XENOMETRIX

Swiss Commitment for Bioassays

XenoScreen XL YES/YAS

Accelerated high-sensitivity microplate assay for the detection of compounds with estrogenic and androgenic agonistic and antagonistic activities

using *Saccharomyces cerevisiae* strains with human estrogen (hERα) and androgen (hAR) receptors

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Instructions for use

For 4 x 96 data points

Art. No. N04-233-Y

Upon receipt of your XenoScreen XL YES/YAS Assay kit, **make sure that all reagents are stored appropriately (see pg. 4 for storage instructions)**. If components are damaged or if any problems occur, please contact Xenometrix by phone: ++41-61-482-14-34; fax: ++41-61-482-20-72, or e-mail: info@xenometrix.ch

Changelog

Date	New version	Changes
17.3.2015	3.01	• 4 T25 flask instead of 2
8.10.2015	3.02	• New order number for Growth Medium
30.5.2016	3.03	• Suggestion to prepare own YES **and** YAS stocks added
20.6.2016	3.04	• Added Art. Nr. of kit version without strains
5.12.2016	3.05	• Removed strains from “Kit contents”; yeast cells have to be purchased separately
16.5.2017	3.06	• Modified freezing procedure for cells in Appendix A
22.5.2017	3.07	• Minor wording optimizations
16.10.2017	3.08	• Minor wording optimizations; modified “Preparation and Dilution of Yeast Cells”, mentioning the Certificates of Analysis
25.05.2018	3.09	• Modified “Preparation and Dilution of Yeast Cells” to take into account the Certificate of Analysis

XenoScreen XL YES/YAS Assay Kit
Table of Contents

Number of Data Points

This test provides a total of 4 x 96 data points (2×96 YES and 2×96 YAS).
We recommend to run test samples in duplicates in 8 concentrations in order to obtain dose-response curves for the calculation of estrogen and androgen agonist and antagonist activities.

The assay description in this manual is based on such a complete analysis which allows to test 4 samples for estrogenic, anti-estrogenic, androgenic and anti-androgenic activities with all necessary controls, the calculation of EEQ and AEQ and the determination of the Limit of Detection LoD and the Limit of Quantification LoQ.

The free Excel calculation workbook available from Xenometrix is based on the plate layout described in this manual.

Principle of the Test

The common Baker's or Brewer's yeast (*Saccharomyces cerevisiae*) was genetically modified to identify compounds that can interact with the human estrogen and androgen receptors hERα and hAR. For this purpose the DNA sequences of hERα or hAR were stably integrated into the main chromosome of yeast cells. Additionally, the cells also contain an expression plasmid carrying the reporter gene lacZ encoding the enzyme β-galactosidase and estrogen (YES) or androgen (YAS) responsive elements (Routledge, E.J. and Sumpter, J.P. 1996. Environ. Toxicol. Chem. 13; 241-248; Sohoni, P. and Sumpter, J.P. 1998. Endocrinol. 158, 327-339)

Upon binding of a ligand, the hERα and hAR interact with the corresponding response elements on the expression plasmid and modulate the transcription of the lacZ reporter gene. The β-galactosidase is secreted into the medium and converts the yellow substrate chlorophenol red-β-D-galactopyranoside (CPRG) into red product which can be quantified colorimetrically at 570 nm. The measured OD_{570} correlates directly with the amount of secreted β-galactosidase and thus with the activity of the test substance which binds to the corresponding receptor.

The XenoScreen XL YES/YAS assay system can identify both activating (agonistic) and inhibitory (antagonistic) activities of test compounds. For the determination of antagonist activities, the samples are incubated in the presence of a fixed concentration of a reference agonist (17-β estradiol for YES and 5α-dihydrotestosterone for YAS). Inhibition of the response relative to this fixed agonist concentration is a sign of antagonist activity.

The assay can be used for either water samples or for samples dissolved in a solvent like DMSO. Samples dissolved in a solvent have to be diluted 100x in the assay in order to have acceptable levels of solvent.

The XenoScreen XL YES/YAS uses lyticase and a detergent (=LYES and LYAS) to facilitate the secretion of the intracellularly synthesized β-galactosidase (Schultis T. and Metzger J.W., 2004. Chemosphere 57, 1649-1655). This allows to reduce the incubation time from 48 hrs in the standard YES/YAS assay to 18 hours. In addition the accelerated protocol leads also to enhanced sensitivities for estrogenic and androgenic compounds.

Assay Description

Growing yeast cells stably transformed with either hERα (YES) or hAR (YAS) and a β-galactosidase reporter system are exposed to serial dilutions of test compound, positive control chemicals (17-β estradiol for YES and 5α-dihydrotestosterone for YAS) and a combination of a fixed concentration of positive control chemical and serial dilutions of the test compound. The cells are incubated for 18 hrs at 31°C. The induced cells are lysed in the presence of the yellow substrate CPRG which turns purple in the presence of β-galactosidase. Yeast cell growth is assessed prior to addition of the lysis buffer at an OD_{690}. The color development is measured at 570 nm and is corrected for diffraction by cells and debris by a simultaneous measurement of OD_{690}. The results are evaluated for estrogenic and androgenic agonistic and antagonistic activities, as well as for yeast growth inhibition or cytotoxicity.

Safety Precautions

- The YES and YAS yeast cells are genetically modified organisms (GMO). Please consult with your institutional and regulatory authorities for the requirements for handling, storage and disposal of such organisms in accordance with directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms (replaces Council Directive 90/219/EEC of 23 April 1990).
- The control chemicals provided in this kit are hormonally active substances. Please consult the Material and Safety Data Sheets (MSDS) for information on handling, disposal and personal protection.
- Not for use in humans and animals. For research purposes only.
- Do not drink, eat, smoke, or apply cosmetics in designated work areas. Wear laboratory coats and gloves when handling specimens and kit reagents. Wash hands thoroughly afterwards. Do not pipette by mouth.

Warnings

» Please observe all highlighted warnings and hints in the text! «

- **Due to the high sensitivity of the XenoScreen XL YES/YAS assay all containers and pipettes coming into contact with the cells or reagents must be absolutely clean and devoid of any residual chemicals such as detergents.**
- **When reusable items are used they should be thoroughly rinsed with distilled water and ethanol (without any additives). We highly recommend to wear gloves also for the handling of glassware and plasticware.**
- **All solvents should be of the highest available purity grade without any additives.**
- **Read the whole Instructions for Use before starting the assay!**

Kit Components

Each XenoScreen XL YES/YAS assay kit contains media and reagents for the analysis of 4 test compounds for agonistic and antagonistic estrogenic (YES) and androgenic (YAS) endocrine activity.

Use your own aliquots of YES and YAS yeast cells or order them separatley: XenoScreen YES Strain, 1 vial, Art. No. N05-230-E; XenoScreen YAS Strain, 1 vial, Art. No. N05-230-A.

The compounds are tested in 8 dilutions. Each assay has its own positive and negative controls.

Alternative plate layouts, dilution schemes or replicate numbers are possible, but are not described in this manual and are not supported by the Excel calculation sheet provided by Xenometrix.

Kit contents:

- Basal medium
- Vitamin solution
- L-aspartic acid solution
- L-threonine solution
- Cu-sulfate solution (500 µl)
- 10x DO medium
- 10x SD medium
- Streptomycin/Ampicillin solution
- DMSO
- Substrate solution CPRG
- 1 vial with 17β-estradiol positive control (“E2”, red label; YES)
- 1 vial with 5α-dihydrotestosterone positive control (“DHT”, blue label; YAS)
- 1 vial with 4-hydroxytamoxifen control antagonist (“HT”, yellow label; YES)
- 1 vial with flutamide control antagonist (“FL”, green label; YAS)
- 1 vial with lyticase
- 1 vial with lacZ lysis buffer
- 1 vial with 2-mercaptoethanol
- 9 96-well plates (2x4 for the assay, 1 for measurement of cell densities)
- 4 gas-permeable plate sealers
- 4 T25 culture flasks with gas-permeable filter caps

To be ordered separately:

- XenoScreen YES Strain, 1 vial, Art. No. N05-230-E
- XenoScreen YAS Strain, 1 vial, Art. No. N05-230-A

Storage Conditions

Each Xenometrix XenoScreen XL YES/YAS kit is shipped at ambient temperature. Please contact Xenometrix if you received the kit later than 10 days after the shipment date indicated on the delivery note (phone: ++41-61-482-14-34; fax: ++41-61-482-20-72, or e-mail: info@xenometrix.ch).

The shipment contains the following components which should be stored **immediately upon arrival** as follows:

-20°C:

- Positive controls before reconstitution (4 vials, E2, DHT, 4-HT, FL)
- Lyticase
- Ampicillin/Streptomycin
- *If ordered separately: Yeast cells on filter discs when stored for more than 1 month*

2 - 8°C

- CPRG substrate solution
- Basal medium
- Vitamin solution
- L-threonine solution
- 2-mercaptoethanol
- 10x DO medium
- 10x SD medium
- Positive controls after reconstitution
- *If ordered separately: Yeast cells on filter discs when used within 1 month*

20 - 25°C (room temperature, liquids protected from light):

- L-aspartic acid solution
- Cu(II)-sulfate solution
- DMSO
- lacZ lysis buffer
- 96-well plates
- Culture flasks
- Plate sealers

Required Equipment and Consumables NOT Included with the Kit

- XenoScreen YES Strain, 1 vial, Art. No. N05-230-E
- XenoScreen YAS Strain, 1 vial, Art. No. N05-230-A
- Incubator (31°C) with rotating platform (orbital movement)
- High-humidity container (e.g. plastic box with a tight lid, and wet paper tissue)
- Microplate reader capable to read at 570 and 690 nm
- Adjustable micropipettes and sterile tips
  (needed volumes: 2, 20-200, 100-1000 µl)
- Adjustable 8-channel pipettes (needed volumes: 20 - 200 µl)
- Serological pipettes (sterile)
- Pipetting reservoirs (sterile)
- Gloves
- Freezing tubes for the establishment of your own yeast stocks
- Glycerol for freezing cells

Recommended:

- Inverted microscope to inspect yeast cultures

Yeast Culture Preparation

It is highly recommended that you prepare your own stock of YES and YAS cells. This ensures a better uniformity and reproducibility of growth and activity characteristics when starting an assay. Please refer to Appendix A for details.

A) With cells on filter discs from Xenometrix (to be ordered separately; YES Strain, Art. No. N05-230-E, YAS Strain, Art. No. N05-230-A)
The start of the culture from the provided filter discs can take several days. It is recommended to start the cultures at least a week before the planned assay date. Once dense cultures of both yeast strains are obtained they can be diluted 1:10 1 day prior to the start of the assay, or stored for several days at 4°C. They will resume growth when diluted in fresh medium and incubated at 31°C with agitation.

For maximum sensitivity it is important that the cells for the YES/YAS assay are taken from a growing culture at 31°C and not directly from a culture kept at 4°C or from a dense culture. Since the assay can be conveniently started in the afternoon we recommend to dilute dense overnight cultures in fresh pre-warmed growth medium 1:4 in the morning and incubate at 31°C for about 6–7 hrs. until the start of the assay. Cultures kept at 4°C should be diluted 1:10 in fresh medium on the evening before the assay and incubated overnight at 31°C; the next morning dilute the dense culture as described above in pre-warmed medium.

If you want to use fresh flasks for sub-culturing please note that the caps should be closed loosely or be equipped with an air permeable filter. Yeast cells need air for optimal growth.

Using sterile technique, prepare a culture of the yeast strains by performing the following steps:

- Prepare growth medium: Add the complete contents of the vials with the vitamin solution, L-threonine solution, L-aspartic acid solution, and add 300 µl of the Cu(II) sulfate solution to the flask with the basal medium. Mix. Keep the remaining Cu(II) sulfate solution for the preparation of the test medium.
  Note: Growth medium should be used within 2 weeks when stored at 4°C. Up to 6 months storage is possible at -20°C.

- Label 2 T25 tissue culture flasks “YES” and “YAS”. Add 5 ml of growth medium to each of the 2 flasks.

- Add the filter discs from the vials labeled “YES” and “YAS” to the corresponding T25 flask. Use sterile forceps if necessary to remove the disks from the vials.

- Tighten the vented filter caps

- Place the flasks on their wide surface (**not** the upright position!) and incubate the yeast cells on an orbital shaker set at 31°C for 2-4 days until clearly turbid. Adjust the orbital frequency such that the liquid is well agitated but is not spilling into the neck of the flask. This ensures an adequate supply of oxygen to support good growth of the yeast cells.

B) With your own aliquots of YES and YAS cells
- Prepare medium and label flasks as above

- Grow your assay cells to a dense culture as per your own experience. Generally, healthy frozen cells grow to dense cultures within 1-2 days. Dilute in fresh medium for the assay as described above.

Plate Layout

Schematic representation of the YES agonist assay plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	E2 $2.1x10^{-12}$	E2 $2.1x10^{-12}$	SP1 Dil 8	SP1 Dil 8	SP2 Dil 8	SP2 Dil 8	SP3 Dil 8	SP3 Dil 8	SP4 Dil 8	SP4 Dil 8	Solv. Control	Solv. Control
B	E2 $6.7x10^{-12}$	E2 $6.7x10^{-12}$	SP1 Dil 7	SP1 Dil 7	SP2 Dil 7	SP2 Dil 7	SP3 Dil 7	SP3 Dil 7	SP4 Dil 7	SP4 Dil 7	Solv. Control	Solv. Control
C	E2 $2.1x10^{-11}$	E2 $2.1x10^{-11}$	SP1 Dil 6	SP1 Dil 6	SP2 Dil 6	SP2 Dil 6	SP3 Dil 6	SP3 Dil 6	SP4 Dil 6	SP4 Dil 6	Solv. Control	Solv. Control
D	E2 $6.7x10^{-11}$	E2 $6.7x10^{-11}$	SP1 Dil 5	SP1 Dil 5	SP2 Dil 5	SP2 Dil 5	SP3 Dil 5	SP3 Dil 5	SP4 Dil 5	SP4 Dil 5	Solv. Control	Solv. Control
E	E2 $2.1x10^{-10}$	E2 $2.1x10^{-10}$	SP1 Dil 4	SP1 Dil 4	SP2 Dil 4	SP2 Dil 4	SP3 Dil 4	SP3 Dil 4	SP4 Dil 4	SP4 Dil 4	Solv. Control	Solv. Control
F	E2 $6.7x10^{-10}$	E2 $6.7x10^{-10}$	SP1 Dil 3	SP1 Dil 3	SP2 Dil 3	SP2 Dil 3	SP3 Dil 3	SP3 Dil 3	SP4 Dil 3	SP4 Dil 3	Solv. Control	Solv. Control
G	E2 $2.1x10^{-9}$	E2 $2.1x10^{-9}$	SP1 Dil 2	SP1 Dil 2	SP2 Dil 2	SP2 Dil 2	SP3 Dil 2	SP3 Dil 2	SP4 Dil 2	SP4 Dil 2	Solv. Control	Solv. Control
H	E2 $6.7x10^{-9}$	E2 $6.7x10^{-9}$	SP1 Dil 1	SP1 Dil 1	SP2 Dil 1	SP2 Dil 1	SP3 Dil 1	SP3 Dil 1	SP4 Dil 1	SP4 Dil 1	Solv. Control	Solv. Control
	E2	E2	SP1	SP1	SP2	SP2	SP3	SP3	SP4	SP4	SC	SC

E2 = 17β-estradiol SP1…4 = Samples1–4 SC = Solvent control

Schematic representation of the YES antagonist assay plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	HT $2.1x10^{-9}$	HT $2.1x10^{-9}$	SP1 Dil 8	SP1 Dil 8	SP2 Dil 8	SP2 Dil 8	SP3 Dil 8	SP3 Dil 8	SP4 Dil 8	SP4 Dil 8	Solv. Control	Solv. Control
B	HT $6.7x10^{-9}$	HT $6.7x10^{-9}$	SP1 Dil 7	SP1 Dil 7	SP2 Dil 7	SP2 Dil 7	SP3 Dil 7	SP3 Dil 7	SP4 Dil 7	SP4 Dil 7	Solv. Control	Solv. Control
C	HT $2.1x10^{-8}$	HT $2.1x10^{-8}$	SP1 Dil 6	SP1 Dil 6	SP2 Dil 6	SP2 Dil 6	SP3 Dil 6	SP3 Dil 6	SP4 Dil 6	SP4 Dil 6	Solv. Control	Solv. Control
D	HT $6.7x10^{-8}$	HT $6.7x10^{-8}$	SP1 Dil 5	SP1 Dil 5	SP2 Dil 5	SP2 Dil 5	SP3 Dil 5	SP3 Dil 5	SP4 Dil 5	SP4 Dil 5	Solv. Control	Solv. Control
E	HT $2.1x10^{-7}$	HT $2.1x10^{-7}$	SP1 Dil 4	SP1 Dil 4	SP2 Dil 4	SP2 Dil 4	SP3 Dil 4	SP3 Dil 4	SP4 Dil 4	SP4 Dil 4	Solv. Control	Solv. Control
F	HT $6.7x10^{-7}$	HT $6.7x10^{-7}$	SP1 Dil 3	SP1 Dil 3	SP2 Dil 3	SP2 Dil 3	SP3 Dil 3	SP3 Dil 3	SP4 Dil 3	SP4 Dil 3	Solv. Control	Solv. Control
G	HT $2.1x10^{-6}$	HT $2.1x10^{-6}$	SP1 Dil 2	SP1 Dil 2	SP2 Dil 2	SP2 Dil 2	SP3 Dil 2	SP3 Dil 2	SP4 Dil 2	SP4 Dil 2	Solv. Control	Solv. Control
H	HT $6.7x10^{-6}$	HT $6.7x10^{-6}$	SP1 Dil 1	SP1 Dil 1	SP2 Dil 1	SP2 Dil 1	SP3 Dil 1	SP3 Dil 1	SP4 Dil 1	SP4 Dil 1	Solv. Control	Solv. Control
	HT	HT	SP1	SP1	SP2	SP2	SP3	SP3	SP4	SP4	SC	SC

E2 at 3.3×10^{-10} M present throughout the plate

HT = 4-hydroxytamoxifen SP1…4 = Samples1–4
SC = Solvent control

Schematic representation of the <u>YAS agonist</u> assay plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	DHT 2.1×10^{-11}	DHT 2.1×10^{-11}	SP1 Dil 8	SP1 Dil 8	SP2 Dil 8	SP2 Dil 8	SP3 Dil 8	SP3 Dil 8	SP4 Dil 8	SP4 Dil 8	Solv. Control	Solv. Control
B	DHT 6.7×10^{-11}	DHT 6.7×10^{-11}	SP1 Dil 7	SP1 Dil 7	SP2 Dil 7	SP2 Dil 7	SP3 Dil 7	SP3 Dil 7	SP4 Dil 7	SP4 Dil 7	Solv. Control	Solv. Control
C	DHT 2.1×10^{-10}	DHT 2.1×10^{-10}	SP1 Dil 6	SP1 Dil 6	SP2 Dil 6	SP2 Dil 6	SP3 Dil 6	SP3 Dil 6	SP4 Dil 6	SP4 Dil 6	Solv. Control	Solv. Control
D	DHT 6.7×10^{-10}	DHT 6.7×10^{-10}	SP1 Dil 5	SP1 Dil 5	SP2 Dil 5	SP2 Dil 5	SP3 Dil 5	SP3 Dil 5	SP4 Dil 5	SP4 Dil 5	Solv. Control	Solv. Control
E	DHT 2.1×10^{-9}	DHT 2.1×10^{-9}	SP1 Dil 4	SP1 Dil 4	SP2 Dil 4	SP2 Dil 4	SP3 Dil 4	SP3 Dil 4	SP4 Dil 4	SP4 Dil 4	Solv. Control	Solv. Control
F	DHT 6.7×10^{-9}	DHT 6.7×10^{-9}	SP1 Dil 3	SP1 Dil 3	SP2 Dil 3	SP2 Dil 3	SP3 Dil 3	SP3 Dil 3	SP4 Dil 3	SP4 Dil 3	Solv. Control	Solv. Control
G	DHT 2.1×10^{-8}	DHT 2.1×10^{-8}	SP1 Dil 2	SP1 Dil 2	SP2 Dil 2	SP2 Dil 2	SP3 Dil 2	SP3 Dil 2	SP4 Dil 2	SP4 Dil 2	Solv. Control	Solv. Control
H	DHT 6.7×10^{-8}	DHT 6.7×10^{-8}	SP1 Dil 1	SP1 Dil 1	SP2 Dil 1	SP2 Dil 1	SP3 Dil 1	SP3 Dil 1	SP4 Dil 1	SP4 Dil 1	Solv. Control	Solv. Control
	DHT	DHT	SP1	SP1	SP2	SP2	SP3	SP3	SP4	SP4	SC	SC

DHT = 5α-dihydrotestosterone SP1…4 = Samples1–4 SC = Solvent Control

Schematic representation of the <u>YAS antagonist</u> assay plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	FL 2.1×10^{-8}	FL 2.1×10^{-8}	SP1 Dil 8	SP1 Dil 8	SP2 Dil 8	SP2 Dil 8	SP3 Dil 8	SP3 Dil 8	SP4 Dil 8	SP4 Dil 8	Solv. Control	Solv. Control
B	FL 6.7×10^{-8}	FL 6.7×10^{-8}	SP1 Dil 7	SP1 Dil 7	SP2 Dil 7	SP2 Dil 7	SP3 Dil 7	SP3 Dil 7	SP4 Dil 7	SP4 Dil 7	Solv. Control	Solv. Control
C	FL 2.1×10^{-7}	FL 2.1×10^{-7}	SP1 Dil 6	SP1 Dil 6	SP2 Dil 6	SP2 Dil 6	SP3 Dil 6	SP3 Dil 6	SP4 Dil 6	SP4 Dil 6	Solv. Control	Solv. Control
D	FL 6.7×10^{-7}	FL 6.7×10^{-7}	SP1 Dil 5	SP1 Dil 5	SP2 Dil 5	SP2 Dil 5	SP3 Dil 5	SP3 Dil 5	SP4 Dil 5	SP4 Dil 5	Solv. Control	Solv. Control
E	FL 2.1×10^{-6}	FL 2.1×10^{-6}	SP1 Dil 4	SP1 Dil 4	SP2 Dil 4	SP2 Dil 4	SP3 Dil 4	SP3 Dil 4	SP4 Dil 4	SP4 Dil 4	Solv. Control	Solv. Control
F	FL 6.7×10^{-6}	FL 6.7×10^{-6}	SP1 Dil 3	SP1 Dil 3	SP2 Dil 3	SP2 Dil 3	SP3 Dil 3	SP3 Dil 3	SP4 Dil 3	SP4 Dil 3	Solv. Control	Solv. Control
G	FL 2.1×10^{-5}	FL 2.1×10^{-5}	SP1 Dil 2	SP1 Dil 2	SP2 Dil 2	SP2 Dil 2	SP3 Dil 2	SP3 Dil 2	SP4 Dil 2	SP4 Dil 2	Solv. Control	Solv. Control
H	FL 6.7×10^{-5}	FL 6.7×10^{-5}	SP1 Dil 1	SP1 Dil 1	SP2 Dil 1	SP2 Dil 1	SP3 Dil 1	SP3 Dil 1	SP4 Dil 1	SP4 Dil 1	Solv. Control	Solv. Control
	FL	FL	SP1	SP1	SP2	SP2	SP3	SP3	SP4	SP4	SC	SC

DHT at 3.3×10^{-9} M present throughout the plate

FL = flutamide SC = Solvent control

SP1…4 = Samples1–4

Sample and Controls Preparation

We recommend to follow the instructions below precisely and to adhere to the proposed pipetting sequence.

Inspect the yeast culture by microscope. If clumps of cells are present use a serological pipette to disperse the yeast cells.

The yeast cultures should be clearly turbid. If in doubt measure the OD_{690} of 200 µl of the yeast culture in a flat bottom 96-well plate. This value should be at least 0.2. Do not proceed unless this criterion is met. If necessary continue the incubation of the yeast cultures.

All manipulations should be done under sterile conditions.

- Prepare the agonist and antagonist controls by adding

  100 µl DMSO to the vial "17β-estradiol" ("E2", red label)
  100 µl DMSO to the vial "5α-dihydrotestosterone" ("DHT", blue label)
  100 µl DMSO to the vial "4-hydroxytamoxifen" ("HT", yellow label)
  100 µl DMSO to the vial "flutamide" (FL", green label)

- Mix all 4 vials thoroughly for ~30 seconds.
  This gives a 1×10^{-6} M stock solution of E2, a 1×10^{-5} M stock solutions of DHT, a 1×10^{-3} M stock solution of HT, and a 1×10^{-2} M stock solution of FL.
- Dissolve non-aqueous test sample(s) in DMSO (preferred), or a different solvent *. Use the highest soluble concentration, or prepare a 10^{-2} M solution. Mix well.
- Unwrap 4 96-well plates.

Label the 96-well plates with "YES Agonist", "YES Antagonist", "YAS Agonist", and "YAS Antagonist" and the date (label the plate and the lid!).

*We strongly suggest to use DMSO as a solvent whenever possible. Other solvents can be used as long as they are not toxic to yeast cells at 1%. Volatile solvents are more difficult to handle in small volumes.

The volumes in the following steps are calculated for half-logarithmic dilution steps. You can adapt the volumes for other dilution schemes, but the final volume of sample dilutions in water should always be 80 µl. The procedure differs for aqueous (water) samples and for samples dissolved in a solvent.

For water samples

- Prepare 40 ml of sterile water with 1% (400 µl) DMSO
- Using a multichannel pipette with 7 tips, transfer 80 µl of 1% DMSO to rows A-G of all plates.
- Add DMSO to your water test samples to obtain a 1% solution. This is to ensure equal solvent concentrations in all samples and controls.
- Add 117 µl of your test samples to row H, column 3+4, 5+6, 7+8, 9+10 (max. 4 samples per plate, duplicates) on all 4 plates.
- Add 80 µl of the 1% DMSO solution to row H, columns 11+12. Columns 11 and 12 are the solvent control.
- Add 200 µl sterile water (NOT 1% DMSO!) to row H, columns 1+2 for the positive controls

(200 µl are used such that 2 µl can be dispensed from the positive control stocks. Smaller volumes require special pipettes and may be less accurate).

- Add 2 µl of the 100× concentrated positive control stocks in DMSO to the wells H1 and H2 on the corresponding assay plates: E2 to the YES agonist plate, HT to the YES antagonist plate, DHT to the YAS agonist plate, and FL to the YAS antagonist plate.
- With a multichannel pipette transfer and mix 37 µl from rows H to G, G to F and so on. Do not transfer and mix columns 11+12 which contain only the solvent control. Remove and discard the last 37 µl from row A such that 80 µl are left in the wells. When using a 8-channel pipette you can do this dilution steps for up to 3 test samples in one run (columns 1-8). When testing 4 samples you need to perform an additional run with 2 tips on columns 9+10.
- Remove and discard 85 µl from wells H1 and H2 on all plates. Now all wells should contain 80 µl.

For concentrated samples dissolved in solvent (DMSO)

- Prepare 40 ml of sterile water with 1% (400 µl) DMSO.
- Using a multichannel pipette with 7 tips, transfer 80 µl of 1% DMSO to rows A-G of all plates.
- Add 80 µl of 1% DMSO to row H, columns 11+12. Columns 11 and 12 are the solvent control.
- Add 200 µl sterile water (NOT 1% DMSO!) to row H, columns 1-10.
- Add 2 µl of the 100× concentrated positive control stocks in DMSO to the wells H1 and H2 on the corresponding assay plates: E2 to the YES agonist plate, HT to the YES antagonist plate, DHT to the YAS agonist plate, and FL to the YAS antagonist plate.
- Add 2 µl of your test samples in DMSO to the corresponding wells in row H an all 4 assay plates (sample 1 in wells H3 and H4 and so on).
- With a multichannel pipette transfer and mix 37 µl from rows H to G, G to F and so on. Do not transfer and mix columns 11+12 which contain only the solvent control. Remove and discard the last 37 µl from row A such that 80 µl are left in the wells. When using a 8-channel pipette you can do this dilution steps for up to 3 test samples in one run (columns 1-8). When testing 4 samples you need to perform an additional run with 2 tips on columns 9+10.
- Remove and discard 85 µl from wells H1–H10 on all plates. Now all wells should contain 80 µl.

Preparation of Test Medium

Combine and mix: 6.25 ml 10× SD Medium + 6.25 ml 10× DO Medium + 7.5 ml water +100 µl Cu- Sulphate 20 mM + 532 µl Ampicillin/Streptomycin. Mix. The medium should have room temperature by the time the cells are added.

Preparation and Dilution of Yeast Cells

Dilute 100 µl of YES and YAS cells with 900 µl growth medium. In a 96-well plate add 300 µl of growth medium to 2 wells. Add 2 x 300 µl of the 1:10 diluted YES and YAS cells to 4 wells and measure the OD_{690}. Calculate the means of the measured duplicate values and subtract the growth medium value from the YES and YAS cells values = OD_{690} net.
Please refer to the Certificate of Analysis of the YES and YAS you are using to calculate the necessary volume of each cell type to be added to test medium.

Transfer of YES and YAS Yeast Cells to Assay Plates

- Add the calculated amount of the original YES culture to 5 ml of test medium, mix and distribute 40 µl to all wells of the YES agonist plate.
- Add the calculated amount of the original YAS culture to 5 ml of test medium, mix and distribute 40 µl to all wells of the YAS agonist plate.
- Add 5 µl of the 1 x 10^{-6} M stock of E2 in DMSO (red label) to 5 ml of test medium, mix = YES Antagonist Test medium = 3.3 x 10^{-10} M final. Add the calculated amount of YES culture, mix and immediately distribute 40 µl to all wells of the YES antagonist plate.
- Add 5 µl of the 1 x 10^{-5} M stock of DHT in DMSO (blue label) to 5 ml of Test medium, mix = YAS Antagonist Test medium = 3.3 x 10^{-9} M final. Add the calculated amount of YAS culture, mix and immediately distribute 40 µl to all wells of the YAS antagonist plate.

Note 1:
Yeast cells settle quite rapidly. Mix the cell suspension frequently during the transfer to the assay plates.

Note 2:
The cells in the antagonist medium should be distributed to the assay plates with the test samples and controls as quickly as possible. Mix and distribute the cells as soon as they have been added to the antagonist test medium.

Note 3:
We recommend to avoid touching the liquid in the wells with the tips and to distribute the cells from right to left in order to minimize cross-contamination of test samples with the positive controls.

Incubation of the Assay Plates

- Seal the plates with a gas permeable foil. Transfer the plates to a plastic container with wet paper towels and close the lid. This ensures a humid atmosphere which prevents evaporation from the wells. Alternatively use a plastic bag (Ziploc or similar) with wet paper towels.
- Incubate for 18 hrs at 31°C with agitation (100 rpm).

Note:
The plates can also be incubated with a plate lid if no plate sealer is available. We have observed a slightly slower growth with lids instead of breathable sealers. Do not use standard plate sealers!
The plastic container should have a tightly closing lid to ensure a constantly humid atmosphere.

Note:
Inspect the cells after 18 hrs of growth with an inverted microscope (this can be done with the plate sealer still on). If the cells in the solvent control wells cover less than 50% of the surface you may continue the incubation of the corresponding plates for several hours to avoid a weak color development.

The YES and the YAS cells may be incubated and read independently of each other, but the agonist and antagonist plates of a given assay should be treated identically!

Addition of Lysis Buffer and Substrate

Note: The lacZ reaction mixture has to be prepared fresh for each experiment

- Prepare the lacZ reaction mixture (this can be done directly in a reservoir; mix carefully as the mixture develops foam bubbles easily):

    - ✓ 20 ml lacZ lysis buffer
    - ✓ 54.4 µl 2–ME
    - ✓ 800 µl CPRG substrate solution
    - ✓ 200 µl of lyticase solution

- Label 4 fresh 96-well plates “YES Agonist”, “YES Antagonist”, “YAS Agonist”, and “YAS Antagonist”.
- Add 50 µl of lacZ reaction mixture to each well of 4 fresh plates.
- Remove the 4 assay plates from the incubator and mix them either for 30-60 sec. on a Vortex mixer at 800 rpm, or - if this is not sufficient - mix the wells with a multichannel pipette. Avoid the formation of bubbles! The yeast cells should be evenly distributed in the wells. Bubbles can be removed with a gas flame.
- Read the plates at 690 nm. These data are used for the calculation of the Growth factor.
- Mix and transfer 30 µl to the freshly prepared 96 well plates. Transfer row-by-row, from right to left.
- Cover with lids
- Incubate for 30 minutes or 1 hr (see below) at 31°C with agitation (100 rpm).

Note:

The rapidity of the color development can vary between tests and between YES and YAS plates. The plates from the YES and YAS assays can be evaluated independently, but agonist and antagonist plates from the same assay (YES or YAS) should be read at the same time point!

If there is a rapid and strong purple color development in the positive control you may read the plates already after 30 minutes of incubation. An $OD_{570} \geq 1.5$ at the highest concentration of the E2 or DHT positive control after 30 minutes indicates that it is not necessary to incubate any further. If the OD_{570} is < 1.5 the plates should be incubated for another 30 minutes and read again after a total time of 1 hr.

Reading the Assay Plates

- Tilt and rotate the plates to distribute the color evenly.
- **Remove bubbles if present** (use a gas flame if necessary)
- Read OD_{570} and OD_{690}

Calculations and Data Evaluation

There are 3 options to evaluate your raw data:

1. With your own method using information provided below under A) and B)
2. Using the comprehensive Xenometrix Excel calculation workbook described under C)
3. Sending the plate reader raw data in an Excel-readable format, including sample dilution information by email to info@xenometrix.ch. We will evaluate your results free of charge and give a short statement towards the validity of the assay based on the positive and negative controls as well as the activities of the tested samples. **This service is free of charge!**

Please note that as an additional option you can also send us your samples and we perform the whole assay for you. Please ask for a quotation!

The XenoScreen XL YES/YAS test kits allows to determine 4 test sample characteristics:

- Estrogenic agonist activity
- Estrogen antagonist activity
- Androgenic agonist activity
- Androgen antagonist activity

A) Definitions

Growth factor G: $G = \frac{A_{690,S}}{A_{690,N}}$

β-galactosidase activity (relative units) U_S: $U_S = \frac{A_{570,S}}{A_{690,S}}$

Induction Ratio I_R: $I_R = \frac{1}{G} \times \frac{A_{570,S}}{A_{570,N}}$

where $A_{690,S}$ is the absorbance of the sample S at 690 nm before lysis
$A_{690,N}$ is the absorbance of the solvent control at 690 nm before lysis

$A_{570,S}$ is the net absorbance of the sample S at 570 nm – 690 nm after lysis
$A_{570,N}$ is the net absorbance of the solvent control at 570 nm – 690 nm after lysis

Limit of Quantification LoQ: Mean of solvent control plus 9 Standard Deviations

Limit of Detection LoD: Mean of solvent control plus 3 Standard Deviations

B) Data evaluation

1. Calculate the delta OD_{570} – OD_{690} of all wells. Use the values read after the final 30 minutes or 1 hr incubation.
2. Calculate the mean values of the standards and samples.
3. Calculate Growth Factor G, β-galactosidase activity (relative units) U_S and Induction Ratio I_R based on the definitions given above.
4. Draw dose-response curves.
5. Inspect the curves and determine the activities of your samples.

Criteria:

Please note: these are suggested values. Other values may be more appropriate in certain circumstances.

I_R sample ≥ I_R10 indicates an agonistic effect (I_R10 is defined as the I_R which is 10% of (I_R max - I_R solvent) above the I_R solvent.

I_R sample ≤ I_R50 indicates a **possible** antagonistic effect (I_R50 is defined as 50% of (I_R control - I_R solvent), where the I_R control is at the corresponding fixed concentration of agonist used: 3.3×10^{-10} M E2 and 3.3×10^{-9} M DHT, respectively).

The evaluation of antagonistic effects may be complicated by non-specific inhibitory effects which reduce the response of the yeast cells by other mechanisms. Parallel inhibition in the YES and YAS assay may be indicative of such non-specific inhibition which is not a true estrogen or androgen antagonistic effect.

You may also want to calculate the estrogen equivalents EEQ of your samples. This corresponds to the concentration of 17β-estradiol (E2) which would give the same activity as your sample. It can be estimated from the horizontal displacement of the test sample dose-response curve relative to the standard curve and factoring in the different overall dilutions. Similarly, androgen equivalents AEQ can be determined.

Definitions: Enrichment factor EF = vol. water / vol. extract
Dilution factor DF = Sample volume added / total assay volume
Relative enrichment factor REF = EF x DF

Example:

You test a 1000× concentrated water sample (= EF) by adding 80 µl sample dilutions to 40 µl assay volume (= DF 0.67). This gives a starting REF of 667 in the assay. You determine the EC_{50} to be at a REF of 7. For the E2 standard in the same solvent you determine an EC_{50} of $1x10^{-10}$ M. This means your original water sample has an EEQ of $1.4x10^{-11}$ M.

C) Data evaluation using the Xenometrix YES/YAS Excel workbook

The workbook is based on the plate layout suggested in the Instructions for Use.

The workbook can be downloaded from our homepage and performs all necessary calculations, draws dose-response curves, calculates EC_{50}'s and IC_{50}'s, and determines EEQ's and AEQ's, LoD's and LoQ's with minimal user intervention.

The workbook is write protected. After entering and analyzing your data you should save the workbook using the "Save as…" command (F12) and save it under a new name.

After filling in the sample names and concentrations in the “Start here” sheet you can Copy-Paste the OD_{570} and OD_{690} raw data into the appropriate fields of the “Rawdata YES” and “Rawdata YAS” sheets. Note that there are 2 different readings for OD690: “OD690 after exposure” is the reading of the first set of plates after 18 hrs of incubation.

You can then inspect the graphs in the two “Diagrams” sheets and perform a few steps to calculate EC_{50}’s and IC_{50}’s and Estrogen and Androgen equivalents (EEQ’s and AEQ’s) in the “Calculation YES” and “Calculation YAS” sheets.

On these sheets, you only have to activate the curve fitting process by clicking on the “Solver” macro button in line 148. The Excel “Solver” function has to be active, perform the following steps:

1. Click the File tab, and then click Options.
2. Click Add-Ins, and then in the Manage box, select Excel Add-ins.
3. Click Go.
4. In the Add-Ins available box, select the Solver Add-in check box, and then click OK.
   Tip If Solver Add-in is not listed in the Add-Ins available box, click Browse to locate the add-in.
   If you get prompted that the Solver add-in is not currently installed on your computer, click Yes to install it.
5. After you load the Solver add-in, the Solver command is available in the Analysis group on the Data tab.

If the curve fitting does not produce a good fit, try first a second click on the “Solver” macro button, then try to change the slope s, the maximum IR and the log EC manually to more realistic values based on your actual data indicated by blue dots in the graph and then start the Solver again. Change these values in the tabs named “Solver_Calc_YES...” and “Solver_Calc_YAS...). Instructions are given in the yellow fields directly in the Calculation Sheet.

The automatic calculation routine tries to select only meaningful data points for the calculation of EEQ’s and AEQ’s. This means, only points between 10% and 90% (10% and 80% in the antagonist assays) of the maximum standard curve induction are considered. Points that yield lower (higher in antagonist assays) induction values than points at a lower concentration are also unlikely to give meaningful values (e.g. with bi-phasic dose-response curves), and are therefore also excluded from the calculation. From all qualifying data points the average (mean) is formed and used to determine the EEQ’s and AEQ’s.

The workbook also analyzes if a test compound reaches an Induction Ratio of $>=I_R10$ and highlights it in green. For antagonist activity the I_R is compared to I_R50 and is highlighted in amber if equal or smaller.

The work book also calculates the Limit of Quantification LoQ = Mean of solvent control + 9 Standard Deviations, and the Limit of Detection LoD = Mean of solvent control + 3 Standard Deviations.

The workbook flags growth factors ≤ 0.5 to draw your attention to possible toxic effects.

The performance of the assays are compared with historical data, and several criteria are either flagged “Within usual limits” or “Outside usual limits”. When all criteria are met, the corresponding assay is evaluated either as “Within usual limits” or “Partially outside usual limits”. Some limited conclusions might still be drawn even when some criteria are not met.

Finally, an algorithm-based summary is calculated to assist you with assigning estrogenic or androgenic activities to test samples.

Before analyzing your data and drawing conclusions please make sure that there is a successful curve fitting. Adjust the solver conditions if necessary as described above and in the Excel sheet.

Please note that the semi-automatically generated values and calculations are intended to support your own conclusions. The final determination of a sample's activities are always the investigator's own responsibility!

Xenometrix declines any liability for conclusions drawn based only on the semi-automatic calculations provided in the Excel calculation workbook!

Validity Criteria

A sample dilution is considered to have agonistic endocrine activity if the Induction Ratio $I_R \geq I_R10$ in either the YES or YAS Agonist assay.

A sample dilution is considered to have antagonistic endocrine activity if the Induction ratio $I_R \leq I_R50$ in either the YES or YAS Antagonist assay.

It should be noted that inhibitory effects may also be caused by non-specific effects which lead to a reduction of the response induced by the fixed concentrations of E2 and DHT. Parallel inhibition in the YES and YAS assay may be indicative of such non-specific inhibition which is not a true estrogen or androgen antagonistic effect.

Appendix A
Storage of Yeast Cells

There are 2 possibilities for storage of yeast cells for future use:

A) Short term storage at 0 – 4°C:
Yeast cell cultures can be stored for up to 2 weeks in growth medium in a refrigerator. Before using in an experiment it is recommended to check the cells 1 day before the planned experiment microscopically if they look healthy and to start an overnight culture by diluting 0.5 ml of stored culture with 5 ml of growth medium* and incubate them at 31°C with shaking. Please also refer to the note on page 6 for re-incubation of stored cells.

B) Frozen storage:
Yeast cells can be stored frozen at –20°C for up to 4 months or at –80°C or in liquid nitrogen for several years.

For freezing, grow the cells in 5 ml growth medium* in a T25 flask to an OD_{690} (200 µl) of ≥0.5. Mix with an equal volume of cold freezing medium (growth medium + 30% glycerol) and freeze in 0.5 ml aliquots, if possible in a –80°C freezer.

1 day prior to a planned experiment thaw a frozen aliquot and - as soon as it is molten - add the contents (500 µl) to 5 ml of growth medium and incubate overnight at 31°C with shaking. Dilute the dense culture as described on page 6 for the experiment.

* Growth medium also available separately from Xenometrix; Art. No. N05-PMY-7 .

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