Standard Operating Procedure

for the

AR-CALUX® *in vitro* test method

to detect (anti)-androgenic activity of chemicals

Joint
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Centre

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This report describes the Standard Operating Procedure (SOP) of the AR-CALUX® *in vitro* test method to detect (anti)-androgenic activity of chemicals. The original version of the SOP was produced by the test method developer Biodetection Systems (The Netherlands) and was subsequently revised during the validation study of the AR-CALUX® method coordinated by the JRC's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM). The SOP revision was led by Ingrid Langezaal on behalf of EURL ECVAM in collaboration with Biodetection Systems.

The AR-CALUX® test method is included in OECD Test Guideline 458 on Androgen Receptor TransActivation assays.

Contact information test method developer and cell line supplier
BioDetection Systems BV
Science Park 406. 1098 XH Amsterdam, the Netherlands
info@bds.nl
www.bds.nl

Contact information validation study
European Commission, Joint Research Centre (JRC), Chemical Safety and Alternative Methods Unit (F3)
Via E. Fermi, 2749. I-21027 Ispra (VA), Italy
JRC-F3-ENQUIRIES@ec.europa.eu

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1 PURPOSE AND APPLICABILITY

AR-CALUX® cells express a functioning human androgen receptor and contain a reporter gene (luciferase) under the control of an androgen responsive element. An increase or decrease of androgenic signalling, results in corresponding changes in the expression of luciferase activity in AR-CALUX® cells, and is measured with a luminometer. Accordingly, AR-CALUX® cells can be used to detect compounds with (anti-)androgenic properties.

1.1 Definitions and Abbreviations

1.1.1 Definitions

Agonist	A compound that can elicit a similar response as the agonist reference compound in the AR-CALUX® bioassay
Antagonist	A compound that can elicit suppression of the response of the agonist reference compound in the AR-CALUX® bioassay
Cross talk	The influence of activity in adjacent wells on the luminescence determination in a specific well.
Cytotoxicity	Cell toxicity.
Lyse	Destruction of the membrane structure of cells by chemical, osmotic, physical, or enzymatic treatment.
Monolayer	A single layer of coherent cells.
Passage number	The number of times a cell line has been sub-cultured after thawing of a new batch of the cell line.
Reference item	A chemical used to provide a basis for comparison with the test item.
Stripping serum	A process to remove interfering substances (e.g. steroids) from the serum by means of dextran-coated charcoal.
Test item	A test chemical that is the subject of a study.
Trypsinate	Enzymatic treatment of cells with trypsin to remove intercellular and surface attachment resulting in a discreet single rounded cell suspension.
Work solutions	Assay medium supplemented with test item or reference item.

1.1.2 Abbreviations

ATP	Adenosine-5'-TriPhosphate
AU	Absorbance Units
BDS	BioDetection Systems, the test developer and owner of AR-CALUX® assay and cells.
CALUX	Chemically Activated LUciferase eXpression
DCC-FCS	Dextran Coated Charcoal treated – Foetal Calf Serum
DF	Dulbecco's modified eagle medium (DMEM) supplemented with F12
DMSO	Dimethylsulfoxide
DHT	Dihydroxy testosterone
DTT	1,4-dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
FCS	Foetal Calf Serum
G-418	Geneticin, an aminoglycoside
HDPE	High Density Poly Ethylene
IF / InhF	Induction Factor / Inhibition Factor
LDH	Lactate dehydrogenase
MEM	Minimum Essential Medium
NC	Negative Control
NEAA	Non-essential amino acids
OD	Optical density
PBS	Phosphate Buffered Saline
PC	Positive Control
RLU	Relative Light Units
SC	Solvent Control
SD	Standard Deviation
TI	Test Item (= Test Chemical)
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
VC	Vehicle Control

1.1.3 Calculation of the Parameters

EC_{10} / EC_{50}	Concentration of a test item at which 10% or 50% of its maximum response is observed
IC_{20} / IC_{50}	Concentration of a test item at which 80% or 50% of its maximum response is observed (20% or 50% inhibition).
PC_{10}, PC_{50}, PC_{80}, PC_{max} or PC_{min}	Concentration of test item where the response is 10%, 50%, 80%, at its maximum or at its minimum with respect to the maximum induction of the reference item
R^2	Square of the correlation coefficient (criterion for specificity control test)
REF EC_{50}	Concentration of the reference item DHT at which 50% of its maximum response is observed
REF IC_{50}	Concentration of the reference item Flutamide at which 50% of its maximum response is observed
REF RPC_{10}, RPC_{50} or RPC_{80}	Response level of the reference item at 10%, 50% or 80%
RPC_{max}	Maximum response level of test item with respect to the reference item DHT.
RPC_{min}	Minimum response level of test item with respect to the reference item Flutamide
S_c	Response of the test/reference item with the specificity control at a specific concentration z
S_c^n	Response of the test/reference item with the normalised specificity control at a specific concentration c
Y_c	Response of test/reference item at a specific concentration c

1.2 Health, Safety and Environment

While working with cells or chemicals, always wear sufficient personal protective equipment, e.g. safety goggles, gloves and laboratory coat.

1.2.1 Chemicals

Always consult the Material Safety Data Sheets before handling chemicals as they may be hazardous.

Chemicals or samples without available hazard information must be treated as if they were 'very toxic' and having 'Carcinogenic, Mutagenic or Reprotoxic' properties i.e. the highest health hazard classification in accordance with the EU classification, labelling and packaging Regulation 1272/2008 applies.

1.2.2 Cells

The AR-CALUX® cells are Genetically Modified Microorganisms (GMMs) to which Directive 2009/41/EC (contained use of genetically modified micro-organisms) or national law applies.

The AR-CALUX® cells must be treated as biohazardous material. Test facility procedures, for the safe handling of biohazardous material, must be applied.

2 METHOD

This section outlines the procedure for the performance of agonist experiments to determine androgenic properties of test items, and antagonist experiments to determine the anti-androgenic properties of test items.

Four different types of experiments are covered by the following procedure:

1) Agonist pre-screen experiments to identify an agonist response, if any.
2) Antagonist pre-screen experiments to identify an antagonist response, if any.
3) Comprehensive testing to determine agonist properties.
4) Comprehensive testing to determine antagonist properties, including confirmation of antagonist response with the specificity control.

During the pre-screen experiments solubility, cytotoxicity and agonist/antagonist response will be taken into account for the selection of the concentration for comprehensive testing.

2.1 Materials

WARNING: Materials, chemicals, reagents and apparatus (luminometer) selected may affect the outcome of the CALUX® assay. Moreover, be aware of drying-ovens used to dry glass-ware that will be re-used that can cause potential contaminations arising from e.g. the plastic/rubber linings and other fixtures in the oven. This extends to the consideration of potential contamination from all lab-ware, and from the local laboratory environment (building material, air, water, etc). The test users should also avoid the use of glassware with any protective coatings. **Testing of new batches of the glass tubes, plastic ware, FBS and DMSO, etc., is required before starting any study.** During the studies, always ensure that RLU values of SC (agonist) and VC (antagonist) are as low as possible and that acceptance criteria are met.

2.1.1 Forms

DAT02-ASY06	AR-CALUX® calculation of results for agonism.
DAT04-ASY06	AR-CALUX® calculation of antagonism pre-screen results
DAT05-ASY06	AR-CALUX® calculation of results for antagonism.
DAT06-ASY06	AR-CALUX® cytotoxicity

2.1.2 Test system

AR-CALUX® cell line (U2-OS-based AR-CALUX® cells). Supplier is BioDetection Systems (BDS).

AR-CALUX® cells originated from the human osteoblastic osteosarcoma U2-OS cell line. Human U2-OS cells were stably transfected with 3xHRE-TATA-Luc and pSG5-neo-hAR.

Only mycoplasma free cultures shall be used. The cell batches to be used must either be certified negative for mycoplasma contamination, or a mycoplasma test should be

performed before use. Mycoplasma testing shall be performed with either a direct culture method or by DNA extraction followed by real-time PCR method.

2.1.3 Chemicals and reagents

Note: If pre-prepared chemicals or kits are not available, see section 2.2 for manual preparation of reagents.

Chemicals and Reagents	Supplier	Cat. Number
Cell maintenance, growth and assay medium		
DF medium with phenol red as pH-indicator	e.g. Gibco	31331-028
Foetal Calf Serum (FCS)	e.g. Gibco	12664-025 or 10099-141 or 10270-106
MEM (100 ×) Non-essential amino acids	e.g. Gibco	11140-035
Penicillin (5000 Units/ml) Streptomycin (5000 µg/ml) solution	e.g. Gibco	15070-063
G-418 disulphate / Geneticin CAS: 108321-42-2	e.g. Sigma	G-8168
Phosphate buffered saline (PBS) pH 7.2, Ca^{2+} and Mg^{2+} free	e.g. Gibco	20012-019
Trypsin without phenol red	e.g. Gibco	27250-018 (powder) 15090-046 (2.5% (10×) solution)
EDTA	e.g. Acros	147855000
Carbon stripped Foetal Bovine serum (or ingredients below for stripping serum)	e.g. Sigma	F6765
DMEM F12 without phenol red as pH-indicator	e.g. Gibco	21041-025
Reference and Control Items		
DHT / Dihydroxy testosterone / [CAS: 521-18-6]	e.g. Steraloids e.g. Sigma	A2570-000 10300
Flutamide [CAS: 13311-84-7]	e.g. Sigma	F9397
Corticosterone [CAS: 50-22-6]	e.g. Sigma	27840
Levonorgestrel [CAS: 797-63-7]	e.g. Sigma	N2260
17α-Methyltestosterone [CAS: 58-18-4]	e.g. Sigma	46444
Linuron [CAS: 330-55-2]	e.g. Sigma	36141
10% Triton X-100 [CAS: 9002-93-1]	e.g. Sigma	X100
Chemicals and reagents needed for stripping serum		
Activated charcoal	e.g. Fluka	05120F
Dextran T500	e.g. Amersham Pharmacia	17.0320.01

2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)	e.g. Boehringer	708976
Chemicals and reagents needed for lysis reagent		
2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)	e.g. Boehringer	708976
Glycerol	e.g. Baker	7044
Dithiothreitol (DTT)	e.g. Duchefa	d 1309
1,2-Diaminocyclohexanetetraacetic acid disodium salt solution (CDTA)	e.g. Sigma	32869
Triton X-100 [CAS: 9002-93-1]	e.g. Sigma	T8787
Chemicals and reagents needed for in-house illuminate mix solution		
N-(tris(hydroxymethyl)methyl)glycine (tricine)	e.g. Sigma	T0377
Magnesium carbonate hydroxide hydrate $C_4H_2Mg_5O_{14}$	e.g., Aldrich	227668
Magnesium sulfate heptahydrate		
NaOH		
HCl		
Dithiothreitol	e.g. DTT, Duchefa	D1309
D-Luciferin [CAS: 2591-17-5]	e.g. Duchefa	L1349
Adenosine 5'-triphosphate (ATP)	e.g. Roche	519987
Ethylenediamine tetraacetic acid (EDTA)	e.g. Acros	167852500
Other reagents		
Cell lysis reagent	e.g. Promega or self-prepared as in SOP	E1531
Cytotoxicity kit using the supernatant of exposed cells (LDH leakage kit) and endpoint detection by absorbance.	Roche	1 644 793 001 (2000 wells)
Luciferase kit	e.g. Promega or self-prepared as in SOP	E1501
Dimethylsulfoxide (high purity)	e.g. Sigma	34869
Sodium hydroxide	e.g. Riedel de Haën	30620
Ethanol 70%		

Table 1: Chemicals and reagents needed for the AR-CALUX® assay

2.1.4 Disposables and equipment

Material	Supplier	Cat. Number
Consumables		
Clean or sterile glass tubes or vials		
Sterile pipettes, 1, 2, 5, 10 and 25 ml		
Reagent-reservoirs	e.g. Costar	4870
Sterile tips, 10, 100, 200, 1000 and 5000 µl		
Flat bottom transparent tissue culture plates, 96 wells	e.g. Greiner	Cell star® 655180
24-well plates	e.g. Greiner e.g. Sigma Aldrich	Cell star® 662160 M8812-100EA
Cell culture flasks 75 cm^2	e.g. Corning	3275 or 430641
Cryovials (1.5 to 2 ml)	e.g. Greiner	121261
Plastic tubes (12-15 ml) with cap, sterile	e.g. Greiner or Falcon	
Plastic tubes (50 ml) with cap, sterile	e.g. Greiner or Falcon	
Filter sterilization unit	e.g. Nalgene	
Glass pipettes, for pipetting DMSO.		
Equipment		
Centrifuge with swing-out rotor for 50 ml centrifuge tubes		
pH meter		
CO_2 incubator set at 37°C +/- 1°C, 5% CO_2 +/- 1% and humidified.		
Inverted microscope (culture microscope) with 4×, 10× and preferably 20× objective		
Luminometer for 96-well plates		
Absorbance plate reader for 96-well plates		
Safety-cabinet or glove box for the safe handling of chemicals.		
Low temperature storage such as liquid nitrogen container or -150 °C freezer, for storage of cryovials.		
Pipettes for accurate pipetting volumes between 1 and 5000 µl		
Multichannel pipette for 30 µl, 100 µl and 200 µl		
Analytical balance suitable to accurately weigh 10 mg.		
Plate shaker for horizontal shaking at 300 rpm.		
Vortex		
Water bath, set at 37°C		
Haemocytometer e.g. Bürker-Türk counting chamber		

Table 2: Consumables and equipment needed for the AR-CALUX® assay

2.2 Preparations

All preparations used for cell culture must be prepared and used under aseptic conditions at all times. Record the date of preparation, ingredients, lot numbers, expiry date, etc.

It is recommended to prepare the following aliquots and freeze them down at -20˚C:

- Trypsin-EDTA or trypsin: 1 to 2 ml of 10 × concentrated solution
- Penicillin-Streptomycin: 1 ml aliquots containing 5000 units penicillin and 5000 µg streptomycin (5000 units penicillin and 5000 µg streptomycin per ml)
- FCS: 41 ml
- DCC-FCS: 27 ml

Aliquots of Geneticin (G418; 2.2 ml; 50 mg/ml) should be stored at 2-8˚C.

2.2.1 Growth Medium

Growth medium for the AR-CALUX® cells consists of DMEM/F12 with phenol red, 7.5% Foetal Calf Serum (FCS), 1% non-essential amino acids, 10 Units/ml Penicillin, 10 µg/ml Streptomycin and 0.02% Geneticin. FCS and penicillin-streptomycin are stored at -20 °C in aliquots for single use only.

- Thaw one tube with 41 ml FCS in a water bath (not warmer than 37°C).
- Open a 500 ml bottle of DMEM/F12 with phenol red in a laminar flow cabinet.
- Add 5.5 ml of non-essential amino acids (MEM 100×).
- Add 41 ml of FCS.
- Add 1 ml penicillin-streptomycin solution.
- Add 2.2 ml Geneticin G418
- Close the flask and mix gently, avoiding foam formation.
- Label the bottle with content, date of preparation and expiry date.
- Store the flask with growth medium at 2 to 8°C.

Growth medium expires 4 weeks after preparation.

2.2.2 Assay medium

Assay medium consists of DMEM/F12 without phenol red 5% Dextran-Coated Charcoal treated FCS (DCC-FCS), 1% non-essential amino acids (MEM 1 ×), 10 Units/ml Penicillin and 10 µg/ml Streptomycin. DCC-FCS is stored at -20°C in aliquots of 26.6 ml for single use only.

- Thaw one tube of 26.6 ml DCC-FCS in a water bath (37°C).
- Open a 500 ml bottle of DMEM/F12 without phenol red in a laminar flow cabinet.
- Add 5.3 ml of non-essential amino acids (MEM 100 ×)
- Add 26.6 ml DCC-FCS.
- Add 1 ml Penicillin-streptomycin solution
- Close the flask and mix gently, avoiding foam formation.
- Label the bottle, indicating content, preparation date and expiry date.
- Store the medium at 2 to 8°C.

Assay medium expires 4 weeks after preparation.

2.2.3 Freezing medium

Freezing medium for the AR-CALUX® cells consists of DMEM/F12 with phenol red, 20% Foetal Calf Serum (FCS), 1% non-essential amino acids and 7.5% DMSO.. FCS is stored at -20°C in aliquots for single use only.

- Open a 500 ml bottle of DMEM/F12 with phenol red in the laminar flow cabinet.
- Remove 142.5 ml from the bottle.
- Add 5.5 ml of non-essential amino acids and mix gently.
- Add 100 ml of FCS.
- Add 37.5 ml of DMSO.
- Mix gently and place 40 ml aliquots in 50 ml plastic bottles.
- Label the plastic tubes indicating content, date of preparation and expiration date (1 year from the day of preparation).
- Store freezing medium at -20°C.
- Following preparation of freezing medium, incubate a 10 ml aliquot of the prepared freezing medium in the CO_2 incubator for 1 week. Check (microscope) after 1 week for contamination. In case of no contamination, the prepared batch of freezing medium can be used.

2.2.4 Trypsin-EDTA solution

Trypsin-EDTA is used to detach the cells from the plastic culture flask and from each other. It must be used sterile at a concentration of 0.05% trypsin.

Manual preparation of the Trypsin-EDTA solution:

- 0.5 gram trypsin should be dissolved in 1000 ml PBS (without Ca^{2+} and Mg^{2+}) containing 0.2 g EDTA per litre.
- Transfer the trypsin solution in 30 ml aliquots to 50 ml tubes.
- Close and label the tubes. The expiry date is 3 months from the day of preparation. Minimally indicate the content, date of preparation and expiring date.

Store the tubes of trypsin-EDTA at -20°C.

Commercially available solutions:

- A Trypsin-EDTA solution must be diluted with PBS without Ca^{2+} and Mg^{2+} to get a final trypsin concentration of 0.05%
- A Trypsin solution (without EDTA) must be diluted with PBS without Ca^{2+} and Mg^{2+} to get a final trypsin concentration of 0.25%

2.2.5 Stripped FCS

Because steroids present in FCS will lead to background activation in the AR-CALUX® assay, charcoal stripped FCS (DCC-FCS) is used for seeding and exposing cells.

26.6 ml of DCC-FCS is added to assay medium. When self-prepared, follow the procedure in this section. Also commercially available DCC-FCS may be used.

2.2.5.1 Preparing the dextran-coated activated charcoal

- Rinse 1 bottle (1 litre) with demineralized water.
- Add 800 ml demineralised water
- Add 11.5 ml 1 M Tris/HCl pH8
- Add 0.625 g Dextran T500
- Make sure all ingredients are dissolved.
- Add 6.25 grams of activated charcoal.
- Fill the bottle completely with demineralized water.
- Close the bottle tightly with a Teflon coated lid.
- Stir overnight at 4˚C.

2.2.5.2 Stripping the serum

- Thaw 1 flask (500 ml) of FCS.
- Heat the FCS for 30 minutes at 56°C in the water bath.
- Whilst the FCS is warming, distribute the dextran-coated charcoal suspension between twenty 50 ml (plastic) centrifuge tubes. The difference in weight between the tubes must not exceed 0.1 gram. Centrifuge for 10 minutes at 2000 g.
- Carefully decant the centrifuge tubes, but do not disturb the dextran-coated charcoal pellets. Keep the centrifuge tubes with opening down on a piece of paper to dry. Remove as much fluid as possible without touching the pellets.
- Add approximately 50 ml of FCS to 10 tubes containing the dextran-coated charcoal pellets and re-suspend the dextran-coated charcoal pellets.
- Pool the FCS serum suspension from all tubes into the original FCS bottles and incubate in water bath at 45°C for 45 minutes while shaking at such a speed that the serum is gently moving. Do not discard the used tubes.
- Fill out the 10 used 50 ml tubes with the incubated charcoal-serum suspension and centrifuge the tubes for 10 min at 2000 g.
- Following centrifugation, carefully transfer the serum into the 10 unused dextran-coated charcoal containing 50 ml tubes and re-suspend the dextran-coated charcoal pellets.
- Pool the FCS serum suspension from all tubes into the original FCS bottles and incubate in water bath at 45°C for 45 minutes while shaking at such a speed that the serum is gently moving.
- Following the second incubation, fill out 10 used 50 ml tubes with the incubated charcoal-serum suspension and centrifuge the tubes for 10 min at 2000 g.
- To remove the last amount of dextran-coated charcoal, transfer the centrifuged serum (supernatant) into 10 clean 50 ml centrifuge tubes and centrifuge for 30 min at 2000 g.
- Pool the serum into a clean 500 ml bottle.
- Filter-sterilize the serum using a bottle top filter sterilization unit (Nalgene).

- Divide the stripped serum into 26.6 ml portions in sterile tubes.
- Label all tubes as DCC-FCS, indicating their content, date of preparation, and expiration date (6 months from the day of preparation).
- Store at -20˚C.

2.2.6 Lysis Reagent

Prepare the lysis reagent according to the procedure below, or use commercially available lysis reagent and follow the supplier's manual.

Lysis mix contains the ingredients given in Table 3.

Compound	Weight[a] (g)	Volume (ml)	Molecular weight[b] (g/mol)	Concentration
Tris	3.0		121.1	25.0 mM
DTT	0.31		154.2	2.0 mM
CDTA	0.73		364.35	2.0 mM
Glycerol		100	-	10%
Triton X-100		10	-	1%

Table 3: Composition of lysis mix

[a]: The amount of compound to weigh out for 1 l lysis mix is given in the second column.

[b]: Check whether the molecular weight of the compounds corresponds to the molecular weight given in the table. The amount of compound needs to be adjusted when the molecular weight does not correspond, based on the molarity given in the fourth column.

- Add 500 ml of demineralized water to a 1 liter glass beaker.
- Add Tris, DTT and CDTA according to Table 3.
- Add 100 ml (or 112.6 g) glycerol.
- Add 10 ml (or 10.7 g) Triton X-100.
- Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.
- Adjust the final volume to 1 L with demineralized water.
- Transfer aliquots of approximately 40 ml into 50 ml tubes.
- Close and label the tubes indicating content, date of preparation and expiration date. Store at -20°C for a maximum of 1 year, store at 4°C for a maximum of 1 month.

2.2.7 Preparation of Tris/HCl solution (1 M)

- Dissolve 121.1 g Tris in 800 ml demineralized water.
- Adjust pH to 8.0 using concentrated HCl.
- Adjust to a final volume of 1 liter using demineralised water.
- Close the flask but not completely to allow air to escape during autoclaving.
- Autoclave at 121°C for 15 minutes.
- Allow Tris/HCl solution to cool down and close the bottle.
- Label the bottle, indicating content, date of preparation and expiration date (3 months from the day of preparation).
- Store at room temperature.

Note: before using the autoclaved Tris/HCl 1 M solution, check the pH.

2.2.8 0.2 M NaOH solution

0.2 M NaOH is used at 100 µl/well to stop the reaction of the illuminate-mix.

- Dissolve 8.0 g NaOH in 1000 ml demineralised water.
- Stir with a magnetic stirrer until the NaOH is dissolved completely.
- Close and label the bottle, indicating content, preparation date and expiry date of 3 months.
- Store at room temperature.

2.3 Maintenance and handling of AR-CALUX® cells

All solutions must be prepared and used under aseptic conditions with sterile plastic ware.

2.3.1 Freezing of AR-CALUX® cells

- Prepare 75 cm^2 or 150 cm^2 culture flasks with cells.
- Start the freezing procedure when cells have reached a minimum of 90% confluence.
- Label sterile 1.5 to 2 ml cryovials indicating the type of cells, passage number, date, and name. From each 75 cm^2 flask, approximately 3 cryovials can be made; from each 150 cm^2 flask, approximately 7 cryovials can be made. Cover the writing with transparent tape to protect it or use cryo proof stickers or pens.
- Trypsinate all cells according to section 2.3.3 and remove trypsin.
- Suspend the cells in growth medium (10 ml for each 75 cm^2 flasks; 15 ml for each 150 cm^2 flask).
- Pool and count the viable cells. Calculate how many cryovials can be prepared at a concentration of 1.5×10^6 cells/vial.
- Divide the cells over 50 ml tubes and centrifuge (250 × g for 5 minutes)
- Open the cryovials carefully and set aside in the laminar flow cabinet (sterile) until filling.
- Following centrifugation, keep the vials and 50 ml tubes on ice at all times and work as quickly as possible to minimise the toxic effect of DMSO at room temperature.
- Discard the growth medium and re-suspend the cells in freezing medium to obtain a final concentration of 1.5×10^6 cells/mL. The volume in ml of freezing medium to be used, is depending on the amount of cells available.
- Divide the re-suspended cells in freezing medium over the number of cryovials calculated (1 ml per cryovial) and close the cryovials.
- Cover the inside of the storage box of the cryovials with tissues to facilitate a graduate freezing of the cells and store at -80°C overnight. Alternatively, use Mr. Frosty freezing containers to achieve a rate of cooling close to 1°C/minute, or a controlled-rate freezing device.
- The next day, transfer cryovials to a -150°C freezer of liquid nitrogen for storage.
- Thaw one cryovial after at least one day of storage in the liquid nitrogen. Culture the cells for at least one week according to the appropriate protocol. Record the confluence after 72h +/- 4 hours culturing. Check the cells for

signs of contamination and check the cell reactivity towards reference items. Cells may be accepted when acceptance criteria in section 2.4 are met.

2.3.2 Thawing of AR-CALUX® cells

- Heat a water bath to 37°C.
- Take a flask of growth medium from the refrigerator and pipet 15 ml of growth medium into a sterile tube (e.g. Greiner). Place the tube back into the refrigerator.
- Heat up the remaining growth medium in a water bath at 37°C.
- Retrieve a cryovial of AR-CALUX® cells from the liquid nitrogen.
  NOTE: Wear safety glasses and protective gloves.
- Wait briefly to be sure that no liquid nitrogen is present in the cryovial.
- Thaw the cells quickly by gently moving the vial in a water bath of 37°C until the ice has almost melted.
- Clean the outside of the cryovial with 70% alcohol.
- Open the cryovial, gently aspire the cell suspension and transfer it into a 15 ml sterile tube (e.g. Greiner).
- Retrieve the tube containing 15 ml of cold growth medium from the refrigerator.
- Transfer 10 ml cold growth medium drop wise into the 15 ml tube with cell suspension.
- Close the tube and gently mix the cell suspension.
- Centrifuge the cell suspension for 5 minutes at 250 g
- Decant or aspire the medium and gently re-suspend the cell pellet into 10 ml warm (37°C) growth medium.
- Open a culture flask (75 cm^2) and pipette the cell suspension into the flask. Label the culture flasks and indicate the type of cells, date of preparation, name and passage number. At thawing, the passage number is 0.
- Place the culture flasks in the CO_2-incubator. When using non-filter lids, ensure the flasks are not closed completely.
- When the flask is 85-95% confluent, sub-culture the cells according to the next section.
- Cells have to be sub-cultured at least 2 times before they may be used (at passage number 3) to assess the agonist and/or antagonist effects of test items in the (anti)AR-CALUX® bioassay.

Quality control to be performed:
Cells that do not show > 75% confluence after 72 +/- 4 hours of incubation following thawing should be discarded. In such case, new cells should be brought in culture.

2.3.3 Sub-culturing of AR-CALUX® cells

Cells must be sub-cultured every three or four days. Seeding densities presented in Table 4, generally lead to the required confluence between 85% and 95% before sub-culturing.

The procedure below is for 75 cm^2 culture flasks. When other sizes are used, adjust the volumes accordingly.

- Remove the medium from the cells.

- Wash the cells carefully two times with 5 ml of pre-warmed (37°C) PBS without calcium and magnesium.
- Rinse the cells with 2 ml of pre-warmed (37°C) trypsin solution and remove it.
- Place the flaks in an incubator and wait until all cells are rounded and have detached from the bottom (after 1-3 minutes).
- Re-suspend the cells in 10 ml of growth medium or, when cells are also needed for seeding into 96-well plates, re-suspend the cells in 10 ml of assay medium.
- Count the cells and determine the cell density.
- Transfer the proper amount of the cell suspension into a new 75 cm^2 culture flask according to Table 4.
- Add growth medium up to a final volume of 10 ml.
- Label the culture flasks. Indicate the type of cells, date of sub-culturing, passage number, seeding density and operator name.
- Incubate the cells in the CO_2 incubator (37°C +/- 1°C, 5% CO_2 +/- 1% and humidified). When using non-filter lids, ensure the flasks are not closed completely to allow transfer of gases.
- The total amount of cell suspension can be used to subculture. The old culture flask and redundant cell suspension must be disposed of as biologically hazardous waste.

Days to next sub-culturing	Seeding density (cells/ml)	Cells per flask (10 ml)
3	110000	1100000
4	70000	700000

Table 4: Seeding density for sub-culturing cells in a 75 cm^2 flask

Requirements for the test system:

The following requirements apply to the cells used in the AR-CALUX® assay:

- Cultured cells must be sub-cultured at least twice a week.
- Cell cultures may not be overgrown. When many detached cells are visible under the microscope, cells must be discarded.
- Cells should have a passage number > 2 and < 30*.
- Cells should be free of bacterial, fungal, yeast and mycoplasma contamination.

* Cells of higher passage numbers may only be used when the response obtained (as measured by relative light units) remains high enough to obtain the minimum induction factor.

Record the following information:

- Date and time of sub-culturing or seeding
- Cell density before sub-culturing (cells/ml)
- Cell density needed for sub-culturing or seeding + volumes required
- Passage number

2.4 Procedure for (anti-)androgenicity pre-screen and comprehensive testing

During pre-screen experiments it is assessed if test items have agonist or antagonist properties. For the determination of an antagonist response, the assay medium used for the work solutions must be spiked with DHT.

If test items show an androgenic response during agonist pre-screen experiments, proceed to comprehensive testing. The only difference between pre-screen and comprehensive testing is the default dilution factor.

If test items show an anti-androgenic response during antagonist pre-screen experiments, proceed to comprehensive testing using a different plate layout, in which the cells are exposed to two different concentrations of DHT. In this way specificity of the antagonist response can be assessed and interference of the test item with the luciferase production excluded.

Short overview of the procedure

Day 1:

- Seed the cells into 96-well plates according to section 2.4.1.

Day 2:

- Examine the cells for contamination and confluence after 16 to 32h incubation according to section 2.4.1.
- Prepare test item stock solutions (2.4.2) and work solutions according to sections 2.4.3 (agonism) or 2.4.4 (antagonism). For antagonism experiments, supplement assay medium according to section 2.4.4.1
- Prepare reference and control item stock solutions (2.4.2) and work solutions (2.4.3) for agonism experiments
- Prepare reference and control item stock solutions (2.4.2) and work solutions (2.4.4) for antagonism experiments
- Expose the cells with test, reference and control items.
  → Agonist experiments (pre-screen and comprehensive testing), see plate layout figure 5 in section 2.4.5.
  → Antagonist pre-screen experiments, see plate layout in figure 7in section 2.4.6.
  → Antagonist comprehensive testing, see plate layout in figure 8 in section 2.4.6.

Day 3:

- Stop exposure after 22 to 26h treatment according to sections 2.4.5 and 2.4.6 (the same for agonism and antagonism experiments).
- Determine cytotoxicity with the LDH leakage kit according to section 2.4.7.
- Lyse the cells and measure luminescence according to section 2.4.8.

Data analysis and decision criteria:

- Verify that acceptance criteria in sections 2.4.5.2 (agonism) and 2.4.6.2 (antagonism) are met. The calculations are found in Appendix A.
- Analyse the valid data according to section 2.5.2 (agonism) and 0 (antagonism).
- Apply the decision criteria for the test item according to section 0.

Note: Induction, as quantified with relative light units (RLU), depends on the type of 96-well plates used, illuminate mix, equipment type, reagents and background RLU values.

2.4.1 Preparation of AR-CALUX® cells for the assessment of androgenicity / Seeding the microtiter plates

- Trypsinate cells which are between 85% and 95% confluent as described in section 2.3.3. **NB: Ensure that all culture medium is washed away with PBS, as phenol red can interfere with the bioassay response**.
- Re-suspend the cells gently in 10 ml assay medium and transfer to a sterile 50 ml tube.
- Count the cells and record the number of living and dead cells.
- Further dilute the cells with assay medium by sterile pipetting until a cell suspension of 100000 viable cells per ml is obtained.
- See section 2.3.3 for the information to record when sub-culturing and seeding AR-CALUX® cells.
- Homogenise the cell suspension in the assay medium by sucking up and releasing the cell-suspension gently with the 8-channel pipette, at least five times. Avoid clump and foam formation.
- Fill 66 wells gently with 100 µl of cell suspension as indicated in Figure 1, so that a final concentration of 10000 cells/well is obtained. Mix the cell suspension carefully from time to time to keep the suspension homogeneous.
- Fill the remaining outer rows with 200 µl of PBS (Figure 1). These wells are not used in the assay.
- Incubate the plates for a minimum of 16 and a maximum of 32 hours in the CO_2 incubator (37°C +/- 1°C, 5% CO_2 +/- 1% and humidified).
- Record the incubation start time.
- To make sure all wells with AR-CALUX® cells have the same conditions during the experiment, the following measures have to be taken:
    1) All microtiter plates must always be placed separately from each other in the incubator, so not stacked.
    2) After seeding the cells and during exposure, the disturbance of the climate in the CO_2 incubator must be kept to a minimum by minimising opening and closing of the door.
    3) After incubation, visually inspect the microtiter plate(s) and check for confluency and cloudy wells as an indicator of contamination.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	I	I	I	I	I	I	I	I	I	I	I	0
C	I	I	I	I	I	I	I	I	I	I	I	0
D	I	I	I	I	I	I	I	I	I	I	I	0
E	I	I	I	I	I	I	I	I	I	I	I	0
F	I	I	I	I	I	I	I	I	I	I	I	0
G	I	I	I	I	I	I	I	I	I	I	I	0
H	0	0	0	0	0	0	0	0	0	0	0	0

Figure 1: Schematic representation of a 96 well microtiter plate after seeding. The grey wells (0) are filled with 200 µl PBS and the clear wells (I) with 100 µl cell-suspension.

The following test system related quality control must be performed before exposure of the cells:

- Confirm that confluence of the cells is between 50% and 90% in all the wells.
- Confirm that there is no contamination in any well. When contamination is observed in any well, the microtiter plate should be rejected.

The following information must be recorded:

- Date
- Acceptance or rejection of the plate. In case of rejection, provide the reason (e.g. confluence < 50% or contamination in 1 well).

2.4.2 Preparation of test, reference and control item stock solutions

Determine the solvent, maximum soluble concentration and mode of preparation according to SOP-ASY15.

Use a glass pipette for the preparation of a vial with solvent.

2.4.2.1 Preparation of test item stock solutions for pre-screen experiments

- Weigh test item into a clear glass vial. Keep containers closed as much as possible and clean the bench surface between weighing of substances to avoid cross contamination.
- Prepare a stock solution of maximum 100 mM, or the highest soluble concentration as determined with SOP-ASY15, in solvent indicated in Table 5. **NB: It is important to perform solubility testing using the conditions applied for the assay (e.g. overnight incubation of stock solutions). Ensure that the solvent is used at Room Temperature and (supplemented) assay medium is pre-warmed to 37°C.**
- Vortex mix for 1 minute.
- Ensure complete solubility by visual inspection.
- Prepare a serial dilution series in glass vials or tubes using the same solvent as used to prepare the stock solution: 1× (C8), 10×, 100×, 1000×, 10000×,100000×, 1000000× and 10000000× dilution (see Table 6). **NB: Change pipette tips each time a new dilution is prepared.**
- Make sure that after preparing each dilution, this dilution is homogenised by thorough vortex mixing before proceeding.

Record the weight, solvent, volume added and final concentration of the test item stock solution.

	Solvent
1st choice	DMSO
2nd choice	H_2O
3rd choice	Ethanol
4th choice	……..

Table 5: Proposed solvents for dissolving test items

Solvents different from DMSO

- Solvents other than DMSO might need to be used to dissolve the test items (e.g. H_2O or ethanol). Always ensure that both solvents (DMSO of reference and control items and the solvent of the test item) are present at the same concentration in all work solutions. E.g. if the test item is dissolved in H_2O or Ethanol, use medium that already contains 0.2% DMSO to prepare the test item work solutions. Similarly, the work solutions of the reference and control items should be prepared using medium that is supplemented with 0.2% H_2O or 0.2% Ethanol.
- The effect of another solvent used must be examined and only concentrations that do not interfere with the receptor binding, activation of the cells, or cell viability may be used.
- Test items that are dissolved in a solvent different from DMSO shall be tested in a separate run with the appropriately prepared work solutions of the reference and control items.

Sample ID	Test item Volume	Solvent volume	Concentration (M)
C8	…. µl stock	…. µl solvent	× 10^{-}
C7	10 µl C8	90 µl solvent	× 10^{-}
C6	10 µl C7	90 µl solvent	× 10^{-}
C5	10 µl C6	90 µl solvent	× 10^{-}
C4	10 µl C5	90 µl solvent	× 10^{-}
C3	10 µl C4	90 µl solvent	× 10^{-}
C2	10 µl C3	90 µl solvent	× 10^{-}
C1	10 µl C2	90 µl solvent	× 10^{-}

Table 6: Test item stock dilution scheme for pre-screening.

2.4.2.2 Preparation of test item stock solutions for comprehensive testing

Following pre-screen experiments, the highest concentration of test item (C8) to be used for comprehensive testing, is determined according to section 2.4.5.1 (agonism) or 2.4.6.1 (antagonism).

- Freshly prepare the test item stock solution in a clear glass vial, according to section 2.4.2.1.

- If needed, pre-dilute the stock solution to the highest dilution to be tested using the same solvent as used to prepare the stock solution. This dilution is indicated as the C8 dilution.
- Prepare a dilution series in glass tubes or vials using the same solvent as used to prepare the stock solution, starting with the highest concentration of test item (C8). Use the dilution scheme as determined in section 2.4.5.1 (agonism) or 2.4.6.1 (antagonism) (see Table 7).
- Make sure that for each dilution prepared, the final volume is approximately 100 µl.
  NB: Change pipette tips each time a new dilution is prepared.
- Make sure that after preparing each dilution, this dilution is homogenised by thorough vortex mixing before proceeding.
- Label the vials to avoid mixing-up dilutions.

Record the following information:
- Test Item ID
- Weight
- Solvent + volume
- Concentration of stock solution (M)
- Concentration of C8

Test chemical	Comprehensive		
	DF 5	DF 3/3.3	DF 2
C1	78,125 x	3,000 x	128 x
C2	15,625 x	1,000 x	64 x
C3	3,125 x	300 x	32 x
C4	625 x	100 x	16 x
C5	125 x	30 x	8 x
C6	25 x	10 x	4 x
C7	5 x	3 x	2 x
C8	1 x	1 x	1 x

Table 7: Test item stock dilution scheme for comprehensive agonistic and antagonistic testing

2.4.2.3 Preparation of agonist reference item stock solutions

Molecular weight of DHT is 290.44 g/mole
- Prepare a 100 mM stock solution of DHT in DMSO to create DHT Stock solution A.
- Prepare a serial dilution series of DHT in DMSO. In Table 8 the required concentrations (in Molar) of the agonist reference compound DHT in DMSO and dilution scheme are provided.
  NB: Change pipette tips each time a new dilution is prepared.
- Record the weight, pipetting volume and final concentration of the stock solution.

NB: Stock solutions may be frozen down as aliquots at -20°C for 3 months. Once an aliquot has been thawed, it may be re-frozen at -20°C and used for an additional 3 weeks.

Sample ID	Reference item weight or volume	Solvent volume	Concentration stock solution
Stock solution A	……. mg DHT	To be calculated on basis of weight	1×10^{-1} M (100 mM)
DHT C8	2 µl stock solution A	1998 µl DMSO	1×10^{-4} M
DHT C7	10 µl C8	90 µl DMSO	1×10^{-5} M
DHT C6	30 µl C7	70 µl DMSO	3×10^{-6} M
DHT C5	10 µl C7	90 µl DMSO	1×10^{-6} M
DHT C4	10 µl C6	90 µl DMSO	3×10^{-7} M
DHT C3	10 µl C5	90 µl DMSO	1×10^{-7} M
DHT C2	10 µl C4	90 µl DMSO	3×10^{-8} M
DHT C1	10 µl C3	90 µl DMSO	1×10^{-8} M

Table 8: Dilution scheme and concentrations of stock solutions of reference item DHT for the agonist AR-CALUX® bioassay.

2.4.2.4 Preparation of antagonist reference item stock solutions

Molecular weight of Flutamide is 276.21 g/mole

- Prepare a 100 mM stock solution of Flutamide in DMSO to create Flutamide Stock solution A.
- Prepare a serial dilution series of Flutamide in DMSO. In Table 9 the required concentrations (in Molar) of the antagonist reference compound Flutamide in DMSO and dilution scheme are provided.
  NB: Change pipette tips each time a new dilution is prepared.
- Record the weight, pipetting volume and final concentration of the stock solution.
  NB: Stock solutions may be frozen down as aliquots at -20°C for 3 months. Once an aliquot has been thawed, it may be refrozen at -20°C and used for an additional 3 weeks.

Sample ID	Reference item weight or volume	Solvent volume	Concentration stock solution
Stock solution A	……. mg Flutamide	DMSO volume to be calculated on basis of weight	1×10^{-1} M (100 mM)
FLU C8	30 µl stock solution A	70 µl DMSO	3×10^{-2} M
FLU C7	30 µl C8	60 µl DMSO	1×10^{-2} M
FLU C6	10 µl C8	90 µl DMSO	3×10^{-3} M
FLU C5	10 µl C7	90 µl DMSO	1×10^{-3} M
FLU C4	10 µl C6	90 µl DMSO	3×10^{-4} M
FLU C3	10 µl C5	90 µl DMSO	1×10^{-4} M
FLU C2	10 µl C4	90 µl DMSO	3×10^{-5} M
FLU C1	10 µl C3	90 µl DMSO	1×10^{-5} M

Table 9: Dilution scheme and concentrations of stock solutions of reference item Flutamide for the antagonist AR-CALUX® bioassay.

2.4.2.5 Preparation of agonist control item stock solutions

Molecular weight of 17α-Methyltestosterone: 302.45 g/mole.
Molecular weight of Corticosterone: 346.46 g/mole.

- Prepare a 100 mM stock solution of positive control item 17α-methyltestosterone and negative control item Corticosterone in DMSO to create Stock solutions A, as in Tables 10 and 11.
- Pre-dilute positive and negative control items in DMSO to create stock solutions B, as in Tables 10 and 11.
- Record the weight, pipetting volume and final concentration of the stock solution.

Sample ID	Control item weight or volume	Solvent volume	Concentration stock solution
17α-methyltestosterone Stock solution A	……. mg 17α-MT	To be calculated on basis of weight	1×10^{-1} M (100 mM)
17α-methyltestosterone Stock solution B	2 µl 17α-MT stock solution A	1998 µl DMSO	1×10^{-4} M

Table 10: Preparation of positive control (PC) stock solutions for the agonist AR-CALUX® bioassay

Sample ID	Control item weight or volume	Solvent volume	Concentration stock solution
Corticosterone Stock solution A	……. mg Corticosterone	To be calculated on basis of weight	1×10^{-1} M (100 mM)
Corticosterone Stock solution B	10 µl Corticosterone stock solution A	990 µl DMSO	1×10^{-3} M

Table 11: Preparation of negative control (NC) stock solutions for the agonist AR-CALUX® bioassay

2.4.2.6 Preparation of antagonist control item stock solutions

Molecular weight of Linuron: 249.09 g/mole.
Molecular weight of Levonorgestrel: 312.45 g/mole.

- Prepare a 100 mM stock solution of positive control item Linuron and negative control item Levonorgestrel in DMSO to create Stock solutions A, as in Tables 12 and 13.
- Pre-dilute positive and negative control items in DMSO to create stock solutions B, as in Tables 12 and 13.
- Record the weight, pipetting volume and final concentration of the stock solution.

Sample ID	Control item weight or volume	Solvent volume	Concentration stock solution
Linuron Stock solution A	……. mg Linuron	To be calculated on basis of weight	1×10^{-1} M (100 mM)
Linuron Stock solution B	10 µl Linuron stock solution A	90 µl DMSO	1×10^{-2} M

Table 12: Preparation of positive control (PC) stock solutions for the antagonist AR-CALUX® bioassay

Sample ID	Control item weight or volume	Solvent volume	Concentration stock solution
Levonorgestrel Stock solution A	……. mg Levonorgestrel	To be calculated on basis of weight	1×10^{-1} M (100 mM)
Levonorgestrel Stock solution B	10 µl Levonorgestrel stock solution A	990 µl DMSO	1×10^{-3} M

Table 13: Preparation of negative control (NC) stock solutions for the antagonist AR-CALUX® bioassay

2.4.3 Preparation of agonist test, reference, control and cytotoxicity positive control work solutions

2.4.3.1 Preparation in glass tubes or vials

2.4.3.1.1 Test item work solutions

Avoid bubble formation at all times when preparing work solutions.

- Fill eight glass tubes or vials with pre-warmed 998 µl assay medium. Label the tubes to avoid mixing-up dilutions.
- For each of the test item concentrations C1 to C8, pipette 2 µl of the test item stock dilution series into 998 µl assay medium.
  NB: Always use pipette tips only once (single use)!
- Carefully homogenise the work solutions by vortex mixing.
- Record test item identification, dilution factor applied, pipetting volumes and final concentrations of the work solutions.
- Ensure solubility by visual inspection. Any signs of insolubility must be recorded e.g. by using the abbreviation 'NS' (not soluble).

2.4.3.1.2 Reference item work solutions

- Fill seven glass tubes or vials with 998 µl and one with 3992 µl pre-warmed assay medium.
- Add 2 µl of reference item concentration C1 to C7 to 998 µl assay medium.
  NB: Always use pipette tips only once (single use)!
- Add 8 µl of reference item concentration C8 to 3992 µl assay medium.
- Carefully mix the work solutions by vortex mixing both after addition of the stock solution to the assay medium and before adding the work solution to the cells.
- In Table 14 the final concentration of reference items in the well is indicated
- Record the preparation of reference item work solutions.

2.4.3.1.3 Control item work solutions

- Prepare work solutions of the solvent control (SC), positive control (PC) and negative control (NC) samples by filling glass tubes or vials with 998 µl pre-warmed assay medium.
- Add 2 µl of PC or NC stock solution B into 998 µl assay medium.
- Prepare the solvent control (SC) samples once for all plates. For six plates, pipette 8 µl solvent into 3992 µl pre-warmed assay medium.
- Carefully homogenise the PC, NC and SC work solution by vortex mixing
- Record preparation of control item work solutions.
- In Table 15 the final concentration of control items in the well is indicated.

Sample ID	Dihydrotestosterone (DHT) concentration in the well (M)
DHT C8	1.0×10^{-07}
DHT C7	1.0×10^{-08}
DHT C6	3.0×10^{-09}
DHT C5	1.0×10^{-09}
DHT C4	3.0×10^{-10}
DHT C3	1.0×10^{-10}
DHT C2	3.0×10^{-11}
DHT C1	1.0×10^{-11}

Table 14: Final concentration of reference item DHT in the well for the agonist AR-CALUX® bioassay

AR CALUX® – positive control (PC) 17α-methyltestosterone Concentration in well (M)	AR CALUX® – negative control (NC) Corticosterone Concentration in well (M)
1×10^{-07} M	1×10^{-06} M

Table 15: Concentrations of positive control (PC) and negative control (NC) items in the well for the agonist AR-CALUX® bioassay

2.4.3.1.4 Cytotoxicity positive control work solutions

Starting from a pre-mixed commercially available 10% Triton X-100 solution, perform the following:

- Pipette 20 µl of 10% Triton X-100 solution into 10 ml pre-warmed assay medium to get a work solution of 0.02% Triton X-100.
- Add 20 µl of DMSO (0.2%).

2.4.3.2 Preparation in a 24-well microtiter plate

Test, reference, control and cytotoxicity positive control work solutions can also be prepared in 24-well microtiter plates. Always make sure that the material used have been evaluated for the absence of background signal.

2.4.3.2.1 Test item work solutions

- Fill 8 wells (Figure 2A) or 16 (Figure 2B) with 998 µl of pre-warmed assay medium, depending on the number of test items to be evaluated.
- For each of the test item concentrations C1 to C8, pipette 2 µl of the test item stock dilution series into 998 µl of assay medium.
- Carefully homogenise the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record test item identification, dilution factor applied, pipetting volumes and final concentrations of the work solutions.
- Ensure solubility by visual inspection. Any signs of insolubility must be recorded e.g. by using the abbreviation 'NS' (not soluble).

2.4.3.2.2 Reference item work solution

- Fill 8 wells with 998 µl of pre-warmed assay medium (Figure 2A).
- For each of the reference item concentrations C1 to C8, pipette 2 µl reference item stock solution into 998 µl of assay medium.
- Carefully homogenise the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of reference item work solutions.

2.4.3.2.3 Preparation of control item work solutions

- Fill 9 wells with 998 µl of pre-warmed assay medium (Figure 2C).
- For the reference item concentration C8 that will be added to each 96-microtiter plate, pipette 2 µl into each of 3 wells with 998 µl of assay medium.
- For the solvent control (SC), pipette 2 µl solvent into each of 4 wells with 998 µl of assay medium.
- For the positive control (PC), pipette 2 µl into 1 well with 998 µl of assay medium.
- For the negative control (NC), pipette 2 µl into 1 well with 998 µl of assay medium.
- Carefully mix the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of reference C8, SC, PC and NC control item work solutions

NOTE: Samples C8 of the reference item and solvent control samples (SC) are used as between-plate controls. Ensure the C8 and SC samples are the same as those used for the dilution series of the reference item, by preparing sufficient solution for all plates at the same time.

2.4.3.2.4 Cytotoxicity positive control work solution

- Fill 4 wells with 998 µl of pre-warmed assay medium (Figure 2C).
- Pipette 2 µl of 10% Triton X-100 solution into each of 4 wells with 998 µl of assay medium to get a work solution of 0.02% Triton X-100.
- Pipette 2 µl of DMSO (0.2%) into the same 4 wells containing 998 µl of assay medium and 2 µl of 10% Triton X-100.

- Carefully mix the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of cytotoxicity positive control work solutions

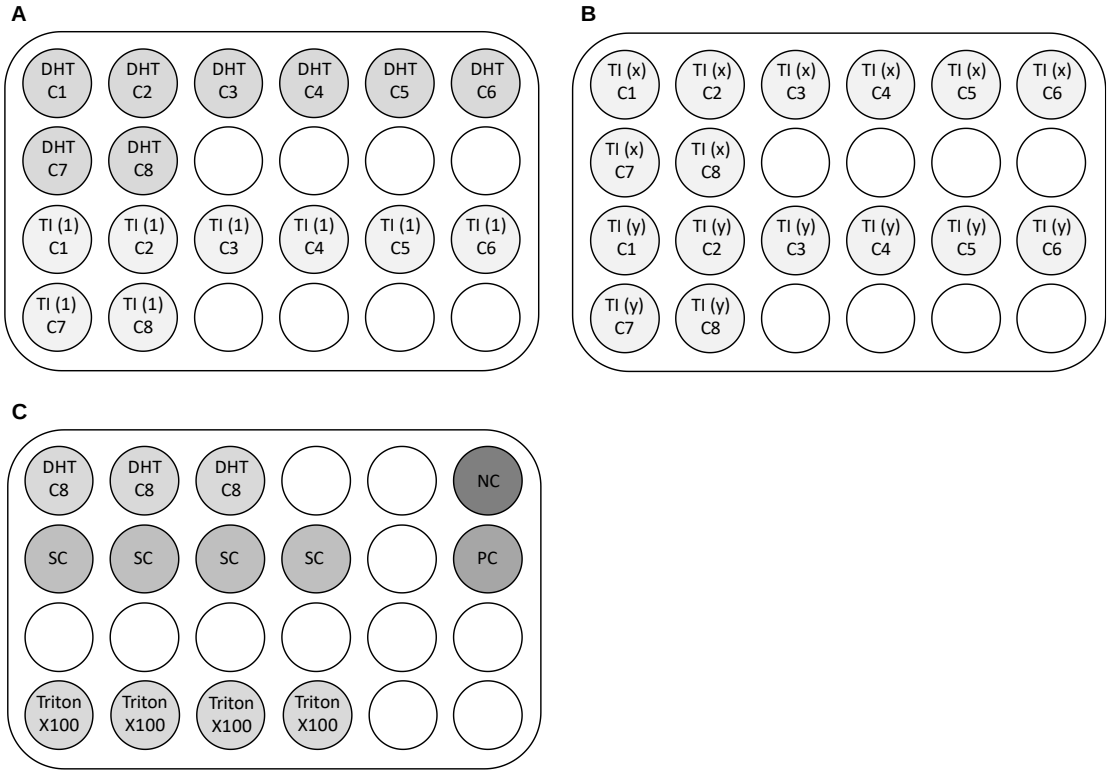


Figure 2: Preparation of reference, control, and test item work solutions in 24-well microtiter plates.

2.4.3.3 Summary of test, reference and control item work solutions to be prepared for the agonist experiments

Sample ID	Amount of assay medium	Volume of item or solvent per work solution	Total volume of assay medium needed
SC	3992 µl (4×998 µl)	8 µl (4×2 µl)	4 ml
DHT (C1 to C7)	998 µl	2 µl	7 × 1 ml
DHT C8	3992 µl (4×998 µl)	8 µl (4×2 µl)	4 ml
PC	998 µl	2 µl	1 ml
NC	998 µl	2 µl	1 ml
Test item (C1 to C8)	998 µl	2 µl	8 × 1 ml
0.02% Triton X-100	4 ml (4 × 998 µl)	8 µl (4×2 µl) 10% Triton 8 µl (4×2 µl) DMSO	4 ml

Table 16: Preparation of the various work solutions for agonism pre-screen and comprehensive testing experiments. Volumes to use for preparation in 24-well plates are placed in brackets.

SC = Assay medium containing 0.1% of solvent.
DHT (C1 to C7) = Serial dilution series of DHT (C1-C7) in assay medium.

DHT C8 = Highest concentration (C8) of the DHT dilution series (1.0×10^{-07} M) in assay medium, inducing maximum luciferase activity.
PC (positive control) = 17α-Methyltestosterone
NC (negative control) = Corticosterone
Test item (C1 to C8) = Serial dilution series of test item (C1 to C8) in assay medium

2.4.4 Preparation of antagonist test, reference, control and cytotoxicity positive control work solutions

For pre-screen experiments, assay medium has to contain 2x EC_{50} concentration of DHT for standard experiments (pre-screen and comprehensive) or 200x EC_{50} concentration of DHT for specificity control experiments (comprehensive). The final exposure media will contain 1× or 100× DHT EC_{50} concentrations.

2.4.4.1 Preparing DHT supplemented assay medium

Use DHT at the EC_{50} concentration of 3×10^{-10} M.

2-times supplemented assay medium containing the 2× EC_{50} concentration of DHT for standard antagonism testing

- Take the 100 mM DHT stock solution (= 1×10^{-1} M) in DMSO
- Dilute 16.7 × in DMSO by pipetting 10 µl into 157 µl DMSO (concentration 6×10^{-3} M)
- Dilute 1000 × in assay medium by pipetting 2 µl into 1998 µl assay medium (concentration 6×10^{-6} M)
- Dilute 10000 × in assay medium by pipetting 10 µl into 100 ml assay medium (concentration 6×10^{-10} M)
- Use this 2-times supplemented assay medium to prepare work solutions for antagonism pre-screen or comprehensive testing.

100-times supplemented assay medium containing 200× EC_{50} concentration of DHT for specificity testing

- Take the 100 mM DHT stock solution (= 1×10^{-1} M) in DMSO
- Dilute 16.7 × in DMSO by pipetting 10 µl into 157 µl DMSO (concentration 6×10^{-3} M)
- Dilute 1000 × in assay medium by pipetting 2 µl into 1998 µl assay medium (concentration 6×10^{-6} M)
- Dilute 100 × in assay medium by pipetting 1 ml into 99 ml assay medium (concentration 6×10^{-8} M)
- Use this 200-times supplemented assay medium to prepare work solutions for antagonism specificity testing.

2.4.4.2 Preparation in glass tubes or vials

2.4.4.2.1 Test item work solutions

Avoid bubble formation at all times when preparing work solutions.

- Fill eight glass tubes or vials with pre-warmed 998 µl supplemented assay medium. Label the tubes to avoid mixing-up dilutions.
- For each of the test item concentrations C1 to C8, pipette 2 µl of the test item stock dilution series into 998 µl supplemented assay medium.
  NB: Always use pipette tips only once (single use)!
- Carefully homogenise the work solutions by vortex mixing.
- Record test item identification, dilution factor applied, pipetting volumes and final concentrations of the work solutions.
- Ensure solubility by visual inspection. Any signs of insolubility must be recorded e.g. by using the abbreviation 'NS' (not soluble).

Note: For pre-screen experiments, test item dilution series must be prepared in 2-times supplemented assay medium. For a comprehensive testing, the test item dilution series must be prepared in both 2-times supplemented assay medium and in 200-times supplemented assay medium (standard comprehensive testing and specificity testing).

2.4.4.2.2 Reference item work solutions

- Fill seven glass tubes or vials with 998 µl and one with 3992 µl pre-warmed supplemented assay medium.
- Add 2 µl of reference item concentration C1 to C7 to 998 µl supplemented assay medium.
  NB: Always use pipette tips only once (single use)!
- Add 8 µl of reference item concentration C8 to 3992 µl supplemented assay medium.
- Carefully mix the work solutions by vortex mixing both after addition of the stock solution to the assay medium and before adding the work solution to the cells.
- In Table 17 the final concentration of reference items in the well is indicated
- Record the preparation of reference item work solutions.

Note: For pre-screen experiments, reference item dilution series must be prepared in 2-times supplemented assay medium. For a comprehensive testing, the reference item dilution series must be prepared in both 2-times supplemented assay medium and in 200-times supplemented assay medium (standard comprehensive testing and specificity testing).

2.4.4.2.3 Control item work solutions

- Prepare work solutions of the solvent control (SC), positive control (PC) and negative control (NC) samples by filling glass tubes or vials with 998 µl pre-warmed 2-times supplemented assay medium.
- Prepare the solvent control (SC) samples once for all plates. For six plates, pipette 8 µl solvent into 3992 µl pre-warmed 2-times supplemented assay medium.

- Add 2 µl of PC or NC stock solution B into 998 µl 2-times supplemented assay medium.
- Carefully homogenise the PC, NC and SC work solution by vortex mixing
- Record preparation of control item work solutions.
- In Table 18 the final concentration of PC and NC items in the well is indicated

Sample ID	Flutamide concentration in the well (M)
FLU C8	3×10^{-5} M
FLU C7	1×10^{-5} M
FLU C6	3×10^{-6} M
FLU C5	1×10^{-6} M
FLU C4	3×10^{-7} M
FLU C3	1×10^{-7} M
FLU C2	3×10^{-8} M
FLU C1	1×10^{-8} M

Table 17: Final concentration of reference item Flutamide in the well for the antagonist AR-CALUX® bioassay

anti AR-CALUX® – positive control (PC) **Linuron** **Concentration in well (M)**	**anti AR-CALUX® – negative control (NC)** **Levonorgestrel** **Concentration in well (M)**
1×10^{-05} M	1×10^{-06} M

Table 18: Concentrations of positive control (PC) and negative control (NC) items in the well, for the antagonist AR-CALUX® bioassay

2.4.4.2.4 Cytotoxicity positive control work solutions

Starting from a pre-mixed commercially available 10% Triton X-100 solution, perform the following:

- Pipette 20 µl of 10% Triton X-100 solution into 10 ml pre-warmed 2-times supplemented assay medium to get a work solution of 0.02% Triton X-100.
- Add 20 µl of DMSO (0.2%).

2.4.4.3 Preparation in a 24-well microtiter plate

Test, reference, control and cytotoxicity positive control work solutions can also be prepared in 24-well microtiter plates. Always make sure that the material used have been evaluated for the absence of background signal.

2.4.4.3.1 Test item work solutions

Pre-screen testing

- Fill 8 wells (Figure 3A) or 16 (figure 3B) with 998 µl of pre-warmed 2-times supplemented assay medium, depending on the number of test items to be evaluated.
- For each of the test item concentrations C1 to C8, pipette 2 µl of the test item stock dilution series into 998 µl of 2-times supplemented assay medium.
- Carefully homogenise the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record test item identification, dilution factor applied, pipetting volumes and final concentrations of the work solutions.
- Ensure solubility by visual inspection. Any signs of insolubility must be recorded e.g. by using the abbreviation 'NS' (not soluble).

Comrehensive testing

- Fill 8 wells (Figure 4B) with 998 µl of pre-warmed 2-times supplemented assay medium (top wells; standard testing))
- Fill 8 wells (Figure 4B) with 998 µl of pre-warmed 200-times supplemented assay medium (bottom wells; specificity testing)
- For each of the test item concentrations C1 to C8, pipette 2 µl of the test item stock dilution series into 998 µl of 2-times supplemented assay medium (top wells) and 2 µl of the test item stock dilution series into 998 µl of 200-times supplemented assay medium (bottom wells).
- Carefully homogenise the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record test item identification, dilution factor applied, pipetting volumes and final concentrations of the work solutions.
- Ensure solubility by visual inspection. Any signs of insolubility must be recorded e.g. by using the abbreviation 'NS' (not soluble).

2.4.4.3.2 Reference item work solution

Pre-screen testing

- Fill 8 wells with 998 µl of pre-warmed 2-times supplemented assay medium (Figure 3A).
- For each of the reference item concentrations C1 to C8, pipette 2 µl reference item stock solution into 998 µl of 2-times supplemented assay medium.
- Carefully homogenise the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of reference item work solutions.

Comrehensive testing

- Fill 8 wells (Figure 4A) with 998 µl of pre-warmed 2-times supplemented assay medium (top wells; standard testing)).
- Fill 8 wells (Figure 4A) with 998 µl of pre-warmed 200-times supplemented assay medium (bottom wells; specificity testing)

- For each of the reference item concentrations C1 to C8, pipette 2 µl of the reference item stock dilution series into 998 µl of 2-times supplemented assay medium (top wells) and 2 µl of the reference item stock dilution series into 998 µl of 200-times supplemented assay medium (bottom wells).
- Carefully homogenise the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of reference item work solutions.

2.4.4.3.3 Preparation of control item work solutions

- Fill 1 well with 998 ul of pre-warmed assay medium (figure 3C or 4C)
- For the vehicle control (VC), pipette 2 µl solvent into each of 1 wells with 998 µl of assay medium.
- Fill 9 wells with 998 µl of pre-warmed 2-times supplemented assay medium (Figure 3C or 4C).
- For the reference item concentration C8 that will be added to each 96-microtiter plate, pipette 2 µl into each of 3 wells with 998 µl of 2-times supplemented assay medium.
- For the solvent control (SC), pipette 2 µl solvent into each of 4 wells with 998 µl of 2-times supplemented assay medium.
- For the positive control (PC), pipette 2 µl into 1 well with 998 µl of 2-times supplemented assay medium.
- For the negative control (NC), pipette 2 µl into 1 well with 998 µl of 2-times supplemented assay medium.
- Carefully mix the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of reference C8, SC, PC, NC and VC control item work solutions

NOTE: Samples C8 of the reference item and solvent control samples (SC) are used as between-plate controls. Ensure the C8 and SC samples are the same as those used for the dilution series of the reference item, by preparing sufficient solution for all plates at the same time

2.4.4.3.4 Cytotoxicity positive control work solution

- Fill 4 wells with 998 µl of pre-warmed 2-times supplemented assay medium (figure 3C or 4C).
- Pipette 2 µl of 10% Triton X-100 solution into each of 4 wells with 998 µl of assay medium to get a work solution of 0.02% Triton X-100.
- Pipette 2 µl of DMSO (0.2%) into the same 4 wells containing 998 µl of 2-times supplemented assay medium and 2 µl of 10% Triton X-100.
- Carefully mix the work solutions by placing the plate on a vortex shaker for at least 10 minutes (300 rpm).
- Record the preparation of cytotoxicity positive control work solutions

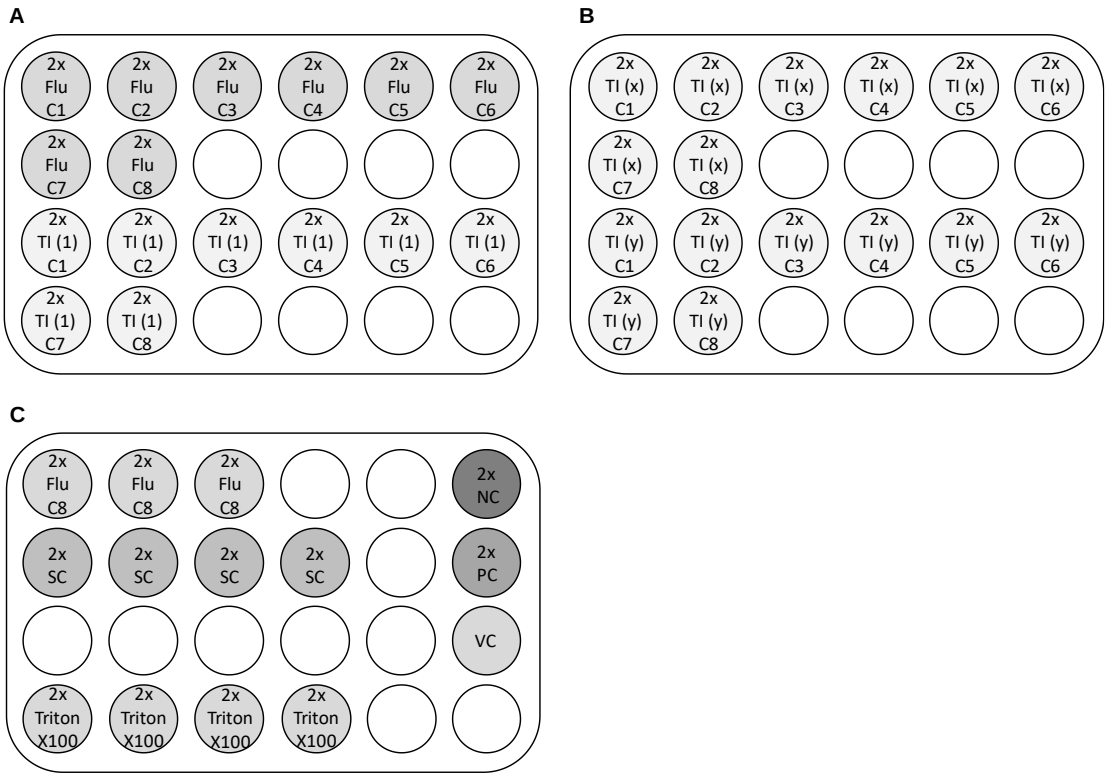


Figure 3: Preparation of reference, control, and test item work solutions in 24-well microtiter plates for pre-screen experiments.

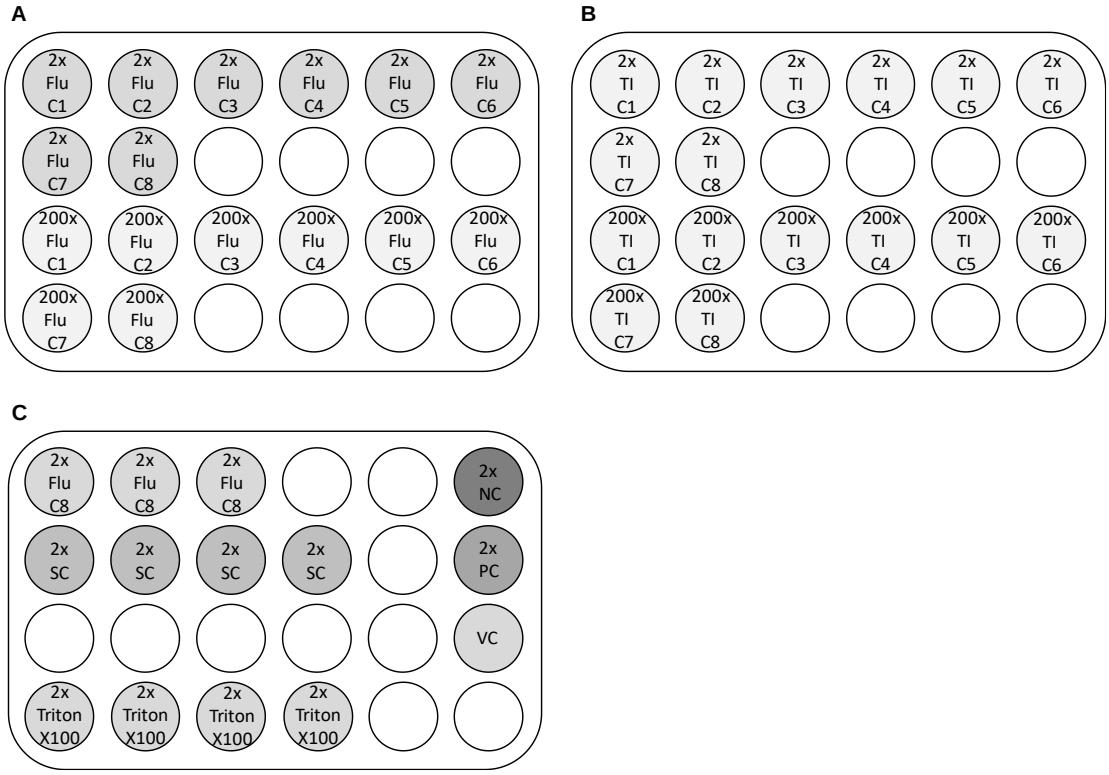


Figure 4: Preparation of reference, control, and test item work solutions in 24-well microtiter plates for comprehensive experiments, including specificity testing.

2.4.4.4 Summary of test, reference and control item work solutions to be prepared for the antagonist experiments

Sample ID	Amount agonist supplemented assay medium	Volume of item or solvent per work solution	Total volume of assay medium needed
SC	3992 µl (4 × 998 µl)	8 µl (4 × 2 µl)	4 ml
Flutamide C1 to C7	998 µl	2 µl	7 × 1 ml
Flutamide C8	3992 µl (4 × 998 µl)	8 µl (4 × 2 µl)	4 ml
PC	998 µl	2 µl	1 ml
NC	998 µl	2 µl	1 ml
Test item (C1 to C8)	998 µl	2 µl	8 × 1 ml
VC	998 µl (No DHT in the assay medium!)	2 µl	1 ml
0.02% Triton X-100	3992 µl (4 × 998 µl)	8 µl (4 × 2 µl) 10% Triton 8 µl (4 × 2 µl) DMSO	10 ml

Table 19: Preparation of the various work solutions for antagonism pre-screen and comprehensive tests, sufficient for 1 series of analysis of 6 plates (11 test items). Volumes to use for preparation in 24-well plates are placed in brackets.

VC (vehicle control) = Assay medium without agonist (DHT), containing 0.1% solvent.
SC = Agonist supplemented assay medium, containing 0.1% solvent used for reference and test item
Flutamide (C1 to C7) = Serial dilution series of Flutamide (C1 to C7) in agonist supplemented assay medium
Flutamide C8 = Highest concentration (C8) of the Flutamide dilution series (3.0×10^{-05} M in well) in agonist supplemented assay medium, causing maximum inhibition of luciferase activity.
PC (positive control) = Linuron
NC (negative control) = Levonorgestrel
Test item (C1 to C8) = Serial dilution series of test item (C1 to C8) in agonist supplemented assay medium

2.4.5 Exposure of the cells for agonist pre-screen and comprehensive testing

Selection of concentrations for comprehensive testing is described in section 2.4.5.1.
Prepare test item stock and work solutions as described in sections 2.4.2 and 2.4.3 respectively.

- Add 100 µl of the work solutions to the cells, according to the plate layout in Figure 5. **NB: Changing pipette tips is not required if cells are exposed to increasing concentrations of the test item. Pipette tips should be changed when exposing cells to a different test item.**
- Add 100 µl of the cytotoxicity control Triton X-100 work solution to 6 wells in row 1, final concentration 0.01%.
- Place the microtiter plate(s) in the CO_2-incubator and incubate for 24 hours +/- 2 hours.
- Record the date and time of incubation start.

- Following exposure, remove microtiter plate(s) from the CO_2 incubator.
- Visually inspect the microtiter plates, using inverted microscopy. Check for cloudy wells as an indicator of contamination and verify solubility. Identify samples with cells showing signs of cytotoxicity, using the examples given in Appendix C. Record observations for the exact position on the plate.
- For a pre-screen experiment, transfer 100 µl to a fresh 96-well plate and perform cytotoxicity testing by LDH leakage (see section 2.4.7),
- Remove all remaining medium and proceed to lysing the cells and measurement of luciferase activity (see section 2.4.8).
- Record the date and time of incubation end at the moment when the cells are lysed.
- Carefully check that the acceptance criteria in Section 2.4.5.2 are met.
- Record observations e.g. as 'NS' for 'not soluble' or 'TO' for 'cytotoxicity' for the exact position on the plate and exclude the results for these samples from the data analysis forms.
- Following a pre-screen experiment, determine the proper concentration range for comprehensive testing as described in section 2.4.5.1.
- Following a comprehensive test, analyse the data to calculate the agonist parameters according to section 2.5.2.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1 DHT	C2 DHT	C3 DHT	C4 DHT	C5 DHT	C6 DHT	C7 DHT	C8 DHT	PC	
C	Triton X-100	SC	C1 DHT	C2 DHT	C3 DHT	C4 DHT	C5 DHT	C6 DHT	C7 DHT	C8 DHT	PC	
D	Triton X-100	SC	C1 DHT	C2 DHT	C3 DHT	C4 DHT	C5 DHT	C6 DHT	C7 DHT	C8 DHT	PC	
E	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	NC	
F	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	NC	
G	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	NC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
C	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
D	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
E	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
F	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
G	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
H												

Figure 5: Plate-layout of the 96-well microtiter plates for pre-screen and comprehensive testing of agonist properties.

C1 to C8 = Concentrations (1 to 8) of test item (TI) and reference item (DHT). DHT is always in rows B, C and D of the first plate.

NC = negative control item Corticosterone.

PC = positive control item 17α-Methyltestosterone.

SC = Assay medium containing 0.1% of the solvent used for reference and test item

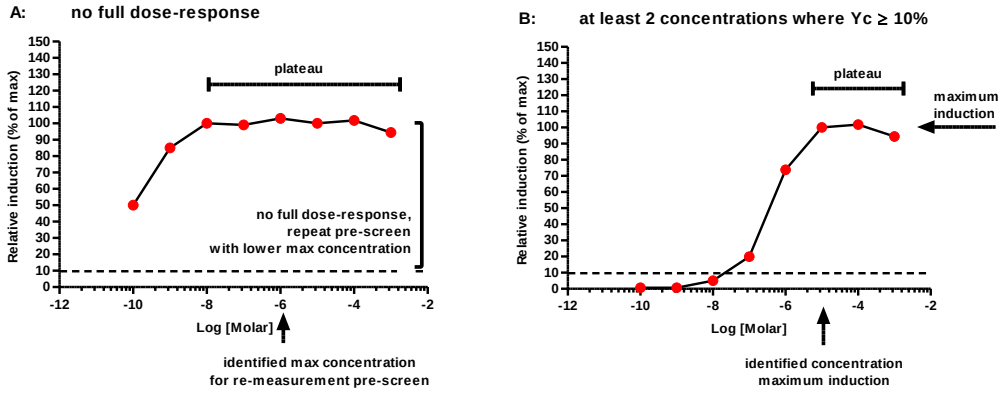
Triton X-100 = 0.01% Triton X-100, the cytotoxicity positive control.

2.4.5.1 Selection of concentrations for agonism comprehensive testing

- Concentrations showing insolubility during the preparation of stock of work solutions for pre-screen experiments, as determined by visual inspection, shall not be selected for comprehensive testing.
- Concentrations showing cytotoxicity after exposure shall not be selected for comprehensive testing.

Following measurement of the luciferase activity and data analysis of the pre-screen experiment(s), the highest concentration of the test item (C8), to be used for comprehensive testing, is selected.

- Transfer the RLU raw data from the agonism pre-screen experiment, into the data analysis form DAT02-ASY06, which automatically determines if the acceptance criteria are met. See calculations in Appendix A.
- Verify the dose response (if any).
- When the test item does not show a full dose response curve, as shown in Figure 6A, repeat the pre-screen with a lower maximum (C8) concentration to ensure the C1 relative induction is at solvent control level or at least below 10%.
- Identify concentrations where the relative induction (Y_c) is equal to or higher than 10% from the solvent control (see calculation 1 in section 2.5.2).
- When there is at least 1 concentration where Y_c is equal to or above 10%, identify the lowest concentration giving maximum relative induction (figures 6 B, C and D).
- Apply one of the following options to select the C8 concentration for the comprehensive test:
    - Prepare a 3 × concentrated solution from the selected concentration giving maximum relative induction.
    - Apply the maximum concentration allowed (100 µM in the well).
- Apply one of the following dilution factors for the comprehensive test:
    - Dilution factor 2 for test items that show response > 10% at the highest concentration only.
    - Dilution factor 5 for test items that do not show any response > 10%.
    - For test items showing a (full) dose response, use the default dilution scheme as in section 2.4.2.2. with alternating dilution factors 3.33 and 3. Only in case the full dose response curve cannot be captured with the default dilution scheme, use dilution factor 5.



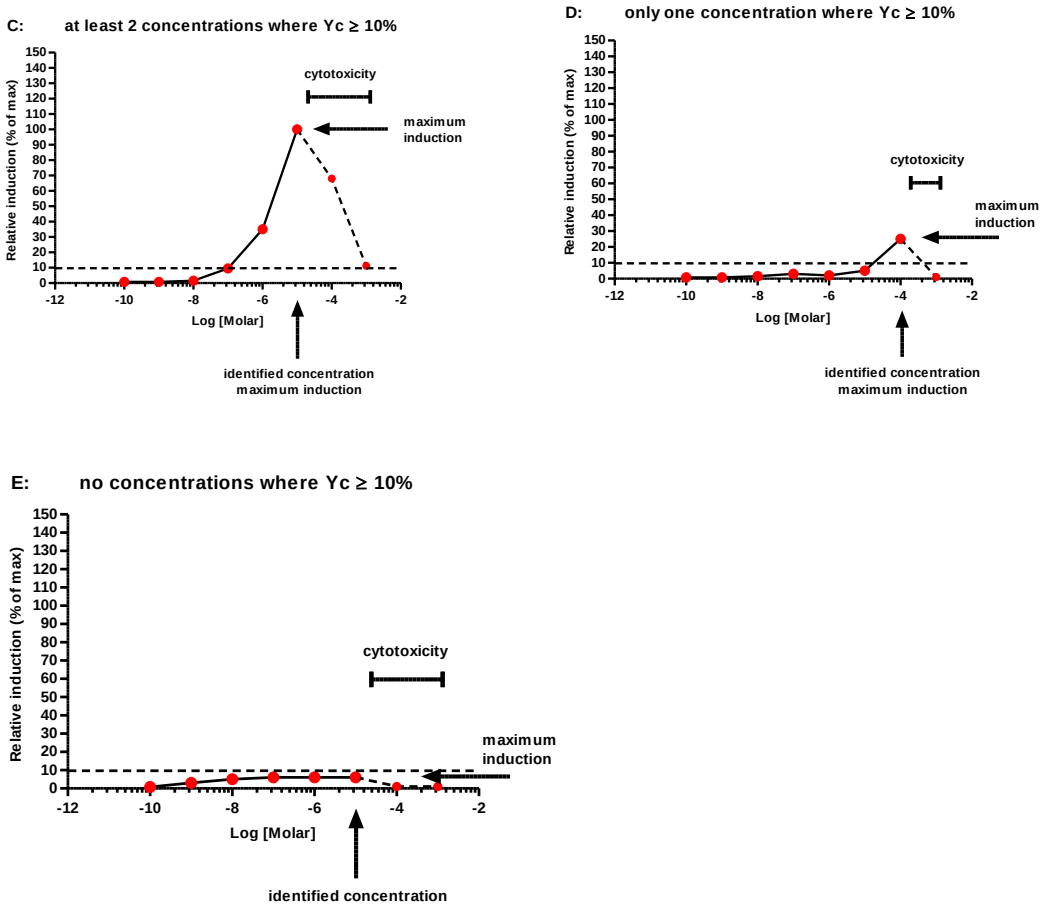


Figure 6: Examples of agonist dose responses (A, B, C, D and E) provided as guidance for the selection of concentrations and dilution factor for agonism comprehensive testing. On basis of the responses shown in the figures, the following dilutions factors shall be chosen: for A, B and C default dilution factor 3.3, for D dilution factor 2 and for E dilution factor 5.

2.4.5.2 Acceptance criteria for agonist experiments

Table 20 summarises the criteria for the acceptance of the whole experiment and individual plates. The application of the acceptance criteria is a stepwise procedure.

1) Whole experiment evaluation: criteria 1 to 7 must be met for the first plate containing reference and control items.
2) Plate evaluation (plates 2 to 6): criteria 6 and 7, using the DHT C8 and solvent control samples on the respective plate, must be met.
3) Test item evaluation: see data analysis section 2.5.1

Moreover:

- maximum two concentrations may be excluded from the reference item dilution series, on basis of operator errors or other information, provided that the acceptance criteria are met.

Number	Acceptance criteria	
1	Sigmoidal curve of reference item DHT	Yes
2	EC_{50} range reference item DHT	1×10^{-10} –1×10^{-9} M
3	|CV| of estimated log(EC_{50}) reference item DHT	< 1.5%
4	Relative induction (%) PC 17α-Methyltestosterone	> 30%
5	Relative induction (%) NC Corticosterone	< 10%
6	Minimum fold induction (IF) of the highest DHT concentration (C8), with respect to the solvent control on each plate	> 20
7	Z-factor calculated with DHT C8 and solvent control samples on each plate	> 0.5

Table 20: Acceptance criteria for agonism pre-screen / comprehensive testing

2.4.6 Exposure of the cells for antagonist pre-screen and comprehensive testing

Selection of concentrations for comprehensive testing is described in section 2.4.5.1.

Prepare test item stock and work solutions as described in sections 2.4.2 and 2.4.4 respectively.

- Add 100 µl of the work solutions to the cells, according to the plate layout in figure 7 (pre-screen) or figure 8 (comprehensive test).
- Add 100 µl of the cytotoxicity control Triton X-100 to 6 wells in row 1, final concentration 0.01%.
- Repeat all handlings for all subsequent plates to be analysed, following the plate setup as given in Figures 7 and 8.
- Place the microtiter plate(s) in the CO_2-incubator and incubate for 24 hours +/- 2 hours.
- Record the date and time of incubation start.
- Following exposure, remove microtiter plate(s) from the CO_2 incubator and visually inspect the microtiter plates using inverted microscopy.
- Check for cloudy wells as an indicator of contamination and verify solubility. Identify samples with cells showing signs of cytotoxicity, using the examples given in Appendix C. Record observations for the exact position on the plate.
- Proceed with cytotoxicity testing by LDH leakage (pre-screen testing only) (see section 2.4.7), lysing the cells and measurement of luciferase activity (see section 2.4.8).
- Record the date and time of incubation end at the moment when the cells are lysed.
- Carefully check that the acceptance criteria in Section 2.4.6.2 are met.
- Record observations e.g. as 'NS' for 'not soluble' or 'TO' for 'cytotoxicity' for the exact position on the plate and exclude the results for these samples from the data analysis forms.
- Following a pre-screen experiment, determine the proper concentration range for comprehensive testing as described in section 2.4.5.1.

- Following a comprehensive test, analyse the data to calculate the antagonist parameters according to section 0.

Compounds showing antagonist response during pre-screen testing have to be tested for specificity of antagonism during comprehensive testing.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
C	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
D	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
E	Triton X-100	NC	C1	C2	C3	C4	C5	C6	C7	C8	PC	
F	Triton X-100	NC	C1	C2	C3	C4	C5	C6	C7	C8	PC	
G	Triton X-100	NC	C1	C2	C3	C4	C5	C6	C7	C8	PC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
C	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
D	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
E	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
F	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
G	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
H												

Figure 7: Plate-layout of the 96-well microtiter plates for pre-screen of antagonist properties.

SC = Assay medium containing 0.1% of the solvent used for the reference and test item + EC_{50} concentration of DHT.

C1 to C8 = Concentrations (1 to 8) of test item (TI) and reference items (Flu) + EC_{50} concentration of DHT. Reference item Flutamide is always in rows B, C and D of the first plate.

NC = negative control item Levonorgestrel + EC_{50} concentration of DHT.

PC = positive control item Linuron+ EC_{50} concentration of DHT.

VC = Assay medium containing 0.1% solvent without DHT.

Triton X-100 = 0.01% Triton X-100, the cytotoxicity positive control.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
C	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
D	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
E	Triton X-100	NC	C1 FLU 100	C2 FLU 100	C3 FLU 100	C4 FLU 100	C5 FLU 100	C6 FLU 100	C7 FLU 100	C8 FLU 100	PC	
F	Triton X-100	NC	C1 FLU 100	C2 FLU 100	C3 FLU 100	C4 FLU 100	C5 FLU 100	C6 FLU 100	C7 FLU 100	C8 FLU 100	PC	
G	Triton X-100	NC	C1 FLU 100	C2 FLU 100	C3 FLU 100	C4 FLU 100	C5 FLU 100	C6 FLU 100	C7 FLU 100	C8 FLU 100	PC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
C	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
D	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
E	Triton X-100	SC	C1 100	C2 100	C3 100	C4 100	C5 100	C6 100	C7 100	C8 100	C8 FLU	
F	Triton X-100	SC	C1 100	C2 100	C3 100	C4 100	C5 100	C6 100	C7 100	C8 100	C8 FLU	
G	Triton X-100	SC	C1 100	C2 100	C3 100	C4 100	C5 100	C6 100	C7 100	C8 100	C8 FLU	
H												

Figure 8: Plate-layout of the of the 96-well microtiter plates for comprehensive testing of antagonist properties.

SC = Assay medium containing 0.1% of the solvent used for the reference and test item + EC_{50} concentration of DHT.

C1 to C8 = Concentrations (1 to 8) of test item (TI) and reference items (Flu) + EC_{50} concentration of DHT. Reference item Flutamide is always in rows B, C and D of the first plate.

C1 to C8 100 = Concentrations (1 to 8) of test item (TI) and reference items (Flu) + 100 × EC_{50} concentration of DHT (specificity controls).

NC = negative control item Levonorgestrel + EC_{50} concentration of DHT.

PC = positive control item Linuron+ EC_{50} concentration of DHT.

VC = Assay medium containing 0.1% solvent without DHT.

Triton X-100 = 0.01% Triton X-100, the cytotoxicity positive control.

2.4.6.1 Selection of concentrations for antagonism comprehensive testing

- Concentrations showing insolubility during the preparation of stock or work solutions for pre-screen experiments, as determined by visual inspection, shall not be selected for comprehensive testing.
- Concentrations showing cytotoxicity after exposure shall not be selected for comprehensive testing.

Following measurement of the luciferase activity and data analysis of the pre-screen experiment(s), the highest concentration of the test item (C8) to be used for comprehensive testing of the antagonist properties of compounds is selected.

- Transfer the RLU raw data from the antagonism pre-screen experiment, into the data analysis form DAT04-ASY06, which automatically determines if the acceptance criteria are met. See calculations in Appendix A.
- Verify the dose response (if any).

- When the test item does not show a full dose response curve (see Figure 9A), repeat the pre-screen with a lower maximum (C8) concentration to ensure the C1 relative induction is at solvent control level or at least above 80%.
- Identify concentrations where the relative induction (Y_c) is equal to or lower than 80% from the solvent control (see calculation 1 in section 2.5.3).
- When there is at least 1 concentration where Y_c is equal to or below 80%, identify the lowest concentration giving maximum inhibition (lowest relative induction) (Figure 9 B, C and D).
- Apply one of the following options to select the C8 concentration for the comprehensive test:
    - Prepare a 3 × concentrated solution of the sample giving maximum inhibition (lowest relative induction).
    - Apply the maximum concentration allowed (100 μM in the well).
- Apply one of the following dilution factors for the comprehensive test:
    - Dilution factor 2 for test items that show response < 80% at the highest concentration only.
    - Dilution factor 5 for test items that do not show any response < 80% in the pre-screen experiment.
    - For test items showing a (full) dose response, use the default dilution scheme as in section 2.4.2.2. with alternating dilution factors 3.33 and 3. Only in case the full dose response curve cannot be captured with the default dilution scheme, use dilution factor 5.

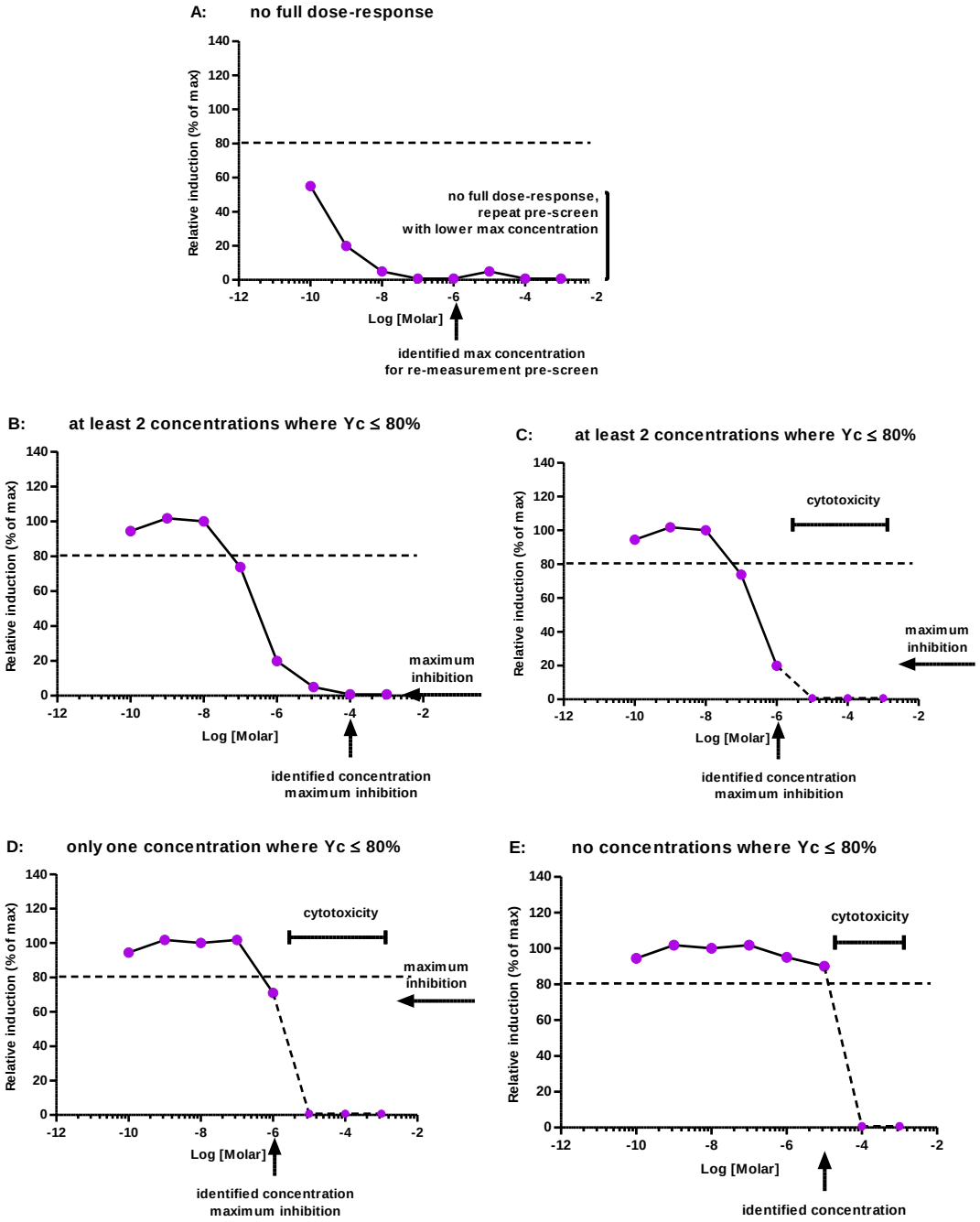


Figure 9. Examples of antagonist dose responses (A, B, C, D and E) provided as guidance for the selection of concentrations and dilution factor for antagonism comprehensive testing. On basis of the responses shown in the figures, the following dilution factors should be chosen: for B and C, the default dilution factor 3.3, for D dilution factor 2 and for E dilution factor 5.

2.4.6.2 Acceptance criteria for antagonist experiments

Table 21 summarises the criteria for the acceptance of the whole experiment and individual plates. The application of the acceptance criteria is a stepwise procedure.

1) Whole experiment evaluation: criteria 1 to 8 must be met for the first plate containing reference and control items.
2) Plate evaluation (plates 2 to 6): criteria 6 and 7, using the FLU C8 and solvent control samples on the respective plate, must be met.
3) Test item evaluation: see data analysis section 2.5.1

Moreover,

- Monitor the relative induction level of the VC. If it is higher than 5%, identify the cause and its impact on the data.
- Monitor the relative induction of the reference item at the lowest concentration C1 falls between 80% and 120%.
- Maximum two concentrations may be excluded from the reference item dilution series, on basis of operator errors or other information, provided that the acceptance criteria are met.

Number	Acceptance criteria	
1	Sigmoidal curve of reference item Flutamide	Yes
2	IC_{50} range reference item Flutamide	$1\times10^{-7} – 1\times10^{-6}$ M
3	|CV| of estimated log(IC_{50}) reference item Flutamide	< 3%
4	Relative induction PC (Linuron)	< 60 %
5	Relative induction NC (Levonorgestrel)	> 85 %
6	Minimum fold inhibition (InhF) of the highest Flutamide concentration (C8) with respect to the solvent control on each plate	> 10
7	Z-factor calculated with Flutamide C8 and solvent control samples on each plate	> 0.5
8	R^2 between S_c^n and Y_c for Flutamide	≤ 0.7

Table 21: Acceptance criteria for antagonism pre-screen (1-7) / comprehensive testing (1-8). Symbols and abbreviations are explained in the calculations in section 2.5.3 and Appendix A.

2.4.7 Cytotoxicity testing

During the pre-screen, cytotoxicity must be determined both visually and by measurement of LDH leakage. During comprehensive testing the LDH leakage is no longer needed but the visual inspection must be carried out.

2.4.7.1 Visual inspection

- Visual inspection must be performed 22 to 26 hours after exposure to the test items, by microscopic observation.
- Observed cytotoxicity must be recorded e.g. as 'TO' for the exact position on the plate.

2.4.7.2 LDH leakage measurement

Note: The procedure described below is for the LDH leakage kit from Roche. Please refer to the manufacturer's manual in case another LDH kit is used. **The enzyme reaction must take place at room temperature and in the dark for all solutions**. Bubbles in the wells increase the OD values and should be avoided.

- Prepare a LDH leakage test plate by transferring 100 µl exposure medium of triplicate samples to a new clear 96-well microtiter plate.
- Reconstitute solution 1 "catalyst" with 1 ml demineralized water for 10 minutes and mix thoroughly (the reconstituted solution is stable for 2 weeks if stored at 2-8^{o}C).
- Thaw 1 bottle of 45 ml solution 2 "dye solution" (the dye solution is stable for 2 weeks if stored at 2-8^{o}C).
- For 100 wells: mix shortly before use 250 µl of solution 1 with 11.25 ml of solution 2 (this reaction mixture cannot be stored and should be used immediately). Avoid bubble formation.
- Add 100 µl reaction mixture to every well of the LDH leakage test plate. Avoid bubble formation.
- Incubate in the dark at room temperature and measure the OD_{490} after 10 minutes. When the OD_{490} values for the solvent control wells (SC and VC samples; see figures 5, 7 and 8) are lower than 0.3, the measurement must be repeated after approximately 2 minutes.
- Check that the wells are free of bubbles. Where applicable, remove any bubbles before the measurement with a needle.
- Read the absorbance at 490 nm (+/-9 nm).
- Transfer the raw data from the LDH leakage experiment, into the data analysis form DAT06-ASY06, which automatically calculates the percentage LDH leakage with respect to the positive control item (regarded as 100% cytotoxic), in accordance with the data analysis section 2.4.7.4.

2.4.7.3 Acceptance criteria for validity of cytotoxicity experiments

- Cytotoxicity positive control item Triton X-100 must visually be cytotoxic to the cells, as observed by decreased confluence and/or rounded cells, with respect to the solvent control.
- Solvent control samples shall visually not show any cytotoxicity, as observed by a confluent monolayer, see Appendix B.
- OD of solvent control wells must be > 0.3 in all wells.

2.4.7.4 Cytotoxicity data analysis

For all concentrations of the test item, calculate the percentage LDH leakage with respect to the cytotoxicity positive control 0.01% Triton X-100 (percentage set at 100%), using the following calculation.

$$\%\ LDH\ leakage = \frac{average\ AU\ test\ chemical\ - average\ AU\ SC}{average\ AU\ positive\ control\ - average\ AU\ SC} \times 100$$

This calculation is performed by data analysis file DAT06-ASY06 (excel format).

The test item is regarded cytotoxic at a specific concentration when:

- Either the average percentage LDH leakage of the test item triplicate sample is higher than 15% with respect to positive control.
- Or, cytotoxicity is observed at visual inspection.

2.4.8 Measuring the luciferase activity

There are several options for the measurement of luminescence. When using a commercial luciferase assay system (either flash or glow luminescence kit) to detect the AR response (firefly luciferase activity), the manufacturer's instructions should be followed. The procedure described below is applicable for the in-house prepared illuminate mix for which equipment with automated injection of the reagents (2 injectors) is needed.

2.4.8.1 Lysing the cells

Note: Ensure that before lysing the cells the appropriate amount of medium is transferred to a fresh 96-well plate for cytotoxicity testing.

- Carefully remove all remaining medium from the cells, either by aspiration or by emptying the plate on absorbing paper in a chemical safety cabinet.
- Add 30 µl lysis reagent to the cells in each well. If another lysis reagent is used, follow the manual of that reagent.
- Shake the plate for at least five minutes (300 rpm) without heating the plate.
- Verify cell lysis by means of microscopic observation. Check one row and one column of each 96-well plate. No cells should be visible.
- Measure luminescence in the plates the same day or store the plates at -20°C for a maximum of 4 weeks.

2.4.8.2 Luminescence measurement

- **Ensure (by temperature measurement) that the illuminate-mix and the plates are fully equilibrated to room temperature (20-25°C) before beginning measurements.**
- Start the measuring program of the luminometer.
- Prime the appropriate injectors of the luminometer with illuminate-mix and 0.2 M NaOH.
- Place the microtiter plate without lid in the luminometer. Make sure the microtiter plate is orientated correctly.
- Enter the name of the plate in the software and start the measurement. Each measurement of a well consists of the injection of 100 µl illuminate-mix, followed by the measurement of emitted light (e.g. 4-5 seconds. Integration time depends on instrument settings) and then injection of 100 µl NaOH to stop the reaction.
- Print the raw data and save the electronic raw data file.
- After the last measurement, empty the injectors and prime/wash them with demineralised water or follow the instructions of the manufacturer.

2.5 Data analysis and reporting of results: (anti-)androgenic properties of test items

Data analysis forms DAT02 (agonism comprehensive testing) and DAT05 (antagonism comprehensive testing) automatically verify if acceptance criteria are met, and, automatically calculate the parameters as described in the following sections. The values for EC_{50}, EC_{10}, IC_{50} and IC_{20} shall be determined via Graphpad prism or similar software and inserted in the data analysis forms DAT02 and DAT05.

2.5.1 Test item data acceptance for agonist and antagonist assays

Test item data are valid when:

- The acceptance criteria for validity of the experiment and plate, as described in section 2.4.5.2 (agonism) and 2.4.6.2 (antagonism), are fulfilled.
- The dose response curve is composed of minimum six concentrations from three replicate wells after excluding samples on basis of insolubility, cytotoxicity, operator errors or other information.

Moreover,

- For test items tested under the antagonist protocol, monitor whether the relative induction of the test item at the lowest concentration C1 falls between 80% and 120%.
- For test items tested under the agonist protocol, monitor whether the relative induction of the test item at the lowest concentration C1 is lower than 10%.

2.5.2 Assessment of agonist properties of test items

1) Transform the test and reference item RLU values into **Relative Induction** (%) with the following calculation:

$$Y_{ic} = \frac{RLU\ of\ item\ (i\ replicate) - average\ RLU\ of\ SC}{average\ RLU\ of\ DHT_{C8} - average\ RLU\ of\ SC} \times 100, \quad i = 1,2,3$$

$Y_c = average\ Y_{ic}$ where the average is taken over the triplicate samples (all from the same plate), *Item* is test or reference item and DHT_{C8} is DHT at concentration C8. The symbol Y_{ic} represents the relative induction at concentration *c* and replicate i and symbol Y_c represents the average relative induction over replicates at concentration *c*.

2) Determine if the test item relative induction (Y_c) is equal to or greater than 10% of the solvent control response (SC) at any concentration.

3) If none of the responses are equal to or greater than 10%, calculations 4 to 7 do not need to be performed.

To get additional potency information proceed with calculations 4 to 7:

4) When one or more Y_c values are equal to or greater than 10%, fit the Hill curve model to the accepted relative induction data Y_{ic} (i.e. the three replicate relative induction values) using Graphpad Prism or other statistical software. The Hill curve model is a logistic regression model (variable slope, 4 parameters) with

$$y = Bottom + \frac{(Top - Bottom)}{\left(1 + 10^{((LogEC_{50} - x) * HillSlope)}\right)}$$

x =	Log of concentration
y =	Relative induction (%)
Top =	Maximum relative induction (%)
Bottom =	Minimum relative induction (%)
$LogEC_{50}$ =	Log of concentration at which 50% of maximum relative induction is observed
HillSlope =	Slope factor of the Hill curve

5) When the Hill curve can be fit well, the model provides estimates of the following parameters and their standard deviations: Top, Bottom, Hillslope and **EC_{50}**. Proceed with calculation of **EC_{10}** estimate according to the following formula with F=10:

$$TI\ EC_{10} = \left(\frac{F}{100 - F}\right)^{1/\,Hillslope} \times TI\ EC_{50}$$

6) Determine the **RPC_{max}**, which is the highest Relative Induction value (%) obtained for the test item, and its corresponding concentration **PC_{max}**.

7) Using Y_c data (i.e the average relative induction values), determine the concentrations **PC_{10}** and **PC_{50}** of the test item at which its Relative Induction is 10% and 50% (REF RPC_{10} or REF RPC_{50}).

The test item **PC** value can be calculated by interpolation between 2 points on the X-Y coordinate, one immediately above and the one immediately below the PC value. Where the data points lying immediately above and below the TI PC value have the coordinates a, b and c, d respectively, then the PC value may be calculated using the following equation:

$PC_{50} = d + (50-c)/(a-c) \times (b-d)$

$PC_{10} = d + (10-c)/(a-c) \times (b-d)$

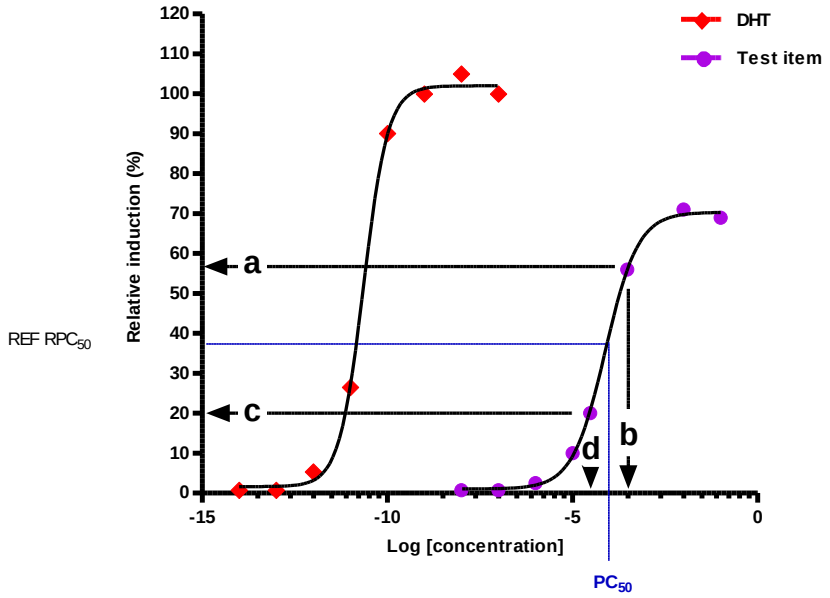


Figure 10: Interpolation of results by selecting the value above and below the point of interest (e.g. PC_{10} and PC_{50}) to be calculated.

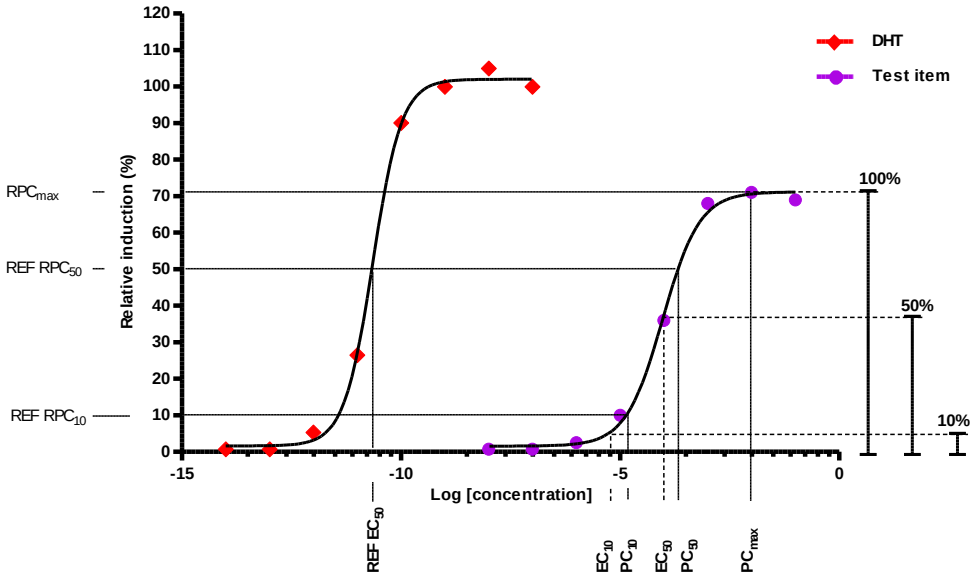


Figure 11: Overview of parameters determined in the agonist assay.

2.5.3 Assessment of antagonist properties of test items

1) Transform the test and reference item RLU values into **Relative Induction** (%) with the following calculation:

$$Y_{ic} \text{ or } S_{ic} = \frac{RLU \text{ of item } (i \text{ replicate}) - average \text{ RLU of } FLU_{C8}}{average \text{ RLU of SC } - average \text{ RLU of } FLU_{C8}} \times 100, \quad i = 1,2,3$$

$$Y_c = average\ Y_{ic}$$
$$S_c = average\ S_{ic}$$

where the average is taken over the triplicate samples (all from the same plate), *Item* is test or reference item and FLU_{C8} is Flutamide at concentration C8.

To distinguish the responses obtained by using two different concentrations of DHT (1 x EC_{50} concentration of DHT for standard analysis and 100 × EC_{50} concentration of DHT for specificity testing), the symbol Y_{ic} represents the relative induction at concentration *c* , replicate *i* when the EC_{50} concentration is used, the symbol S_{ic} represents the relative induction at concentration *c* , replicate *i* when the 100 × EC_{50} concentration is used. S_{ic} is the relative induction of specificity control and is only calculated with data from comprehensive testing.

2) Determine if the test item relative induction (Y_c) is less than or equal to 80% at any concentration (See Figure 12 A).

3) If none of the Y_c values are less than or equal to 80%, calculations 4 to 10 do not need to be performed. Proceed to the decision-making.

The following data analysis steps (4 and 5) can only be performed with data obtained in the specificity control experiments:

4) Calculate S_c^n as follows: normalise the relative induction of the specificity control S_c for all concentrations tested (C1 to C8) by setting the C1 concentration of the S_c at 100% i.e.:

$$S_c^n = 100 \times \frac{S_c}{S_{c_1}}, \qquad c = c_1, \ldots, c_8$$

Calculate the square of the correlation (R^2), where the sample correlation coefficient is-between the relative induction of the test item (Y_c) and the relative induction of its normalised specificity control (S_c^n) (See figure 12 B).

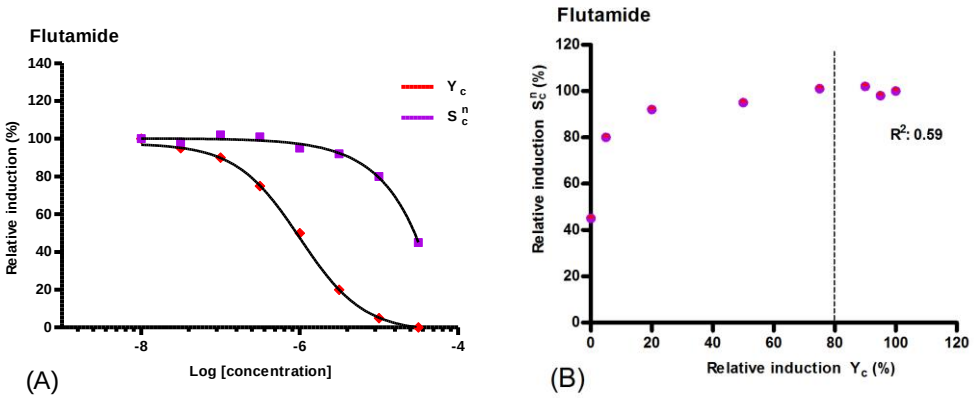


Figure 12: A: Dose response of a test item when tested in a standard experiment with calculation of Y_c values (shown in red) and in a specificity control experiment with calculation of S_c^n values (shown in purple), where the C1 of the specificity control is set to 100%. B: Plot of the response of Flutamide S_c^n (specificity control) versus Y_c (standard) with indication of R^2 value.

To get additional potency information proceed with calculations 6 to 9:

5) When one or more Y_c responses are less than or equal to 80%, fit the Hill curve model to the accepted relative induction data Y_{ic}. Use Graphpad Prism or other statistical software. The Hill curve model is a logistic regression model (variable slope, 4 parameters) with

$$y = Bottom + \frac{(Top - Bottom)}{\left(1+10^{((LogIC_{50}-x)*HillSlope)}\right)}$$

x =	Log of concentration
y =	Relative induction (%)
Top =	Maximum relative induction (%)
Bottom =	Minimum relative induction (%)
$LogIC_{50}$ =	Log of concentration at which 50% of maximum response is observed
HillSlope =	Slope factor of the Hill curve

Add the S_c^n values to the plot for visualisation of both Y_c and S_c responses.

6) When a proper hill curve fit can be obtained, the model provides estimates of the following parameters and their standard deviations: Top, Bottom, Hillslope and **IC_{50}**. Proceed with calculation of **IC_{20}** estimate according to the following formula with F=80:

$$TI\ IC_{20} = \left(\frac{F}{100-F}\right)^{1/Hillslope} \times TI\ IC_{50}$$

7) Determine the **RPC_{min}**, which is the lowest Relative Induction value (%) obtained for the test item, and its corresponding concentration **PC_{min}**.

8) Using Y_c data determine the concentration **PC_{80}** and **PC_{50}** of the test item at which its Relative Induction is 80% and 50% (also defined as REF RPC_{80} or REF RPC_{50}).

The antagonist **PC** values can be calculated by interpolation between 2 points on the X-Y coordinate, one immediately above and the one immediately below the PC value. Where the data points lying immediately above and below the PC value have the coordinates a, b and c, d respectively, then the PC_{80} and PC_{50} value may be calculated using the following equations:

$$PC_{50} = b + (a-50)/(a-c) \times (d-b)$$

$$PC_{80} = b + (a-80)/(a-c) \times (d-b)$$

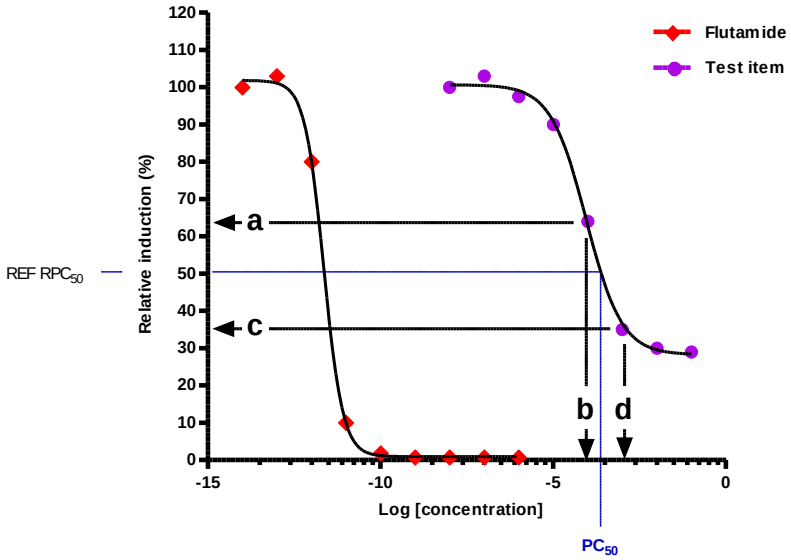


Figure 13: Interpolation of results by selecting the value above and below the point of interest (e.g. PC_{80} and PC_{50}) to be calculated.

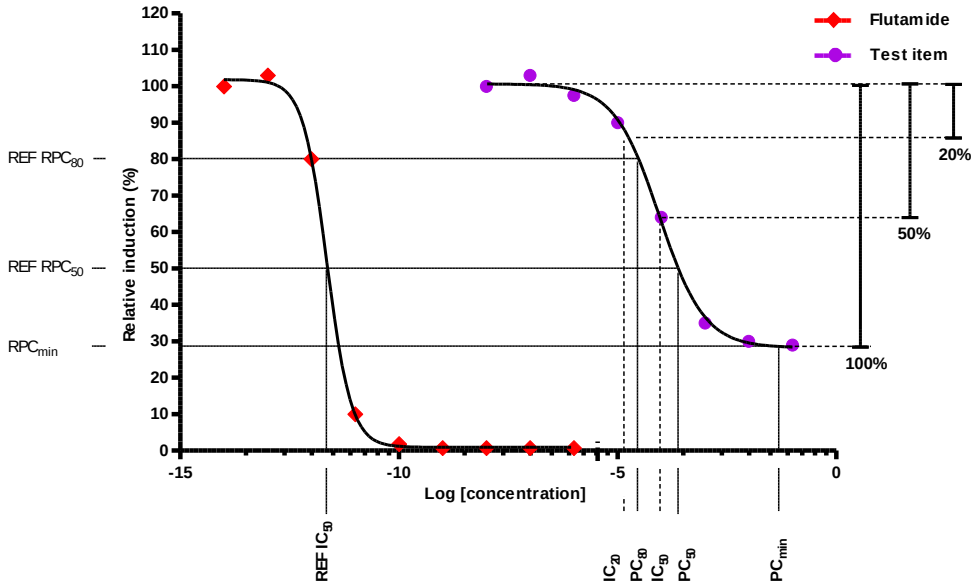


Figure 14: Overview of parameters determined in the antagonist assay.

2.5.4 Reporting of results

The following shall be reported for the test, reference and control items:

Samples	Agonism	Antagonism
Reference item	• EC_{50} (M) • PC_{50} (M) • PC_{10} (M) • CV of Log EC_{50} (%)	• IC_{50} (M) • PC_{50} (M) • PC_{20} (M) • CV of Log IC_{50} (%)
Positive control item	• Relative Induction 17β-methyltestosterone (%)	• Relative Induction linuron (%)
Negative control item	• Relative Induction corticosterone (%)	• Relative Induction levonorgestrel (%)
Test item	• RPC_{max} (%) • PC_{max} (M) • EC_{50} (M) • CV of Log EC_{50} (%) • EC_{10} (M) • PC_{50} (M) • PC_{10} (M) • Graph visualising relative induction (y-axes) at concentrations tested (x-axes) • Cytotoxicity observations by microscope and LDH leakage + concentration • Insolubility observations + concentrations	• RPC_{min} (%) • PC_{min} (M) • IC_{50} (M) • CV of Log IC_{50} (%) • IC_{20} (M) • PC_{50} (M) • PC_{80} (M) • Graph visualising relative induction (y-axes) at concentrations tested (x-axes) • Graph visualising correlation between Y_c and S_c^n (i.e. the relative inductions of normal

	• Final result (Positive or Negative)	response versus specificity control) and value for R^2 • Cytotoxicity observations by microscope and LDH leakage + concentration • Insolubility observations + concentrations • Final result (Positive or Negative)

Table 22: Parameters to be reported for test, reference and control items.

2.6 Decision criteria for agonist and antagonist properties

Agonism

For each run, a test item is considered

A. **Positive** when the relative induction (Y_c) of the test item is ≥ 10% (REF RPC_{10}) for two or more consecutive concentrations.

B. **Negative** in all other cases.

Antagonism

For each run, a test item is considered

A. **Positive** (competitive antagonist) when the relative induction (Y_c) of the test item is ≤ 80% (REF RPC80) for two or more consecutive concentrations and

   Either
   - The relative induction of the test items normalised specificity control s_c^n > 80% at all concentrations

   Or when the following two conditions are met:
   - The relative induction of the test items normalised specificity control at the highest concentration s_{c8}^n is ≤ 80%,
   - The square of the correlation coefficient (R^2) between Y_c and S_c^n is ≤ 0.9.

B. **Negative** in all other cases.

Explanation:

The result of the antagonist test is negative when the relative induction (Y_c) of the test item is > 80% (REF RPC80) at all concentrations or only one concentration is < 80%. The negative result also applies when the following 2 conditions are met:
- the relative induction (Y_c) of the test item is ≤ 80% (REF RPC80) for at least 2 consecutive concentrations and the relative induction of the test item's specificity control (S_c) at the highest tested concentration s_{c8}^n is ≤ 80%,
- the square of the correlation coefficient (R^2) between Y_c and S_c^n is > 0.9 (false positive)

3 REFERENCES

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4 APPENDICES

4.1 Appendix A: Calculations

The following calculations are performed by data analysis forms DAT02-ASY06, DAT04-ASY06 and DAT05-ASY06.

Calculations related to the acceptance criteria for agonism pre-screen and comprehensive test

Induction Factor: Calculate the induction factor (IF) for all microtiter plates tested, according to the following formula:

$$IF_{plate\ number} = \frac{average\ RLU_{plate\ number}\ [DHT\ C8]}{average\ RLU_{plate\ number}\ [SC]}$$

where for the highest DHT concentration C8, the average is taken over 3 replicate RLU values on plate 1 and over 6 replicates for all other plates; for the solvent control SC, the average is taken over 6 replicates[1] on all plates.

Z-factor: Calculate the Z-factor for all microtiter plates:

$$Z - factor_{plate\ number} = 1 - 3 * \frac{(stdev\ RLU_{plate\ number}\ [SC] + stdev\ RLU_{plate\ number}\ [C8\ DHT])}{abs(average\ RLU_{plate\ number}\ [SC] - average\ RLU_{plate\ number}\ [C8\ DHT])}$$

The averages and sample standard deviations (stdev) are constructed in the same way as described for the Induction Factor.

DHT EC_{50} value: For reference item DHT, estimates of the EC_{50} value and related coefficient of variation of the log(EC_{50}) are obtained from the fit of the Hill curve model to the responses, using the Y_{ic} values (i.e. the three replicate relative induction values)

$$y = Bottom + \frac{(Top - Bottom)}{\left(1 + 10^{((LogEC_{50} - x) * HillSlope)}\right)}$$

x =	Log of concentration
y =	Relative induction (%)
Top =	Maximum relative induction (%)
Bottom =	Minimum relative induction (%)
$LogEC_{50}$ =	Log of concentration at which 50% of maximum response is observed
HillSlope =	Slope factor of the Hill curve

The fit can be made by using statistical software (such as GraphPad Prism).

[1] The average is taken over responses of the two different test item solvent controls. This is only allowed when the solvents of test and reference items are the same. When using DMSO and water as solvents, the work solutions are always supplemented with appropriate DMSO concentrations.

The estimated coefficient of variation of $\log EC_{50}$ is then obtained via formula:

$$CV\ of\ \log EC_{50} = abs\left(\frac{stdev\ \log EC_{50}}{\log EC_{50}}\right)$$

Relative Induction of positive and negative control item: Calculate the relative induction of positive and negative control items with respect to the reference item DHT on plate 1.

$$relative\ induction\ of\ the\ PC\ or\ NC = \frac{average\ \ RLU\ \ [PC\ or\ NC] - average\ \ \ RLU\ \ [SC]}{average\ \ RLU\ \ [DHT\ C8] - average\ \ \ RLU\ \ [SC]} \times 100$$

Calculations related to the acceptance criteria for antagonism pre-screen and comprehensive test

Inhibition Factor: Calculate the inhibition factor (InhF) for every microtiter plate tested, according to the following formula:

$$InhF_{plate\ number} = \frac{average\ RLU_{plate\ number}[SC]}{average\ RLU_{plate\ number}[Flutamide\ C8]}$$

where for the highest Flutamide concentration C8 and it's solvent control, the average is taken over 3 replicate RLU values on plate 1 and over 6 replicates[2] on all other plates.

Z-factor: Calculate the Z-factor for all microtiter plates:

$$Z - factor_{plate\ number} = 1 - 3 * \frac{(std\ RLU_{\ plate\ number}\ [SC] + std\ RLU_{\ plate\ number}[C8\ Flutamide])}{abs(average\ RLU_{\ plate\ number}\ [SC] - average\ RLU_{\ plate\ number}[C8\ Flutamide])}$$

The averages and sample standard deviations (stdev) are constructed in the same way as described for the Inhibition Factor.

Flutamide IC_{50}: For reference item Flutamide, estimates of the IC_{50} value and related coefficient of variation of the log(IC_{50}) are obtained from the fit of the Hill curve model to the responses, using the Y_{ic} values (i.e. the three replicate relative induction values)

$$y = Bottom + \frac{(Top - Bottom)}{\left(1 + 10^{((LogIC_{50} - x) * HillSlope)}\right)}$$

x =	Log of concentration
y =	Relative induction (%)
Top =	Maximum relative induction (%)
Bottom =	Minimum relative induction (%)

[2] The average is taken over responses of the two different test item solvent controls. This is only allowed when the solvents of test and reference items are the same. When using DMSO and water as solvents, the work solutions are always supplemented with appropriate DMSO concentrations.

$LogIC_{50}$ = Log of concentration at which 50% of maximum response is observed

HillSlope = Slope factor of the Hill curve

The fit can be made by using statistical software (such as GraphPad Prism).

The estimated coefficient of variation of $\log IC_{50}$ is then obtained via formula:

$$CV\ of\ \log IC_{50} = abs\left(\frac{stdev\ \log IC_{50}}{\log IC_{50}}\right)$$

Relative induction of positive and negative control item: Calculate the relative induction of positive and negative control items with respect to the reference item Flutamide.

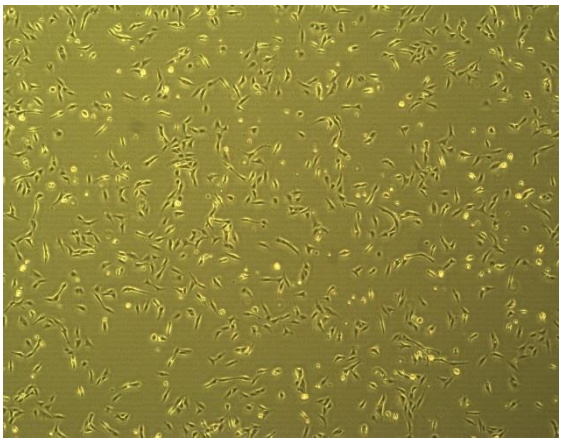
$$relative\ induction\ of\ the\ PC\ or\ NC = \frac{average\ \ RLU\ \ [PC\ or\ NC] - average\ \ RLU\ \ [Flutamide\ C8]}{average\ \ RLU\ \ [SC] - average\ \ RLU\ \ [Flutamide\ C8]} \times 100$$

Additional calculation related to the antagonism pre-screen and comprehensive test (no acceptance criterion)

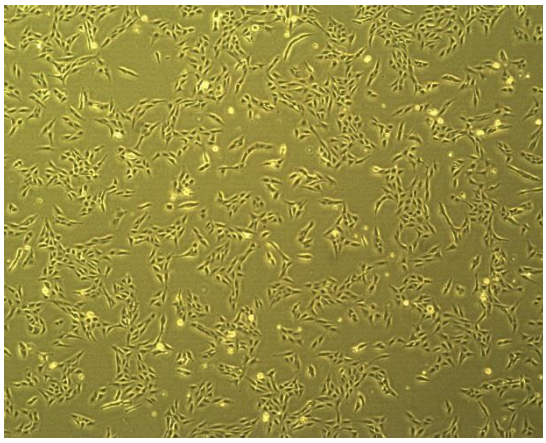
Relative induction of vehicle control: Calculate the relative induction of the vehicle control with respect to the solvent control.

$$relative\ induction\ of\ the\ VC = \frac{average\ \ RLU\ \ [VC] - average\ \ RLU\ \ [Flutamide\ C8]}{average\ \ RLU\ \ [SC] - average\ \ RLU\ \ [Flutamide\ C8]} \times 100$$

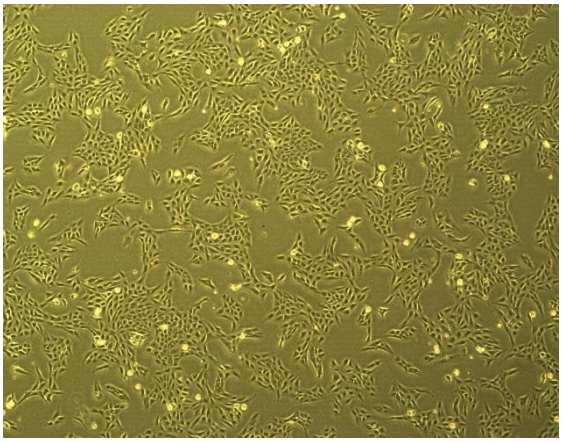
4.2 Appendix B: Scoring of confluency



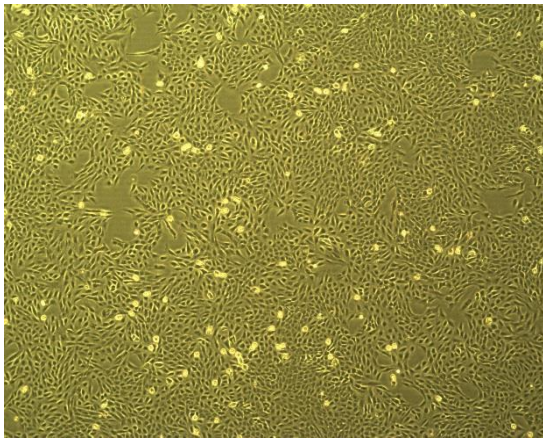
10-20 % confluency



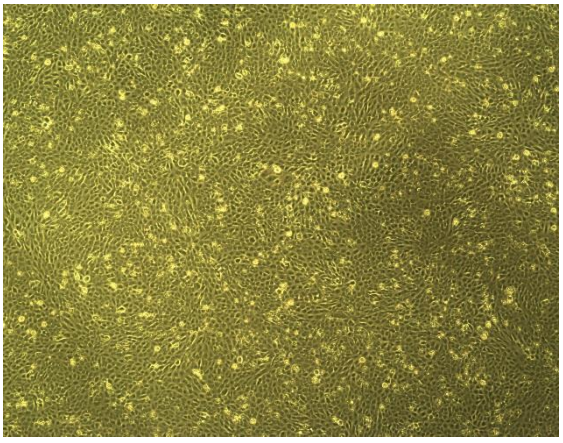
50-60 % confluency



70-80 % confluency



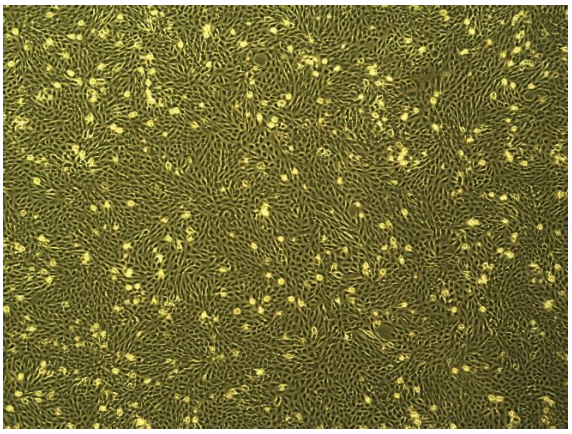
85-95 % confluency



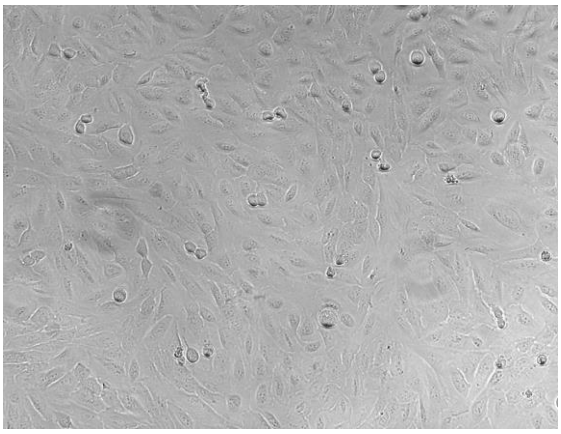
>100% (Overgrown)

4.3 Appendix C: Examples of cytotoxicity

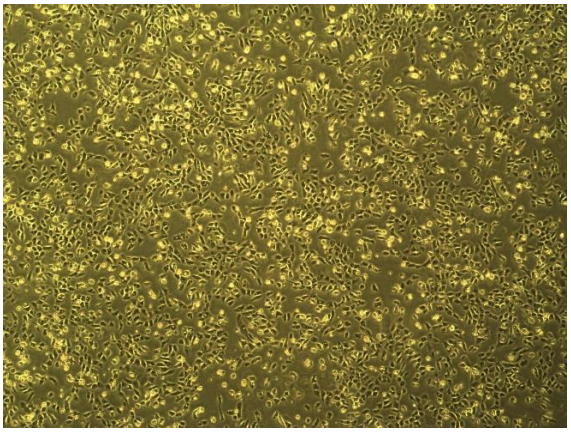
Visual inspection to detect morphological change as a measure of cytotoxicity, 24 hours after treatment.



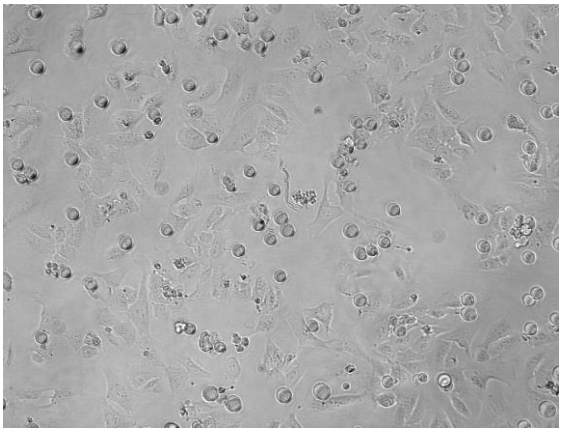
> 95% confluency in **solvent control**. Cells are densely packed and start to overgrow.



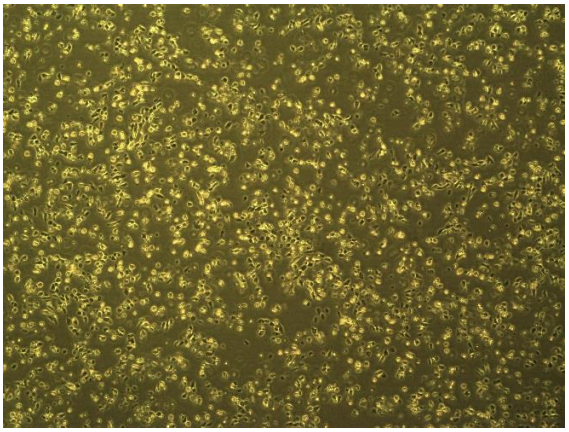
> 95% confluency in **solvent control**. Cells are densely packed and start to overgrow.



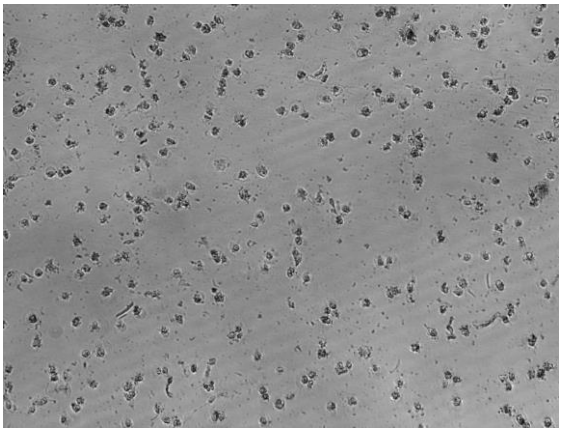
Cells become detached and contact between cells decreases. Cells are rounded. Result: 'cytotoxicity'



Cells become detached and contact between cells decreases. Cells are rounded. Result: “cytotoxicity'



Cells are fully detached and contact between cells is broken. Cells are rounded. Result: “cytotoxicity”



Cells are fully detached and contact between cells is broken. Cells are rounded. Result: “cytotoxicity”

4.4 Appendix D: Preparation of illuminate mix

Illuminate mix solution is added to the cells to measure the luciferase activity. It is added to each well immediately before the measurement of luminescence.
Illuminate mix contains the ingredients given in Table 1.

Compound	Weight[a] (g)	Molecular weight[b] (g/mol)	Molarity
Tricine	3.580	179.2	20.0 mM
$(MgCO_3)4Mg(OH)2.5H_2O$	0.520	485.69	1.07 mM
$MgSO_4 \times 7\ H_2O$	0.658	246.48	2.67 mM
EDTA	0.037	372.23	0.10 mM
DTT	0.231	154.2	1.50 mM
D-Luciferin	0.151	280.3	539 µM
ATP	3.026	551.1	5.49 mM

Table 1: Composition of illuminate-mix

[a]: The amount of compound needed for 1 l illuminate-mix is given in the second column. One litre of illuminate-mix is sufficient for approximately 150 microtiter plates.

[b]: Check whether the molecular weight of the compounds used corresponds to the molecular weight given in the table above. If the molecular weight does not correspond, adjust the amount of compound needed based on the molarity given in the fourth column.

- Add 500 ml demineralised (demi) water to a one litre glass beaker.
- Add tricine and magnesiumhydroxidecarbonate pentahydrate, according to Table 1, and stir with a magnetic stirrer until the solution is clear (this will take approximately one hour).
- Take D-luciferin from the freezer and adjust to room temperature.
- Add magnesiumsulphate-heptahydrate, EDTA, DTT and D-luciferin, according to Table 1. From this point on preparing the illuminate-mix should be performed in the dark and may last no longer than half an hour due to the instability of the compounds used.
- Add ATP according to Table 1.
- Add 400 ml demineralized water and adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.
- Adjust the final volume to 1 L with demi water and mix carefully.
- Divide the illuminate mix into 100 ml portions in HDPE bottles.
- Close and label the bottles indicating content, date of preparation and expiration date. Store at -20°C for a maximum of 3 months or at -80°C for one year.
- Check the activity of the flash-mix with luciferase standards.

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