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A high-throughput screening assay to identify kidney toxic compounds

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Abstract

Kidney toxicity due to drugs and chemicals poses a significant health burden for patients and a financial risk for pharmaceutical companies. However, currently no sensitive and high-throughput *in vitro* method exists for predictive nephrotoxicity assessment. Primary human proximal tubular epithelial cells (HPTECs) possess characteristics of differentiated epithelial cells, making them a desirable model to use in *in vitro* screening systems. Additionally, heme oxygenase 1 (HO-1) protein expression is upregulated as a protective mechanism during kidney toxicant-induced oxidative stress or inflammation in HPTECs and can therefore be used as a biomarker for nephrotoxicity. In this article, we describe two different methods to screen for HO-1 increase: A homogeneous time resolved fluorescence (HTRF) assay and an immunofluorescence assay. The latter provides lower throughput but higher sensitivity due to the combination of two readouts, HO-1 intensity and cell number. The methods described in the protocol are amendable for other cell types as well.

Keywords

high-throughput; screening; *in vitro*; biomarker; heme oxygenase 1; primary human proximal tubular epithelial cells; HTRF

Introduction

One of the major challenges in developing safe and effective agents is the accurate prediction of human toxicity for new drug candidates and industrial chemicals (Astashkina et al., 2012). The advent of high-throughput screening capacities in academic, government, and industry facilities provides scientists with the opportunity to screen thousands of small

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Internet Resources

<http://iccb.med.harvard.edu> (Homepage of the ICCB-L screening facility at the Harvard Medical School with available compound libraries)

<http://www.enzolifesciences.com/ENZ-LIB100/screen-well-nephrotoxicity-library/>

(Focused nephrotoxicity library used for 8-point dose-response curves of 65 compounds)

molecules for their toxic effects. However, there is still no *in vitro* model available that has been validated and accepted by regulatory authorities to screen for kidney toxicity. We previously characterized primary human proximal tubular epithelial cells (HPTECs) as having structural and functional characteristics of differentiated epithelial cells. These include features such as polar architecture, junctional assembly, expression and activity of transporters, ability to synthesize endogenous antioxidants like glutathione, and increased activity of γ -glutamyl transferase (Adler et al., 2015). Using these cells as a desirable *in vitro* system, we then applied toxicogenomic profiling and identified heme oxygenase 1 (HO-1) as a gene that is significantly upregulated after incubation with various nephrotoxic compounds (Adler et al., 2015). HO-1 expression was found to correlate (FDR<0.01) with increasing dose for six of nine kidney toxic compounds and significant HO-1 protein deregulation could be confirmed in all of the nine nephrotoxicants. This is consistent with the literature, which reports that HO-1 is ubiquitously expressed in unstressed cells at low levels but is highly inducible in response to cell injury mediated by oxidative or pro-inflammatory stress, heavy metals, ischemia and hypoxia (Agarwal and Bolisetty, 2013; Nath, 2014).

This unit will describe methods for measuring HO-1 induction in HPTECs. Prior to commencing Basic protocols 1 and 2, readers are advised to read the methods for seeding and expanding the HPTEC cells detailed in Support Protocol 1 and addition of compounds for dose-response curves detailed in Support Protocol 2. Basic Protocol 1 is a 1-day procedure, compatible with automation and robotics and is designed to screen thousands of compounds. Basic Protocol 2 is a more time-consuming, yet more sensitive version of Basic Protocol 1 that can be done either in a semi-automated fashion or by an individual with instrumentation available in a typical academic laboratory. Thus, Basic Protocol 1 is designed to identify highly toxic compounds while Protocol 2 can be used to confirm non-toxic “hits” obtained from the high throughput screen (HTS).

Basic Protocol 1: High-throughput HTRF assay for HO-1 in kidney cells

Introduction

Basic Protocol 1 provides details on measuring the deregulation of HO-1 using a time resolved fluorescence resonance energy transfer (TR-FRET) between two epitopically distinct anti-human HO-1 antibodies that are labeled either with Europium Cryptate as the donor fluorochrome or d2 as the acceptor fluorochrome. If HO-1 is present in the cell lysate, the two dyes are brought into close proximity with each other and excitation of the donor with a light source triggers a FRET toward the acceptor. This emission fluorescence signal can be detected after incubation for 4 h and is proportional to the amount of human HO-1 present in the cell lysate.

Information on culturing and seeding of primary human cells (Day 0) can be found in Support Protocol 1. When cells are grown to confluency (Day 3), they are treated with potentially nephrotoxic compounds for 24 h (see Support Protocol 2). Finally, at Day 5, cells are lysed, incubated with a cocktail of the two epitopically distinct antibodies to HO-1 that are labeled with acceptor or donor fluorophores respectively, and the fluorescence signal is detected after 4 h using a microplate fluorescence reader.

Materials

Pin transfer robot equipped with 384 pipetting heads (Seiko)

EL 406 washer dispenser & Biostack 3 microplate stacker (Bio-Tek)

1 μ l cassette with 8-tips and plastic tubes for peristaltic pump of EL406 plate washer and dispenser

HTRF lysis buffer (LB1) (Cisbio Bioassays)

100x protease inhibitor cocktail (Cell Signaling Technology)

Microseal Foil (BioRad)

Titer plate shaker (Lab Line Instruments Inc.)

Allegra X-14R Centrifuge (Beckmann Coulter)

HTRF detection buffer (Cisbio Bioassays)

Europium cryptate (k)-labeled rabbit monoclonal HO-1-antibody (CST #5853-k) (Cell Signaling Technology)

D2-conjugated rabbit polyclonal anti HO-1-antibody (CST #5061-d2) (Cell Signaling Technology)

White low volume 384-well plates (Greiner Bio One)

MicroLab STARlet liquid handling system (Hamilton)

SpectraMax Paradigm (Molecular Devices)

Protocol Steps

1. As described in Support Protocols 1 and 2, HPTECS (5000/ well) are grown to confluency over 3 days and then treated for one day with 8 different concentrations of the toxic compounds in 4 replicates.
2. Prepare lysis buffer (LB1) 24 h after treatment by adding protease inhibitor cocktail: 156 μ l of 100x PIC in 3.9 ml of 4x LB1 are sufficient for 1x 384-well plate. Additional 1.2 ml of 4x LB1 (+ 48 μ l 100x PIC) should be prepared as dead volume for automated dispensing.
3. Stack plates in the stacker of an automated dispenser, prime the low volume (1 μ l) peristaltic pump tubing with 1.2 ml of prepared 4x LB1 and dispense 10 μ l of lysis buffer directly in the 50 μ l medium in each well. This step could also be performed manually with an 8-channel pipette with a capacity of 10 μ l.

An alternative cell lysis approach is to aspirate the 50 μ l medium in each well first and then add 40 μ l of a 1x LB1 buffer: 160 μ l of 100x PIC in 16 ml of 1x LB1 (= 4 ml 4x LB1 + 12 ml water) are sufficient for 1x 384-well plate. An additional 1.2 ml of 1x LB1 (+12 μ l 100x PIC) must be prepared as dead volume for

automated dispensing. The low volume dispensing cassettes can be used for volumes up to 50 μ l.

4. After addition of the lysis buffer, seal the plates with microseal foil, place them on a plate shaker at high speed for 5 min to assure complete lysis, and then spin the plates down for 20 seconds using the pulse function of a centrifuge.

In case the HO-1 measurement is not performed on the same day of the cell lysis, sealed plates can be stored at -80°C until use.

5. Prepare four assay controls according to the following template:
 - a. Buffer Control (**BuffCtrl**): 16 μ l lysis buffer + 4 μ l detection buffer
 - b. K Control (**KCtrl**): 16 μ l lysis buffer + 2 μ l detection buffer + 2 μ l k-labeled HO-1 antibody (AB)
 - c. Negative Control (**NegCtrl**): 16 μ l lysis buffer + 2 μ l k-labeled HO-1 AB + 2 μ l d2-labeled HO-1 AB
 - d. Positive Control (**PosCtrl**): 4 μ l lysate of cells treated with 50 μM cadmium chloride for 24 h + 12 ml water + 2 μ l k-labeled HO-1 AB + 2 μ l d2-labeled HO-1 AB
6. Next, prepare the HO-1 antibody cocktail: for 1x 384-well plate mix 1.6 ml detection buffer + 40 μ l of k-labeled anti-HO-1 (=0.85 ng/well) + 40 μ l of d2-labeled anti-HO-1 (=10 ng/well).
7. Figure 2 shows the layout of one replicate plate for HTRF detection, which is optimally achieved in white low volume 384-well plates. Transfer 4 μ l of each sample lysate to the white reaction plates using a liquid handling robotic system with disposable pipette tips (or an 8-channel pipette with a capacity of 0.5 – 10 μ l) and dilute 1:4 by adding 12 μ l water.

If the extent of HO-1 increase in the cells is expected to be rather low, the 1:4 dilution can be reduced to 1:2, or 16 μ l of the undiluted lysate can be used in order to stay in the linear range of the fluorescent emission.
8. Dispense 4 μ l of the HO-1 antibody cocktail into each of the sample wells already containing 16 μ l of cell lysate dilution.
9. Finally, add 20 μ l of pre-mixed controls to the plate according to the layout in Figure 2.
10. Incubate the plate for 4 h in the dark at RT.

If the expected HO-1 increase is very high, it is possible to decrease the incubation time to 2h which will result in a reduction of signal intensity by ~25%. In case the signal is very

weak, the incubation can be prolonged to overnight (21h), which leads to an increase of signal intensity.

HTRF measurement

1. To read HTRF, the SpectraMax microplate reader must be equipped with the Paradigm Cisbio HTRF cartridge. The excitation at 337 nm enables the simultaneous measurement of donor emission (620 nm) and acceptor emission (665 nm). The HTRF detection cartridge has been optimized for assays that use a Europium cryptate donor and uses a 616-nm filter for donor emission. Any references to '620 nm' measurements in this protocol correspond to 616 nm measurements made using the HTRF detection cartridge.

Emission at 620 nm is used as internal reference, while emission at 655 nm is proportional to the amount of HO-1. A ratio of the two fluorescence intensities (acceptor/donor) then allows the calculation of Delta F (%), i.e. the relative energy transfer rate for each data point.

2. The measurement conditions should be set up in the DTX Multimode Detection software according to the following indications:

Cartridge:	PARADIGM Cisbio HTRF
Number of flashes:	15 pulses
Delay (Lag time):	20 μ s
Integration time:	200 μ s
Excitation time:	50 μ s

Optimal z-position: volume and plate format dependent. This parameter must be optimized before each new configured measurement using the labware optimization procedure of the software.

3. Data analysis can be performed automatically when samples in the assay plate are assigned to pre-configured groups using the Template Editor. In the Template Editor window, accessed through the Plate section, assign the assay positive control (50 μ M CdCl₂) to the 'PosCtrl' group, and the negative control to the 'NegCtrl' group in the template window. Assign experimental samples to the 'Samples' group. In this standard assay protocol, the negative control is used to calculate 'Ratio Neg' (see calculations below) and '% Delta F' values are calculated accordingly.

In addition, separate zero-concentration controls (0.5% DMSO controls) can be designated as negative controls for each sample group by using the 'Assay Optimization' protocol.

If assay samples have been assigned to groups in the Template Editor, analyze raw data after the read is complete to obtain the HTRF Ratio and %Delta F values.

$$\text{HTRF Ratio} = (\text{Signal } 665 \text{ nm} / \text{Signal } 620 \text{ nm}) * 10,000$$

$$\% \Delta F = ((\text{Sample or Standard Ratio} - \text{Ratio Neg}) / \text{Ratio Neg}) * 100$$

The fluorescence ratio associated with HTRF readout is a correction method developed by Cisbio international and covered by patents from Cisbio international. Rights to use the patented equation are granted when an HTRF reagent kit is purchased from Cisbio.

4. % Delta F values of the samples can then be normalized to the average % Delta F of the 0.5% DMSO zero-concentration control wells to calculate a fold change that allows comparison between different plates and different experiments. Figure 3 shows fold change results of HO-1 measurements using the HTRF assay in the positive control (50 μM CdCl₂), and four different concentrations of one non-toxic compound (aspirin) and three kidney toxic compounds (cisplatin, citrinin, and arsenic trioxide).

Basic Protocol 2: An immunofluorescent-based screen to identify toxic compounds

Introduction

Basic Protocol 2 provides details on measuring the HO-1 protein expression using an immunofluorescent method which can be either used to confirm results obtained from the HTRF assay in Basic Protocol 1 or as a screening method on its own. Information on culturing and seeding of primary human cells can be found in Support Protocol 1. The generation of 8-point dose-response curves of a library of 68 compounds and methods to transfer them on the cells are described in Support Protocol 2.

Basic Protocol 2 is a more time-consuming, yet more sensitive version of Basic Protocol 1. Not only it can be combined with the analysis of changes in cell number in the same well, the automated microscopy-based imaging technique also allows detection of HO-1 expression in 10,000 – 15,000 cells per well which produces very narrow windows of variability. Briefly, 24h after compound treatment and on day 4 after seeding, cells are fixed and permeabilized for subsequent immunofluorescent staining with an anti-HO-1 antibody and nuclei are stained with Hoechst. Image analyses and toxicity evaluation can be performed on day 5.

Materials

Paraformaldehyde (PFA) (37%) (Sigma)

Methanol (Sigma)

EL 406 washer dispenser & Biostack 3 microplate stacker (Bio-Tek)

Straight 24-place stainless steel Manifold for Microtest Plates for 384-well plates (Drummond)

Odyssey blocking buffer (LiCor)

Mouse monoclonal anti-HO-1 antibody (Santa Cruz)

Goat anti-mouse antibody (Alexa 555) (Life Technologies)

Mouse IgG Isotype control (Thermo Fisher Scientific)

Microseal Foil for IF (BioRad)

Operetta high-content microscope with plate handler (Perkin Elmer)

Fixing, permeabilizing, and staining cells with Hoechst and HO-1 antibody

1. As described in Support Protocols 1 and 2, HPTECS (5000/ well) are grown to confluency over 3 days and then treated for one day with different concentrations of the toxic compounds in 4 replicates.
2. After 24 h of treatment, add 15 μ l of undiluted 37% Paraformaldehyde (PFA) into the 50 μ l medium per well (= 8% PFA). Fix cells for 20 min at RT. *Adding the PFA directly into the medium prevents loss of only lightly attached cells due to vacuum aspiration of medium and dispensing PFA on the cells.*
3. Place plates in the stacker and use an automated plate washer and dispenser or, as a manual alternative, a 24-place aspiration manifold and a multi-channel pipette to wash the plates 4x with 90 μ l 1x PBS.

The following settings were used for our plate washer and 384-well plates from Corning. "Aspiration" is very gentle and leaves ~15 μ l in the well. "Final Aspiration" has a lower distance to the carrier and aspirates all but ~5 μ l so that buffers or antibodies added in small volumes won't be diluted. The settings have to be optimized before use in different systems or different plates, so that the needles are not touching the sides or bottom of the wells.

- a. Aspiration: Travel rate = 7.3 mm/sec; Z-Offset= 4.45 mm above carrier; X-Offset = 0.91 mm left of center; Y-Offset = 0.74 mm front of center
 - b. Dispense: Travel rate = 2 mm/sec; Z-Offset= 13.09 mm above carrier; X-Offset = 0.46 mm left of center; Y-Offset = 1.11 mm front of center
 - c. Final Aspiration: Travel rate = 7.3 mm/sec; Z-Offset= 4.07 mm above carrier; X-Offset = 0.91 mm left of center; Y-Offset = 0.74 mm front of center
4. Permeabilize the cells by adding 50 μ l ice-cold methanol and allow the plates to incubate at RT for 10 min. Then, aspirate methanol and wash again 4x with 90 μ l 1x PBS and aspirate at the end.
 5. Block for unspecific binding by adding 30 μ l Odyssey blocking buffer and incubate at RT for 1 h.

6. Aspirate the buffer (“final aspiration” settings) then add 30 μ l of the primary antibody (mouse monoclonal anti-HO-1, 1:250 dilution) in Odyssey blocking buffer and incubate at 4 °C overnight. Wash 4x with 90 μ l 1x PBS and aspirate at the end.

To control for binding specificity, include a negative control that contains a mouse IgG Isotype control in the same concentration as the mouse anti-HO-1 primary antibody.
7. Add 30 μ l of the secondary antibody (goat anti-mouse, Alexa555, 1:250 dilution) in Odyssey blocking buffer and incubate in the dark at RT for 1 h. Wash 4x with 90 μ l 1x PBS and aspirate at the end.
8. Prepare 1:10,000 dilution of Hoechst 33342 (1 mg/ml stock) in 1x PBS and incubate cells with 50 μ l staining solution for 30 min at RT in the dark. Wash 4x with 90 μ l 1x PBS and don’t aspirate at the end.
9. Seal plates with Microseal Foil for IF and store them in the dark at 4 °C until imaging.

If possible, plates should be imaged as soon as possible.
However, if stored in the dark, the Alexa555 signal intensity is usually high enough, so that they can still be measured up to 1 month after staining.

High-throughput imaging and image analysis

The following analysis requires a high-content fluorescence microscope with the capability to screen 384 multi-well plates and, if possible, an attached robotic plate handler (e.g. ImageXpress Micro by Molecular Devices, Evotec Opera by Perkin Elmer). We used the PerkinElmer Operetta High Content Imaging System, and exposure times and scanning heights would have to be optimized before use in different systems.

1. Operate the microscope using Harmony software with the following settings:

10x long WD objective, 2 fields/ well,

Channel 1: Hoechst 33342 (ex 360–400/ em 410–480),
exposure time = 40 ms, height (z) = –6.0 μ m

Channel 2: Alexa546 (ex 520–550/ em 560–630), exposure
time = 300 ms, height (z) = 0.0 μ m

Before loading the sealed plates in the plate stacker,
wipe the bottom of the plates with 70% ethanol and
dry them with pressured air to ensure that no
fingerprints or attached dust particles are affecting the
analysis.
2. Determine the scanning time for one 384-well plate (at Operetta ~45 min) and program the robotic plate handler to change plates accordingly.

3. At the end of each run, captured images are automatically uploaded to the Perkin Elmer Columbus Image Analysis software. After the measurement of all plates is complete, set up the image analysis pipeline in Columbus software to calculate well averages of i) number of nuclei and ii) HO-1 intensity per cell.

For the segmentation of nuclei based on Hoechst 33342, use Method B and the following settings:

- Common Threshold = 0.45
- Area > 30 μm^2
- Split Factor = 7
- Individual Threshold = 0.45
- Contrast > 0.25

For the segmentation of cytoplasm based on HO-1 (Alexa 546) use Method C and the following settings:

- Common Threshold = 0.45
- Individual Threshold = 0.15

4. Export results of image analysis as csv files and calculate fold changes of drug-treated wells to the average of 0.5% DMSO-treated control wells.
5. Generate combined toxicity score by calculating the ratio of HO-1 mean intensity to the change in number of cells:
6. Toxicity score ($\log_2$) = [HO-1 Intensity (treated) / Mean HO-1 Intensity (controls)] x [Mean Cell Number (controls) / Cell Number (treated)]

Figure 4 shows the comparison between the “traditional” assay for cell viability, measuring ATP concentrations in the cells (CellTiter Glo), and the immunofluorescence image analysis of HO-1 and cell number after treatment with a toxic compound for 24h (cadmium chloride). Our method lowers the limit of detection of significant CdCl_2 -induced toxicity from 50 μM using the traditional CellTiter Glo assay down to 12.5 μM with HO-1 quantification. Additional improvement can be achieved by combining HO-1 and cell number to a toxicity score in a $\log_2$ scale, which further lowers the detection of significant CdCl_2 toxicity from 12.5 μM down to 6.25 μM .

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps.

Collagen IV solution

Dilute 5 mg collagen IV (Sigma) in sterile distilled water at 25 $\mu\text{g}/\text{ml}$ by addition of 200 ml water.

Dilute at least 2 h at RT or overnight at 4 °C, then sterile filter into a glass bottle.

Make sure all of the collagen is diluted, keep it 1 h at 37 °C if overnight is not sufficient

Growth medium

Prepare hormone-defined growth medium by adding the following supplements to 500 ml of DMEM/ HAM F12 with GlutaMAX (Invitrogen)

- a. 5 ml P/S (Penicillin/Streptomycin) (Gibco) (=100 UI/ml)
- b. 500 µl HC (Hydrocortisone 21-Hemisuccinate sodium salt) (Sigma) (=36 ng/ml)

Dilution of 1 mg Hydrocortisone in 1 ml 100% ethanol (VWR). Add 27 ml autoclaved 1x PBS (= 1 mg/28 ml). Store aliquots of 520 µl HC in 1 ml tubes at −20°C.
- c. 500 µl hEGF (human epidermal growth factor) (Promega) (=10 ng/ml)

Prepare 20 ml of 10 mM acetic acid containing 0.1% BSA by diluting 200 µl acetic acid (1 N) (Alfa Aesar) and 20 mg BSA (bovine serum albumin, fraction V) (Sigma) in 20 ml water. Mix well, filter sterilize into a sterile tube. Dilute 100 µg hEGF with 10 ml of this 10 mM acetic acid containing 0.1% BSA to a concentration of 10 µg/ml. Store aliquots of 520 µl in 1 ml tubes at −20°C; stable for 6 months.
- d. 1 ml ITS (Insulin, Transferrin, and Selenium) (500x) (Lonza) (=1x)
- e. 50 µl of 40 ng/ml T3 (Triiodothyronine) (Sigma) (=4 pg/ml)

Prepare 20 µg/ml stock solution by adding 1 ml 1N NaOH (Sodium hydroxide, 1N) (VWR) to 1 mg T3. Gently swirl to dissolve, then add 49 ml sterile medium. Further dilute T3 stock 1:500 to 40 ng/ml solution using DMEM/F12 medium. Store aliquots at −20°C.

FCS medium

Prepare FCS medium by adding 25 ml FCS (Heat inactivated fetal calf serum) (Lonza) to 500 ml of DMEM/ HAM F12 with GlutaMAX (Invitrogen) (= 5% serum).

Support Protocol 1: Thawing, expansion, and seeding of kidney cells

Characterization of the purchased, cryoconserved primary human proximal tubular epithelial cells (HPTECs) regarding their structural and functional characteristics has been described elsewhere (Adler et al., 2015). However, we include a supporting protocol on how to seed and expand HPTECs in culture, in order to maintain the characteristics of differentiated epithelial cells.

Materials

Primary human proximal tubule epithelial cells (HPTECs) (Biopredic)
(alternative: Lonza, ATCC)

Collagen IV solution (see recipe)

25 cm² and 75 cm² flasks (Sigma)

“Growth medium” based on DMEM/ HAM F12 with GlutaMAX (Invitrogen)
(see recipe)

“FCS medium” based on DMEM/ HAM F12 with GlutaMAX (Invitrogen) (see
recipe)

1x PBS (phosphate buffered saline) (–/–) (Corning)

0.25% Trypsin / EDTA (1x) (Gibco)

Automated Cell Counter (TC20) (Biorad)

384-well plates (Poly-D-lysine-coated, black, clear bottom) (Corning)

Multidrop Combi Reagent Dispenser (Thermo Scientific)

50 ml sterile reagent reservoirs (Corning)

10–100 µl 12-channel Multi-Channel Pipette (Eppendorf)

Standard Tube Dispensing Cassette (Thermo Scientific)

Straight 24-place stainless steel Manifold for Microtest Plates for 384-well
plates (Drummond)

Plastic storage boxes with lid (Sterilite)

Thawing cryoconserved primary cells (P1), splitting and propagation to P3, preparation of cell suspensions, and seeding cells in 384-well microplates

1. Add 2 ml collagen IV solution to 25 cm² flasks, 6 ml to 75 cm² flasks, and incubate plastic ware at 37 °C for at least 2 h in an incubator. Afterwards, aspirate the collagen solution, rinse the coated supports with sterile distilled water, and store for up to 2 weeks at 4 °C.
2. Pre-warm FCS medium at 37 °C and transfer the cryovial directly from the liquid nitrogen into a 37 °C pre-warmed water bath.

Work as quickly as possible, as a slow temperature gradient will reduce the cell viability.

3. Quickly transfer the vial content into 10 ml of warm FCS medium as soon as only a small ice cube is left in the cryovial and centrifuge the cell suspension at 300 x g for 5 min at RT.
4. Aspirate the FCS medium supernatant and resuspend the pellet in 500 μ l FCS medium. Transfer the resuspended cell solution in a 25 cm² collagen-coated flask containing 5 ml pre-warmed growth medium. Incubate the cells under standard cell conditions.
5. Change medium in 25 cm² flask 24 h after seeding and then every 2 days with fresh growth medium.
6. Split cells from P1 to P2 when they reach 90–100% confluency at day 3: aspirate media and wash once with 5 ml pre-warmed 1x PBS. Add 1 ml of 0.25% Trypsin / EDTA (1x) and incubate at 37 °C for 3 –5 min.

Closely monitor detachment of cells under inverted light microscope. Slightly tap sides of the flask if they are still not detaching after 5 min or use a cell scraper.

7. Stop trypsin activity by adding 6 ml FCS medium and wash bottom of plate with pipette to ensure complete detachment and single-cell suspension.
8. Centrifuge the cell suspension at 300 x g for 5 min at RT. Aspirate the FCS medium supernatant and resuspend the pellet in 5 ml growth medium. Use 10 μ l of the cell suspension to determine cell density using a hemocytometer or an Automated Cell Counter.

Common cell yield after 3 days is $\sim 4 \times 10^6$ cells from one 25 cm² flask.

9. Pipette an aliquot of the cell suspension equivalent to 1×10^6 cells in 15 ml growth medium in a 75 cm² flask. Usually enough cells for 4 flasks. Incubate cells and continue as described under 4.
10. Split cells from P2 to P3 when they reach 90–100% confluency at day 3 after seeding in 75 cm² flasks. Detach cells using trypsin, resuspend in pre-warmed medium and count cells as described under 5. - 7. but increase volumes: 2.5 ml of 0.25% trypsin, deactivate with 10 ml FCS medium, pool pellets from multiple flasks and resuspend pellet in 10 ml growth medium.

Common cell yield after 3 days is $\sim 8 \times 10^6$ cells from one 75 cm² flask. The cell yield can be increased further by splitting cells after 4 days -> up to 40×10^6 cells from pooled flasks available to seed in P3.

11. Prepare a cell suspension with 1×10^5 cells/ml growth medium (= 5000 cells/well/50 μ l) and seed cells in pre-warmed black poly-D-lysine-coated 384-well plates. For seeding either pour an aliquot of the cell suspension into a 50 ml reservoir and use a 12-channel multi-pipette to add 50 μ l into each well or use a Multidrop™ Combi Reagent Dispenser with standard tube dispensing cassette.

Calculate how many cells are needed based on how many 384-well plates should be seeded ($\sim 2 \times 10^6$ cells in 20 ml medium per plate; seeding with reagent dispenser requires at least 8–10 ml of additional cell suspension due to dead volume of tubing: 40×10^6 cells in 400 ml growth medium are sufficient for 19 plates).
12. Change growth medium in 384-well plates 24 h after seeding by removing media using a 24-place aspiration manifold and adding 30 μ l of fresh pre-warmed growth medium per well using the automated reagent dispenser or a 12-channel multi-pipette.
13. HPTECs should be 90–100% confluent after 3 days in growth medium. If the confluency is less than 80% 72h after seeding 5000 cells, it is recommended to wait one more day as low confluency can lead to higher variability in the toxicity assays.

Support Protocol 2: Addition of compound libraries for dose-response curves

There is a wide range of commercially available compound libraries, such as the collections from Selleck, Lopicol, Tocris, or Enzo, that contain annotated small molecules which can be screened in high-throughput assays. Those libraries are usually pre-dissolved in DMSO at a maximum of 10mM and can therefore be screened only up to relatively low doses ($\sim 10 - 50 \mu$ M). However, many toxic compounds might require higher concentrations (up to 0.5 – 1 mM) and dose-response curves of 6 - 8 concentrations to determine their IC₅₀ values *in vitro*. To achieve the necessary high stock solutions of 100 mM, we purchased 2 mg of 68 compounds of a nephrotoxic screening library rather than pre-dissolved 10 mM stocks.

Materials

68 compounds from SCREEN-WELL Nephrotoxicity library (Enzo) (#ENZ-LIB100–0100)
DMSO 100% (Sigma)
96-well library screening plates (ABGene)
Microlab STARlet liquid handling system (Hamilton)
8-Channel Transferpette 0.5–10 μ l (BrandTech)
384-well pin transfer plates (V&P Scientific)
Pin transfer robot equipped with 384 pipetting heads (Seiko)

The 68 compounds include mainly nephrotoxic compounds (Cadmium chloride was considered a positive control), but also a limited number of hepatotoxic compounds (e.g. Troglitazone), and 10 non-toxic controls (e.g. Dexamethasone, Carboplatin, Methylparaben, Atenolol, Aspirin).

1. Dissolve the 2 mg of all 68 compounds manually with the respective volume of DMSO (based on molecular weight) to stock solutions of 100 mM and store at -20°C .

Due to limited solubility of some compounds in DMSO library preparation requires special attention, including repeated vortexing, and warming up to 37°C , to ensure complete dilution of the compounds.

2. Based on the layout in Figure 1, manually pipette 30 μl of the 100 mM compound stocks as well as 30 μl DMSO controls into 96-well library plates.
3. To prepare three additional library dilution plates in DMSO (10 mM, 1 mM, and 0.1 mM) use an 8-channel multi-pipette or a tip-based liquid handling system to transfer 3 μl from the first stock plate (100 mM) into 27 μl DMSO in the second stock plate (10 mM), and so forth (Figure 1).

All robotic compound transfers and library dilutions were performed at the Institute of Chemistry and Cell Biology—Longwood (ICCB-L) at the Harvard Medical School, Boston, MA.

4. Next, generate an 8-point concentration curve of each compound on two 384-well plates with HPTECs. As depicted in Figure 1, combine the four 96-well dilution plates on a first 384-well plate (A) by adding a total of 166 nl of compound stocks (0.1 – 100 mM) to 30 μl medium in each well in plate A using a pin transfer system with 384 pipetting heads (concentrations in plate A: 0.55 – 553.3 μM). On a second 384-well plate (B), transfer 100 nl of compound stocks from the four 96-well plates (concentrations in plate B: 0.33 – 333.3 μM). After the transfer, the plates are placed in a humidity controlled incubator.

The concentration range and spacing could be increased or decreased; however, using a pin transfer system comes with certain limitations as depending upon the pin diameter the specific pin array used in our screening facility is calibrated to only transfer volumes of 33 nl or 100 nl into a 384-well assay plate volume of 30 μl .

5. Perform each treatment in 4 technical replicates. Therefore, eight 384-well plates (4x plate A and 4x plate B) are required for the screening of 68 compounds in 8-point concentration curves.

The automated transfer of compounds using a calibrated, robotic pin transfer system is the preferred option for screening assays, as it assures high reproducibility and low variability. However, if the appropriate equipment for a pin transfer of compounds in the nM range is not available, a manual alternative is briefly described.

1. Starting from 100 mM stock solutions in 100% DMSO, prepare four additional library dilution plates in 10% DMSO (10 mM, 1 mM, 0.1 mM, and 0.01 mM) using an 8-channel multi-pipette and transfer 3 μ l from the first stock plate (100 mM) into 27 μ l water with 10% DMSO in the second stock plate (10 mM), and so forth.
2. Then generate the 8-point concentration curve on two 384-well plates containing cells by combining the four 96-well dilution plates in water with 10% DMSO. On the first 384-well plate (A), add 1.75 μ l of compound stocks (0.01 – 10 mM) to 30 μ l medium in each well in plate A using a 8-channel multi-pipette with a capacity of 0.5 – 10 μ l (concentrations in plate A: 0.55 – 551 μ M). On a second 384-well plate (B), transfer 1.05 μ l of compound stocks from the four 96-well plates (concentrations in plate B: 0.33 – 338 μ M).

Commentary

Background Information

There is a tremendous need to identify new biomarkers that can sensitively and specifically identify kidney toxic compounds in cell-based screening approaches. Although biomarkers such as Kidney Injury Molecule-1 (KIM-1) have been qualified by the FDA and EMA for preclinical studies, its translation to high throughput *in vitro* screening has been challenging (Huang et al., 2014). We did not observe KIM-1 upregulation in HPTECs cultured in 2D consistent with a previous study (Li et al., 2013), or even consistently when HPTECs were cultured in the microphysiological 3D kidney system (Adler et al., 2015).

Heme oxygenase is responsible for the physiological degradation of heme to bile (Maines, 1988), i.e. it cleaves the heme ring to form carbon monoxide, free iron, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. HO-1 is ubiquitously expressed in unstressed cells at low levels but is highly induced in response to cell injury mediated by oxidative or pro-inflammatory stress, heavy metals, ischemia and hypoxia (Agarwal and Bolisetty, 2013; Nath, 2014). Its cytoprotective and antiapoptotic properties are mediated by degradation of the pro-oxidant heme into iron, biliverdin and CO via induction of p38 MAPK and PI3K/AKT signaling pathways (Gozzelino et al., 2010). In contrast, pronounced chemical or genetic inhibition of HO-1 is associated with increased cell death and tissue necrosis in models of Alzheimer's disease (Takahashi et al., 2000), as well as in mice models of cisplatin-induced toxicity (Bolisetty et al., 2015). These effects have partly been explained by a lowered antioxidative capacity (Regehr et al., 2007) and cells being more susceptible to damaging agents during HO-1 inhibition (Berberat et al., 2005), a property that has been used to sensitize cells for cancer therapy (Abraham and Kappas, 2008). Given that deregulation of HO-1 in HPTECs correlated well with the known

toxic effects of the tested compounds in humans, changes in cellular HO-1 might indicate a cellular process to protect from further damage. HO-1 has previously been found to be upregulated in the urine of patients with acute kidney injury (Zager et al., 2012) and tubulointerstitial damage (Yokoyama et al., 2011). Additionally, HO-1 expression was significantly upregulated in rat kidneys following tubular toxicity and in “human kidney-on-a-chip” system following CdCl₂ exposure (Adler et al., 2015).

Critical Parameters and Troubleshooting

The protocols described in this unit rely on primary human cells and some of the potential sources of variability that have to be controlled for include a) cell culture related factors, b) compound and reagent related issues, and c) robotic and instrument settings.

Cell culture related factors

- 1 As HPTECs are primary cells rather than an immortalized cell line, it is possible that donor-to-donor variability affects changes in cell density directly after thawing of a new vial or cell growth during the propagation phase. In order to account for those potentially longer culture times, it is recommended to thaw one test vial of every new cell donor before this batch is used for a big screening experiment. Calculation of the number of living and attached cells 24 h after seeding, as well as the doubling time in passage 3 allows implementation of potential changes (such as higher seeding densities or longer period between splitting of cells) into the screening protocol.
- 2 The number of cells in each well should be uniform. Using an automated dispenser for cell suspensions is very helpful in this regard. However, the resuspension of the cell pellet after centrifugation has to be performed very thoroughly as cell aggregates might block the dispenser nozzles and lead to uneven cell numbers. Additionally, placing the freshly seeded plates on a flat surface and moving the plates swiftly in an 8-shaped movement helps to prevent the cells from attaching only in one corner of the well.
- 3 The cell culture plates are cultivated in an open system, where the culture medium is in equilibrium with the ambient air. If humidity in the incubator is not tightly controlled (e.g. because the water pan ran empty) and falls below 95% for a longer period, 384-well plates are especially susceptible to increased evaporation of water from the cell culture medium. This not only changes the osmotic balance in the cells, it also induces “edge effects” in the plates, as edge wells are more prone to evaporation, and additionally this could lead to an uneven concentration of the screening compounds in the wells. To avoid these issues, either use humidity controlled incubators, or place the 384-well plates in a secondary container that was sprayed and wiped clean with 70% ethanol (e.g. plastic storage boxes), then cover plates with wet sterile tissues, put lid on top without sealing the box from gas exchange, and place box in the incubator.

- 4 Great care should be executed to prevent contaminations of the cell cultures, especially when working in screening core facilities shared with a large number of other investigators.

Compound and reagent related issues

- 5 The screening compounds should undergo quality control (e.g. via LC-MS/MS) when implemented in the library and DMSO stock solutions stored appropriately. The library plates should only contain an aliquot of the stock compound to prevent repeated thaw-freeze-cycles.
- 6 The final DMSO amount that the cells are exposed to should not exceed 0.5%, as it might impact cell viability and biochemical reactions.
- 7 After addition of nl amounts of stock solutions in 100% DMSO to the medium, the wells should be briefly inspected to ensure complete solution of the compounds. Formation of crystals in the well due to oversaturated compounds can produce false toxicity results.
- 8 If the screening library contains many compounds with auto-fluorescent properties at 620 or 665 nm the medium cannot be mixed with the lysis buffer due to potential interference of the compounds with the HTRF emission signal. In this case the alternative approach, to aspirate the 50 µl medium in each well and add 40 µl of a 1x LBI buffer, can be used.
- 9 A decrease in signal in the immunofluorescent HO-1 assay might be due to inappropriate storage of the secondary Alexa555-labelled antibody. The fluorescent conjugate is very light sensitive and should be aliquoted to avoid multiple thaw-freeze cycles and stored in the dark.

Robotic and instrument settings

- 10 Washing and dispensing steps using an automated plate washer have to be carefully optimized as settings that aspirate and dispense too quickly or forcefully might wash off cells from the well bottom and bias the assay.

Anticipated Results

Basic Protocol 1 provides the framework to set up and perform a cell-based HTS for small molecule drugs and chemicals that have the potential to induce kidney toxicity. Because the primary HTRF screen is often performed at single concentrations, it should be used as an exclusion step for toxic compounds while the non-toxic compounds can be followed up in a secondary screen using multiple doses as described in Support Protocol 2. When screening compound libraries, it is hard to predict typical “hit” rates of toxicity-inducing compounds, because this is highly dependent of the concentration used and the type of library (e.g. biologically active compound libraries vs. libraries spanning the chemical space). A recently performed screen of a biologically active compound library, containing active pharmaceutical agents, chemotherapeutic agents, as well as some natural products, used at a concentration of 10 µM, yielded a “hit” rate of toxicity-inducing compounds of roughly 37% (i.e., for every 10,000 compounds screened, 3700 could be excluded from further

consideration if their renal concentrations *in vivo* are likely to reach the concentration used for the screening).

Time Considerations

The amount of time required to screen for nephrotoxic compounds in primary renal cells can be divided into three phases: (1) set-up of the compound transfer, (2) biological preparation, (3) transfer and HO-1 assay.

It usually takes weeks to months to set up a cell-based HTS (Basic Protocol 1) at an academic or government-sponsored core facility. Protocols, including cells, reagents, and measurement settings have to be tested in preliminary studies, and pilot experiments with core facility staff members have to be performed to ensure the desired reproducibility and robustness of the assay.

The preferred compound library has to be selected and an initial concentration agreed upon. Once established, the time needed for the actual HTS depends on the number of compounds to be screened. Using Basic Protocol 1, the transfer of a medium size compound screen of 10,000 compounds at a single dose and time point could be performed on thirty-two 384-well plates in one day. For the screening of less compounds in multiple concentrations, as described in Support Protocol 2, the dilution of 68 compounds in DMSO as 100 mM stocks takes ~1 day. Preparation of the subsequent 96-well stock dilution plates can be done in just 1 h using automated pipetting.

The preparation of enough primary cells requires about 1.5 weeks. On day 1, one vial of HPTECs is thawed and seeded in a small flask. After cell adherence on day 2, the medium is changed. On day 4, the cells are split into 4 medium flasks and medium is replaced on day 5. Two days later, on day 7, cells are split again and seeded in 384-well plates and medium is replaced on day 8. Cells should be confluent on day 9 and ready to be treated with the compound libraries. Cells are incubated with the test compounds at 37 °C for 24 h and on day 10, changes in HO-1 expression are determined using the HTRF assay.

The HTRF assay can be completed in 6–7 h, including 4 h incubation with the antibody mixture and HTRF reading time using the SpectraMax Paradigm, which requires about 2 min to read a 384-well plate. Faster read speeds may be attained by using fewer pulses or On the Fly detection. The immunofluorescent HO-1 assay required one additional day to incubate with the primary HO-1 antibody overnight, 2 h the next day to incubate with secondary antibody and Hoechst, and another hour to image one 384-well plate in 2 channels using the Operetta microscope.

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Significance Statement

There is an urgent need for a reliable, reproducible and high throughput *in vitro* model that allows rapid screening of compounds that are potentially toxic to the kidneys. We previously reported that primary human proximal tubular epithelial cells (HPTECs) represent a suitable *in vitro* model as they retain many of the phenotypic as well as functional characteristics of the human tubular epithelial cells. In the same study we also demonstrated that in comparison to currently used assays of cell viability/death, heme oxygenase-1 (HO-1) was a more specific and sensitive biomarker for predicting compound toxicity in HPTECs. The HO-1 based screening protocol, using HPTECs, outlined here allows rapid and robust screening of potential kidney toxic compounds, supporting chemical risk assessment and facilitating elimination of drug candidates early in the development process.

Figure 1.

Overview of protocol to generate 8-point dose-response curves for a compound library in a concentration range from 0.3 up to 0.55 mM. Serial dilutions of 100 mM stock solutions in a 96-well plate containing 68 drugs generate three additional stock plates (10, 1, and 0.1 mM) in 100% DMSO. Transfer of either 166 (A) or 100nl (B) from each of the 4 stock plates into two 384-well plates with cells yields 8 different dilutions of each drug. These transfers are performed on 8x 384-well plates in total to generate four technical replicates.

Figure 2.
Plate layout of one replicate of a HO-1 measurement plate using the HTRF assay.

Figure 3.

Representative results of HO-1 measurements using the HTRF assay. The positive control (50 μ M CdCl₂) and three kidney toxic compounds (cisplatin, citrinin, and arsenic trioxide) show dose-dependent, significant increase in HO-1 fold change compared to 0 μ M control. The non-kidney toxic compound (aspirin) does not show an increase in HO-1 protein expression. Results are presented as mean $\pm$ SD (n=4). * p<0.05; ** p<0.01; *** p<0.001 (ANOVA, Dunnett's).

Figure 4.

Increased sensitivity in detection of CdCl₂-induced toxicity by the HO-1 measurement compared with the standard ATP-based cell viability assay. Red line indicates lowest concentration of CdCl₂ with significant increase/ decrease of the readout compared to control. Dashed lines show the variability of the control as 0 μM ± 3* SD. Results are presented as mean ± SD (n=4). * p<0.05; **** p<0.0001 (ANOVA, Dunnett's).