

Multiplex Immunoassay to Monitor Drug Toxicity in Renal Proximal Tubule Chips

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Abstract

Organ-on-chip technology can mimic complicated structures and provide researchers with physiologically relevant three-dimensional (3-D) tissue models. The Nortis ParVivo Human Kidney Chip utilizes human telomerase reverse transcriptase (hTERT)-immortalized kidney cells to generate renal proximal tubules (RPTs) for use in drug toxicity studies. In this study, biomarkers associated with tubular nephrotoxicity were monitored using the six-target Bio-Plex™ Pro RBM Human Kidney Toxicity Panel 1, and the data were compared to traditional ELISA assays. We show that while the data are similar between the two methods, the Bio-Plex Pro Multiplex Assay uses significantly less volume per well, conserving precious sample and enabling analysis of multiple biomarkers simultaneously.

Introduction

Animal models and traditional two dimensional (2-D) in vitro culture systems used in preclinical screening of compounds are limited by their poor translational predictability of human physiological outcomes, contributing to the high attrition rate of drugs in early stages of clinical trials (Cook et al. 2014, Morgan et al. 2018, Tiong et al. 2014). A significant contributor to the high dropout rate of drug candidates is safety concerns due to drug-induced nephrotoxicity that goes undetected in preclinical screening (Phillips et al. 2020). Microphysiological systems such as organ-on-chip technologies give rise to organ-specific microenvironments that mimic in vivo structural and biochemical conditions, facilitating their use in drug discovery research.

Nortis, Inc. is a recognized leader in organ-on-chip technology, revolutionizing traditional drug development and discovery processes. The company's ParVivo Organ-on-Chip Platform comprises patented microfluidic chips and a corresponding perfusion system (Figure 1). The Nortis ParVivo Platform provides biomedical researchers with physiologically relevant 3-D tissue models. To simulate the in vivo environment, the tissue models are surrounded by a biologically relevant matrix and are continuously perfused.

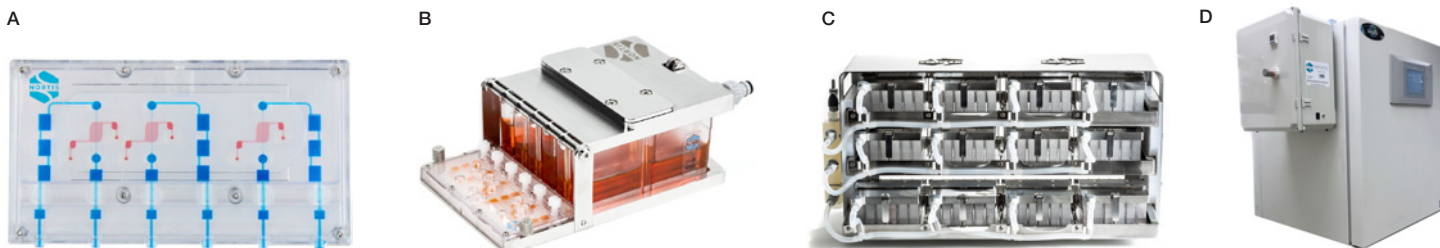


Fig. 1. The ParVivo Platform modules and components. **A**, ParVivo Microfluidic Chips are provided with relevant matrix and cells of choice; **B**, ParVivo Perfusion Module provides in vivo-like unidirectional fluid flow through the chips, providing nutrients to the microtissues and enabling perfusion of test compounds; **C**, Culture Station provides locations to house multiple perfusion modules; **D**, standard CO₂ cell culture incubator with perfusion pump harbors the culture station during growth and development of the tubules.

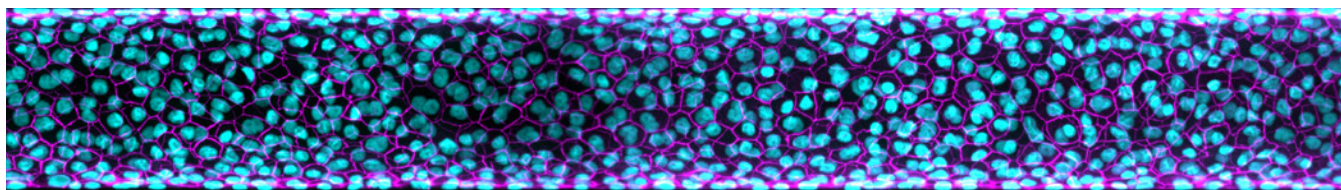


Fig. 2. Immunohistochemistry image of a human kidney proximal tubule segment within a ParVivo Human Kidney Proximal Tubule SC3 Chip. A human kidney proximal tubule segment grown from TERT1/RPTEC cells in the ParVivo SC3 chip stained with antibodies against the tight junction protein zonula occludens (ZO-1) (■) and the nuclear stain Hoechst 33342 (■). Image was taken using a point-scanning confocal microscope.

One tissue particularly prone to toxicity risk is the human kidney proximal tubule due to its high rate of xenobiotic traffic. Nortis manufactures the ParVivo Human Kidney Proximal Tubule (RPT) SC3 Chip (Immortalized) which contains pre-established kidney proximal tubules on-chip where three human kidney proximal cell tubule tissue segments, each surrounded by a collagen matrix, are individually and unidirectionally perfused. This recapitulates critical in vivo conditions but with the benefits of in vitro status without coming in contact with any inorganic surfaces, in contrast to standard 2-D models. A proximal kidney tubule grown in these conditions gives rise to structures resembling in vivo polarity of proteins in distinct apical and basolateral compartments, and expression of key proteins such as the tight junction protein zonula occludens (ZO-1), shown in Figure 2.

To characterize this organ-on-chip model, it is useful to subject the model to a known nephrotoxin such as polymyxin B and test for analytes associated with nephrotoxicity using the workflow shown in Figure 3. U.S. Food and Drug Administration (FDA)–qualified biomarkers (U.S. Food and Drug Administration 2021), such as KIM-1 and clusterin have demonstrated significant elevations in urinary concentration early after acute kidney injury in animals and humans (Griffin et al. 2019). Traditional testing of these analytes is done via ELISA, which allows for a single analyte test per well and requires a 50–100 μ l sample volume. In a model system where sample volumes are intrinsically low, finding a method that conserves sample and allows for multiple analytes to be detected simultaneously is highly beneficial. Here, we study a six-target multiplex immunoassay for kidney toxicity using the Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 and Bio-Plex 200 System. Both are based on the Luminex xMAP bead-based multiplex technology.

Materials and Methods

3-D Cell Culture

Eight ParVivo Human Kidney Proximal Tubule SC3 Chips ($n = 24$ tissue segments) (Nortis, Inc., catalog #800001) were perfused with DMEM: F12 Medium (ATCC, #30-2006) supplemented with the hTERT Immortalized RPTEC Growth Kit (ATCC, #ACS-4007) and G418 (Geneticin) (InvivoGen, #ant-gn-5), and then coated with Collagen IV (MilliporeSigma, #C5533-5MG) at a concentration of 15 μ g/ml. Kidney hTERT RPTEC/TERT1 cells (Evercyte GmbH, #CHT-003-0002) were collected at passage 13 and used to seed the prepared chip with a cell density of 12 million cells/ml. The cells were incubated in a biosafety cabinet (BSC) at room temperature for 45 min before reattaching the port plug array, opening the

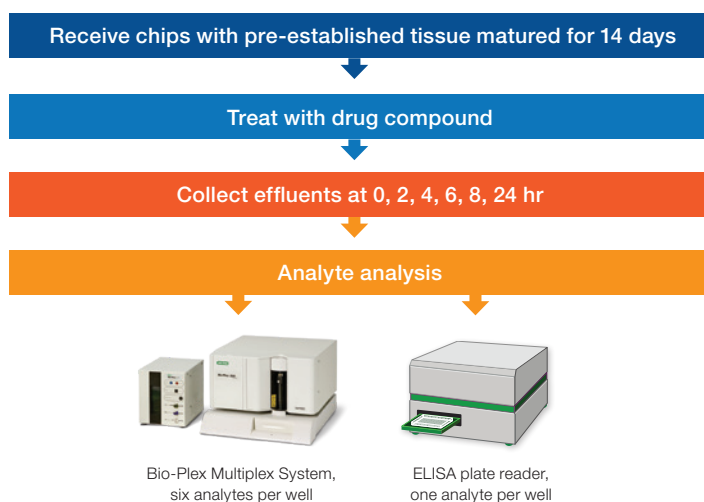


Fig. 3. Experimental design. Workflow steps include cell culture, cell seeding in the ParVivo SC3 Chip, monitoring 3-D tubule formation, perfusion of test compounds, and waste effluent collection followed by effluent sample analysis using the Bio-Plex 200 Multiplex System and an ELISA plate reader.

appropriate valves of the ParVivo System modules and Culture Station (Nortis, Inc., #900001), and then incubated under perfusion at 37°C and 5% CO₂. Cell media was changed twice a week, with careful attention given to cell morphology and cell health, over two weeks as the 3-D hTERT tissues formed. Cultured cells were stained with -CellStain- Hoechst 33342 solution (Dojindo Molecular Technologies, Inc. #H342) and the primary antibody ZO-1 Antibody (Cell Signaling Technology, Inc., #5406) at 1:100, and with secondary antibody Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) (Abcam plc., #150077) at 1:150, and imaged using a Leica Microsystems SP8 STELLARIS Confocal Microscope.

Compound Treatment and Sample Collection

On day 14, four of the eight 3-D cultured tubules were treated with a 200 μ M Polymyxin B Solution (MilliporeSigma, #92283-10ML) for 2 hr in culture. The remaining four cultured tubules were left untreated and used as controls. All cultured tubules were monitored before and after treatment to ensure they remained healthy and confluent.

Table 1. Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 assay results for RPT chip effluent samples. Data collected using the Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 on the Bio-Plex 200 System. Time-course study over a 24 hr period comparing data from six analytes* on both untreated (no compound) and treated (200 μ M polymyxin B) tubules. OOR, out of range; RPT, renal proximal tubule.

Time, hr	KIM-1, ng/ml		Clusterin, ng/ml		Calbindin, ng/ml		GST- π , ng/ml		MCP-1, ng/ml	
	No Compound	200 μ M Polymyxin B	No Compound	200 μ M Polymyxin B	No Compound	200 μ M Polymyxin B	No Compound	200 μ M Polymyxin B	No Compound	200 μ M Polymyxin B
0	0.01	0.01	0.29	OOR <	0.29	0.29	0.3	0.18	0.13	0.11
2	0.01	0.02	0.32	0.35	0.42	0.27	0.17	8.06	0.17	0.15
4	0.01	0.28	0.19	1.18	0.25	0.67	0.07	8.58	0.11	0.2
6	0	0.16	0.19	0.66	0.3	0.82	0.06	6.84	0.14	0.31
8	0	0.13	0.19	0.69	0.28	0.65	0.07	9.17	0.11	0.28
24	0	0	0.19	0.14	0.28	OOR	0.12	1.16	0.09	0

* IL-18 was not detected.

Effluent samples were collected at 0 hr from all samples before the polymyxin B compound treatment. To treat the four cultured tubules with the 200 μ M polymyxin B solution for 2 hr, the hTERT media was removed and treatment media was added to the source reservoir and then perfused. Effluent samples were collected from a collection reservoir containing a 0.5 ml microcentrifuge tube every 2 hr for 8 hr. One more effluent sample was collected 24 hr post-treatment. Samples were pooled, mixed, and aliquoted for each time point. Effluent samples were immediately stored at -80°C after collection and pooling. Samples were divided for testing at two locations: via traditional ELISA at Nortis, and via Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 Assays at Bio-Rad™ Laboratories, Inc. Aliquots (400 μ l) of pooled samples were shipped on dry ice to Bio-Rad for each treatment at each of the six time points.

Analyte Analysis

Human Serum TIM-1/KIM-1/HAVCR Quantikine ELISA Kits (R&D Systems, Inc., #DSKM100) and Human Clusterin Quantikine ELISA Kits (R&D Systems Inc., #DCLU00) were used to quantify analytes from the effluent samples. ELISA assays followed the manufacturer's recommended protocols. One 120 μ l pooled aliquot from each treatment and time point was thawed for each ELISA. All standards and samples were run in duplicate, with 50 μ l of sample added to each well. The signal was read on a Spectramax i3x Multi-Mode Microplate Reader with SoftMax Pro Software (Molecular Devices, LLC., #i3x) using a 540 nm absorbance for background subtraction and 450 nm absorbance for optical density (OD) reading.

Bio-Plex Pro Multiplex Assays

The Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 (Bio-Rad, #171ATR1CK) detects six kidney toxicity markers (calbindin, clusterin, GST- π , IL-18, KIM-1, and MCP-1), including two of the analytes, KIM-1 and clusterin, tested by ELISA. All panel instructions were followed with modifications made for input volume and dilution. The panel was originally designed to accept 30 μ l of urine at a 1:4 dilution. A pilot study was conducted previously using renal proximal tubule (RPT) chip effluent. It was determined that 40 μ l of undiluted sample resulted in analyte concentrations within the linear dynamic range of quantification. Several dilutions, volumes, and incubation conditions were tested in the pilot study.

** Bio-Plex Pro RBM Canine Toxicity Panel is discontinued.

All wash steps were done on a Bio-Plex Handheld Magnetic Washer (Bio-Rad, #171020100). All standards, controls, and samples were run in duplicate. The Bio-Plex 200 System with HTF (Bio-Rad, #171000205) was calibrated according to the protocol, and data acquisition and analysis were performed using Bio-Plex Manager 6.2 Software (Bio-Rad, #171STND01).

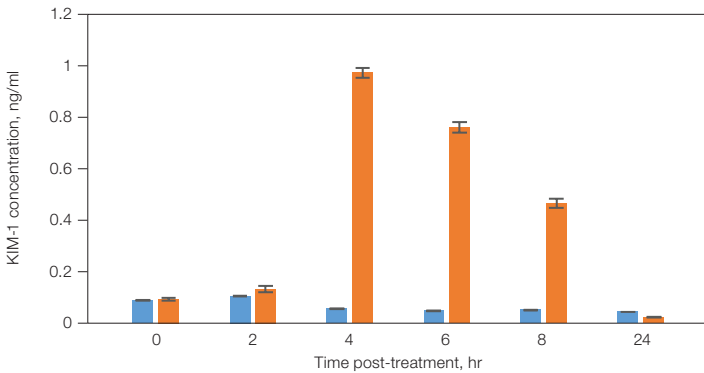
Results

The Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 was originally designed to measure important biological markers expressed in human kidneys by analysis of urine samples (Bio-Rad bulletin 6400**). This study provides evidence that RPT chip effluents can also be used as input samples to measure these analytes of interest. The panel measures the abundance of six analytes in the sample (clusterin, GST- π , KIM-1, MCP-1, IL-18, calbindin). Two sets of samples were run in duplicate: a control set and a set treated with 200 μ M polymyxin-B. Of the six analytes, one, interleukin 18 (IL-18), was not detected in the treated or untreated samples, as expected. IL-18 is a marker of acute kidney injury (Lin et al. 2015). The RPT chip models the proximal tubule epithelium and does not have a basolateral endothelial interface with circulating inflammatory cells to build up the IL-18 response after injury, as opposed to urinary IL-18 in vivo, where macrophages are the main contributors (Griffin et al. 2019).

Comparing the ELISA results obtained for KIM-1 (Figure 4A) with Bio-Plex results (Figure 4B) reveals values within an order of magnitude of each other, with maximal KIM-1 expression at 4 hr post-treatment, dropping at the 6 and 8 hr time points, and not detectable at 24 hr. Figure 4 shows the detection of clusterin over the time course in the effluent of treated and untreated RPT chips; ELISA data (Figure 5A) and Bio-Plex data (Figure 5B) are comparable.

Table 1 shows the concentrations of Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 analytes in RPT chip effluent. Five of the six analytes were detected in both untreated and treated effluent. Notably, IL-18 was not detected. GST- π was elevated above untreated levels in treated samples at all time points tested beyond 0 hr. GST- π showed a peak at 4 hr post-treatment, then a dip at 6 hr with another peak at 8 hr; at 24 hr post-treatment, the concentration of GST- π in treated effluent was still considerably higher than in untreated samples at the same time point. The calbindin and MCP-1 concentrations appear to peak at 6 hr post-treatment.

A. ELISA for KIM-1



B. Bio-Plex Pro Assay for KIM-1

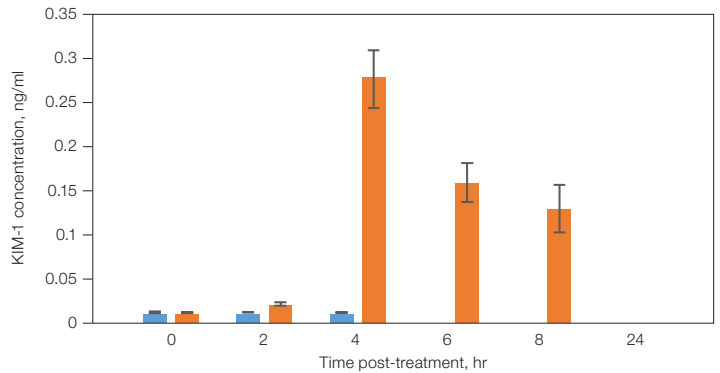
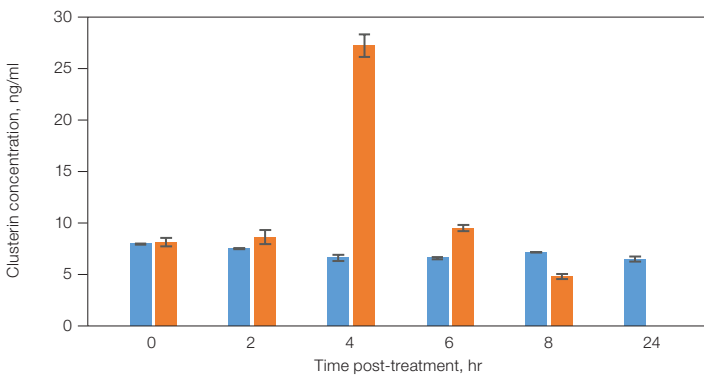


Fig. 4. Analysis of KIM-1 in effluents of RPT chips. Immunoassays for KIM-1 performed by **A**, ELISA and **B**, Bio-Plex Pro RBM Human Kidney Toxicity Panel 1. Effluents were collected at time points post-treatment with polymyxin B (■) and untreated (■).

A. ELISA for clusterin



B. Bio-Plex Pro Assay for clusterin

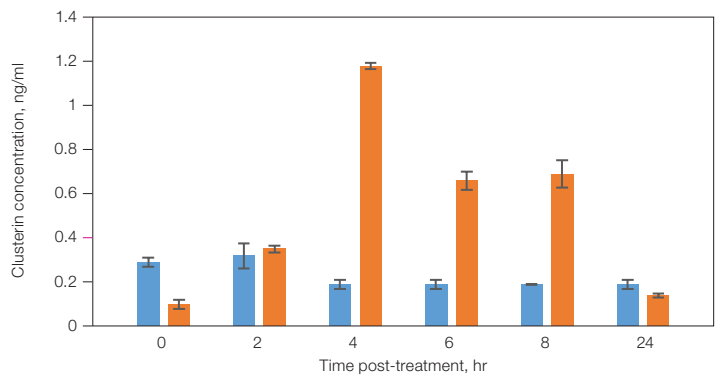


Fig. 5. Analysis of clusterin in effluents of RPT chips. Immunoassays for clusterin performed by **A**, ELISA and **B**, Bio-Plex Pro RBM Human Kidney Toxicity Panel 1. Effluents were collected at time points post-treatment with polymyxin B (■) and untreated (■).

Conclusions

The Bio-Plex Multiplex Assay results in hTERT immortalized kidney proximal tubule cell effluent are comparable to the corresponding ELISA results. We report here a different input sample for the Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 — RPT chip effluent — which was a previously untested sample type. Although a larger volume of effluent is required per well (40 µl) than urine (30 µl, diluted 1:4), we were able to detect five of the six analytes simultaneously in the Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 in the same sample. The multiplex assay format allows the conservation of precious sample; testing for six analytes in duplicate on the Bio-Plex 200 System requires a total of 80 µl effluent, whereas running the same six individual assays in duplicate using ELISA requires a total of 600 µl.

References

Cook D et al. (2014). Lessons learned from the fate of AstraZeneca’s drug pipeline: A five-dimensional framework. *Nat Rev Drug Discov* 13, 419–431.

Griffin BR et al. (2019). Biomarkers of drug-induced kidney toxicity. *Ther Drug Monit* 41, 213–226.

Lin X et al. (2015). Urine interleukin-18 in prediction of acute kidney injury: A systemic review and meta-analysis. *J Nephrol* 28, 7–16.

Morgan P et al. (2018). Impact of a five-dimensional framework on R&D productivity at AstraZeneca. *Nat Rev Drug Discov* 17, 167–181.

Phillips JA et al. (2020). A pharmaceutical industry perspective on microphysiological kidney systems for evaluation of safety for new therapies. *Lab Chip* 20, 468–476.

Stephen L et al. (2013). Profiling of human, canine, and rat urine samples using Bio-Plex Pro RBM Kidney Toxicity Assays. *Bio-Rad Bulletin* 6400.

Tiong HY et al. (2014). Drug-induced nephrotoxicity: Clinical impact and preclinical in vitro models. *Mol Pharm* 11, 1,933–1,948.

U.S. Food and Drug Administration (2021). List of Qualified Biomarkers. [fda.gov/drugs/biomarker-qualification-program/list-qualified-biomarkers](https://www.fda.gov/drugs/biomarker-qualification-program/list-qualified-biomarkers), accessed April 4, 2023.

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