	Ago? Antag?	-
L-limonene – 1	-	-	Antag?	-
Oleic acid – 2	Antag?	x	Antag?	-
Ginsenoside Rg1 – 3	x	Ago?	Ago? Antag?	-
Triclosan – 2	x	x	Antag?	Int
Tolylfluanid – 4	Antag?	-	Ago? Antag?	-
Butyl paraben – 2	-	-	Ago?	-
Propyl paraben – 2	unclear, metabolites	-	Ago?	-
λ-Cyhalothrin – 1	-	Ago?	Antag?	-
Cypermethrin – 2	-	Ago?	Antag?	-
Methoxychlor – 1	Antag?	Ago?	Antag?	-
Arsenic				
Monocrotophos – 1	-	-	Ago?	-
Benzo[a]pyrene – 0	x	x	x	-
2-Methylresorcinol – 1	-	-	-	-
PFOS – 1	-	Ago?	-	-
Cortisol – 13	Ago	Ago	Ago	Ago
Budesonide – 6	Ago	Ago	Ago	Ago
Fluticasone propionate – 8	Ago	Ago	Ago	Ago
Aldosterone – 4	Ago	Ago	Ago	Ago
Progesterone – 6	Antag? metabolites	Ago	Ago? Antag?	Int
21-Hydroxyprogesterone – 2	Antag?	Ago	Ago?	Int
Mifepristone (RU-486) – 3	Antag	Ago?	Antag	Antag
Ketoconazole – 3	Ago? metabolites	Ago?	Antag?	-
Spironolactone – 3	x	x	Antag?	Antag
17β-estradiol – 3	x	x	Antag?	Int
Fulvestrant – 1	x	x	Antag?	Int
Lithium Chloride – 1	x	-	Antag?	-
Sucrose – 1	x	x	-	-
Sodium nitrite – 1	x	x	-	-
Sodium azide – 1	x	x	-	-
Sodium arsenite	x	x	Ago? Antag?	Int?

6.2 Comparison of expected and obtained results

Most compounds were found to be neither agonists nor antagonists/interference substances in the GR-TA. Four expected compounds apart from DEX were clearly tested agonists as expected, while only two compounds were deemed antagonists. Here Mifepristone was expected as this is a well describe and reference antagonist. The other was Spironolactone that was expected to be a partial antagonist. All these compounds belong to the family of steroids. A further 5 compounds were classified as interfering in the assay rather than being antagonists. The predictions for these were quite mixed but for 17β-estradiol, Triclosan, and Fulvestrant the suggestions from literature were toward antagonism, which would be in line with the data.

Since the number of compounds with known activity toward GR was very low, the sensitivity of the GR-TA was not analyzed in any detail. For those with established mode of action the GR-TA correctly identified all (100%). It is encouraging that none of these were missed and there was consensus among labs.

Since many substances were chosen from ED literature, they also represent a lot of different chemical families. In the GR-TA mainly steroids (natural or pharmaceuticals) were showing effects. Only Triclosan and in the supplementary phase, arsenite (one lab), were shown to have interference effect. Thus, the GR-TA appears very selective toward steroids and should prove valuable for de-selecting in pharmaceutical discovery/development or in other chemistry with steroid or steroid-like substances. Furthermore, smaller chemical substances with substructures that mimic steroids may gain access to the GR binding site and interfere with the function. The interference mode is useful for this type of activity and together with agonist and antagonist modes can be used for a number of environmental samples (water, soil, waste or similar) or to exclude chemistry with GR effects in agro-chemicals, petrochemicals, preservatives, paints, cosmetics and more. This assay could effectively complement testing with the ER and AR TA assays (OECD TG 455 and TG 458).

6.3 Availability of the results

All data from the labs in all phases were compiled in data management Excel sheets that are available through Pepper. This allows for full data comparison in and between labs, pr compound or several compounds at once. There is also access to invalid data. The figure below shows a screen short with an example of data drill-out with curves. There are ample options to do additional analyses and comparisons from all the data.

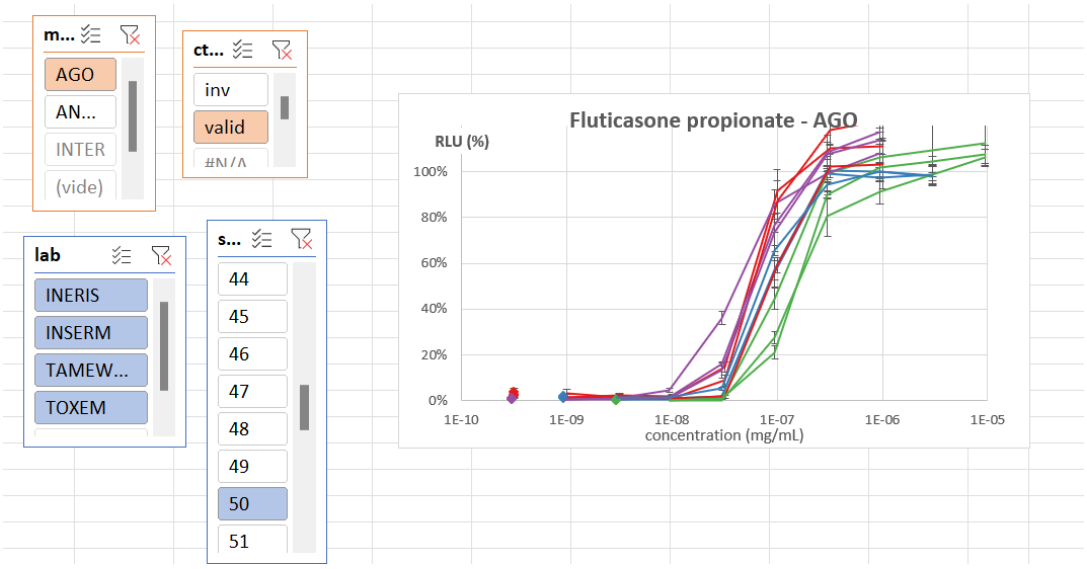


Figure 89: Example of data choice from the Excel data management tool of agonistic compound from all labs with Dose-response figure.

7. Conclusions

The aim of the project was to transfer the human glucocorticoid receptor (hGR) transcriptional activation assay for the detection of agonist and antagonist activity of chemicals towards hGR, based on the HMLN-hGR cells to naïve labs, and assess its relevance and reproducibility.

During this exercise, the SOP of the method has been refined, and its limitations defined. The method was transferred successfully to the naïve labs without any difficulty (without the need for an on-site training). The within-laboratory and between-laboratory reproducibility was exceptionally high (96% (64 of 68) and 100% respectively). An additional experimental phase was conducted in two labs re-testing some chemicals using higher concentrations. Different outcomes in the antagonist/interference mode were observed for some chemicals (in agonist mode results were the same). This raises concern about the maximum concentration to be tested in the GR-TA. High concentrations may trigger toxicity related pathways that may influence the outcome. The transfer of the GR-TA improved the SOP, e.g. by introducing a range finding and running antagonist as well as interference on the same plates.

The predictive capacity with comparison to literature data proved difficult. This is likely a general issue for test methods that target mechanism, for which experimental evidence is limited. With proper assessments of the literature, supported by in silico tools, only the well know agonists and one antagonist were assigned their function with high certainty. All labs found the correct mode of action for these. It is noteworthy that none of the unrelated chemistry (expected not to have effect), showed any responses in the main test phase. This is a significant advantage, as false positives have been identified as a key concern in the ED detection area.

The validation demonstrated that the GR-TA is a well-designed assay which can be transferred easily, and which has good reproducibility within and between labs. The assay successfully determined the mode of action of all compounds with known activity and did not falsely identify unrelated substances. This robustness and reliability are a testament to a well-designed cell line and SOP. In the context of other steroid assays this would be a valuable addition to the scarcely populated level 2 (in vitro, OECD GD 130) of test guidances with OECD.

Key point:

- The GR-TA assay proved to be easily transferable to naïve labs and generated the same results in a highly reproducible fashion in the labs and between labs.

- To the extent possible (due to the few compounds with known activity) the results proved to be in line with expectations. The possibility to distinguish between antagonist and interference is a valuable addition which in this case shed light on some suggested antagonists turning out to be interference compounds. The assay also appears to be non-promiscuous implying that false positives can be avoided. Most of the chemistry that showed activity were from the steroid family. It is possible to imagine simpler chemicals to be able to affect GR, but very few such tested chemicals had any effect.

- The fact that all expected compounds were identified also suggest that the number of false negatives could be low, however, this can only be confirmed as the number of compounds with well described GR activity increases.

- GR is involved in a multitude of biological processes by regulating gene expression in many tissues during development and in adulthood, as demonstrated by experimental animal models, the human genetic disorder and gene expression analyses (see Introduction and objectives).

- GR along with other steroid hormone receptors are major players in ED biology/toxicology. Furthermore, GR activity is the MIE or Key event (KE) for several AOPs in the area of ED biology. Thus, detection of chemistry with GR activity is crucial in ED toxicology.

- The main applicability areas are expected to be pharmaceuticals (particularly in the steroid family or related) to avoid unwanted GR activity. However, many industries can also use the assay to screen for unwanted GR activity (agro, petro, cosmetics, food, and other industries). Additionally, the assay can be applied to testing of environmental (or other) samples and mixtures, to assess the GR activity of such samples. In both cases the assay could also be applied together with other steroid assays, like the ER and AR assays mentioned, and is not considered a "stand-alone assay".

- For the pharmaceuticals and industrial chemicals, it is expected that data/results from the assay (being approved) can be used when regulations require toxicity information in the ED space. This may happen along with data from other approved assays in the area.

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Annex 1: Information on selected substances

The identification of substances that might have an action on the Glucocorticoid Transactivation Assay has involved several steps for selection, starting with a larger coverage of literature.
The information on the relevant references has been compiled, discarding those that were too far from the scope (e.g. *in vivo* study with no clear measurements toward the method). In the end, relevant references were not that numerous because the GR activation is not readily studied. Information from the references were documented on specific issues such as the cell types, exposure conditions and other available characteristics. Results are displayed in the following table.
As can be noticed, for some substances, information is scarce (e.g. only one reference). Some substances are reported as both agonist and antagonist. For many substances the literature is not consistent. Thus, there are several question marks when interpretations are different in papers, or the findings are not convincing or consistent. AGO means agonist, ANT means antagonist (though interference may be a possibility), NEG means negative in both modes, - means no activity found.

#	Substance name	CAS N°	Model and exposure conditions	Effects	Ref.	Literature
28	Bisphenol A (BPA) Molar mass = 228,29 g/mol	80-05-7	- 3T3-L1 cell line - Exposure during 24h - 1 µmol/L (2.28.E-4mg/mL) to 100 pmol/L (2,28E-08 mg/mL)	–BPA stimulates GR-mediated luciferase expression only at 1 µmol/L (2.28E-4mg/mL) –BPA stimulates lipid accumulation only at 100 nmol/L (2.28E-5 mg/mL)	(Sargis et al., 2010) PMID: 19927138	AGO
			Xenopus laevis tails cultured *ex vivo*, exposure to BPA (100, 1000 and 10,000 nmol/L) (2.28E-5, 2.28E-4 and 2.28E-3 mg/mL) for 24h	Concentration-dependent very low agonistic activity of BPA compared to control, the established assay could be sensitive for detecting GR-related biology.	(Chen et al., 2018 PMID: 29628076	AGO?
			5-week-old male and female C57BL/6J mice exposed to four dosages of BPA (5, 50, 500, and 5000 µg/kg/d) by oral intake for 30 days co-exposure with RU486	BPA exposure promoted adiposity in a non-monotonic dose-response way in low fat diet mice (as low as 5 µg/kg/d) BPA exposure increased the expression of genes related to adipogenesis and lipogenesis in vivo **BPA exposure induced the differentiation of adipose progenitors partially by GR activation *in vitro*** BPA treatment increased systemic and local inflammation in the CD mice but not in the HFD-induced obese mice BPA is associated with circulating inflammation factors in lean female subjects but not in lean male subjects or in both sexes with overweight/obesity	(Yang et al., 2016) PMID: 27145005	AGO?
			Recombinant yeast stably expressing GR: agonist mode: 10 nM (2.28E-6 mg/mL) – 300 µM (6.8E-2 mg/mL) antagonist mode: 1 nM (2.28E-7 mg/mL) –300 µM 24h at 30°C	**Yeast cells stimulated by DEX (60 µM) are concentration-dependently inhibited (decrease of fluorescence) by BPA with IC_{50} values of 67 µM (1.53E-2 mg/mL)** **No agonist effect**	(Roelofs et al., 2015) PMID: 25576683	ANT?

			A549 lung epithelial cell line 1-1000 nM (2.28E-7 to 2.28E-4 mg/mL) 24h of treatment Study of ENaCY by RT-qPCR and WB	Suppression of ENaCY expression and protein expression (target gene of GR) following 10 nM (2.28E-6 mg/mL) BPA exposure This inhibitory effect is blocked by DEX and transient siRNA-mediated knockdown of GR ➔ proves that the inhibition occurs via GR	(Hijazi et al., 2017 PMID: 27522653	ANT?
			BPA administered to pregnant mice from embryonic day 7.5 to 18.5 - 5, 25 or 50 mg/kg of BPA (1.2 to 2.6 ng/mL in maternal plasma) (2,74 to 5.94E-07 (mg/mL) Analyse of structure and protein expression by WB of key molecular markers of lung maturation (including 2 well known-glucocorticoid target genes: epithelial sodium channel (ENaCY) and glutathione peroxidase (GPX) in fetal lung at E18.5	Decrease of ENaCY and GPX protein expression with BPA with 25 mg/kg of proteins	(Hijazi et al., 2015) PMID: 26283537	ANT?
29	Diethylhexyl phthalate (DEHP) Molar mass = 390,56 g/mol	117-81-7	MDA-kb2 cell line, express glucocorticoid receptor and stably transfected with luciferase reporter gene 1nM (3,90E-7 mg/mL) and 10 nM (3,90E-6 mg/mL) (alone or in mixture with propylparaben and butylparaben) Exposition for 24h	Alone: glucocorticoid-like activity at 1 nM (3,90E-7 mg/mL) (1.50-fold over the control) (increase of luciferase activity) Mixture: glucocorticoid-like activity with some mixture at 1 nM and 10 nM (increase of luciferase activity)	(Klopčič et al., 2015) PMID: 25448277	AGO
			Receptor plasmids encoding for fin whale ligand binding domains (LBD) of GR COS7 cell line Agonist mode: 8 nM to 50 µM (3.12E-06 to 2E-02 mg/mL) Antagonist mode: 40 nM to 50 µM (1.56E-05 to 2E-02 mg/mL) 24h	Agonist mode: Slight non significative decrease of basal luciferase activity of GR from 1 µM (3.9E-04 mg/mL) no decrease in activity was observed in the presence of the agonist dexamethasone	(Routti et al., 2021) PMID: 33677245	NEG

30	Phthalic acid mono-2-ethylhexyl ester (MEHP) Molar mass = 278.34 g/mol	4376-20-9	Epidemiological study Maternal blood MEHP measurement by LC/MS Glucocorticoids measurement in the umbilical blood right after delivery by LC/MS	MEHP levels is inversely associated with cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio Decrease of glucocorticoids concentrations with increasing concentrations of MEHP	(Araki et al., 2017) PMID: 28043700	?
31	Tetrabromo-bisphenol A (TBBPA) Molar mass = 543,9 g/mol	79-94-7	Human HEK-293 cell-based reporter gene assay: Incubation for 24h 10 µM (5.44E-3 mg/mL) or 1 µM (5.44E-4 mg/mL) cell-free receptor binding experiments: Incubation for 4h 10 µM (5.44E-3 mg/mL)	TBBPA has no direct effect on GR ligand binding and does not affect GR transcriptional activity at 10 µM TBBPA was not able to induce GR-mediated activation of the luciferase reporter gene, even at the high concentration of 10 µM an interference of TBBPA with GR-mediated transactivation could not be demonstrated in this human cell-based assay	(Beck et al., 2016) PMID: 27693315	NEG
			Recombinant yeast stably expressing GR, agonist mode: 10 pM (5.44E-9 mg/mL) –100 mM (54 mg/mL), antagonist mode: 1 nM (5.44E-7 mg/mL)–100 µM (5.44E-2 mg/mL), 24h at 30°C	TBBPA showed clear antagonistic properties towards GR with IC50 values of 22 nM (1.2E-05 mg/mL)	(Roelofs et al., 2015) PMID: 25576683	ANT
			3T3-L1 preadipocytes GR competitive binding assay: 1nM to 100 µM (5.44E-07 to 5.4E-02 mg/mL)	TBBPA binds to GR with IC_{20} = 0.5 µM (2.7E-04 mg/mL)	(Liu et al., 2020) PMID: 32314580	?
32	Butylated hydroxyanisole (BHA) Molar mass = 180,24 g/mol	121-00-6	MDA-kb2 reporter cell line 1E-14 (1,8024E-15 mg/mL) to 1E2 µM (1.8E-02 mg/mL) for agonist mode 1E-4 (1.8024E-5 mg/mL) to 1E2 µM (1.8E-02 mg/mL) for antagonist mode Incubation for 24h	BHA showed Glucocorticoid-like activities by inducing the expression of luciferase with EC_{50} values 0.1 nM (1.8E-08 mg/mL). Maximum of induction at 0,5 µM (9E-05 mg/mL) BHA showed anti-glucocorticoid-like activities with IC_{50} values of 0.81 µM (1.5E-04 mg/mL). Maximum of inhibition at 50 µM (9E-03 mg/mL)	(Klopčič & Dolenc, 2017) PMID: 28115641	AGO?/ANT?
33	L-limonene Molar mass: 136,24 g/mol	5989-54-8	Measurement of GR translocation (fluorescence) in HeLa cells	Binds to GR from 0.5E01 µM (6.8E-04 mg/mL)	(Ryu et al., 2021) PMID: 33947115	ANT?

			GR binding assay (fluorescence polarization): 1E-6 to 1E2 µM GR-mediated transactivation: no concentrations data	selected GR agonist (SEGRA), i.e. activator of GR-mediated trans-repression and inhibitor of GR-mediated transactivation (antagonist-like activity in transactivation assay)		
34	Oleic acid Molar mass = 282,47 g/mol	112-80-1	*In vitro* effect of fatty acids on [3H]triamcinolone acetonide (TA) binding to the cytosolic glucocorticoid receptor in L2 cells, a cell line cloned from the adult rat type II cell (10-500 µM) (2.82E-3 - 1.41 mg/mL)	Oleic acid markedly inhibited [3H]triamcinolone acetonide specific binding at 500 µM (1.41 mg/mL) with an IC50 of 2.2E-4M Don't highlight the effect of oleic acid itself on GR	(Viscardi & Max, 1993) PMID: 8212085	?
			In vitro heat activation of mouse hepatic glucocorticoid receptor complex, as assessed by binding to DNA-cellulose and purified nuclei 25°C for 45 min 0 to 200 µM (0 to 5.6E-02 mg/mL)	38 % inhibition at 40 µM (1.13E-2 mg/mL)	(Ranhotra & Sharma, 2004) PMID: 22900281	ANT?
35	Ginsenoside Rg1 Molar mass = 801 g/mol	22427-39-0	FTO2B cells Receptor binding assay: 2h of incubation with 5 nM of [3H]DEX Transactivation assay with luciferase: 30h of incubation Concentrations?	Receptor binding assay: Maximum competition at 50 µM (0,04 mg/mL) of G-Rg1 Activation of GR at 15 µM (0.012 mg/mL) (increase of reporter activity)	(Lee et al., 1997) PMID: 9406859	AGO?
			- zebrafish larval model - amputation of the tail fin used to assess drug effects on inflammation - Exposure: 2h before amputation and 4h after amputation - 30 to 180 µM (2.4E-02 g/L to 1.44E-01 g/L) - macrophages were detected based on the red fluorescence of their mCherry label, and neutrophils were detected based on their green-fluorescent GFP label 4h after amputation	– Rg1 attenuates neutrophilic inflammation at the amputation site at 120 µM (9.61E-02 g/L) and higher – Mutation of GR abolishes the anti-inflammatory effect of Rg1 ⇨ **Effects of Rg1 via GR** - **Low affinity of Rg1 for hGR** - **High doses are required for Gr-mediated effects** Significant upregulation of both genes, but Rg1 did not affect the transcription rate of pck1 and fkpb5, in line with the hypothesis	(He et al., 2020) PMID: 32365641	?

			- grs357 mutant zebrafish line used has a point mutation in the gene encoding the GR which makes the receptor transcriptionally inactive **Study of pck1 and fkpb5 (GR target genes known to be activated by transactivation)**	that the Rg1-activated GR **does not have transcriptional activity ➔ antagonist effect or selective agonist (SEGRA)?**		
			RAW264.7 and A549 cell lines NF-Kb reporter gene assay (luciferase activity) 10 µM (8E-03 mg/mL) for 8h	The inhibitory effect of Rg1 on NF-kB is abrogated by knockdown of GR effects similar to effects obtained with DEX **(agonist effect) (we do not study the direct activation of GR there!)** Ginsenoside triggers a decrease of NF-Kb linked luciferase activity **➔ effects of Ginsenoside occurs via GR**	(Du et al., 2011) PMID: 21666059	?
			Luciferase reporter gene containing two copies of a glucocorticoid response element was constructed and transiently transfected into FTO2B rat hepatoma cells 30h of exposure 0 to 10 µM	G-Rg1 gradually induced the enzyme activity with peak values of ninefold at doses higher than 5 µM (4E-3 mg/mL), and the effect was maintained up to 10 µM (8E-3 mg/mL) Inhibition of this induction by RU486	Chung and al., 1998 PMID: 9654649	AGO?
36	Triclosan Molar mass = 289.54 g/mol	3380-34-5	In silico: using the Endocrine Disruptome and VirtualToxLab™ version 5.8 in silico tools In vitro: agonist and antagonist activities in cell-based luciferase reporter assays in vitro in AR-EcoScreen, hERa-HeLa-9903, MDA-kb2, and GH3.TRE-Luccelllines	Triclosan showed antagonist effects on GR at 10 µM (2.9E-3 mg/mL) and TR at 5 µM.	(Kenda et al., 2020) PMID: 33064576	ANT
			MDA-kb2 cell line Incubation for 24h 5 µM (1.45E-3 mg/mL)	Decreases the luciferase activity by >20% at 5 µM (1.45E-3 mg/mL), weak antagonist?	(Kolšek et al., 2015) PMID: 25192815	ANT?

37	Tolylfluanid Molar mass = 347,3 g/mol	731-27-1	Primary adipose tissue obtained from male C57BL/6 mice (8 wk of age) 1nM (3.47E-7 mg/mL) to 1µM (3.47E-4 mg/mL) 48h of incubation	**Induction of GILZ gene expression at 100 nM and 1 µM (3.47E-4 mg/mL) (The GILZ promoter contains multiple GREs)** 4 hours of treatment with TF (100nM) resulted in synchronous transcriptional effects **100 nM TF induces IR-S gene expression** **TF promotes the activating phosphorylation and nuclear translocation of GR at 100 nM (3.47E-5 mg/mL)** **Recruitment of GR to IRS-1 and GILZ GREs at 100 nM (Chip experiment)** Increase of relative IRS-1 protein expression with 100 nM after 4h incubation Increases expression of ACC, SCD-1, and HK2 (insulin-induced lipogenesis genes) with 100 nM after 4h incubation TF is able to intercalate into the ligand binding pocket and bind GR +	(Neel et al., 2013) PMID: 23340252	AGO?
			3T3-L1 cell line Exposure during 24h 1 µmol/L (3.47E-4 mg/mL) to 100 pmol/L (3.47E-8 mg/mL)	TF stimulates GR-mediated luciferase expression at 1 µmol/L (3.47E-4 mg/mL) TF enhances adipogenesis at concentrations as low as 100 pmol/l (increase of anti-insulin receptor subunit β, anti-CCAAT/enhancer binding protein α, and anti-adiponectin protein expression) stimulates lipid accumulation at 100 nmol/L	(Sargis et al., 2010) PMID: 19927138	AGO
			Reuber rat hepatoma H-II-E-C3 cell line 1 nM (3.47E-7 mg/mL) -100 µM (3.47E-2 mg/mL) for competitive binding studies ([3H]-dexamethasone competition) 0.05 (1.7E-05 mg/mL) –10 µM (3.47E-3 mg/mL) for 18h for tyrosine aminotransferase assay	TF can bind to GR with IC_{50} values of 1-10 µM Lack of agonist effect Antagonist effect: reduce the dexamethasone-induced tyrosine aminotransferase specific activity in a dose-dependent manner	(Johansson et al., 2005) PMID: 15755314	ANT
			Chinese hamster ovary K1(CHO-K1) cells (Luciferase Reporter Assays for hGRα):	No agonist effect (no increase of luciferase activity)	(Zhang et al., 2016) PMID: 26647222	ANT?

			Agonist mode : 1E−5 (3.47E-3 mg/mL) or 1E−6 M (3.47E-4 mg/mL) Antagonist mode: 1E−9 (3.47E-7 mg/mL) to 1E−6 (3.47E-4 mg/mL) or 1E−5M (3.47E-3 mg/mL) Exposure for 24h Rat hepatoma cells H4IIE, and mouse macrophage cells J774A (RT-qPCR) : 1E−6 M (3.47E-4 mg/mL) for 24 h	Antagonistic (decrease of luciferase activity) activity against GR at 10-6 M (3.47E-4 mg/mL) with RIC20 lower than 10−6 M (2.46E-07 M), effect not convincing 10-5-10-6 M: non-toxic concentrations H4IIE cells: decrease of TAT expression (Glucocorticoid-responsive gene) at 10-6 M J774A: decrease of cortisol-stimulated GILZ expression at 10-6 M		
38	Butyl paraben Molar mass = 194,23 g/mol	94-26-8	MDA-kb2 cell line Incubation for 24h 25 µM (4.86E-3 mg/mL)	Increases the luciferase activity by over 50% at 25 µM (4.86E-3 mg/mL), however cells were treated with antagonist RU486, no direct evidence	(Kolšek et al., 2015) PMID: 25192815	AGO?
			MDA-kb2 cell line, express glucocorticoid receptor and stably transfected with luciferase reporter gene 10E-08 to 10E3 µM (1.94E-06 to 1.94E05 mg/mL) (alone or in mixture) Exposure for 24h	Toxicity at concentrations higher than 75 µM (1.46E-2 mg/mL) Induces an increase of luciferase activity (low induction) with an EC_{50} = 1.75 µM (3.4E-4 mg/mL) and concentration for maximum induction = 10 µM (1,9E-03 mg/mL)	(Klopčič et al., 2015) PMID: 25448277	AGO?
39	Propyl paraben Molar mass = 180.2 g/mol	94-13-3	MDA-kb2 cell line Incubation for 24h 25 µM (4,5E-3 mg/mL)	Increases the luciferase activity by over 50% at 25 µM (4,5E-3 mg/mL), cells treated with antagonist (as above)	(Kolšek et al., 2015) PMID: 25192815	AGO?
			MDA-kb2 cell line, express glucocorticoid receptor and stably transfected with luciferase reporter gene 1 nM (1.8E-7 mg/mL) and 10 nM (1.8E-6 mg/mL) (alone or in mixture) Exposure for 24h	Toxicity at concentrations higher than 125 µM Induces a small increase of luciferase activity with an EC_{50} = 13.01 µM (2.34E-6 mg/mL)	(Klopčič et al., 2015) PMID: 25448277	AGO?
40	λ-Cyhalothrin Molar mass = 449.85 g/mol	91465-08-6	Luciferase reporter gene assay in CHO-K1 Study of the expression of glucocorticoid-responsive genes in H4IIE and J774A.1 cells: 24h	No agonist effect (no increase of luciferase activity) Decreases luciferase activity with RIC20 value at 7.35*10-7M (3,3E-4 mg/mL) and RIC50 at	(Zhang et al., 2016) PMID: 26647222	ANT?

			1E-5 M (4,5E-3 mg/mL)	2.84*10-6M (1,3E-3 mg/mL). Percent decrease of cortisol response = 63.1% Reduces GILZ expression in J774A.1 cells at 1E-5M. Effects at high concentrations.		
41	Cypermethrin Molar mass = 416.3 g/mol	52315-07-8	CHO-K1 cells (transfected with plasmids for Dual-luciferase reporter gene assay) 1E-6 M (4,2E-4 mg/mL) to 10-4 M (4,2E-2 mg/mL) Exposure for 24h H295R cell (for qRT-PCR and hormones measurement) 1E-4 M for RT-qPCR and 1E-4-1E-5 M for hormones detection Exposure for 48h	CP11 shows antagonist activity at 10-4 and 10-5M with a decrease of luciferase activity CP11 induces an increase of CYP11A1, CYP11B1, CYP17, CYP21, StAR, and 3bHSD expression levels at 10-4 M CP11 increases cortisol levels at 10-4 and 10-5M and aldosterone levels at 10-4 M	(Ji et al., 2019) PMID: 30611075	ANT?
			Chinese hamster ovary K1(CHO-K1) cells (Dual-Luciferase Reporter Assays for hGRα): Agonist mode: 1E−5 (4,2E-3 mg/mL) or 1E−6 M Antagonist mode: 1E−9 (4,2E-7 mg/mLl) to 1E−6 or 10−5M Exposition for 24h Rat hepatoma cells H4IIE, and mouse macrophage cells J774A (RT-qPCR): 1E−5 M for 24 h	No agonist effect (no increase of luciferase activity) Antagonistic (increase of luciferase activity) activity against GR at 1E-5 M (4,2E-3 mg/mL with RIC20 lower than 1E−6 M H4IIE cells: decrease of TAT expression (Glucocorticoid-responsive gene) at 10-5 M J774A cells: No effect on GILZ expression	(Zhang et al., 2016) PMID: 26647222	ANT?
42	Methoxychlor (no other references identified) Molar mass = 345,65 g/mol	72-43-5	Chinese hamster ovary K1(CHO-K1) cells (Dual-Luciferase Reporter Assays for hGRα): Agonist mode: 1E−5 (3.46E-3 mg/mL) or 1E−6 M (3.46E-4 mg/mL) Antagonist mode: 1E−9 (3.46E-7 mg/mL) to 1E−6 or 1E−5M Exposition for 24h	No agonist effect (no increase of luciferase activity) Antagonistic (decrease of luciferase activity) activity against GR at 10-5 M (3.46E-3 mg/mL with RIC20 lower than 10−6 M, untypical D/R curve H4IIE cells: decrease of TAT, Arg and PEPCK expression (Glucocorticoid-responsive genes) at 10-5 M	(Zhang et al., 2016) PMID: 26647222	ANT?

			Rat hepatoma cells H4IIE, and mouse macrophage cells J774A (RT-qPCR) : 10−5 M for 24 h	J774A: decrease of cortisol-stimulated GILZ expression at 10-5 M		
43 Arsenic Then 63 Sodium arsenite	Arsenic Molar mass = 74,92 g/mol Sodium arsenite	7440-38-2 7784-46-5	H4IIE rat hepatoma cells	0.3-3.3 µM (2.2E-5 to 2.4E-4 mg/mL) significantly decreased dexamethasone-induced expression of transiently transfected luciferase, indirect assay	Kaltreider and al., 2001	ANT?
			Rat EDR3 hepatoma cells Endpoints: the endogenous tyrosine aminotransferase (TAT) gene and the reporter genes containing TAT glucocorticoid response elements	Agonist effect: 0.05−1 µM (3.7E-6 to 7.4E-5 mg/mL) Antagonist effect: 1−3 µM (7.4E-5 to 2.2E-4 mg/mL) Transient effects	Bodwell and al., 2004	AGO?/ANT?
			- In ovo whole chick embryo culture: embryos < 10 days of age eggs were incubated with Arsenic or Arsenic + inhibitor of GR pathway from day 3 to day 6 of incubation **0.1 µM of arsenic (7.5E-6 mg/mL) embryos were removed and analyzed for gross malformations and scored according to established morphological scoring criteria** - Chick embryo midbrain micro mass (MM) culture in vitro assay **1, 2, or 5 µM** of Arsenic (or Arsenic + inhibitor of GR) for 5 days	Observed morphological defects included abnormalities in the head fold region, microcephaly, anterior neural tube defects, and gross facial deformities with Ar with the in ovo assay. Embryos exposed to iAs plus cortexolone showed no gross structural malformations and displayed normal growth parameters. Significant decrease in the viability of embryonic mid brain cells treated with 2 and 5 µM iAs. Cortexolone-treated cells, with inhibited signaling of the GR pathway, were protected from cytotoxicity induced by 2 **µM** (**1.5E-4 mg/mL**) iAs. At the highest iAs concentration (**5 µM) (3.7E-4 mg/mL**), midbrain MM cultures were partially protected from iAs-induced cytotoxicity. Highly indirect evidence	Ahir and al., 2013	AGO?
44	Monocrotophos Molar mass = 223.2 g/mol	6923-22-4	Adult male rats (CFT - Wistar strain Then, administration of Monocrotophos at 1.8 mg/k.g.b.w for 2h	Single dose of Monocrotophos to rats elicits increase in liver TAT activity via glucocorticoid-receptor activation, indirect evidence	(Joshi et al., 2012) PMID: 22305719	AGO?

			Measure of tyrosine aminotransferase (TAT) in liver (enzyme induced by GC via GR)			
45	Benzo[a]pyrene Molar mass = 252.31 g/mol	50-32-8			No bibliography found	-
46	2-Methylresorcinol (2MR) Molar mass = 124,14 g/mol	608-25-3	MDA-kb2 reporter cell line (stably transformed with murine mammalian tumor virus (MMTV)-luciferase neo reporter construct) 1E-15 to 1E-3 µM (1.24E-7 mg/mL) for agonist mode 1E-4 (1.24E-8 mg/mL) to 10E3 µM for antagonist mode Incubation for 24h	2MR showed Glucocorticoid-like activity by inducing the expression of luciferase with EC_{50} values 1.24 µM. Maximum of induction at 0,01 µM (1.24E-6 mg/mL) 2MR showed anti-glucocorticoid-like activities with IC_{50} values of 4435 µM. Maximum of inhibition at 175 µM (2.17E-2 mg/mL), these data are basically ridiculous, curves are mostly flat and incoherent	(Klopčič & Dolenc, 2017) PMID: 28115641	-
47	Perfluorooctane sulfonic acid (PFOS)	1763-23-1	Recombinant U2OS cells stably expressing the human GR (U2OS-GR) 0.0147 to 0.2943 mg/mL Exposure for 48h	Agonist effect: Enhances nuclear transcriptional activity of GR with the two lowest concentrations (0.0147 mg/ml and 0.0294 mg/ml) Antagonist effects: reduction in the TGRM-Luc nuclear receptor transcriptional activity at the two highest concentrations (0.1471 and 0.2943 mg/mL)➔ cytotoxicity with these concentrations (way too high)	(Wilson et al., 2016) PMID: 26599974	AGO?/ANT?
			10 and 20 mg/kg in mice	Decrease of GR mRNA at day 7 in liver with both concentrations and at day 14 in white adipose with the highest concentration	Wang and al., 2020 https://pubs.acs.org/doi/10.1021/acs.estlett.0c00048	?
			Offspring of pregnant Sprague Dawley rats exposed to dexamethasone (Dex), perfluorooctane sulfonate (PFOS) 18.75mg/kg/day	Significant but small increase of GR expression in offspring (less important than dex)	Rogers and al., 2013 PMID: 24218149	?

48	Cortisol (Hydrocortisone) Molar mass = 362,46 g/mol	50-23-7	Measurement of GR translocation (fluorescence) in HeLa cells GR binding assay (fluorescence polarization) GR-mediated transactivation	Cortisol is able to bound to GR Cortisol induces nucleus GR translocation with increasing concentrations up to 10 nM (3.62E-07 mg/mL) before saturation 1 min after cortisol stimulation Cortisol induce GR-mediated transactivation; ➔ **agonist**	(Ryu et al., 2021) PMID: 33947115	AGO
			15 male volunteers 10 days 9α-fludrohydrocortisone : 1,5 mg Hydrocortisone: 60 mg Measure of urinary ammonium excretion	Increase of ammonium excretion from day 9 with 9α-fludrohydrocortisone and from day 7 with hydrocortisone The coadministration with the GR inhibitor spironolactone did not alter the urinary ammonium excretion. The coadministration with the GR inhibitor RU486 attenuated the hydrocortisone-induced increase in urinary ammonium excretion.	(Waybill et al., n.d.) PMID: 8025226	?
			Healthy middle-aged men (n=16) and women (n=25) pulsatile iv infusion of normal saline (low cortisol clamp) or soluble hydrocortisone hemisuccinate (Solucortef) (pulsatile cortisol clamp) over **12 hours** (10:00 PM to 10:00 AM) at the young-adult cortisol production rate of **0.525 mg/m2 / h** **Measure ACTH secretion in response to pulsatile infusions of physiological amounts of cortisol**	During pulsatile infusion of cortisol (in the three different experiments), plasma ACTH concentrations decreased clearly With mifepristone (GR inhibitor), plasma ACTH secretion is higher	(Roelfsema et al., 2016) PMID: 27548106	AGO?
			Reporter assay yeasts expressing human GR and MR Wild yeasts and mutant yeasts (deletion of the CWP1, CWP2, PDR5, and/or PDR10 genes)	Agonist mode: increase of induction of B-galactosidase activity with EC_{50} 31,6 µM (1.14E-2 mg/mL) with wild yeast and induction with EC_{50} 0.78µM (2.8E-4 mg/mL) with mutant yeast No effect in antagonist mode	(Ito-Harashima et al., 2015) PMID: 26070404	AGO

			0,0001 (3,6E-7 mg/mL) to 100 (3,6E-2 mg/mL) µM Incubation for 18h at 30°C	higher sensitivity with mutant yeasts		
			UAS-bla HEK293T or UAS-bla GripTite™master reporter cell lines (Invitrogen) with Gal4 DBD fused to the LBD of the receptor Incubation for 16h at 37°C 10 mM to 0.318 nM (3.62 to 1.15E-07 mg/mL) (decrease in half-logs)	Agonist effect: increase of beta-lactamase activity with EC_{50} = 9.863 nM No Antagonist effect	(Wilkinson et al., 2008) PMID: 18753690	AGO
			Transiently transfected COS-7 cells with YFP-GR for translocation assay Exposition for 1h 100 nM(3.6E-05 mg/mL) HeLa cells stably transfected with a chimera receptor joining the Gal 4 DNA binding domain and human GR ligand binding domain for reporter assay Exposition for 18h	Induces GR translocation at EC50 = 13,3 nM (increase of nuclear-cytoplasm intensity difference) Agonist mode: increase of fluorescence at EC50=118,1 nM No effect in Antagonist mode	(Agler and al., 2007) PMID: 17989426	AGO
			Identification of several EDCs (and their concentrations) in Water samples in Czech Republic and Switzerland GR-CALUX model of GR activation challenged with the EDCs found in both waters including cortisol (>1E-08 mg/mL) concentration–response curves in sup data	agonist activity in GR-CALUX model No antagonist activity	(Macikova et al., 2014) PMID: 25269596	AGO
49	Budesonide Molar mass = 430,53 g/mol	51333-22-3	Transiently transfected COS-7 cells with YFP-GR for translocation assay Exposition for 1h 100 nM (4.3E-5 mg/mL) HeLa cells stably transfected with a chimera receptor joining the Gal 4 DNA	Induces GR translocation at EC50 = 3,1 nM (1.33E-6 g/L) (increase of nuclear-cytoplasm intensity difference) Agonist mode: increase of fluorescence with EC50=0,5 nM (2.15E-7 g/L) No effect in Antagonist mode	(Agler et al., 2007) PMID: 17989426	AGO

			binding domain and human GR ligand binding domain for reporter assay Exposition for 18h From ??? to 100 nM			
			7 subjects 400 µg twice daily 6 weeks Biopsy samples of the bronchial mucosa are obtained after each treatment and analysed for the DNA binding activity of GR, CREB, and NFκB by electrophoretic mobility shift	Increase of GR binding on GRE by a mean of 52%	(Hancox et al., 1999) PMID: 10335001	?
			34 asthmatic children COS-1 cells transfected with GR receptor, a coactivator protein and reporter plasmid containing GR response elements upstream of the luciferase gene 10 microliters of human serum are added directly to the cell culture to measure glucocorticoid bioactivity from the serum **Children are treated with 800 µg/d of budesonide for 2 months**	Increase of luciferase activity in COS-1 cells with BUD treatment (from 0,1 to 12,1 nM cortisol equivalents in the serum) (3.62E-08 to 4.39E-06 mg/mL cortisol equivalents in the serum) Increase of luciferase activity linked to increase of serum cortisol levels	(Biomedicum Helsinki, 2002) PMID: 12161504	AGO
			Bile fistula rats, isolated intrahepatic bile duct units (IBDUs), and purified cholangiocytes Study of the effect on biliary bicarbonate excretion and Cl-/HCO3- transport processes by mRNA measurements, western blot and Measurement of the Activities of Cl-/HCO3- Transport Processes 2 days 0,15 mg 3 times daily	Treatment for 2 days with budesonide increased ($P < 0.05$) biliary bicarbonate concentration and secretion, which were blocked by the specific GcR antagonist, RU-486 **proves that budenoside effects occurs via GR**	(Alvaro et al., 2002) PMID: 11910357	?

			Primary culture of human neutrophils 16 h 0,1 to 1000 nM (4.3E-7 mg/mL to 4.3E-4 mg/mL) for IP (Iodure propidium) staining and 1 µM (4.3E-4 mg/mL) for Annexin V staining 1 µM (4.3E-3 mg/mL) of BUD with RU486 Analyse of neutrophil apoptosis with IP and Annexin V by flow cytometry	Inhibition of neutrophil apoptosis (decrease of IP staining) from 10 nM (4.3E-5 mg/mL) Inhibition of neutrophil apoptosis (decrease of Annexin V staining) at 1 µM (4.3E-4 g/L) **RU486 reduces inhibition of apoptosis by 1 µM of BUD (increase of IP staining)** ➔ Inhibition of apoptosis via GR	(X. Zhang et al., 2001) PMID: 11730731	?
			Identification of several EDCs (and their concentrations) in Water samples in Czech Republic and Switzerland GR-CALUX model of GR activation challenged with the EDCs found in both waters including Budenoside > 10 ng/L (1E-08 g/L)	Higher GR-agonist activity than DEX (Concentration-response curve in sup. Data) No antagonist activity	(Macikova et al., 2014) PMID: 25269596	AGO
50	Fluticasone propionate Molar mass = 444,51 g/mol	80474-14-2	34 asthmatic children COS-1 cells transfected with GR receptor, a coactivator protein and reporter plasmid containing GR response elements upstream of the luciferase gene 10 microliters of human serum are added directly to the cell culture to measure glucocorticoid bioactivity (GBA) from the serum **Children are treated with 500 µg/d of fluticasone for 2 months**	No significant increase of GBA (corresponding to luciferase activity) with the serum of Fluticasone-treated children This study doesn't prove directly that fluticasone is GR agonist or antagonist	(Biomedicum Helsinki, 2002) PMID: 12161504	NEG?
			Primary culture of human neutrophils 16 h 0,1 (4,44E-8 mg/mL)to 1000 (4,44E-4 mg/mL) nM for IP (Iodure propidium) staining and 1 µM for Annexin V staining	Inhibition of neutrophil apoptosis (decrease of IP staining) from 1 nM (4,44E-7 g/L) Inhibition of neutrophil apoptosis (decrease of Annexin V staining) at 1 µM (4,44E-4 g/L) **RU486 reduces inhibition of apoptosis by 1 µM of BUD (increase of IP staining)**	(X. Zhang et al., 2001) PMID: 11730731	?

			1 µM of BUD with RU486 Analyse of neutrophil apoptosis with IP and Annexin V by flow cytometry	➔ Inhibition of apoptosis via GR		
			Identification of several EDCs (and their concentrations) in Water samples in Czech Republic and Switzerland GR-CALUX model of GR activation challenged with the EDCs found in both waters including Fluticasone propionate > (1E-08 mg/mL)	GR-mediated luminescence >100% induction of Dex No antagonist activity	(Macikova et al., 2014) PMID: 25269596	AGO
			UAS-bla HEK293T or UAS-bla GripTite™master reporter cell lines (Invitrogen) with Gal4 DBD fused to the LBD of the receptor Incubation for 16h at 37°C 10 mM (4.44 mg/mL) to 0.318 nM (1,41E-7 mg/mL) (decrease in half-logs)	Agonist effect: increase of beta-lactamase activity with EC50 = 0,0032 nM (1,42E-9 mg/mL) No Antagonist effect	(Wilkinson et al., 2008) PMID: 18753690	AGO
51	Aldosterone Molar mass = 360,44 g/mol	52-39-1	MDA-MB-453 stably transformed with the MMTV promoter to set up MDA-kb2 cell line Overnight exposition 10E-12 to 10E-05 M (3.6E-10 to 3.6E-03 mg/mL)	Agonist activity: Induction of luciferase with LOEC = 0,05 µM (1.8E-05 mg/mL)	(V. S. Wilson et al., 2002) PMID: 11861974	AGO
			reporter assay yeasts expressing human GR and MR Wild yeasts and mutant yeasts (deletion of the CWP1, CWP2, PDR5, and/or PDR10 genes) 0,0001 (3,6E-8 mg/mL) to 100 µM (3.6E-2 mg/mL) Incubation for 18h at 30°C Measure of the expression of the lacZ reporter	Agonist activity: weak induction of GR with EC50 =31,6 µM (1.14E-2mg/mL) for wild yeasts and EC50= 1,76 µM (6.34E-4mg/mL) for mutant	(Ito-Harashima et al., 2015) PMID: 26070404	AGO
			Mouse fibroblast cell line 1471 (permanently transfected with	Decrease of [3H]aldosterone binding with increasing concentrations of RU486 (which	(Couette et al., 1992) PMID: 1309341	Impossible to conclude

			chloramphenicol acetyltransferase (CAT)) 4h of incubation 1194 +/- 83 fmol/mg protein Use of [3H]aldosterone to study aldosterone binding to the GR	has high affinity for GR) ➔ prove that aldosterone binds to GR		more than binding
			UAS-bla HEK293T or UAS-bla GripTite™master reporter cell lines (Invitrogen) with Gal4 DBD fused to the LBD of the receptor Incubation for 16h at 37°C 10 mM (3.6E-2 mg/mL) to 0.318 nM (1.15E-7 mg/mL) (decrease in half-logs) Measure of fluorescence activity of Beta-galactosidase	Agonist effect : EC50 = 440 nM (1.6E-4 mg/mL) No Antagonist effect	(Wilkinson et al., 2008) PMID: 18753690	AGO
52	Progesterone Molar mass = 314,46 g/mol	57-83-0	Corpus luteum of Sprague-Dawley female rats obtained on Day 15 of pregnancy Exposition for 4h 1E-8 (3,14E-6 mg/mL) to 1E-5 M (3,14E-3 mg/mL) Temperature sensitive SV-40 transformed luteal cell line (CG-CL) that expresses the GR and 20a-HSD mRNA (supposed to be decreased by Pg via GR) Exposition for 24h 1E-8 to 1E-6 M (3,14E-4 mg/mL)	Dose related decrease in 20a-HSD mRNA in both models **Addition of the GR/PR antagonist RU486 to Pg reverses in a dose-dependent manner, the inhibitory action on 20a-HSD mRNA expression** Weak indirect evidence	(Sugino et al., 1997) PMID: 9322971	AGO?
			MDA-MB-453 stably transformed with the MMTV promoter to set up MDA-kb2 cell line expressing luciferase Overnight exposition 1E-13 to 1E-5 M (3.14E-11 to 3.14E-03 mg/mL)	Agonist activity: Induction of luciferase with LOEC = 0,1 µM (3.14E-05 mg/mL) Unclear if AR or GR mediated	(Wilson et al., 2002) PMID: 11861974	AGO?

			Transiently transfected COS-7 cells with YFP-GR for translocation assay Exposition for 1h 100 nM (3.14E-5 mg/mL) HeLa cells stably transfected with a chimera receptor joining the Gal 4 DNA binding domain and human GR ligand binding domain for reporter assay Exposure for 18h	Induces GR translocation at EC_{50} = 90,1 nM (increase of nuclear-cytoplasm intensity difference) No Agonist mode Antagonist mode: decrease of fluo at EC_{50} > 666 nM (2.09E-4mg/mL)	(Agler et al., 2007) PMID: 17989426	ANT?
			HeLa cells for competitive binding assay (competition with ^{3}H-cortisol) Transfection of COS-7 cells with a GFP-tagged hGR for nuclear translocation of the hGR **10-6 M(3.14E-04 mg/mL) for 60 min** COS-7 cells and HEK 293 cells after cotransfection with hGR and luciferase reporter vectors using a dual luciferase assay for transactivation assay **10-9 to 10-4 M** (3.14E-07 to 3.14E-02 mg/mL) **for 24h**	P4 binds to the hGR with relative binding affinity of 43 % Partial translocation of GFP-tagged hGR with P4 at 10-6 M COS-7: EC_{50} = 3,10E-6 M for transactivation HEK293: EC_{50} = 2,3.10E-6 M for transactivation Low sensitivity compared to control	(Pijnenburg-Kleizen et al., 2015) PMID: 26207344	AGO
			Reporter assay yeasts expressing human GR and MR Wild yeasts and mutant yeasts (deletion of the CWP1, CWP2, PDR5, and/or PDR10 genes) 0,01 to 100 µM (3.14E-06 to 3.14E-02 mg/mL) Incubation for 18h at 30°C	Agonist mode: Increase of induction of B-galactosidase with both mutant (EC_{50} = 3,16 µM) and wild (EC_{50}= 31,6 µM) yeasts in a dose-dependent manner, indirect measure	(Ito-Harashima et al., 2015) PMID: 26070404	AGO?
			UAS-bla HEK293T or UAS-bla GripTite™master reporter cell lines (Invitrogen) with Gal4 DBD fused to the LBD of the receptor Incubation for 16h at 37°C	No agonist effect Antagonist effect: decrease of beta-lactamase activity with EC_{50} = 18,02 nM (5.67E-06 mg/mL)	(Wilkinson et al., 2008) PMID: 18753690	ANT?

			10 mM to 0.318 nM (3.14 to 1E-08 mg/mL) (decrease in half-logs)			
53	21-Hydroxyprogesterone Molar mass = 330,46 g/mol	64-85-7	Rat-1 cells, a rat fibroblast cell line known to express glucocorticoid receptor, and to be glucocorticoid responsive Transfected with the promoter region of human IL-6 (luciferase activity) Exposition to 21-Hydroxyprogesterone for 24h 1000 nM (3.3E-04 mg/mL)	Inhibition of IL-6-luc by 50% after 24h of treatment ➔ Ability of this steroid to activate the GR. ⇨ No showed antagonist activity in this assay Indirect evidence	(McMaster et al., 2008) PMID: 18434350	AGO?
			Identification of several EDCs (and their concentrations) in Water samples in Czech Republic and Switzerland GR-CALUX model of GR activation challenged with the EDCs found in both waters including 21-Hydroxyprogesterone > 10 ng/L (1E-08 g/L)	GR-agonist activity of 21-hydroxyprogesterone with 37% induction of maximal activity induced by DEX (Concentration-response curve in sup. Data) No antagonist activity Possible partial agonism	(Macikova et al., 2014) PMID: 25269596	AGO?
54	Mifepristone Molar mass = 429,6 g/mol	84371-65-3	Bile fistula rats, isolated intrahepatic bile duct units (IBDUs), and purified cholangiocytes 2 days 0,15 mg 3 times daily	GcRs expressed in rat cholangiocytes RU486 blocks increase of biliary bicarbonate concentration and secretion induced by dexamethasone RU486 blocks the increase of activity of the Na+/H+ exchanger (NHE1 isoform) and Cl-/HCO3- exchanger by dexamethasone Used as GR antagonist	(Alvaro et al., 2002) PMID: 11910357	ANT
			15 male volunteers 10 days 9α-fludrohydrocortisone : 1,5 mg Hydrocortisone: 60 mg RU486 : 300 mg every 6h at days 6 and 7 only Measure of urinary ammonium excretion	Mifepristone markedly inhibits the hydrocortisone-induced increase In urinary ammonium excretion.	(Waybill et al., n.d.) PMID: 8025226	ANT

			Reporter assay yeasts expressing human GR and MR Wild yeasts and mutant yeasts (deletion of the CWP1, CWP2, PDR5, and/or PDR10 genes) 0,01 to 100 µM (4.3E-6 mg/mL to 4.3E-2 mg/mL) Incubation for 18h at 30°C	Agonist mode: increase of induction of B-galactosidase activity with EC_{50} = 0,32 µM (1.37E-4 mg/mL) with wild yeast and induction with EC_{50} = 0.03 µM with mutant yeast Antagonist mode: Decrease of induction of B-galactosidase activity with EC_{50} = 1,89 µM (8.1E-4 mg/mL) with wild yeast and induction with EC50 =6,09 µM (2.6E-3 g/L) with mutant yeast **Mifepristone has agonistic and antagonistic effects**	(Ito-Harashima et al., 2015) PMID: 26070404	AGO?/ANT
55	Ketoconazole Molar mass = 531,43 g/mol	65277-42-1	- Reuber rat hepatoma H-II-E-C3 cell line - 1 nM (5,31E-7 mg/mL) -100 µM (5,31E-2 mg/mL) for competitive binding studies ([3H]-dexamethasone competition) - 0.05 (2,66E-4 mg/mL) –10 µM (5,31E-3 mg/mL) for 18h for tyrosine aminotransferase assay	KTZ can bind to GR with IC_{50} values of 50 µM (2,66E-2 mg/mL) Lack of agonist effect Antagonist effect: reduce the dexamethasone-induced tyrosine aminotransferase specific activity in a dose-dependent manner, high concentrations	(Johansson et al., 2005) PMID: 15755314	ANT?
			Double-blinded experiment on a 44-year man 200 mg/day with the dose gradually increased to 1000 mg/day 2 weeks Study of depression symptoms linked to cortisol levels in response to KTZ	KTZ declines cortisol levels at 8:00 AM, followed subsequently by a slow upward trend indicating compensation by the hypothalamic-pituitary-adrenal (HPA) axis (KTZ is a GR antagonist) from 800 mg/day Decrease of cortisol levels by KTZ reduces depression symptoms	(Anand et al., 1995) PMID: 7748987	?
			Primary culture of human hepatocytes (for RT-PCR and western blot) Stably transfected HLMN cells line (HeLa cell line with the glucocorticoid responsive reporter gene MMTV-LUC-SVNeo): Exposure for 4h	Human hepatocytes: Decrease of TATmRNA (targets of GR) levels by KTZ Human hepatocytes: KTZ affected neither the accumulation of the GR protein nor of the mRNA HLMN cells: In the presence of dexamethasone, ketoconazole inhibited the	(Duret et al., 2006) PMID: 16608920	ANT?

			Transiently transfected HepG2 or HeLa cells with 10 ng of expression plasmid (pSG5-hGR, pSG5-hPXR) or 100 ng of pBSEN-hCAR together with 100 ng of luciferase reporter constructs pGL3-CAR(-4711/+144)-LUC or pGL3(CYP3A4/XREM-7800-7200/-262/+11)-LUC COS-1 cells transfected with Psg56Hgr expression plasmid for ligand binding assay ([3H]-dexamethasone assay) 24h Exposure concentrations?	reporter gene activity in a concentration-dependent manner with a IC_{50} of approximately 30 µM (1.59E-2 mg/mL), very high concentration COS-1 cells: decrease of the amount of [3H]-dexamethasone with a IC_{50} of approximately 100 µM (5.3E-2 mg/mL) of KTZ, very high concentration		
			Hepatoma tissue culture (HTC) cell TAT activity was assessed by a spectrophotometric method after 20h of treatment with or without Dex	Inhibition IC_{50} = 12 µM (6.4E-3 mg/mL) 10µg/mL	Loose and al, 1983 PMID: 6135709	ANT?
56	Spironolactone Molar mass = 416,57 g/mol	52-01-7	Mouse fibroblast cell line 1471 (permanently transfected with chloramphenicol acetyltransferase (CAT)) Incubation for 4h for binding assay Incubation for 19h for CAT activity 1E-9 (54,16E-7 mg/mL) to 1E-6 M (54,16E-4m g/mL) for agonist activity 1E-7 (54,16E-5 mg/mL) to 1E-5 M (54,16E-2 mg/mL) for antagonist activity	Competes with dexamethasone for GR binding with an ED_{50} of about 2 µM (8.3E-4 mg/mL) No agonist activity Significant antagonist activity, with a 70% decrease in CAT induction at a 10 µM (54,16E-2 mg/mL) concentration. ED50 at 8 µM (3.33E-3 mg/mL), at high conc then	(Couette et al., 1992) PMID: 1309341	ANT?
			Reporter assay yeasts expressing human GR and MR Wild yeasts and mutant yeasts (deletion of the CWP1, CWP2, PDR5, and/or PDR10 genes) 0,01 to 100 µM (4.2E-6 mg/mL to 4.2E-2 mg/mL) Incubation for 18h at 30°C	Agonist mode: increase of induction of B-galactosidase activity with EC_{50} 3,16 µM (1.33E-3 mg/mL) with mutant yeast and no induction with wild yeasts Antagonist mode: inhibition of relative activity with IC_{50} 0.39 µM (1.62E-4 mg/mL) (maximum inhibition reaches 52%) and IC_{50}	(Ito-Harashima et al., 2015) PMID: 26070404	AGO?/ANT?

				2,69 µM (1.12E-3 mg/mL) (maximum inhibition reaches 22%) with wild yeasts higher sensitivity with mutant yeasts		
			UAS-bla HEK293T or UAS-bla GripTite™master reporter cell lines (Invitrogen) with Gal4 DBD fused to the LBD of the receptor Incubation for 16h at 37°C 10 mM (4.16 mg/mL) to 0.318 nM (1,32E-7 mg/mL) (decrease in half-logs)	No agonist effect Antagonist effect: decrease of beta-lactamase activity with IC_{50} = 592,6 nM (2.47E-4 mg/mL)	(Wilkinson et al., 2008) PMID: 18753690	ANT
57	E2 (17β-estradiol) Molar mass = 272,4 g/mol	50-28-2	MCF-7 cells stably transfected with the GR responsive pMTV-CAT reporter (MCF-7–MTV cells) Chronic treatment for 16 days or E2 withdrawal with 5 days with E2 and withdrawal of E2 from Day 6 to 16 10 nM of E2 (2,724E-6 mg/mL) MCF-7 cells transiently transfected with [(GRE)3-Luc] reporter plasmid 1 nM of E2 (2,724E-7 mg/mL) GR abundance determined using [3H]DEX binding assays Exposure for 6 days with 10 nM of E2 Northern blot to target GR mRNA Exposure for 6 days with 0,1 (2,724E-8 mg/mL) or 10 nM (2.72E-7 mg/mL) of E2	Reduction of dexamethasone (DEX)-induced chloramphenicol acetyl transferase (CAT) (decrease of CAT levels by ELISA) No recovery of CAT levels with the withdrawal of E2 Suppression of DEX-induced luciferase Decrease of GR levels (decrease of [3H]DEX binding) Decrease of GR nMRA with both concentrations of E2 All indirect evidence	(Krishnan et al., 2001) PMID: 11358672	ANT?
			Transiently transfected COS-7 cells with YFP-GR for translocation assay Exposition for 1h 100 nM (2,724E-5 mg/mL) HeLa cells stably transfected with a chimera receptor joining the Gal 4 DNA binding domain and human GR ligand binding domain for reporter assay Exposure for 18h	Low affinity for GR and very low translocation Agonist mode: No effect Antagonist mode: EC_{50} = 1,65 uM (4.5E-4 mg/mL), binding data poor	(Agler et al., 2007) PMID: 17989426	ANT?

			UAS-bla HEK293T or UAS-bla GripTite™master reporter cell lines (Invitrogen) with Gal4 DBD fused to the LBD of the receptor Incubation for 16h at 37°C 10 mM (2.724 mg/mL) to 0.318 nM (to 8,66E-8 mg/mL) (decrease in half-logs)	Antagonist effect: decrease of of beta-lactamase activity with IC_{50} = 1,2 uM (3.2E-4mg/mL) No agonist effect	(Wilkinson et al., 2008) PMID: 18753690	ANT?
58	Fulvestrant Molar mass = 606,77 g/mol	129453-61-8	mRNA and protein expression of the GR were investigated by reverse transcription polymerase chain reaction and Western blotting respectively, in the ERa-positive MCF-7 breast cancer cell line Wild type cells and fulvestrant-resistant cells 7 days 100 nM (6.1E-05 mg/mL)	Both fulvestrant -treated and -resistant MCF-7 cells exhibited increased GR mRNA and protein expression	(Fakes et al., 2021) DOI: 10.18573/bsdj.184	?
59	Lithium Chloride (LiCl) Molar mass = 42,39 g/mol	7447-41-8	Chinese hamster ovary K1 (CHO-K1) cells (dual-luciferase reporter gene assay for hGR) 24h of exposure 1E-9 (4,24E-8 mg/mL) to 1E-5 M (4,24E-4 mg/mL) mouse macrophage cells J774A.1 (rtPCR with expression of GILZ gene) 24h Exposure at the non-toxic concentration: 10-5 M (4,24E-4 mg/mL)	CHO-K1 cells: no agonist activity and antagonist activity at RIC20 = 3,4E-6 (1.44E-4 mg/mL) (decrease of relative luciferase activity) J774A.1: decrease of GILZ expression level at 1E-5 M	(Zhang et al., 2018) PMID: 29957541	ANT?
			Primary culture of mouse thymocytes (from 5 to 500 µmol/L) Study of caspase 3 4h	Strong antagonistic effect of LiCl on DEX-induced apoptosis at 5 (2.1E-4 mg/mL) and 100 (4.2E-3 mg/mL) µmol/L (Dex effect almost completely prevented at this concentration) Indirect evidence	Tiang and al., 2003	ANT?

60	sucrose	57-50-1	Study of competition of [3H]dexamethasone binding to the GR cytosol receptor with RU486 and dexamethasone using a sucrose density gradient analysis Skin fibroblasts	RU486 abolishes a specific [3H]dexamethasone binding peak in the cytosol	(Nawata et al., 1988) PMID: 3347052	NEG
61	Sodium nitrite	7632-00-0	30 patients Effects on lung function in asthmatic patients 15 mg twice daily	No adverse effects 120 min and 12 weeks after the beginning of the treatment Increase of the forced expiratory volume	(Sriboonyong et al., 2021)PMID: 33338662	NEG
62	Sodium azide	26628-22-8	Study of GR translocation in response to cortisol Rainbow trout (Oncorhynchus mykiss) hepatocytes	Use of sodium azide as a buffer The cells were incubated with a blocking solution (2% BSA with **sodium azide** in Medium A) for 3 h at 37 ◦C followed by overnight incubation with anti-trout GR antibody at 4°C, little relevance	(Das & Vijayan, 2023) PMID: 36829586	NEG

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Annex 2: Standard operating procedure for the GR-TA assay

The following is the final SOP for the GR-TA assay as it was developed throughout the validation effort. There were some changes between phases to clarify specific steps, the data processing and other details. This has been outlined in the report. Following the review a few further updates were included.

Stably transfected human glucocorticoid receptor hGR transcriptional activation assay for detection of agonistic and antagonist activity of chemicals towards hGR

The HMLN-hGR assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to establish signal activation (agonism) or blocking of the hGR (antagonism) caused by a hGR ligand. In HMLN-hGR cell line, when a chemical substance binds to hGR, together they form homodimers with other liganded hGR and recruit cofactors to modulate luciferase transcription through the expression of the MMTV-Luc reporter gene. The expression of luciferase is thus directly linked to the concentration of the effector ligand.

Once the substrate of luciferase, luciferin, is added to the cells, it is possible to assess transactivation quickly and inexpensively by measuring cell luminescence in living cells. Luciferase activity can also be evaluated quickly with a number of commercially available test kits in lysing cells. The test system for the assessment of the relative hGR activation, inhibition or transcriptional interference by test chemicals provided here utilises the HMLN-hGR cell line.

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ABBREVIATIONS AND GLOSSARY

Acceptability criteria: minimum standards for the performance of experimental controls. All acceptability criteria should be met for an experiment to be considered valid.

Agonist: a substance that produces a response, e.g. transcription, when it binds to a specific receptor.

Antagonist: a type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Assay: uses interchangeably with Test.

Cytotoxicity: harmful effects to cell structure or function that can ultimately cause cell death and can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

hGR: human Glucocorticoid Receptor

DBD: DNA Binding Domain

DCC: dextran-coated charcoal

DF: dilution factor

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethylsulfoxyde

EC_{50}: half maximal effective concentration

GRE: Glucocorticoid Response Elements

IC_{50}: half maximal inhibitory concentration

LBD: Ligand Binding Domain

MMTV: Mouse Mammary Tumor Virus

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

Range finding: experiment that evaluates the dose response, usually carried out with a large dilution factor (e.g., 10) in order to capture the full dose response (if possible). It serves to determine the range of concentrations to be used in a following comprehensive run.

R^2: the square of the correlation coefficient between the Relative Induction of the test chemical at concentration C and the Relative Induction of its specificity control at concentration C.

RI: Relative Induction

RLU: Relative Light Unit

Run: an individual experiment that evaluates chemical action on the biological outcome of the test method. Each run is a complete experiment performed on replicate wells of cells plated from a common pool of cells at the same time. ("run" and "experiment" or "repetition" can be used interchangeably but have to be strictly differentiated from "replicate").

Independent run: a separate, independent experiment that evaluates chemical action on the biological outcome of the test method, using cells from a different pool, freshly diluted chemicals, conducted on different days or on the same day by different staff.

2 CONTEXT

The glucocorticoid receptor (GR) is a ligand-activated transcription factor of the steroid receptor family and the nuclear receptor superfamily. It is involved in a wide range of key physiological processes, including stress response, immune system, and metabolism.

Its expression is ubiquitous and its ligands include endogenous and synthetic glucocorticoids (GCs) such as cortisol, dexamethasone and prednisolone. Once activated by a ligand, GRs form homodimers and bind to GREs (glucocorticoid response elements), specific regions on the DNA of target genes, to modulate their transcription.

Because of the involvement of GR in key physiological processes, it is important to assess the ability of chemicals to interfere with this specific nuclear receptor.

For this purpose, we developed a stably transfected cell line HMLN-hGR expressing the reporter gene luciferase specifically activated by GR in the presence of an effector ligand.

This document describes an *in vitro* transactivation assay for the evaluation of agonistic and antagonistic activity of chemicals towards human glucocorticoid receptor hGR using the HMLN-hGR cell line.

3 DESCRIPTION OF THE CELL LINE

3.1 ESTABLISHMENT OF THE CELL LINE

The establishment of the HMLN-hGR cell line from HeLa cells was described in Grimaldi *et al.*, 2019.

HeLa cells endogenously express several nuclear receptors (NRs): retinoic acid receptors (RAR) α, β and γ, retinoid receptors (RXR) α, β and γ, vitamin D receptor (VDR) and GR. HeLa cells also express aryl hydrocarbon receptor (dioxin receptor) (AhR). However, HeLa cells do not express androgen receptor (AR), progesterone receptor (PR), mineralocorticoid receptor (MR) or estrogen receptors (ER) α and β.

Because they do not express AR, PR and MR and they are easy to transfect and cultivate, HeLa cells were chosen to establish a glucocorticoid responsive cell line. HeLa cells were stably co-transfected with a glucocorticoid responsive gene, MMTV-Luciferase-SV-Neomycin, and a glucocorticoid receptor alpha (GRα) expressing plasmid (pSG5-hGR-puromycin) to obtain HMLN-hGR cells. The transfection of glucocorticoid receptor expressing plasmid (pSG5-hGR-puromycin) enabled to increase the expression of GRα.

The HMLN-GR cell line can be obtained from the INSERM U1194, IRCM laboratory, upon signature of a Material Transfer Agreement.

3.2 CELL CULTURE CONDITIONS

3.2.1 PLASTICS

Plastics used for cell culture are either polystyrene or polypropylene. Cells are grown in cell culture flasks treated for adherent cells, (*i.e.* T75 flasks Sarstedt Inc 83.3911.002 with 10 mL culture medium or T175 flasks Sarstedt Inc 83.3912.002 with 20 mL culture medium).

There are no specific recommendations regarding the plastic that should be used. If the basal activity of the cells is too high (higher than 4% of the maximal activity induced by dexamethasone 100 nM), it could be suspected that glucocorticoid receptor agonists are present in the plastics. In that case, plasticware (plates, flasks, pipettes, tips, tubes, etc) should be tested by extracting in DMSO and ran in the assay.

3.2.2 MEDIA

Cells are grown in culture medium, *i.e.* Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) with phenol red (Gibco 31331-028) supplemented with 10% of fetal bovine serum (FBS, Eurobio CVFSVF00) and 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL) in a 5% CO_2 humidified atmosphere at 37°C. Culture medium is supplemented with 1 mg/mL geneticin (Invivogen ant-gn 100 mg/mL) and 0.5 µg/mL puromycin (Sigma P8833) as selection antibiotics in order to maintain stable transfections.

Exposures are made in test medium, *i.e.* phenol red-free DMEM/F-12 medium (Gibco 21041-025) supplemented with 5% dextran-coated charcoal (DCC)-treated FBS and 1% v/v penicillin/streptomycin (Gibco 15070-63 5000 U/mL). The composition of the media is summed up in Table 1. The suppliers and references are listed as an indication, but equivalent products can be used.

There are no specific recommendations regarding the DCC-treated FBS. If the basal activity of the cells is too high (higher than 4% of the maximal activity induced by dexamethasone 100 nM), it could be suspected that the serum is not correctly depleted.

Luminescence measurements are done in luminescence medium, *i.e.* test medium containing 0.3 mM D-luciferin (Perkin Elmer 122799) in Tris buffer 10 mM pH 8.0. D-luciferin is dissolved at 10^{-2} M and stored at -20°C, then diluted extemporaneously to 0.3 mM in test medium. D-luciferin at 10^{-2} M can be stored at -20°C for one year.

Table 27: Media composition. The suppliers and references are listed as an indication, but equivalent products can be used.

Culture medium	- DMEM/F-12 with phenol red (Gibco 31331-028) - 10% FBS (Eurobio CVFSVF00) - 1% v/v penicillin/streptomycin (Gibco 15070-63) - 0.5 µg/mL puromycin (Sigma P8833) - 1 mg/mL geneticin (Invivogen ant-gn)
Test medium	- DMEM/F-12 without phenol red (Gibco 21041-025) - 5% DCC-treated FBS - 1% v/v penicillin/streptomycin (Gibco 15070-63)
Luminescence medium	- DMEM/F-12 without phenol red (Gibco 21041-025) - 5% DCC-treated FBS - 1% v/v penicillin/streptomycin (Gibco 15070-63) - 0.3 mM D-luciferin (Perkin Elmer 122799)
Freezing medium	- FBS (Eurobio CVFSVF00) - 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich D2650)

FBS is treated with DCC to deplete steroids and avoid interferences during exposures. DCC-treated FBS is obtained as follows: FBS is incubated with 10 g/L activated charcoal Norit (Sigma 97876) and 1 g/L Dextran (Sigma D8821) for 30 min at 56°C on magnetic stirrer then centrifugated for 40 min at [9000-9100] x g and 4°C. The supernatant is incubated again with 10 g/L activated charcoal Norit and 1 g/L Dextran for 30 min at 40°C on magnetic stirrer then centrifugated for 2 hours at [9000-9100] x g and 4°C. The supernatant is then filtered into sterile vessels in two steps through a sterile single use vacuum filter unit with a 0.45 µm membrane then a sterile single use vacuum filter unit with a 0.22 µm membrane.

3.2.3 PASSAGING

When the flask reaches 90-100% confluence, cells should be passaged. Confluent flasks are rinsed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco 14190250), trypsin 0.05% (trypsin-EDTA (0.5%, Gibco 15400054 diluted to 0.05% in DPBS) is added to the flask (2 mL if T75, 4 mL if T175) which is then put in the incubator at 37°C. After 2 to 3 minutes, when cells are detaching, culture medium is added to the flask (8 mL if T75, 6 mL if T175) and cells are counted and percent viability is estimated using a hemocytometer or cell counter and Trypan blue exclusion. Cells are then diluted to the wanted density and put into a new flask in fresh culture medium.

As an indication, a confluent flask diluted at 1/50 takes about a week to reach confluence again (around 300 000 cells in a T75 flask).

The maximum number of passages for using this cell line is 50, as long as all the acceptance criteria are met (see chapter 5.8 Acceptance criteria).

3.2.4 FREEZING AND THAWING

Cells are frozen as follows: after trypsinization and resuspension in culture media, cells are counted and percent viability is estimated using a hemocytometer or cell counter and Trypan blue exclusion. A volume of cell suspension containing the number of cells wanted (*e.g.* to freeze aliquots of 5 million vial) is sampled and centrifuged at 135 x g for 5 minutes. After centrifugation, the supernatant is carefully aspirated and discarded. The pellet is gently resuspended in cold freezing medium (FBS, Eurobio CVFSVF00 and 10% dimethyl sulfoxide DMSO, Sigma-Aldrich D2650, see Table 1) for a concentration of 5 million cells in 1 mL freezing medium. 1 mL aliquots of this cell suspension are dispensed into cryogenic storage vials then frozen in a controlled rate freezing chamber (*e.g.* isopropanol chamber). The chamber is stored at -80°C overnight then cryovials are transferred into a liquid nitrogen tank.

For thawing, the needed amount of culture medium (*e.g.*, if thawing of one cryovial containing 1 mL of cell suspension with 5 million cells, the needed amount is 9 mL) must be pre-warmed at 37°C. The cryovial containing frozen cells is placed into a 37°C water bath and gently swirled for under 1 minute until the ice starts to thaw. The vial is then wiped with 70% ethanol and put under the laminar hood. 400 µL of pre-warmed culture medium is added dropwise into the cryovial, then cells are gently resuspended into the remaining pre-warmed culture medium, put into a T75 flask and incubated at 37°C and 5% CO_2.

3.2.5 MYCOPLASMA

Only cells tested as mycoplasma-free should be used in the assays. The cells can be tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit from Lonza (LT07-118) or the RT-PCR detection method as described in OECD (2018) Guidance Document on Good In Vitro Method Practices (GIVIMP), Series on Testing and Assessment No. 286. This detection shall be done every time a vial is thawed and an example of the results of the test should be provided.

4 CHEMICALS

Reference agonist ligand dexamethasone (DEX) (CAS number 50-02-2) and reference antagonist ligand mifepristone (RU 486) (CAS number 84371-65-3) can be obtained from Sigma-Aldrich Chemical Co. (Saint-Quentin Fallavier, France).

5 INSTRUMENTATION

The assay described herein requires a luminometer able to read 96 well-plates with detectors from above the wells (for example the plate reader MicroBeta Wallac luminometer from Perkin-Elmer). No injector is needed.

6 DESCRIPTION OF THE ASSAY

6.1 PRINCIPLE

When a chemical binds to hGR, it forms homodimers, translocate to the nucleus, and recruits cofactors to modulate luciferase transcription through the expression of the MMTV-Luc reporter gene. The expression of luciferase is thus directly linked to the concentration of the effector ligand. Once the substrate of luciferase, luciferin, is added to the cells, it is possible to assess transactivation by measuring cell luminescence. The transcriptional assay using HMLN-hGR cell line thus allows to assess the activities of tested chemicals towards hGR.

The steps of an independent run of the assay are presented in Figure 1.

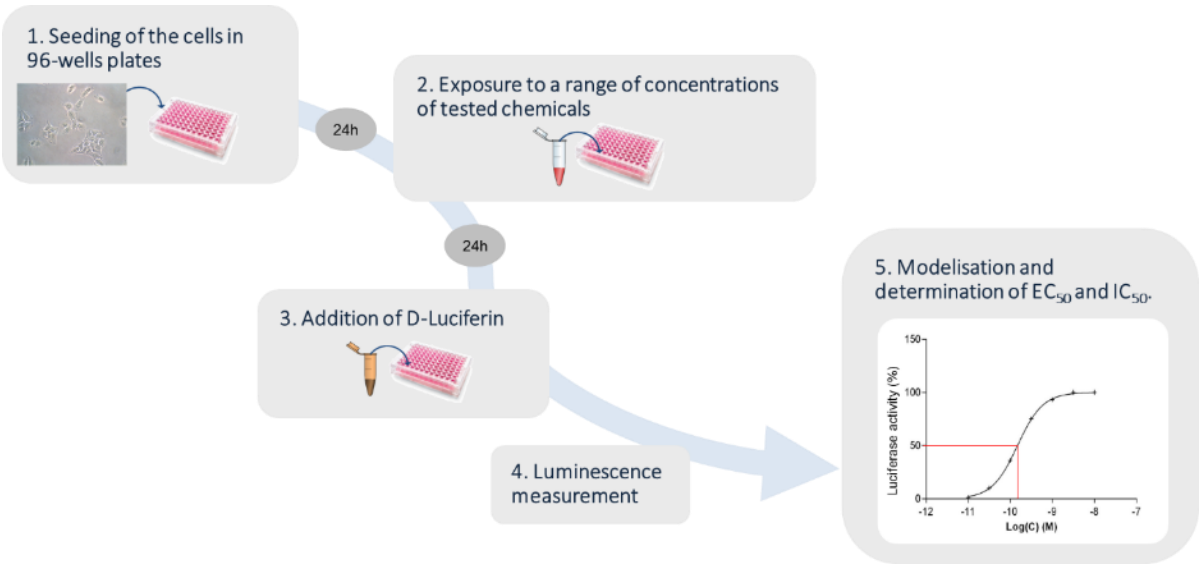


Figure 1: Steps of an independent run

6.2 SOLUBILITY ASSAY

The solubility test procedure is based on attempting to dissolve a test item in various solvents with increasingly rigorous mixing techniques. DMSO is the recommended solvent for the assay. If an alternative solvent is utilized, the following should be determined: a) the solubility of the test substance in the solvent; and b) the cytotoxicity as a function of the concentration of solvent.

Determination of whether a test item is properly dissolved can be based on visual observation using a microscope and/or by centrifugation at ~10 000 g of e.g. 1-2 mL of solvent with test item and evaluating the possible presence of a pellet. A test item has dissolved if the solution is clear and shows no signs of cloudiness or precipitation, and if no pellet is visible upon centrifugation.

If the test item does not dissolve in the solvent, the volume of solvent is increased so as to decrease the test item concentration, and then the sequence of mixing procedures are repeated in an attempt to solubilise the item at the lower concentration. If a test item dissolves in more than one solvent, then the choice of solvent should be, in order of priority, DMSO, water, and EtOH. If the solvent is not DMSO, the medium for test items should anyway be spiked with DMSO to make conditions identical between test item and controls. Similarly, if EtOH is used as solvent, the reference and controls in the assay should also be spiked with the same final concentration of EtOH.

Determination of Solubility Using the Step-Wise (Tiered) Procedure (from OECD guidance document 129):

Tier 1: Weigh out 20-30 mg of the test item into a glass tube/vial. Add appropriate volume of DMSO into the tube to get a concentration of 200 mg/mL. Mix the solution by vortexing. If complete solubility is achieved, then additional solubility procedures are not needed. If the test item is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2.

Tier 2: Weigh ~10 mg of the test item into a glass tube/vial. Add appropriate volume of DMSO into the tube to get a concentration of 60 mg/mL. Mix the solution by vortexing. If complete solubility is achieved, then additional solubility procedures are not needed. If the test item is insoluble in Tier 2 at 60 mg/mL, proceed to Tier 3.

Tier 3: Weigh ~10 mg of the test item into a glass tube/vial. Add appropriate volume of DMSO into the tube to get a concentration of 20 mg/mL. Mix the solution by vortexing. If complete solubility is achieved, then additional solubility procedures are not needed. If the test item is insoluble in Tier 3 at 20 mg/mL, proceed to Tier 4.

Tier 4: If the item is insoluble in DMSO, repeat the sequence from Tier 1 and onwards using water as solvent. If the test item dissolves, no additional solubility procedures are necessary. If the test item does not dissolve, continue with Tier 5.

Tier 5: If the item is insoluble in DMSO or water, repeat the sequence from Tier 1 and onwards using ethanol as solvent. If the test item dissolves, no additional solubility procedures are necessary. If the test item does not dissolve, continue with Tier 6.

Tier 6: Weigh out new aliquots of test item at ~10 mg each and evaluate higher dilutions in all three solvents in parallel following the mixing procedures below.

Mixing Procedures

The following hierarchy of mixing procedures will be followed to dissolve the test item:

a) Gently mix at room temperature by vortexing for 1-2 minutes.

b) If test item has not dissolved, use water-bath sonication for up to 30 minutes.

c) If test item has not dissolved after sonication, then warm solution to 37°C for up to 60 minutes in a water-bath or in a CO_2 incubator. The solution may be stirred during warming.

d) Allow the sample to stand for ~1h on the bench.

e) Proceed to Tier 2 (and Tiers 3-6, if necessary and repeat mixing procedures a - d).

Determination of Solubility in Complete assay Medium

After that the highest soluble concentration of test item in appropriate solvent has been determined, proceed with diluting the stock solution of test item in cell culture medium to a final solvent concentration of 0.1%. Evaluate solubility by microscopy and/or centrifugation. If the test item is not soluble in medium, dilute the stock solution 3.162-fold with solvent, perform a new dilution in cell culture medium to 0.1% solvent concentration, and repeat the evaluation of solubility. Continue until a concentration of test item that is soluble in cell culture medium is determined.

6.3 CYTOTOXICITY ASSAY

A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any cytotoxicity problems. Initially, chemicals are tested up to the maximum soluble concentration in test medium with log dilution factor and 7 concentrations to cover a large concentration range which should be adjusted (if necessary).

A positive control for cytotoxicity is added in three solvent control wells per plate: 4 wells containing solvent control are emptied, rinsed in PBS and replaced by 200 µL Triton X-100 1% v/v then incubated for 5 minutes.

Cytotoxicity is primarily assessed by checking one well per column of the plate by microscopy, to estimate cell number/viability issues. Anomalies in the wells can be documented by taking pictures. If the morphology of the cells is not normal, the concentration is excluded (even if the well passes the MTT assay). Cytotoxicity assay should be done in clear-bottom plates.

The method used to assess cytotoxicity during the validation of the HMLN-hGR assay is the tetrazolium-based colorimetric (MTT) assay[1]. However, other quantitative methods for the determination of cytotoxicity can be used, like resazurin assay or lactate dehydrogenase (LDH) leakage test in combination with qualitative visual inspection of cells following exposure to test chemicals.

In the MTT assay, the reagent used is the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma M2128). The tetrazolium ring it contains is reduced to formazan by mitochondrial succinate dehydrogenase from active living cells. In the mitochondria of the cells, a violet precipitate is formed, the amount of which is proportional to the amount of living cells. After incubation of the cells with MTT, they are lysed in 100% DMSO and the formazan crystals are dissolved. The optical density measurement by spectroscopy then makes it possible to determine the relative quantity of living cells.

The MTT test is performed as follows:

- Preparation of MTT 10X (5 mg/mL) in sterile water (this solution can be stored for about ten days at 4°C),
- Dilution to 1X (0.5 mg/mL) in test medium,
- Wells are emptied and rinsed with PBS,

[1] MTT assay should be done in clear-bottom plates. If the transactivation assay was done in white-bottom plates, the resazurin assay can be done instead of the MTT.

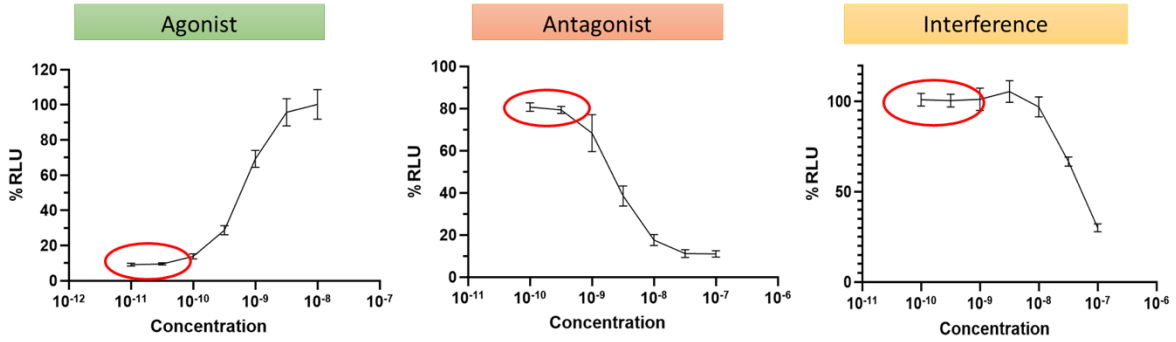
- 100 µL of MTT 1X is added per well,
- Plates are incubated for 3 hours at 5% CO_2 ± 37°C,
- Wells are gently emptied with a vacuum pomp or by flicking plates,
- 100 µL of 100% DMSO is added per well (other solutions such as acidified isopropanol is also suitable),
- Plates are shaken for 10 minutes,
- Plates are read with a spectrophotometer at 570 nm and 640 nm.

The OD values measured at 640 nm are subtracted from the OD values measured at 570 nm (OD570 - OD640). Values obtained for the solvent control (average of technical replicates) is considered to be 100% of viability. Test chemicals (average of technical replicates) must not exceed 20% mortality or they are considered to be cytotoxic and must be tested again at lower concentrations. Chemicals/concentrations exceeding 20% cytotoxicity must be excluded from the analysis for hGR (ant-)agonism.

Once concentrations range has been determined, cytotoxicity is assessed in the agonist and antagonist test method protocol. After each run, viability of the cells in the plates is checked to ensure that there is no cytotoxic effect at the concentrations tested.

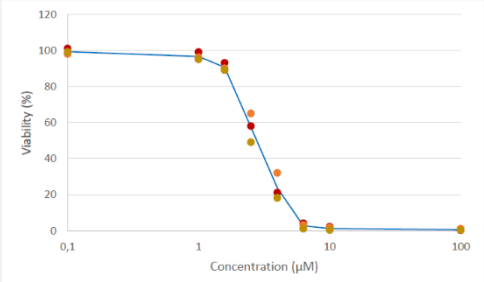
6.4 RANGE FINDING AND CONCENTRATION SELECTION

Based on the extent of cytotoxicity observed in the cytotoxicity test, a first range-finding run should test the chemical starting at the maximum soluble non-toxic concentration with a large dilution factor (1:10). Concentrations in the second, and if necessary third run may be adjusted as appropriate to better characterise the concentration-response curve, *i.e.* the curve should cover no-effect concentrations (at least two concentrations with no effect), intermediate effect concentrations, and maximum effect concentration of the chemical (see below).



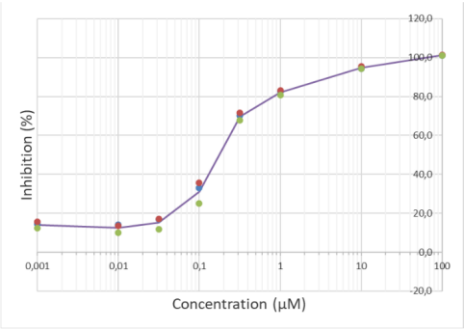
Examples of potential dilution schemes to better characterise the concentration-response curve are presented below:

Item A	DMSO stock solutions		
	Concentration	Test item volume	DMSO volume
C8	50.0x10^{-2}M	100µL stock solution	100µL
C7	5.00 x10^{-2}M	20µL C8	180µL
C6	3.15 x10^{-3}M	106µL C7	62µL
C5	1.99 x10^{-3}M	106µL C6	62µL



C4	1.26 x10^{-3}M	106µL C5	62µL
C3	7.92 x10^{-4}M	106µL C4	62µL
C2	5.00 x10^{-4}M	106µL C3	62µL
C1	5.00 x10^{-5}M	20µL C2	180µL

Item B	DMSO stock solutions		
	Concentration	Test item volume	DMSO volume
C8	1.0x10^{-1}M	200µL stock solution	0µL
C7	1.0 x10^{-2}M	20µL C8	180µL
C6	1.0 x10^{-3}M	20µL C7	180µL
C5	1.0 x10^{-4}M	20µL C6	180µL
C4	5.0 x10^{-6}M	10µL C5	190µL
C3	2.5 x10^{-7}M	10µL C4	190µL
C2	1.25 x10^{-8}M	10µL C3	190µL
C1	6.25 x10^{-10}M	10µL C2	190µL



Stock solutions of reference chemicals and test chemicals are prepared in DMSO (Sigma-Aldrich D2650) for a concentration of 10^{-4} M for DEX and 10^{-3} M for RU 486.

Each chemical is dissolved in DMSO at 1000X the maximum final concentration wanted in the well as long as there is no cytotoxicity and no lack of solubility.

Aliquots of stock solutions can be stored at -20°C in amber glass vials up to one year. Volume of aliquots should be adapted to limit the number of freezing/thawing cycles to a maximum of five (5).

Fresh dilutions of test chemicals in medium are prepared extemporaneously to test a range of concentration with an appropriate dilution factor. The final DMSO concentrations in the wells must not exceed 0.2% (v/v) of the test medium.

The exposures are done as described in chapter 5.5 and should be done with the same plate layout (figure 3 for agonist range-finding runs and figure 4 for antagonist range-finding runs).

During the range finding run, 1:10 serial dilutions of the test chemical are tested. An appropriate refined concentration range for test chemicals is derived from the pre-screen results (first run), to be tested during the comprehensive runs (second run and on). The dilution factor (DF) to be used for comprehensive testing should be as follows: in case a positive response is observed (RI ≥10% for agonism or RI ≤80% for antagonism), an alternating DF 3/3.3 is applied; in case only the highest tested concentration is above the 10% threshold (in agonism testing) or below the 80% threshold (in antagonism testing), DF 2 is applied. The dilution factor must be adjusted according to the effect induced by the test item. Thus, the dilution factor is test item dependent. Another possibility to better characterize the concentration-response curve consists in making adjusted dilution curves, *e.g.,* 10-fold dilutions for the first and last concentrations and a narrower range around EC_{50} or IC_{50} (see examples above).

Following the validation study and review a highest test concentration of 100 µM for test chemicals is recommended for this assay. There is considerable concern that higher test concentrations are unphysiological and may trigger pathways apart from that of GR. It is of course possible to push higher in case this makes biological meaning, and no significant cytotoxicity is encountered.

6.5 *IN VITRO* TRANSCRIPTIONAL ACTIVATION ASSAY

Day 1

Confluent flasks are rinsed with DPBS then trypsin 0.05% is added (2 mL if T75, 4 mL if T175) and the flask is incubated at 37°C. After 2 to 3 minutes, when cells are detaching, culture medium is added to the flask (8 mL if T75, 6 mL if T175) and cells are counted, and percent viability is estimated using a hemocytometer or cell counter and Trypan blue exclusion. Cells are then diluted to reach a concentration of 0.7 x 10^6 cells/mL and seeded in 96 well plates flat bottom white polystyrene wells with micro-clear bottom (Greiner Bio-One 655098, CellStar; Dutscher, Brumath, France) or with white opaque bottom (Greiner Bio-One 655083)[2] to reach a density of 100,000 cells per well in 150 µL culture medium.

Plates are shaken and incubated for 20 minutes on the bench to let the cells settle, then incubated at 5% CO_2 ± 37°C for 22 ± 2 h. Stacking of the plates in the incubator should be avoided.

The details of operator, passage number and any observation regarding the cells are recorded in a reporting sheet (see Excel templates "Reporting_by_Plate.xlsx").

Day 2

After 22 ± 2 h, the cells are observed under a microscope to verify even seeding, normal morphology and confluency. At this stage, the cells should cover 100% of the well.

- ***Agonist assays: testing of chemicals in agonist mode, i.e., chemicals are tested alone in a range of concentrations.***

[2] If cells are seeded in white-bottom plates, a few control wells should also be seeded in a transparent-bottom plate to visually inspect the cells and check the proper seeding and growing of the cells in parallel.

STEP 1

- Tested chemicals and controls are prepared as follows:
- Tested chemicals: the 1000X stock solution of the tested chemical (in solvent) is diluted to 4X[3] the final test concentration in test medium (for example, 4 µL of stock solution 3 mg/mL added to 996 µL of test medium (without solvent), resulting in $3x10^{-3}$ M mg/mL in 0.4% (v/v) solvent).
- A range of concentrations is then done by adapted serial dilutions (default dilution factor is 10, except if results from range-finding suggest otherwise) in test medium containing 0.4% v/v solvent (for example, 95 µL of diluted chemical "4X" added successively to 205 µL of test medium) (Figure 2). Tips must be changed between each dilution step. Chemicals are tested in a run using the 2 substances-layout. After the first run (range-finding test), concentrations and the dilution factor must be adjusted as needed (see above).
- Controls:
  - Solvent control: solvent is diluted to 0.4% (v/v) in test medium (for example, 4 µL of solvent added to 996 µL of test medium).
  - Positive control DEX: the 10^{-4} M stock solution of DEX (in solvent) is diluted to 4X in test medium (for example, 4 µL of stock solution 10^{-4} M added to 996 µL of test medium (without solvent), resulting in $4x10^{-7}$ M in 0.4% (v/v) solvent). For one plate, 800 µL of this solution will be needed (50 µL x 16 wells).

STEP 2

- Culture medium is flicked from the plates above a liquid waste container then plates are tapped on a paper towel. Alternatively, wells can be emptied using a vacuum system. Once it has been checked that all wells are emptied, 150 µL of test medium is quickly added (this must be done rapidly to avoid drying out the cells).
- Tested chemicals: 50 µL of the 4X diluted chemicals are distributed to the wells containing 150 µL of test medium without solvent, in quadruplicates (*i.e,*. 4 wells per concentration), resulting in a final concentration of 1X and a final solvent content set at 0.1% v/v (Figure 2).
- Controls (Figure 3):
  - Medium control: 50 µL of test medium are distributed to the designated wells of the assay plate containing 150 µL of test medium, for a final volume of 200 µL test medium.
  - Solvent control: 50 µL of the 4X diluted solvent are distributed to the designated wells of the assay plate containing 150 µL of test medium, for a final volume of 200 µL test medium and a final solvent content set at 0.1% v/v.
  - Positive control DEX: 50 µL of the 4X diluted DEX are distributed to the designated wells of the assay plate containing 150 µL of test medium, for a final volume of 200 µL test medium, resulting in a final concentration of 10^{-7} M and a final solvent content set at 0.1% v/v.

[3] A 4X dilution is an example of what can be done. It is also possible to prepare chemicals at a 3X concentration before making a 1/3 dilution.

- Plates are incubated at 5% CO_2 ± 37°C for 22 ± 2 h.
- Reference stocks may be prepared in bulk, *i.e.*, they should be prepared in sufficient amount so that all plates of one run get the same working solution distributed to them in order to be compared to each other.
- 4X solutions of test chemicals should be prepared fresh before each experiment.
- For each run in agonist mode, there is a need to test the reference agonist DEX to check the repeatability of the dose-response curve and Hill model parameters (the range of DEX must not be modified) (Figure 3).

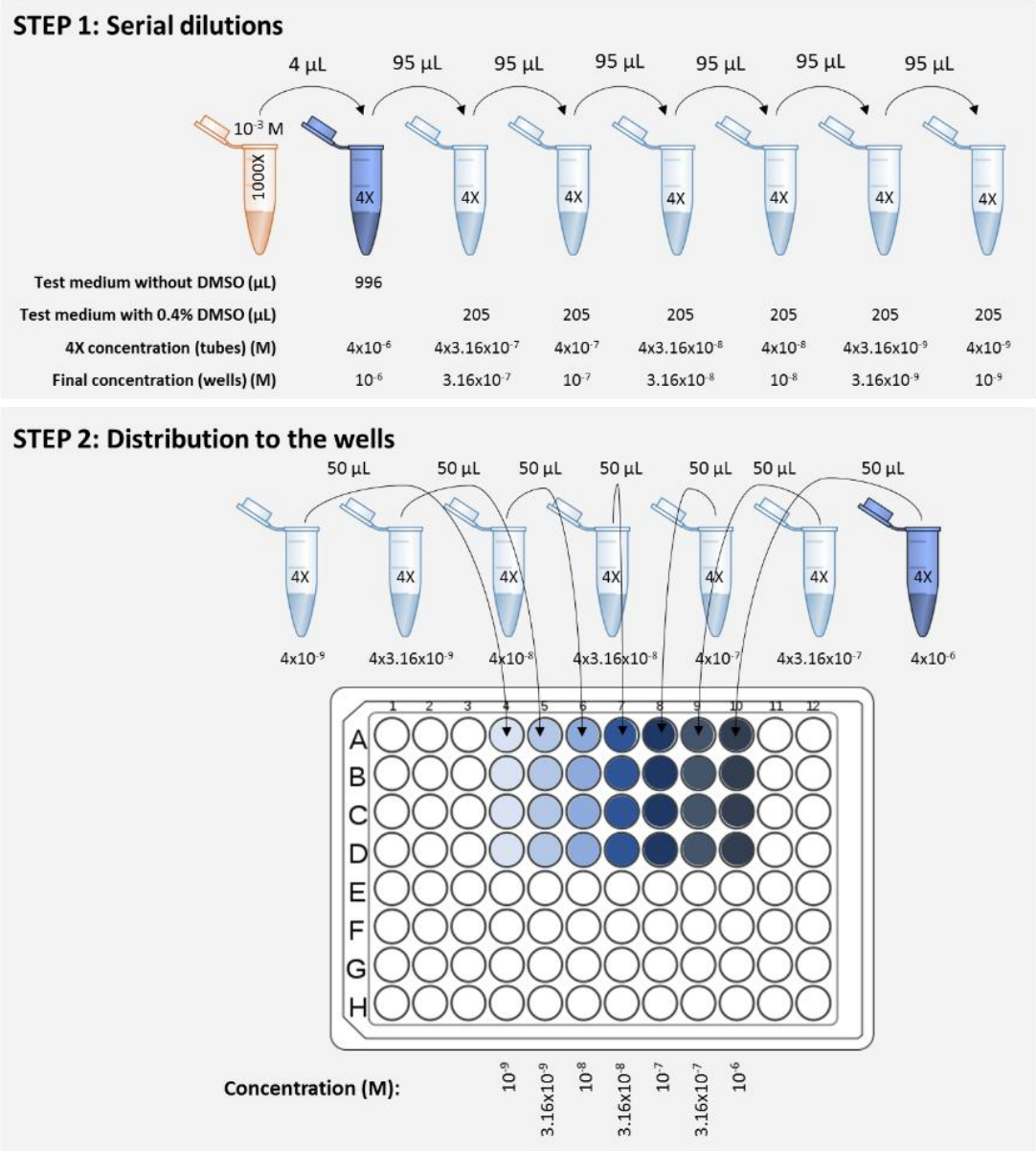


Figure 2: Dilution of test substances in solvent and exposure of the cells.

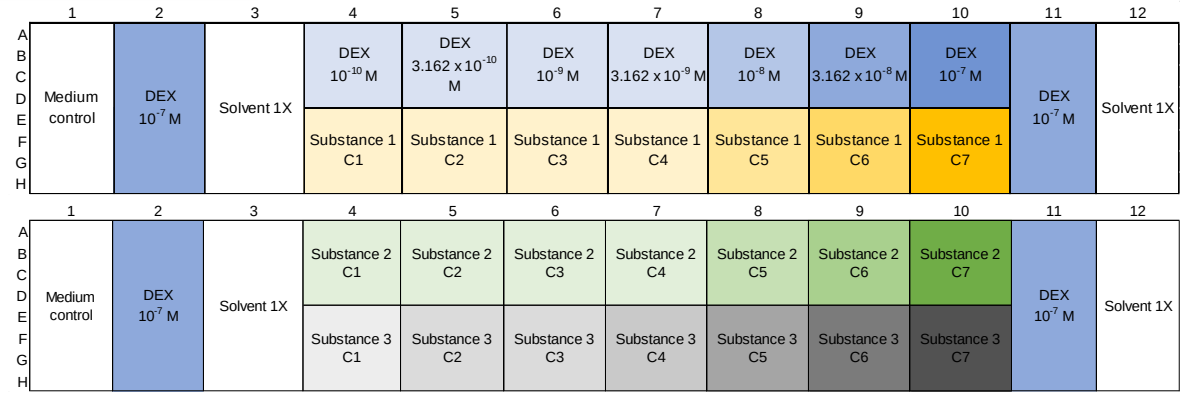


Figure 3: Plate layouts for agonist assays: top plate contains the testing of DEX as a control for the whole independent run, and other chemicals can be tested in the bottom half of the plate and in additional plates.

- ***Antagonist assays: testing of chemicals in antagonist mode, i.e., chemicals are tested in a range of concentrations and co-exposed with DEX at a concentration yielding 80% of the maximum response.*** *The concentration at which this percentage is obtained may vary between laboratories and should be identified by each laboratory when establishing their historical data. We aim at 80% [65%-90%] of the maximum response. As an indication, in our laboratory, this concentration is reached at DEX 8.10^{-9} M. In antagonism assays, because of the co-exposure of two chemicals, the final solvent content will reach 0.2% v/v.* Note: test items that showed antagonist activity during the range finding should be tested in three independent antagonist/interference runs (figure 5).
  - Interference assay: In case a chemical inhibits luminescence in an assay, and is tested negative in the cytotoxicity assays, then it must be tested in an interference assay, *i.e.* in co-exposure with the reference agonist chemical (DEX) at a concentration yielding 100% (±15%) of the response and being 100 to 333 times the concentration used in the antagonist assay (*i.e.*, if the concentration used is the antagonist assay is 3 nM, then the concentration used in the interference assay can be 1000 nM). Concentration of DEX used must not exceed 10^{-6} M. The interference mode has to be tested using the same concentration range than the antagonist mode and must be done on the same plate as the antagonist mode (figure 5). To quantify the shift between the antagonism response and the interference response, we use the R^2 criterion. This criterion is based on the assumption that the decrease of the test chemicals response which is not due to competitive antagonism is proportionally the same as the decrease of the specificity control response at all (non-cytotoxic) concentration. The criterion R^2 is the square of the correlation coefficient between the Relative Induction of the test chemical at concentration C and the Relative Induction of its specificity control at concentration C. The following criteria are applied:
    - Competitive antagonist: $R^2 \leq 0.9$.
    - False positive antagonist: $R^2 > 0.9$.

    The Excel template provided to report the data automatically calculates this R^2.

For the R^2 criteria analysis to be optimal, it is required that the antagonist and interference modes were performed under the same conditions. Thus, antagonist and interference assays must be made on the same plate (see figure 5). Concentrations and dilution factor must be adjusted if needed according to the results obtained with the pre-screen test (see agonist section above).

STEP 1

- Tested chemicals and controls are prepared as follows:
- Tested chemicals: the 1000X stock solution of the chemical (in solvent) is diluted to 4X the final test concentration in test medium (for example, 4 µL of stock solution 3 mg/mL added to 996 µL of test medium (without solvent), resulting in $3x10^{-3}$ M mg/mL in 0.4% (v/v) solvent).
- A range of concentrations is then done by adapted serial dilutions in test medium containing 0.4% v/v solvent (for example, 95 µL of diluted chemical "4X" added successively to 205 µL of test medium) (Figure 2). Tips must be changed between each dilution step. Chemicals are tested in a run using the 2 substances-layout. After the first run (range-finding test), concentrations and the dilution factor must be adjusted as needed (see the agonist part above).
- Controls:
  - Solvent control: solvent is diluted to 0.8% (v/v) in test medium (for example, 8 µL of solvent added to 992 µL of test medium).
  - Positive control DEX: the 10^{-4} M stock solution of DEX (in solvent) is diluted to 4X in test medium containing 0.4% (v/v) solvent (for example, 4 µL of stock solution 10^{-4} M added to 996 µL of test medium containing 0.4% (v/v) solvent, resulting in $4x10^{-7}$ M in 0.8% (v/v) solvent). For one plate, 400 µL of this solution will be needed (50 µL x 8 wells).
  - 80% DEX control for antagonist assay: the $4x10^{-7}$ M stock solution of DEX is diluted to $3.2x10^{-8}$ M in test medium containing 0.4% (v/v) solvent, which corresponds to 4 times $8x10^{-9}$ M, the concentration yielding 80% of the maximum luciferase activity. For example, for one plate, 400 µL of this solution will be needed (50 µL x 8 wells).
  - 100 % DEX control for interference assay only in the half bottom plate (figure 5) of the comprehensive assay from the run 2 and on: the $4x10^{-7}$ M stock solution of DEX is diluted to $4x10^{-6}$ M in test medium containing 0.4% (v/v) solvent, which corresponds to 4 times 10^{-6} M, the concentration yielding 100% of the maximum luciferase activity.
  - Antagonism control RU 486: the 10^{-3} M stock solution of RU 486 (in solvent) is diluted to 4X in test medium (for example, 4 µL of stock solution 10^{-3} M added to 996 µL of test medium, resulting in $4x10^{-6}$ M in 0.4% (v/v) solvent). For one plate, 400 µL of this solution will be needed (50 µL x 8 wells).
- 80% DEX for co-exposure: the $4x10^{-7}$ M stock solution of DEX is diluted to $3.2x10^{-8}$ M in test medium containing no solvent, which corresponds to 4 times $8x10^{-9}$ M, the concentration yielding 80% of the maximum luciferase activity. For example, for one plate, 3200 µL of this solution will be needed (50 µL x 8 columns wells x 8 line wells).

STEP 2

- Culture medium is flicked from the plates above a liquid waste container then plates are tapped on a paper towel. Alternatively, wells can be emptied using a vacuum system. Once it has been checked that all wells are emptied, 100 µL of test medium is quickly added (this must be done rapidly to avoid drying out the cells).
- 50 µL of the 4X diluted chemicals are distributed to the wells containing 100 µL of test medium without solvent, in quadruplicates (*i.e.*, 4 wells per concentration).
- 50 µL of the 4X diluted reference chemical yielding 80% of the maximum luciferase activity is added to the same wells (50 µL of the 4X diluted DEX without solvent), resulting in a final concentration of 1X and a final solvent content set at 0.2% v/v).
- Controls (Figure 4):
    - Medium control: 100 µL of test medium are distributed to the designated wells of the assay plate containing 100 µL of test medium, for a final volume of 200 µL test medium.
    - Solvent control: 50 µL of the 0.8% (v/v) diluted solvent are distributed to the designated wells of the assay plate containing 100 µL + 50 µL of test medium, for a final volume of 200 µL test medium and a final solvent content set at 0.2% v/v.
    - Positive control DEX: 50 µL of the $4x10^{-7}$ M diluted DEX are distributed to the designated wells of the assay plate containing 100 µL + 50 µL of test medium, for a final volume of 200 µL test medium, resulting in a final concentration of 10^{-7} M and a final solvent content set at 0.2% v/v.
    - 80% DEX control: 50 µL of the 4X diluted DEX (with 0.8% v/v solvent) are distributed to the designated wells of the assay plate containing 150 µL of test medium, for a final volume of 200 µL test medium, resulting in a final concentration of $8x10^{-9}$ M and a final solvent content set at 0.2% v/v.
    - 100% DEX control for the interference assay only in the half bottom plate of the comprehensive assay from the run 2 and on : 50 µL of the 4X diluted DEX (with 0.8% v/v solvent) are distributed to the designated wells of the assay plate containing 150 µL of test medium, for a final volume of 200 µL test medium, resulting in a final concentration of 10^{-6} M and a final solvent content set at 0.2% v/v.
    - Antagonism control RU 486:
        - 50 µL of the $4x10^{-6}$ M diluted RU 486 are distributed to the designated wells of the assay plate containing 100 µL of test medium,
        - 50 µL of the diluted 80% DEX ($3.2x10^{-8}$ M in our laboratory) (without solvent) are distributed to the same wells,
        - 50 µL of the diluted 100% DEX ($4x10^{-6}$ M) (without solvent) are distributed to the wells of the half bottom part of the plate of the comprehensive test from the run 2 and on,
        - for a final volume of 200 µL test medium, resulting in a final concentration of 10^{-6} M RU 486 and $8x10^{-9}$ M DEX in our laboratory, and a final solvent content set at 0.2% v/v.

- Plates are incubated at 5% CO_2 ± 37°C for 22 ± 2 h.
- Reference stocks may be prepared in bulk, *i.e.*, they should be prepared in sufficient amount so that all plates of one run get the same working solution distributed to them in order to be compared to each other.
- 4X solutions of test chemicals should be prepared extemporaneously before each experiment.
- For each run in antagonist mode, there is a need to test the reference antagonist RU 486 in antagonist mode, to check the repeatability of the dose-response curve and Hill model parameters (the range of RU 486 must not be modified) (Figure 4).

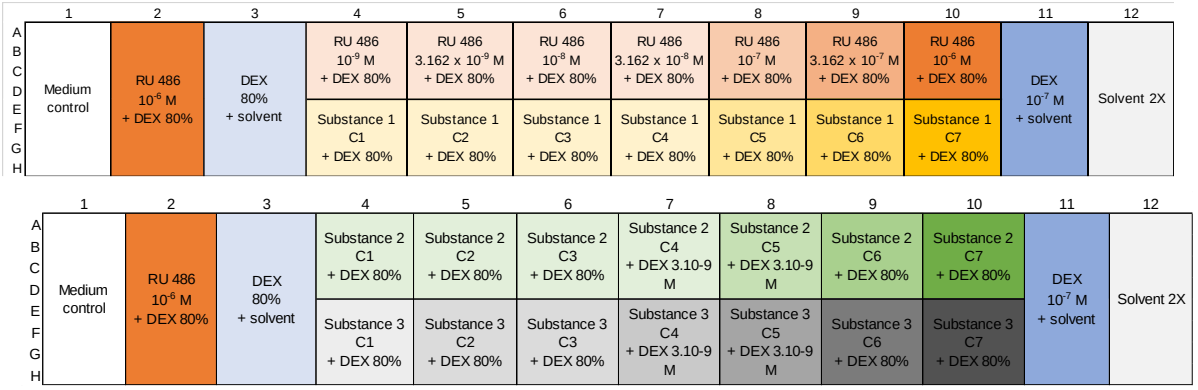


Figure 4: Plate layouts for antagonist assays: top plate contains the testing of RU 486 as a control for the whole independent run, and other chemicals can be tested in the bottom half of the plate and in additional plates.

For the comprehensive test, the control plate layout is shown in figure 5, where the RU 486 control was duplicated.

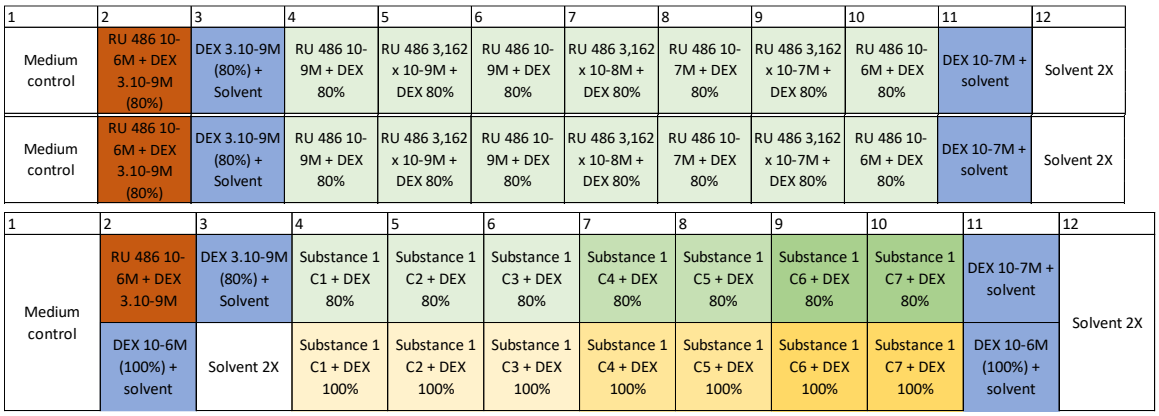


Figure 5: Control plate (above) and plate layout (below) for an antagonist and an interference assay of the comprehensive test from the run 2 and on.

Day 3

At the end of the incubation of 22 ± 2 h, the wells are inspected visually to check for contamination, confluence and potential cytotoxicity at the highest concentration tested. Test medium is flicked from the plates above a liquid waste container then plates are tapped on a paper towel. Alternatively, wells can be emptied using a vacuum system. Once it has been

checked that all wells are emptied, 50 µL per well of luminescence medium is quickly added (this must be done rapidly to avoid drying out the cells) (described in Chapter 2.2.2 Media and Table 1). Luminescence signal is measured in living cells for 2 s per well using a luminometer. Optimal reading time is up to 20 minutes after the addition of luminescence medium.
It is not necessary to lyse the cells before reading luminescence.

Each chemical substance must be tested in at least three independent runs. In case the results re discordant, see the decision tree in Figure 6.

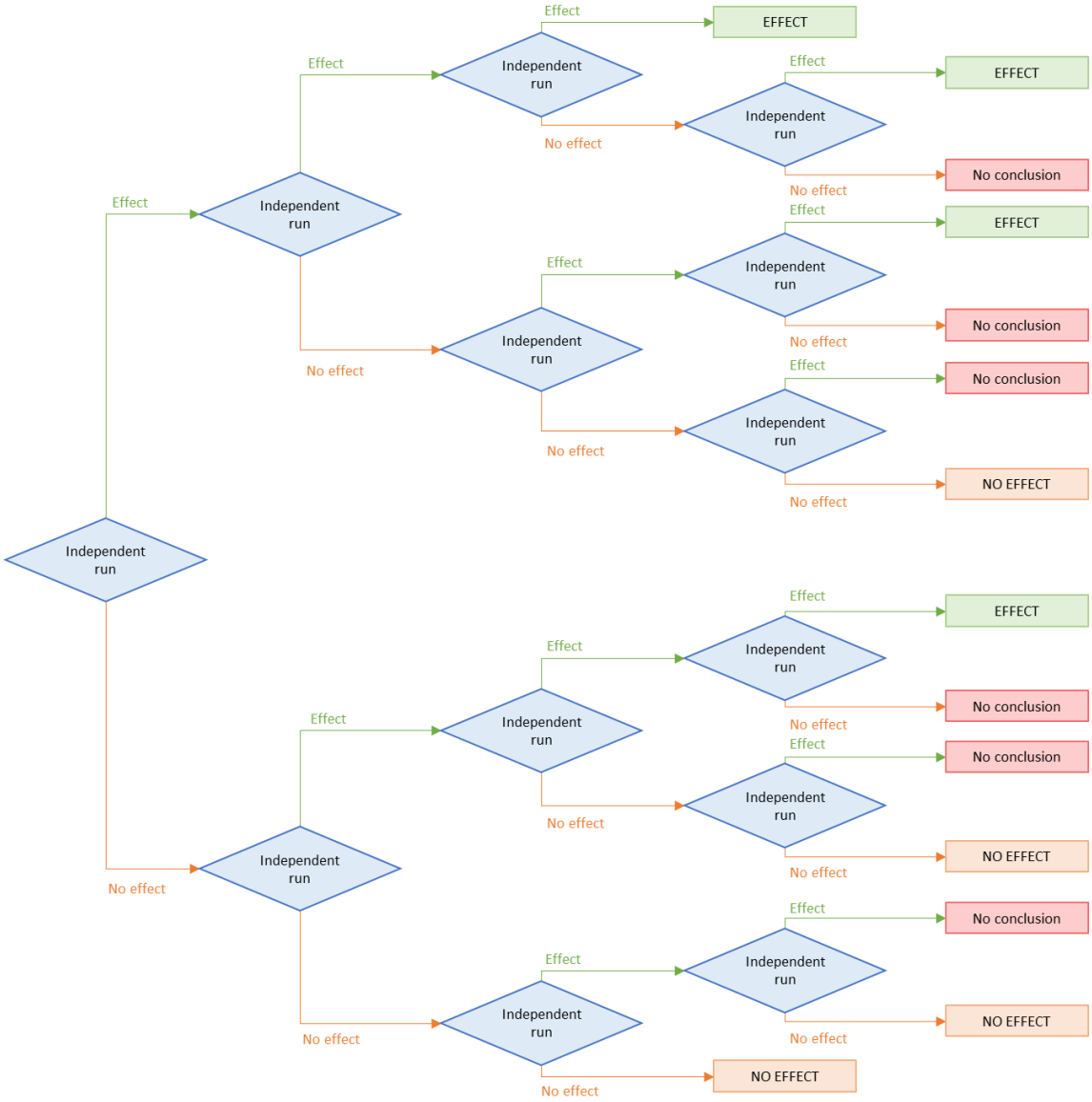


Figure 6: Decision tree for the number of independent runs needed to complete an assay testing one chemical.

6.6 DATA ANALYSIS

Every chemical is tested for a range of concentrations in agonist assays and/or antagonist assays. Examples of typical responses are given in the figure 7.

The mean of the relative light units (RLU) replicates is calculated, and the positive control 10^{-7} M DEX is set at 100%. Results are expressed as the percentage of the maximum luciferase activity induced by the reference chemical: 10^{-7} M DEX according to the following formula:

$$Mean\ luciferase\ activity\ (\%) = \frac{mean\ RLU(Tested\ compound\ Cx)}{mean\ RLU(DEX\ 10^{-7}M)}$$

Where:

- Mean luciferase activity (%): the mean luciferase activity of the tested compound at a given concentration,
- mean RLU (Tested compound Cx): the mean of four RLU replicates exposed to the tested compound at a given concentration,
- mean RLU (DEX 10^{-7} M): the mean of all RLU replicates on the plate exposed to the reference chemical DEX at the concentration 10^{-7} M.

For the wells above basal activity (4%), replicate CVs of >30% are an indication of a potential aberrant replicate. In this case, it must be investigated if the high CV is due to an outlier (and not just due to dispersed values). If it is indeed an outlier, the value can be excluded (maximum 1 out of the 4), and an explanation for the discrimination must be provided. Otherwise, the entire quadruplicate is deleted.

For substances tested in the interference mode, calculate the square of the correlation coefficient between the Relative Induction of the test chemical at concentration C and the Relative Induction of its specificity control at concentration C, using the appropriate excel template.

6.7 PREDICTION MODEL

The basal activity of the cells is < 4% of the maximal activity. A chemical is considered to have an agonistic effect when it increases luciferase activity above 10% of the basal activity of the plate (which is maximum 4%) for at least two consecutive concentrations or the highest concentration (Figure 7A). If not, the chemical is considered as non-agonist (Figure 7B).

For antagonist assays, test chemicals are co-exposed with the reference chemical at the concentration inducing 80% of the maximum activity (*i.e.* 8.10^{-9} M DEX in developing laboratory). A chemical is considered to have an antagonistic effect when it decreases luciferase activity by more than 20% for at least two consecutive concentrations, in the presence of the reference chemical at the concentration inducing 80% of the maximal response (Figure 7C). If not, the chemical is considered as non-antagonist (Figure 7D). The threshold of positiveness for the antagonist mode will be calculated using the ref 80% value of each plate.

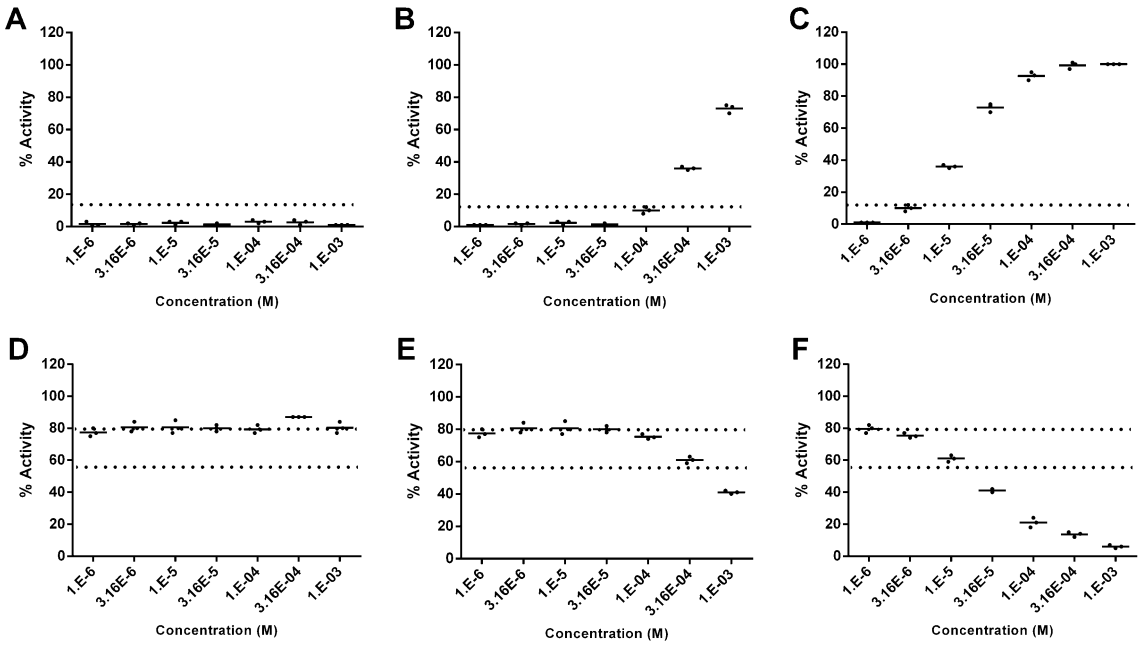


Figure 7: Typical responses of agonistic and antagonistic chemicals obtained in a transactivation assay. (A) Absence of agonist effect, (B) incomplete agonist effect, (C) agonist effect, (D) absence of antagonist effect, (E) incomplete antagonist effect, (F) antagonist effect. Results are expressed as a percentage of the maximum effect obtained with the reference chemical. Bars represent means and each dot represents a replicate.

If the graphical display of data indicates a sigmoidal concentration-response curve, the curve can be fitted using the sigmoidal dose-response function of a graphics and statistics software program (*e.g.*, the Regtox 7.0.6 Microsoft Excel TMmacro http://www.normalesup.org/~vindimian/fr_index.html or GraphPad Prism 6, GraphPad Software Inc.) (Figure 8). Effective concentrations (EC) and inhibitory concentrations (IC) are derived from the following four-parameter Hill equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\lg EC_{50} - X)\text{HillSlope}}}$$

where Y = response (i.e. RLUs); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; lg EC50 (or lg IC50) = the logarithm of X as the response midway between Top and Bottom; and Hillslope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, Hillslope, and IC50 and EC50 parameters.

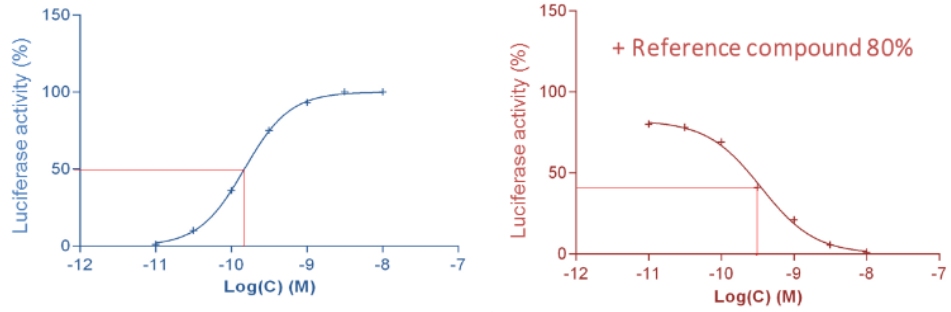


Figure 8: Typical modelled responses of agonist and antagonist chemicals obtained in a transactivation assay. (A) Agonist effect, (B) antagonist effect. Results are expressed as a percentage of the maximum effect obtained with the reference chemical. EC_{50} and IC_{50} are indicated by red lines.

For agonist chemicals, EC_{10}, EC_{50} and maximum luciferase activities are reported in a reporting sheet. For antagonist chemicals, IC_{30}, IC_{50} and minimum luciferase activities are reported in a reporting sheet.

In case the Hill function cannot be fitted, significance of the effect must be assessed by checking for normal distribution (*e.g.*, Shapiro-Wilk test), equal variances (*e.g.*, Fisher's F test) and performing ANOVA one-way. In case the normality and equal variances conditions are not met, significance of the effect is assessed with a non- parametric test, for example the Kruskal-Wallis test.

6.8 ACCEPTANCE CRITERIA

For a run to be considered acceptable, the following criteria must be met:

- The cells must have normal morphology.
- The cells must have 100% confluence when exposed.
- The cells must be mycoplasma-free (tested every time a vial is thawed).
- Induction factor: in each run, the average luciferase activity of the positive control (DEX 10^{-7} M) must be at least 10 times that of the solvent control.
- The basal activity must be $\leq$ 4% of maximum luminescence.
- The coefficient of variation (CV) between replicated wells must be under 30% for the points above basal activity (4%). One outlier should be removed per condition, or the condition excluded entirely.
- Chemicals must be tested in the concentration ranges not inducing cytotoxicity. The highest tested concentration must be the highest soluble non-toxic concentration.
- Response of the cells to the reference chemical must be consistent throughout the runs qualitatively (expected effect of reference substances (*i.e.* sigmoid aspect of the curves)/no effect of solvent control) and quantitatively (EC_{50} must be in the range of values expected for the effect or reference substance, *i.e.* DEX tested from 10^{-10} to 10^{-7} M results in an EC_{50} between log -8.9 and -7.9 ; and RU 486 tested from 10^{-9} to 10^{-6} M results in an IC_{50} between log -8.5 and -7.5).

- For active chemicals, the concentration-response curve should cover no-effect concentrations (at least two concentrations with no effect), intermediate effect concentrations, and maximum effect concentration of the chemical (excluding toxicity). A test item is considered as positive if two consecutive positives concentrations, or the highest one, are observed. If this condition is not met, the stock solution should be diluted with half-log-serial dilutions to adjust the concentration range and runs should be repeated three times. If the half-log dilutions are not relevant, the dilution factor must be adjusted according to the effect induced by the test item. Thus, the dilution factor can be test item dependant.
- For antagonist assays, the 80% DEX activity and the consideration for no effect level (i.e., at the two lowest concentrations) should be between 65% and 90% of the maximum luciferase activity.
- For interference assays, the DEX activity and the considerations for no effect level (i.e., at the two lowest concentrations) should be between 100% ± 15% of the maximum luciferase activity.
- Goodness of fit: when fitting a curve with the 4 parameters Hill model, the calculated R^2 should be above 0.95.

6.9 DATA REPORTING

An Excel and Word templates are provided to report the data.

The report must be the clearer as possible and be self-supporting. Following information must appear.

General information:

- Name and address of the sponsor, test facility and study director;
- Reference to this SOP and to the test method;
- Reference to the solubility method.

Reference standards (reference chemical, positive and negative control) and test chemical:

- Source, batch/lot number, expiry date, CAS number if available;
- Purity and chemical identity of impurities as appropriate and practically feasible;
- Physical appearance, water solubility, molecular weight and additional relevant physicochemical properties to the extent available;
- Storage conditions and stability to the extent available;
- Choice of solvent/vehicle.

Test method conditions:

- Cell line used, its source, storage and maintenance conditions, passage number and level of confluence of cells used for testing; mycoplasma testing;
- Luminometer used, including instrument settings. Luciferase substrate used (product name, supplier, lot);
- Type of plates and their supplier and code;
- Application procedure and exposure time as specified in the protocol;
- List of the acceptability criteria to be met;

- Description of any modification of the test procedure;
- Reference to the cytotoxicity procedure.

Results:

The fulfilment of the acceptance criteria must be clearly expressed for each run. In the same way, any derivation of them must be detailed and justified.

For the reference standards, tabulation of the normalized data of luminescent signals; results of the application of the acceptability criteria; Measure of error (sd, % CV).

For the test chemicals, tabulation of the following results:

- Solubility data and stability if known;
- An example of mycoplasma testing results;
- Measurement of precipitate in the culture medium to which the test chemical was added, as appropriate;
- For each run:
    - Cytotoxicity data;
    - Concentrations tested;
    - Normalized data of luminescent signals and a measure of error (SD, % CV);
    - Agonist testing: log EC_{50} value if appropriate, the maximum fold induction level;
    - Antagonist testing: log IC_{50} value if appropriate, the maximum fold inhibition level;
    - Interference testing: log IC_{50} value if appropriate, the maximum fold inhibition level; and R^2 criterion for the determination of antagonist or interference activity;
    - The result (positive or negative) per chemical after application of the decision criteria.
    - The conclusion (positive or negative) per chemical (based on the result of three runs)
- Number of runs performed;
- Graphs depicting the concentration-response relationship of reference chemicals and test chemicals;
- Rationale on which runs have been identified as non valid;
- Description of any other relevant observation.

Conclusion

The SOP has been updated following the validation study with 4 involved laboratories. Acceptance criteria are based on the study and may not all apply in following studies but should remain a strong consideration. The validation study support that the SOP can be updated with a weaker agonist in antagonist mode (Cortisol recommended). This will allow for detection of weaker antagonists as compared to the current setup with DEX.

REFERENCES

OECD (2005) Guidance Document on the validation and international acceptance of new or updated test methods for hazard assessment. Series on Testing and Assessment

OECD (2016) Test Guideline No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists

OECD (2016) Test Guideline No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals

OECD (2018) Guidance Document on Good In Vitro Method Practices (GIVIMP), Series on Testing and Assessment No. 286

Grimaldi, M, Boulahtouf, A, Toporova, L, Balaguer, P, 2020. Functional profiling of bisphenols for nuclear receptors. Toxicology 420, 39–45 .

Neale, P, Grimaldi, M, Boulahtouf, A, Leusch F, Balaguer, P, 2019. Assessing species-specific differences for nuclear receptor activation for environmental water extracts. Water Research 185, 116247.

ANNEXES

ANNEXE I: Summary of the consumables used for cell culture and transactivation assay. The suppliers and references are listed as an indication, but equivalent products can be used.

Product	Supplier	Reference
96 well plates flat bottom white polystyrene wells with micro-clear bottom	Greiner Bio-One	655098
96 well plates flat bottom white polystyrene wells with white opaque bottom	Greiner Bio-One	655083
Activated charcoal Norit	Sigma	97876
D-luciferin	Perkin Elmer	122799
Dextran	Sigma	D8821
Dimethyl sulfoxide (solvent)	Sigma-Aldrich	D2650
DMEM/F-12 with phenol red	Gibco	31331-028
DMEM/F-12 without phenol red	Gibco	21041-025
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	14190250
Fetal bovine serum (FBS)	Eurobio	CVFSVF00
Geneticin	Invivogen	ant-gn
MycoAlert Mycoplasma Detection Kit	Lonza	LT07-118
Penicillin/streptomycin	Gibco	15070-63
Puromycin	Sigma	P8833
T75 flasks	Sarstedt Inc	83.3911.002
T175 flasks	Sarstedt Inc	83.3912.002
Trypsin-EDTA 0.05%	Gibco	15400054

Annex 3: Overview of GR related AOPs and toxicology.

Here are the GR relevant AOPs from AOP wiki with details of their potential impact and outcomes. This overview is meant as a guide for regulators and scientists that aim to further understand the potential impact of disrupting normal GR regulation.

AOP 64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility.

In this AOP the activation of GR is the MIE. Through GR activation (MIE 122) there is a link to Repressed expression of steroidogenic enzymes (KE 495), Increased apoptosis, decreased of adult Leydig cells (KE 496), Testosterone synthesis reduction in Leydig cells (KE 413), Decrease in circulating testosterone levels (KE 1690), Decreased sperm quantity or quality in the adult (KE 520), and decreased fertility (AO 406).

AOP 318 - Glucocorticoid Receptor activation leading to hepatic steatosis.

In this AOP the activation of GR is the MIE. Through GR activation (MIE 122) there is a link to Decreased Acyl-CoA dehydrogenases (KE 1834), Decreased mitochondrial Fatty Acid Beta Oxidation (KE 860), Accumulation of Triglycerides (KE 291), Increase in Liver Steatosis (AO 459).

AOP 14 - Glucocorticoid Receptor Activation Leading to Increased Disease Susceptibility.

In this AOP the activation of GR is the MIE. Through GR activation (MIE 122) there is a link to Inhibition of Nuclear factor kappa B (NF-kB) (KE 202), Suppression of Inflammatory cytokines (KE 152), Decreased, of lymphocytes (KE 168), Induction of IKB inhibitory protein (KE 145), Suppression of Immune system (KE 403), leading to Increase in Disease susceptibility (AO 323).

AOP 334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration.

In this AOP the activation of GR is the MIE and is related to fish physiology. Through GR activation (MIE 122) there is a link to Altered Cell Differentiation Signaling (KE 2245), Altered Bone Cell Homeostasis (KE 2089), Inhibition of Fin regeneration (KE 1761), Reduced swimming performance (KE 1005), Decrease in Population growth rate (AO 360).

Annex 4. The project chart with RACI model for individual tasks.

This is the project chart document that outlined the tasks and involved parties before the initiation of the project, put together after the selection of method and laboratories (RACI means responsible, actor/accountable, consulted and informed).

Human glucocorticoid receptor trans-activation assay: **GR-TA**

Project chart

The scope of the chart

The chart describes the process of the GR-TA Validation Project, states the tasks and roles of the participants using a RACI model (Responsible/actor(accountable)/consulted/informed), and identifies the main deliverables and milestones.
It does not have contractual value but may serve as a basis for contracts with the labs, data manager, statistician and other relevant partners.

The validation process and its scope

The Validation Project started when the method was selected by the Pepper governance (Relevance Committee) to support a ring trial validation study. The scope is to evaluate the transferability of the method from the developing lab to other (naïve) labs and then to evaluate the between-labs and inter-lab reproducibility of the method using "blinded" chemicals. A successful transfer and validation is then reported (Validation report) and reviewed by external experts to ultimately become an approved method for ED testing (e.g. test guideline under OECD/EU).

The naïve labs were chosen by Pepper after scrutiny of applications including their budget. Initial meetings also established whether the chosen labs had the right set of experiences and equipment. Pepper will contract the naïve labs based on their application and is also responsible for the data management and statistical analyzes following the lab work.
To govern the project, Pepper will populate and contract experts in EDs, GR biology, assay validation, chemical selection and regulatory questions. These experts will constitute the Validation Management Group (VMG) that are asked to approve the SOP and updates, list of chemicals, and other relevant validation decisions.

Three naïve laboratories will be involved in the generation of data. Laboratories will be trained by representatives of INSERM.

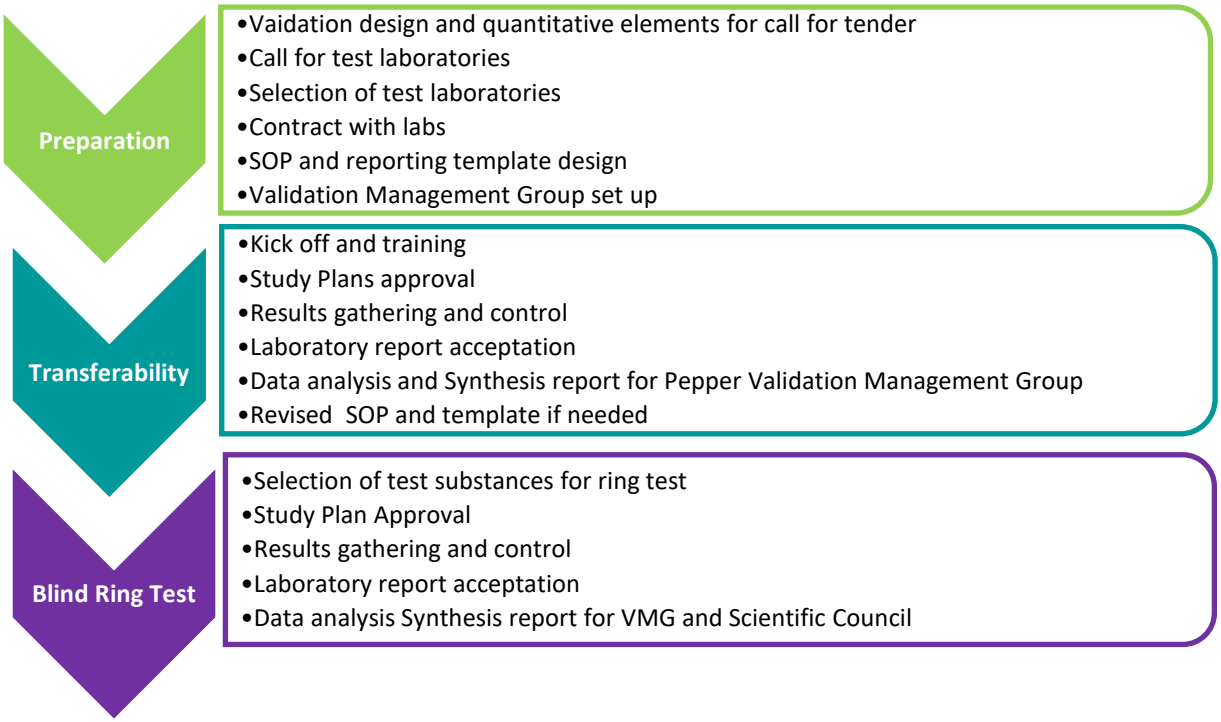
The generation of data is divided into 2 phases. In phase 1 (transfer) data will be generated for 3 test items (and a blank control). This phase is expected to be finalized in Q2-2021.

Phase 2 will be run on a set of 35 (blinded) chemicals decided by the VMG. We expect to discuss options between labs, and to consult experts in ring-trail chemical selection. We have in other projects asked CEHTRA to find references from the literature that are relevant to the biology and assay in question and populate a chemical list that the VMG can chose from. This phase is expected to be finalized in Q2-2023.

Method and Project steps

The method itself was developed at INSERM in Montpellier under Prof. Patrick Balaguer who has been contracted by Pepper to represent the developing lab (INSERM) and support transfer to naïve labs, writing and updating the SOP and participate in the validation effort in phase 1 and 2 for testing of chemicals.

The validation effort is outlined below and shows the major phases with key steps. This represents a project flow and is amenable to changes based on the experiences we have underway.



After this “technical validation” process, there are other tasks such as writing a full “Validation report” and organizing/participation a of peer review process. These tasks are not part of the project depicted here.

Actors, responsibilities and working packages

Actors in a Pepper supported standard validation process are usually

- A validation Project manager (Pepper) that is the key manager of the entire project as outlined in this chart
- The Pepper team to support the project manager during the whole process
- A developer laboratory (INSERM), responsible for SOP updates, training, help for reporting templates, generation of data in the phases and reporting
- Naïve laboratories involved in the validation are Ineris, Tame-Water and Toxem, they are also responsible for data generation and reporting in phases, and support SOP updates
- A data manager, is contracted for the data management flow: template design + data basis buildup from individual experimental reports + analyses (ad hoc extractions, simulations and statistics for reproducibility etc.)
- A biostatistician is contracted and involved in key steps such as interpretation, advice on the choice of statistics, DIP and acceptance criteria evolutions

- An external expert for the choice of reference substances is contracted
- A laboratory is contracted for blinding and sending substances to the labs in phase 2
- The Validation Project Team (Pepper, INSERM, Ineris, Tame-Water and Toxem representatives)
- A "Validation Management Group (VMG)" set up for governance of the validation effort
- The "Scientific Council" of Pepper that can settle scientific questions that arise and will approve the project after finalization as well as the Validation report

After initial meetings with the labs and contracts for the study have been signed, Pepper will establish the Project Team and arrange a kick-off meeting with the aim to outline and agree the project steps and dates of the transfer.

Pepper will lead the establishment of the VMG early in the project to include members from the science community with relevant experiences covering: EDs, chemical selection, cell assay validation, GR signaling and biology and regulatory questions.

A project team is established, with participants from the developing laboratory from INSERM, and the three naïve labs from Ineris, Tame-Water and Toxem. In addition, the Data Manager and the Biostatistician are contracted by Pepper.

The 3 main work packages are depicted below with action at the various steps outlined.

	Validation steps and management	INSERM support	Data Management	Biostatistician
Preparation	Validation design and quantitative elements for call for tender Call for test laboratories Selection SOP and reporting template design Validation Management Group set up	Initial SOP and tuning Versioning SOP Initial Template	Reporting Template V1 w ability to be compiled Database design for template aggregation	Critical look at the SOP At template (e.g. statistical tool embedded)
Transferability	Kick off and training Study plan approval Results gathering and control Lab report acceptance Data analysis and Synthesis report for Validation Management Group (VMG) Revised SOP and template if needed	In situ Kick off and training Study plan QC Lab report QC Understanding of experimental mishaps Revised SOP and template if needed	Aggregation of Reporting Templates Perform planned analyses; and unplanned (e.g. sensitivity to DIP change Preparation of data set for biostatistician	Request of test implementation (e.g. reproducibility index) Judgement on the reproducibility Advice on changes if needed (acceptance criteria, DIP criteria)
Blind Ring Test	Selection of test substances for ring test Study Plan Approval Results gathering and control Laboratory report acceptation Data analysis Synthesis report for VMG and Scientific Council	Suggestion test substances for ring test Study Plan QC Results gathering and control Laboratory report acceptation QC	Same as above ... with 10 times more template	Criteria for judgements Judgement on blinded reproducibility and accuracy

		Data analysis assistance in Synthesis report for VMG and Scientific Council		

Steps and actions

Preparations

Validation design and quantitative elements for call for tender

Quantitative assessment of needs for participation (hours, consumables, equipment number of substances and runs at each phase). The number of substances is decided in the validation design.

- ⇨ Document: need of resources for a run

Validation design: Decision simultaneous (number of substances, and of labs)

- ⇨ Document: validation design

Call for tender launch: Pepper

- ⇨ Document: Call

Selection of laboratories:

- ⇨ Contracts and/or NDAs with laboratories

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Validation design Call writing	Pepper	Pepper		INSERM
Call dissemination	Pepper	Pepper	INSERM	
Laboratory selection	Pepper's Board	Pepper		
Contracts with partner laboratories	Pepper's director	Pepper VPM	External: legal	Peppers board

VMG

Invitations by Pepper, proposals from UU and Pepper Scientific Committee are possible.

- ⇨ Document: List of members
- ⇨ Meetings: all through the process.

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
VMG set up	Pepper	Pepper	Pepper SC	Labs and Pepper SC
VMG meetings	Pepper	Pepper VMG	Ad hoc relevant involved persons	Labs

SOP V1 design versioning and dissemination.

Initial version by INSERM is based on the procedure available.

The SOP is based on the application of the method and tuned to allow naïve labs to follow the main protocol and still understand all preparations and subsequent analysis. Later changes and versioning may be approved by the VMG in case of significant updates.

- ⇨ Document: SOPV1

Versioning by INSERM. Go from Pepper, dissemination to laboratories is done by Pepper.

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
SOP V1 design	Pepper	INSERM	VMG Labs Pepper	Labs
SOP Dissemination to labs	Pepper	Pepper	Labs	VMG
SOP Versioning and storage	Pepper	INSERM	Labs	VMG

Reporting template V1 design

The labs will report data in a common template format, usually a reporting Excel sheet designed for importing raw data and make initial calculations. This should also include acceptance criteria specific for the assay. The aim is to develop a reporting template that can feed the data management process for full processing, database storage and statistical analyzes.

Initial versions are designed together with UU. Final design is set up by the data manager with biostatistician consultation, readouts from laboratories (import, embedded controls, ability for RT be compiled in Database).

⇨ Document: Reporting template (RT) V1

Pepper determines when it is ready and does the dissemination to the other labs.

It cannot be modified regarding the application of the methods, but the document must be tuned following analyses from several points of views (possible ambiguities, ease of understanding, easiness for data basis handling and more), by data manager, VMG, Labs.

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
RT V1 design	Pepper	INSERM	VMG Labs Pepper Data Manager	Labs
RT Dissemination to labs	Pepper	Pepper		VMG
RT Versioning and storage	Pepper	Data Manager	UU	Labs

Transferability

Training and kick-off

A kickoff will take place before the occasion of training. Due to the pandemic, onsite training is not possible, but we are confident the instructions from INSERM are sufficient due to the good level of assay experience in the naïve labs. Online training will likely be a few meetings.

Training targets method implementation but also Reporting Template use. Participation of test laboratories is included in the support contract from Pepper.

Follow up: Validation Project Team, VMG, and interlaboratory meetings

Interlab meetings and VMG meetings take place all through the process. Regularity of interlab meetings matters for sharing of information, experiences and results, but are particularly important when an issue arises relating to the performance/results, SOP, or practical matters.

VMG meetings are scheduled for the end of transferability, substance choice approval, and when an answer to unexpected issues is warranted.
The Validation Project team must decide upon venues and items for the agenda in the meetings.

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Validation Project Team (VPT) meetings	Pepper	Pepper	Labs	Labs
Interlaboratory meetings	Pepper	Pepper Labs	INSERM Pepper SC	Pepper SC Labs
VMG meetings	Pepper	Pepper VMG	Possibly external experts	Pepper (SC) governance Labs

Substance selection and acquisition

For transferability, test labs buy the substances. These are agreed beforehand by the VPT.

Study Plan

A study plan is requested from laboratories before starting experiments. It allows for verification that the SOP will be properly applied. In Pepper validation projects, the verification is done by two people. Pepper must give formal approval to the study plans.
Experience showed that the process may require iterations.

- ⇨ One study plan per lab
- ⇨ Accept of study plan by Pepper

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Study Plan writing	Labs	Labs	Validation Project Team	Pepper
Study Plan acceptance	Pepper	Pepper		Labs

Results gathering and control

The data management process must be established both from “technical” (e.g. circuit of RT, checking processes, compilation processes) aspects and responsibility aspects (control and storage). It requires optimization with the data manager (e.g. automatized controls vs manual controls).
The ultimate responsibility is with Pepper (INSERM may participate in data control) according to the process established with data manager (e.g. when to reject non-acceptable files, how to store, how to control …).
Documents:

- ⇨ Data Management Plan
- ⇨ Tool for compilation of Reporting Templates
- ⇨ Reporting Template files from laboratories
- ⇨ Compiled & cleaned database

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Data management plan	Pepper	Pepper and Data Manager	Statistician	Labs

Reporting Template from Labs	Labs	Labs	Pepper and/or DM	
Data collection	VPT	Pepper Data Manager	Pepper SC	Pepper SC
Reporting Template control	VPT	Pepper		Pepper governance
Integrated cleaned Data base	VPT	Data manager	Pepper	

Laboratory report acceptation

Laboratory reports synthetize the results, interpretations and explanations for key results, any mishaps or odd results in the experiments. One laboratory report per lab.

Pepper formally approves the laboratory reports.

- ⇨ Formal approval of report for each lab

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Laboratory transferability report writing	Labs	Labs	VPT	Pepper
Laboratory report acceptance and analysis	Pepper	Pepper		Labs

Data analysis and Synthesis report for Validation Management Group (VMG)

The analysis is to be made for all the laboratories and the transferability phase as such. The goal is to show reproducibility and identify possible needs for SOP enhancements, changes in acceptance criteria and prediction model. Pepper is responsible for the report. In case of poor transferability, a plan for further activities is proposed.

Documents:

- ⇨ Data from Statistical analysis
- ⇨ Transferability report

Decision on acceptance, changes, are prepared within the Validation Project Team, Pepper, statistician and submitted to the VMG.

Further actions may include

- ⇨ Suggestions for change in SOP & RT

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Statistical analysis	Pepper	Data Manager and biostatistician	Pepper, INSERM	Labs
Transferability report	VPT	Pepper with Data Manager	INSERM	VMG
Submission to VMG	Pepper	Pepper		Labs SC

SOP and reporting template for blind ring test

There might be slight changes, and even if not the case, versions are to be disseminated.

- ⇨ Document SOP v2
- ⇨ File RT v2

Process is similar to process for V1.

Blinded ring test

Substances: selection, acquisition, dissemination

The selection of substances with approval by the VMG, is based on proposals from Pepper and labs, but may require input from expert CRO. All involved can propose substance and the list must be reinforced by literature and availability.

A subcontractor is required to anonymize and send substances.

- ⇨ Confidential list of substances
- ⇨ Selection of contractor by Pepper
- ⇨ Shipment to each lab

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
Selection of substances	Pepper	Pepper	VMG Labs	Labs
Contract	Pepper Board	Pepper		
Blinding and shiping substances	Pepper	Contractor		Labs

Study Plan approval

As for the transferability phase, the labs send their study plans for approval. It can be based on the previous plan with updates and adapted to the phase.

- ⇨ One laboratory study plan per lab
- ⇨ Formal approval per lab

***Follow up**: Validation Project Team, VMG, and interlaboratory meetings*

To be continued as for transferability.

Results gathering and control

As for transferability …at a different scale, maybe change in organizational patterns, and with no need to rewrite a data management plan.

- ⇨ Reporting templates from laboratories
- ⇨ Data from Statistical analysis
- ⇨ Transferability report

Laboratory report acceptation

Same as for transferability adapted to the phase

- ⇨ One laboratory report per lab
- ⇨ Formal approval of report for each lab

Data analysis and Synthesis report for Validation Management Group (VMG) & SC

As for transferability but very different scale. Explanations to find out in the laboratory reports. Probably need from external sources.

- ⇨ Data from Statistical analysis
- ⇨ Validation report

Task	Responsible	Actor/Accountable	Consulted/contribute	Informed
VPT meetings	Pepper	VPT		Labs
Interlaboratory meetings	Pepper	Pepper	Labs	(VMG)
VMG meetings	Pepper	Pepper	Possibly external experts	Labs (SC)
Reporting Templates from Labs	Labs	Labs	Pepper	
Reporting Templates control	VPT	Pepper		Pepper
Integrated data base	VPT	Data manager	Pepper	Labs
Laboratory full validation report writing	Labs	Labs	VPT	
Lab report analysis and acceptance	Pepper	Pepper		Labs
Statistical analysis	Pepper	Data Manager and biostatistician	Pepper, INSERM	Labs
Validation Report	Pepper	Pepper with Data Manager	Pepper SC	Labs
Submission to VMG	Pepper	Pepper	VMG	
Submission to OECD (after project)	Pepper	Pepper INSERM	Pepper SC	Labs