Assay Endpoint ID: 804

Tox21_TR_LUC_GH3_Antagonist

Assay Name: Tox21 GH3 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Antagonists of the Thyroid Receptor (TR) Signaling Pathway by Monitoring Thyroid Response Element Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Thyroid receptor (TR), a nuclear hormone receptor, plays an important role in development, proliferation, differentiation, metabolism, brain function, and cardiovascular system. TR-interacting compounds have been shown to disrupt thyroid homeostasis [1]. To profile the Tox21 compound library's potential to interfere in TR signaling pathways, a cell-based GH3-TRE-Luc assay was used to measure the inhibition of TR following xenobiotic exposures. Activity was measured in GH3 (rat pituitary tumor) cells stably expressing a TR activity sensor consisting of two TR response elements and a luciferase reporter gene in 1536-well plates following 24-hour incubation with test chemicals. Increased luciferase activity indicates elevated levels of TR transactivation, and to detect TR antagonism, this assay is designed to monitor for loss-of-signal against thyroid hormone (T3) agonists. The cytotoxicity of the Tox21 compound library against the GH3.TRE-Luc cell line was tested in parallel by measuring the cell viability using CellTiter-Fluor assay (Promega, Madison, WI) in the same wells, using tetraoctylammonium bromide as a positive control for cytotoxicity.

1.2. Assay Definition

Assay Throughput:

GH3.TRE-Luc cells are aliquoted into 1536-well microtiter plates (1500 cells/5 µL/well) and incubated for 4 hours prior to 24-hour exposure to test compounds in the presence of T3 (agonist). Antagonistic activity is monitoring by measuring decreased luminescence resulting from xenobiotic-repression of TR gene expression.

Experimental System:

GH3 cell line was derived from rat pituitary tumor cells and has been routinely employed for studying effects of TH disruption using the T-screen assay [2, 3] and is reported to retain unique characteristics of the original differentiated tissue such as production of growth hormone and prolactin [3-5]. Moreover, this cell line endogenously expresses both TH receptor isoforms (α and β) in very high amounts and they respond to physiological concentrations of TH by proliferating [6]. The GH3.TRE-Luc cell line, developed in the laboratory of Dr. Albertinka J. Murk (Wageningen University) is derived from GH3 cells and stably expresses a modified firefly luciferase reporter gene under the regulation of a TR activity sensor consisting of a pair of thyroid hormone response elements (TREs). [7].

Xenobiotic Biotransformation Potential:

GH3 cells display an increased level of cell proliferation and growth hormone secretion in response to physiologic levels of thyroid hormones. TRE-LUC cells are activated by the thyroid hormone triiodothyronine (T3) and all trans retinoic acid but not RAR or LXR specific ligands. [2] CYP1A1 and 1B1 (but not 1A2) have been shown to be inducible by PCBs in GH3 cells [3].

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM:F12	Invitrogen	Gibco, 10565

Fetal Bovine Serum	Hyclone	Hyclone, Sh30071.03
Pen/Strep	Invitrogen	Invitrogen, 15140
Insulin	Sigma	Sigma, I6634
Ethanolamine	Sigma	Sigma, E0135
Sodium Selenite	Sigma	Sigma, S5261
Human Apotranferin	Sigma	Sigma, T2036
Bovine Serum Albumin	Sigma	Sigma, A9647
TrypLE Express	Invitrogen	Invitrogen, 12605
PBS w/o Calcium And Magnesium	Invitrogen	Invitrogen, 14190
Recovery Cell Culture Medium	Invitrogen	Invitrogen, 12648
Centrifuge	Sorvall Legend Xtr	Thermo Fisher Science 75004520
Bioraptr Microfluidic Workstation	Beckmen	-
Pintool	Kalypsys	-
White, TC, Sterile 1536-Well Assay Plates	Greiner Bio-One	Greiner, 789173-F
Viewlux Plate Reader	Perkinelmer	-
T3 (Agonist control compound)	Calbiochem	Calbiochem, 642511
DMSO	Amresco	Kd Medical, Rge-3070
Cell Titer Glo	Promega	Promega, G7572
Tetraoctylammonium bromide	Sigma	Sigma, 294136

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Culture Medium	-	-	-	100%
DMEM: F12	90%	100%	90%	-
Fetal Bovine Serum	10%	-	10%	-
Pen/Strep	100U/mL-100µg/mL	-	100U/mL-100µg/mL	-
Insulin	-	10µg/mL	-	-
Ethanolamine	-	10µM	-	-
Sodium Selenite		10ng/mL	-	-
Human Apo-transferrin	-	10µg/mL	-	-
Bovine Serum Albumin	-	500µg/mL	-	-

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a T75 flask

1.2.2 Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
1.2.3 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.
1.2.4 Transfer the vial contents drop-wise into 10 mL of Thaw Medium in a sterile 15-mL conical tube
1.2.5 Centrifuge cells at 1000 rpm for 4 minutes
1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37°C/5% CO2 incubator
1.2.7 Switch to growth medium at first passage.
1.3. Propagation method
1.3.1 Aspirate medium, rinse once in DPBS, add TrypLE Express (3 mL for a T75 flask and 5 mL for a T175 flask and 7.5 mL for T225 flask) and swirl to coat the cell evenly.
1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.
1.3.3 Centrifuge cells at 1000 rpm for 4 minutes and resuspend in Growth Medium.
1.3.4 Cell should be passage or fed at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture in Growth Medium and resuspend in assay medium
2.2 Dispense 1500 cells/5 µL/well into 1536-well tissue treated white solid plates using a BioRAPTR dispenser.
2.3 After the cells were incubated at 37°C for 4 hours, 23 nL of compounds dissolved in DMSO, positive controls or DMSO were transferred to the assay plate by a PinTool
2.4 Dispense 1µL of T3 or buffer control using BioRaptr
2.5 Incubate the plates for 23.5 hours at 37°C.
2.6 Add 5µL of Cell Titer Glo to each well using a BioRAPTR dispenser and incubate the plate at room temperature for 30min.
2.7 Measure luminescence using Viewlux

GH3.TRE-Luc cells were dispensed at 1500 cells/4 uL/well into 1536-well white solid bottom plates using a Multidrop Combi dispenser (Thermo Fisher Scientific Inc., Waltham, MA). After the assay plates were incubated at 37 °C for 4 hours, 23 nL of library compound or DMSO controls was transferred to the assay plates by a pintool station (Kalypsys, San Diego, CA), followed by addition of 1 ul of T3 (1 nM, final concentration in the wells) to stimulate TR transactivation. The assay plates were incubated at 37 °C for 23.5 h, and then 1 uL of CellTiter-Fluor reagent (Promega, Madision, WI) of measuring cytotoxicity was added to each well using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The plates were incubated at 37 °C and 5% CO2 for additional 30 min, and fluorescence intensity was measured by a ViewLux plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [4]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote thyroid receptor mediated gene expression, and is intended to provide

information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Freitas, J., Cano, P., Craig-Veit, C., Goodson, M. L., Furlow, J. D., & Murk, A. J. (2011). "Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay". Toxicol In Vitro 25(1), 257-266.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 thyroid receptor luciferase GH3 antagonist assay screened a large library of diverse environmental compounds to probe for xenobiotic activity and potential to suppress thyroid-dependent transcription, monitored through decreased luciferase reporter gene signal activation using a TR-luciferase reporter gene construct stimulated by 20μM of the thyroid hormone T3. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 24-hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent to detect TR inhibition. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in antagonist mode using luciferase-coupled ATP detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 μM. Loss-of-signal due to antagonism was distinguished from cytotoxicity by Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

TH is essential for normal brain development both before and after birth and has profound effects on cellular metabolism in almost all organs. One potential mechanism by which endocrine disrupting chemicals may produce toxic effects is by interfering with the ability of thyroid hormones (T3, triiodothyronine and T4, thyroxine). Thyroid hormone interfering compounds can result in neurological disorders by interfering with normal developmental and metabolic processes. Thyroid hormones also have important roles in the initiation and proliferation of central nervous system and cardiovascular tissues. An important component of an endocrine disruptor screening program should be the inclusion of assays designed to screen TH disrupting compounds. The GH3_TRE_LUC cell line is a TR responsive system which is a sensitive and reliable method to evaluate the potential for xenobiotic compounds to act as interfering ligands to the thyroid hormone receptor. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous thyroid signaling by monitoring the increase in luminescent signals relative to a thyroid hormone (T3), positive control and indicator of receptor activation.

Method Development Reference:

Gutleb, A. C., Meerts, I. A., Bergsma, J. H., Schriks, M., & Murk, A. J. (2005). "T-Screen as a tool to identify thyroid hormone receptor active compounds". Environ Toxicol Pharmacol 19(2), 231-238. (PMID: 21783481)

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Thyroid receptor ligand-binding and antagonism, measured by monitoring decreased luminescence resulting from repressed thyroid response element-driven expression of luciferase

Analytical Elements:

The Tox21_TR_LUC_GH3_Antagonist assay was monitored for decreased luminescence (loss-of-signal) relative to 0.001 mM T3 (positive control, 100% activity) signal, using DMSO (negative control) as a signal baseline, and response was reported as a percent of positive control (T3) activity. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Thyroid receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up
ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_up
LTEA_HepaRG_THRSP_dn
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Agonist

3.2. Assay Performance

Assay Performance Measures:

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [9].

4. Assay Documentation

4.1. References

[1] Crofton, K. M. (2008). Int J Androl 31(2): 209-223.
[2] Gutleb, A. C., et al. (2005). Environ Toxicol Pharmacol 19(2): 231-238. (PMID: 21783481)
[3] Hohenwarter, O., et al. (1996). Anal Biochem 234(1): 56-59.
[4] Ghisari, M. and E. C. Bonefeld-Jorgensen (2005). Mol Cell Endocrinol 244(1): 31-41. (PMID: 16221524)
[5] Samuels, H., et al. (1988). J Clin Invest 81(4): 957.
[6] Freitas, J., et al. (2011). Toxicol In Vitro 25(1): 257-266.
[7] Freitas, J., et al. (2014). Current Chem Genom Transl Med 8: 36.
[8] Gauger, K. J., et al. (2007). Environ Health Perspect: 1623-1630.
[9] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
T3, Triiodothyronine
T4, Thyroxine
TF, Transcription Factor
TH, Thyroid Hormone
TR, Thyroid Receptor
TRE, Thyroid Hormone Response Elements

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

3 August 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information