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Fall 2011

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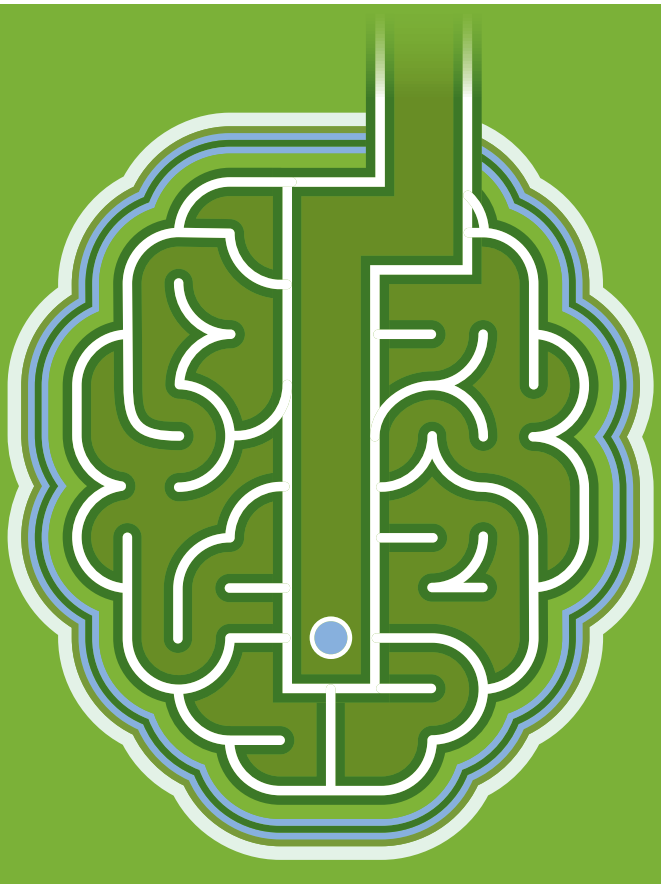
In vitro model for ligand mediated ion channel investigation	3
Automated cell signaling assays using primary human umbilical vein endothelial cells	6
4D-Nucleofector™ Y unit – enabling adherent Nucleofection™ in 24-well culture plates	10
The StellARray® system supports adherence to MIQE guidelines	12
A serum-free chemically defined medium for the expansion of adult normal human dermal fibroblasts	14
New PAGE buffers yield faster migration, improved resolution and decreased blotting time	16
An air-liquid interface culture system for small airway epithelial cells	17
Cell-based assays using biosensors in human mesenchymal stem cells	20

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In vitro model for ligand mediated ion channel investigation

By Monica Siegenthaler, Jason Sharp and Gabriel Nistor, California Stem Cell, Inc., Irvine, CA, USA

Introduction

Motor neuron (MN) diseases are a group of neurological disorders that affect the neurons located in the spinal cord that induce muscle activation. Approximately 5,600 MN disease cases are diagnosed each year in the U.S. One of these MN diseases, amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, has been extensively studied in mouse models and results suggest a role for excitotoxicity and cell death via apoptosis. In conjunction with animal studies, increased levels of fasting glutamate have been detected in the serum and plasma of people with ALS. In addition, cultured MNs are demonstrated to be susceptible to calcium dependent glutamate toxicity. The primary drug used to treat ALS, riluzole, functions by blocking the effects of the neurotransmitter glutamate. However, use of this drug is also associated with multiple side-effects and lifespan is only expanded by months. As part of an ALS Association-sponsored project (TREAT-ALS), this study is aimed to develop a high-throughput screening assay for compounds that can affect glutamate induced excitotoxicity in MNs as part of a drug discovery effort for new treatments for ALS.

Functional high-throughput and high-content screens are designed for use in drug discovery and *in vitro* predictive ADMET to evaluate compound libraries for multiple drug-cell interactions. These screens commonly test these compounds on animal or human primary cells, immortalized and/or cancerous cell lines, which might not provide an accurate indication of the effects of these compounds on normal, specific human cell types. The ability to use high-purity, normal, specific human cell types for high-throughput screening would be expected to improve the accuracy of this process for drug discovery and ADMET. Thus, the emerging alternative to these primary and immortalized or cancerous cell types is the use of high-purity, specific cell types derived from pluripotent stem cells (PSCs). PSCs can easily be expanded to meet the quantities required for drug screening and can be directed to develop into the multiple cell types used for discovery and *in vitro* ADMET.

Use of PSC-derived, high-purity MNs is expected to enable a more relevant drug screen model for discovery of potential ALS therapeutics. The MotorPlate™ is a 96-well plate format pre-seeded with high-purity (>75% TUJ1), PSC-derived human MN progenitors (hMNPs) shipped live and ready to use. There are two versions of the MotorPlate™ – standard and mature. The latter version are matured to yield adult-like human MNs. Matured hMNPs have been characterized for expression of MN specific markers, glutamate toxicity, and electrophysiological activity. Immunocytochemical characterization showed that cultures are positive for MN markers HB9 and SMI32 (Figure 1). Immunocytochemical staining with SMI32 reveals >30% expression, demonstrating matured hMNPs in culture. Functional characterization of matured hMNPs via whole-cell patch-clamp electrophysiology showed inward currents related to an influx of sodium and calcium upon application of glutamate (Figure 3). Accordingly, exposure of matured hMNPs to increased glutamate concentrations results in toxicity. This immunocytochemical and electrophysiological characterization, accompanied by glutamate sensitivity, confirms that the cells closely resemble MNs and are a suitable cell type for drug discovery of compounds that can potentially affect the excitotoxicity-induced MN cell death that is the focus of treatment for ALS.

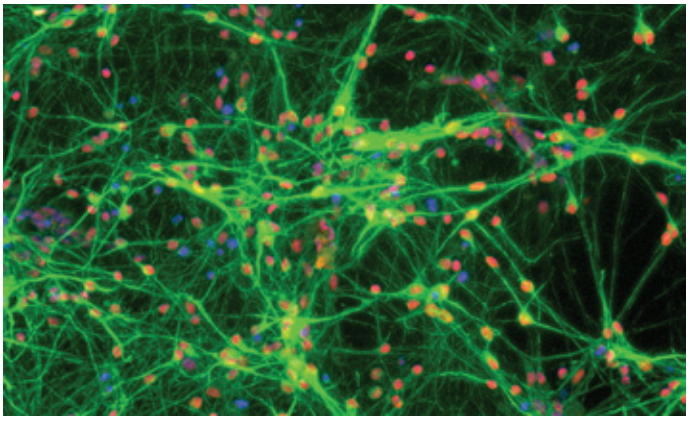


Figure 1
MotorPlate™ mature hMNPs stained with HB9 (red), SMI32 (green), and DAPI nuclear counterstain (blue). The image demonstrates the expression of motor neuron specific markers, HB9, with the expression of a more mature marker of motor neurons, SMI32.

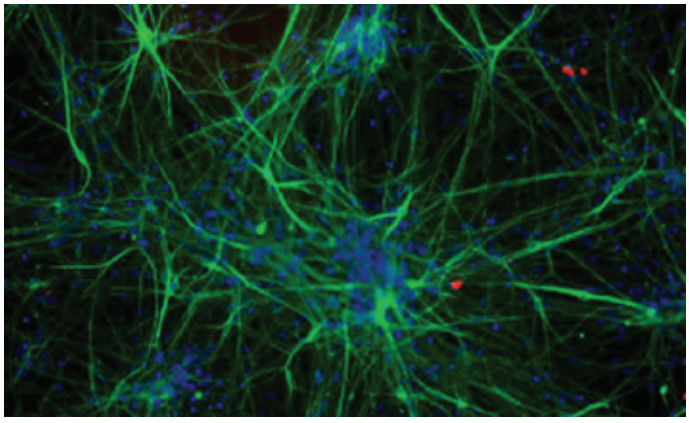


Figure 2
MotorPlate™ mature hMNPs stained with SMI32 (green), a marker of adult MNs, Annexin V (red), a marker for apoptotic cells, and Hoechst (blue) nuclear counterstain. Image presented on Siegenthaler *et al.*, 2009 poster, "Maturation of motor neuron progenitors derived from hESCs for use in a high throughput assay for ALS target discovery" at ISNR Conference.

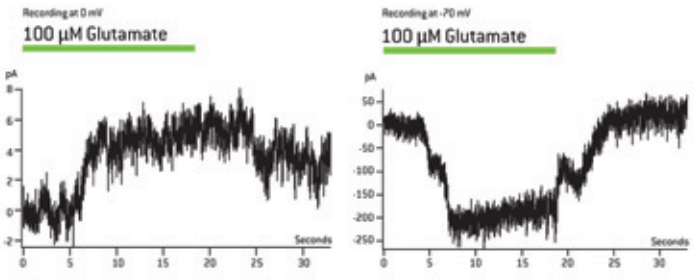


Figure 3

Patch clamp recording of glutamate exposure to MotorPlate™ mature hMNPs. In a gigaseal patch clamp system, exposure to 100 µM glutamate at 0 mV mediated a small outward current and exposure to 100 µM glutamate at -70 mV mediated a large inward current. Figure produced by BioFocus, and published in Rossi *et al.*, 2010, "Histological and functional benefit following transplantation of motor neuron progenitors to the injured rat spinal cord" PLoS ONE5(7): e11852. doi:10.1371/journal.pone.0011852.

In this study, we tested an alternate method to examine the calcium permeability of matured hMNPs that can be readily applied to the MotorPlate™ mature for screening compounds that affect this permeability and excitotoxicity. Together, these experiments demonstrate the adaptability, suitability, and feasibility of using assay ready 96-well plate formats of high-purity, PSC-derivates, and in particular MotorPlate™ mature, to screen compounds for drug discovery and *in vitro* ADMET.

Materials and methods

Glutamate stimulation of cobalt uptake

This method replaces calcium (Ca^{2+}) with cobalt (Co^{2+}) in the media such that exposure of the hMNPs to glutamate stimulates the AMPA and/or NMDA ligand-gated Ca^{2+} ion channels to open and permit the passage of Co^{2+} into the cells. The intracellular Co^{2+} ions are then precipitated and stained (Yin *et al.*, 1994, Turetsky *et al.*, 1994).

MotorPlate™ mature plates were maintained in the media formulation in which they were further matured for 2 weeks (this can also be performed using MotorPlate™ standard further matured for 5 weeks). After 2 weeks, plates were exposed to buffer containing 1,000 µM glutamate and 2.5 mM $CoCl_2$. The uptake buffer consisted in 139 mM sucrose, 57.5 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM glucose, and 10 mM hepes. Live cells were maintained in this mixture for 10 minutes at room temperature, washed in the same uptake buffer containing 2 mM EDTA to remove extracellular Co^{2+}. Immediately after wash, the live cells were exposed to 0.05% $(NH_4)_2S$ for 5 minutes to precipitate intracellular Co^{2+} and rinsed twice in phosphate-buffered saline (PBS). Finally, the cells were fixed in 4% paraformaldehyde for 20 minutes.

The intracellular Co^{2+} was visualized using a modified Timm's stain procedure as described in (Yin *et al.,* 1994). The cultures were incubated in the dark in a solution consisting of one part of solution A (0.1 M $AgNO_3$), 20 parts of solution B (2% hydroquinone and 5% citric acid in water), and 100 parts of solution C (20% gum arabic, in water). This solution was periodically refreshed for better contrast. The staining was terminated by washing in water when a good contrast existed between stained and unstained neurons.

Glutamate-mediated toxicity

MotorPlate™ mature plates were maintained in the media formulation in which they were further matured for 2 weeks. Following 2 weeks of maturation, cells were challenged to glutamate exposure at 0, 500, and 2,000 µM for 24 and 72 hours. A stock solution of 100 mM glutamic acid was diluted to either 500 µM or 2,000 µM. These dilutions, or 0 µM glutamic acid, were applied to these MotorPlate™ mature wells for 24 hours before performing cell viability assessment. Propidium iodide (PI) and Annexin V were used to visualize the dead or apoptotic cells on different plates. A stock staining solution of PI and Hoechst was made by adding 15 µl Hoechst and 15 µl PI to 720 µl PBS. For staining, 10 µl of the PI and Hoechst staining solution was added to each well for 30 minutes at room temperature. Wells were imaged immediately after the incubation using the ImageXpress® imager (Molecular Devices). A stock staining solution of Annexin V and Hoechst was made by adding 40 µl Hoechst and 100 µl Annexin V-A647 to 2,000 µl PBS. For staining, 20 µl of the stock staining solution was added to each well for 30 minutes at room temperature. Following incubation, cells were fixed with 8% PFA for 15 minutes. The cells were then washed with PBS three times for 5 minutes each. Immunocytochemical staining for SMI32 was then performed to label matured MNPs. The wells were imaged using the ImageXpress® imager (Molecular Devices). PI and Annexin values were obtained from four separate wells per glutamate concentration, averaged, and presented as a percentage of Hoechst-positive cells. Analysis was performed to determine % PI-positive cells/total Hoechst-positive cells and % SMI32-positive cells/total Hoechst-positive cells.

Results

Effects on ligand-mediated channel openings can be detected in MotorPlate™ mature using Co^{2+} staining

Although MotorPlate™ mature is amenable to most electrophysiological recording techniques, such methods tend to be low-throughput and require specialized personnel and equipment. Thus, we assessed the use of an alternative method to demonstrate the presence and functionality of glutamate-mediated Ca^{2+}-equivalent permeable channels on hMNPs.

Using this method, we found that the ligand-mediated ion channels are progressively expressed, only on very young neurons and on a majority of the neurons after about 2 weeks of maturation (Figure 4). This method demonstrates the ability to measure Ca^{2+} permeability for drug discovery in a non-electrophysiological manner in MotorPlate™ mature.

Glutamate toxicity assay in MotorPlate™ mature

Matured MNPs were tested for toxicity following exposure to glutamate. Exposing matured MNPs to increasing concentrations of glutamate for 24 and 72 hours resulted in toxicity, as evidenced by the percentage of PI-positive cells. Exposure to increasing concentrations resulted in increasing amounts of MNP death. The percentage of PI-positive cells was 11.36 +/- 1.25% for 0 µM glutamic acid, 9.68 +/- 2.2% for 500 µM glutamic acid, and 17.47 +/- 1.3% for 2,000 µM glutamic acid (Figure 5). There was also an increase in PI-positive cells with duration of exposure, as is evidenced in the images in Figure 5. The percentage of Annexin V-positive cells was not significantly different ($p > 0.01$) than the percentage of PI-positive cells.

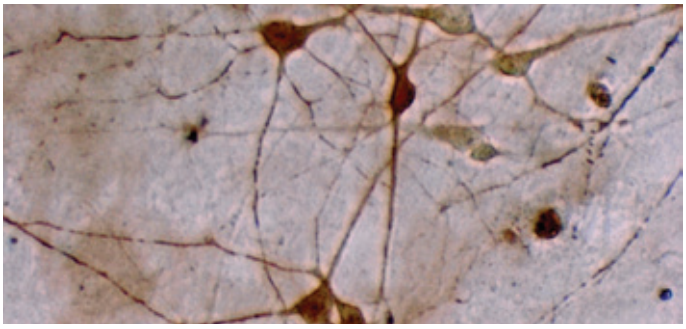


Figure 4

Timm's staining of MNPs exposed to glutamate in Co^{2+} solution. The darker staining is equivalent to increased ligand mediated ion channel expression, therefore with more mature stage of neuronal development. In the same picture, less intense staining of intermediate maturity or no staining (phase contrast only) of immature neurons can be observed.

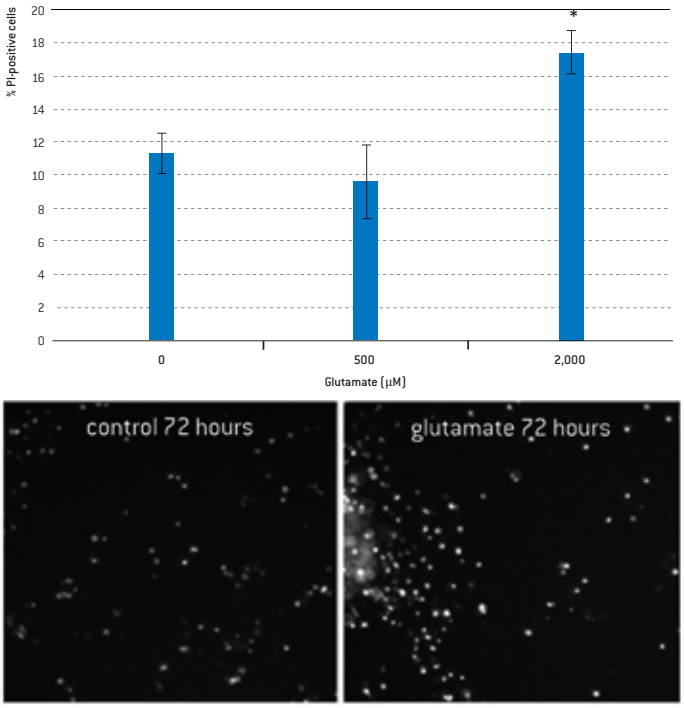


Figure 5

Propidium iodide (PI) staining after glutamate exposure. Cell death in motor neuron cultures challenged with 0, 500, and 2,000 µM glutamate. Cell death was measured by automated counting of PI-positive cells using the IN Cell Analyzer 1000 imaging platform. Bars represent mean ± s.e.m. from n=4 wells per condition. The asterisk indicates significant difference from control, 0 µM (Student's T-test; $p < 0.05$). Figure generated by BioFocus, and is representative of data reported in Rossi *et al.*, 2010, "Histological and functional benefit following transplantation of motor neuron progenitors to the injured rat spinal cord" PLoS ONE5(7): e11852. doi:10.1371/journal.pone.0011852.

Conclusion

MotorPlate™ mature is one of two PSC-derived high-purity, specific, human cell-type plates offered by Lonza. High-purity, human cell plates in a live, ready-to-use format can allow for the most rapid and robust results from high-throughput drug discovery and *in vitro* ADMET assays. The results shown here, directly demonstrate the applicability of MotorPlate™ products for ALS drug discovery targeting glutamate excitotoxicity. In addition, through the use of alternative methods, this study demonstrates the ability to adapt a low-throughput technique, such as electrophysiology, to a higher-throughput staining method to examine ion permeability using MotorPlate™ mature MNPs.

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Automated cell signaling assays using primary human umbilical vein endothelial cells

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Introduction

The serine/threonine kinase AKT, also known as protein kinase B (PKB), mediates cell survival, therefore inhibiting apoptosis, or cellular death. It also regulates many cellular functions, such as cell proliferation and differentiation, cellular migration, glucose and intermediary metabolism, and transcription. Additionally, AKT induces protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy, tumor development and general tissue growth. Its implication for cancer research and that of other disease states makes AKT an important target for drug discovery campaigns.

AKT is a key downstream intracellular convergence point for many cell signaling pathways. These diverse signaling pathways are activated by growth factors including vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1). One or more signaling pathways may be abnormally activated in cancer patients, resulting in deregulated cell proliferation, tumor angiogenesis, and abnormal cell metabolism.

Here we demonstrate a homogeneous HTRF® phospho-AKT (Ser473) assay from Cisbio US (Bedford, MA) for measuring endogenous AKT phosphorylation at serine residue 473 (Ser473) using Lonza primary human umbilical vein epithelial cells (HUVECs). Primary cells are neither altered nor transformed, for less risk of functional or phenotypical changes compared to cell lines. Endothelial cells, such as these, provide robust and biologically relevant data for angiogenesis and cancer development, macromolecule and cell adhesion and transport, clotting, cell signaling pathway analysis, and other pharmacological studies. HUVECs, lining the umbilical vein, serve as the selective barrier between circulating blood and the underlying smooth muscle, and as they closely simulate *in vivo* environments, they are well suited for *in vitro* cellular research. Additionally, cells from multiple donors can be analyzed, allowing for a good estimation of physiological responses across human populations.

Validation and pharmacology data demonstrate that the combination of the Cisbio assay, BioTek instrumentation, and Lonza primary cells are sufficiently sensitive to detect endogenous phosphorylation of this key signal transduction pathway in a system applicable for automated, high-throughput screening.

Materials and methods

Materials

Cryopreserved HUVEC (Cat. No. CC-2517, Lot 152470), EBM™ (endothelial basal medium) (Cat. No. CC-3121), and EGM™ SingleQuots™ (Cat. No. CC-4133) were attained from Lonza. Albumin (human), USP, 25% Solution (Cat. No. 1500233) was purchased from Baxter. Collagen, type I (Cat. No. 354236) was purchased from BD Biosciences. Recombinant human VEGF 165 (Cat. No. 293-VE-010/CF) was purchased from R&D Systems. PI-103 hydrochloride (Cat. No. 2930) and LY294002 hydrochloride (Cat. No. 1130) were purchased from Tocris Bioscience. The HTRF® phospho-AKT (Ser473) kit (Cat. No. 64AKSPEG) was attained from Cisbio US. 96-well Clear, Flat Bottom, Polystyrene TC-treated Microplates (Cat. No. 3598) and 384-well Low Volume, White, Round Bottom, Polystyrene Non-treated Microplates (Cat. No. 3674) were attained from Corning Life Sciences. The EL406™ microplate washer dispenser, and Precision™ automated microplate pipetting system, as well as the Synergy™ H4 hybrid multi-mode microplate reader were donated from BioTek Instruments.

Methods

Assay principle

The HTRF® phospho-AKT (Ser473) assay (Figure 1) is designed to detect and study activated AKT directly in whole cells, and is based on a sandwich immunoassay principle. Upon receptor activation, the kinases are subsequently activated, thus phosphorylating AKT kinase. After cell membrane lysis, an anti-kinase d2 labeled monoclonal antibody (Anti AKT-d2) and an anti-phospho-kinase Eu(III)-cryptate labeled monoclonal antibody (Anti pAKT-K) are introduced. Upon Eu(III)-cryptate excitation, phosphorylated AKT transfers energy to the d2 molecule, and emission is detected at 655 nm. In the absence of phosphorylated AKT, no energy is transferred, and emission from the Eu(III)-cryptate is detected at 620 nm.

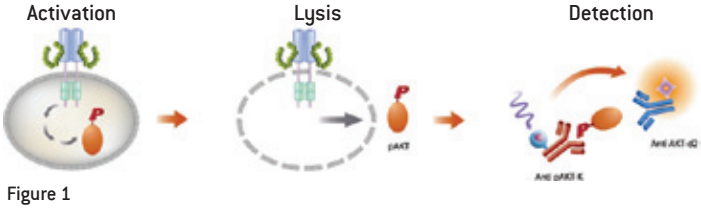


Figure 1

Cisbio HTRF® phospho-AKT (Ser473) assay. Surface receptor stimulation leads to phosphorylation of AKT within the cell. Following cell lysis, Anti pAKT-K antibody binds phospho-AKT. When excited at 330 nm, this antibody transfers energy to Anti AKT-d2 which is detected at 655 nm.

Instrumentation

The Precision™ was used for all serial titrations, compound transfers and 96-well to 384-well aliquot transfers. Cell, agonist EC_{80} and HTRF® assay component dispensing, media removal and plate washing were performed with the EL406™. Assay data was detected and analyzed using the HTRF®-certified Synergy™ H4.

Cell processing

Cryopreserved HUVEC were thawed, resuspended in complete Clonetics™ EGM™ endothelial cell growth medium, and propagated in culture flasks until the cells reached 60 - 70% confluency. Cell concentrations of 6.5×10^4 cells/ml were then plated in 200 µl aliquots into clear, collagen-coated, tissue culture-treated 96-well microplates and incubated at 37°C/5% CO_2 for 40 hours. The complete media was then removed, the cells were washed with 200 µl DPBS wash, 100 µl of serum starvation media (EBM™ with 0.1% human serum albumin (HSA)) was added, and the cells were again incubated for 4 hours at 37°C/5% CO_2.

Assay procedure

Agonist assay (Figure 2A): 50 µl of 3X titrated agonist was added to the 96-well plate, and incubated for 10 minutes at 37°C/5% CO_2. Media was removed from the well, 50 µl of 1X lysis buffer was added, and the plate was incubated for 20 minutes at room temperature on a plate shaker set at 750 - 1,000 RPM. Following incubation, 16 µl aliquots were transferred from the 96-well cell plate to an LV384-well assay plate. 4 µl of detection buffer containing the Anti AKT-d2 and Anti pAKT-K antibodies were then added to the well, the plate was covered, and incubated at room temperature for 4 hours. The fluorescent signal was then read using: 330 nm (Ex)/620 (Em)/665 (Em).

Antagonist assay (Figure 2B): 25 µl of 6X titrated antagonist was added to the 96-well plate, and incubated for 60 minutes at 37°C/5% CO_2. 25 µl of 6X agonist (EC_{80}) was added to the 96-well plate, and incubated for 10 minutes at 37°C/5% CO_2. The remainder of the procedure is as described for the agonist assay.

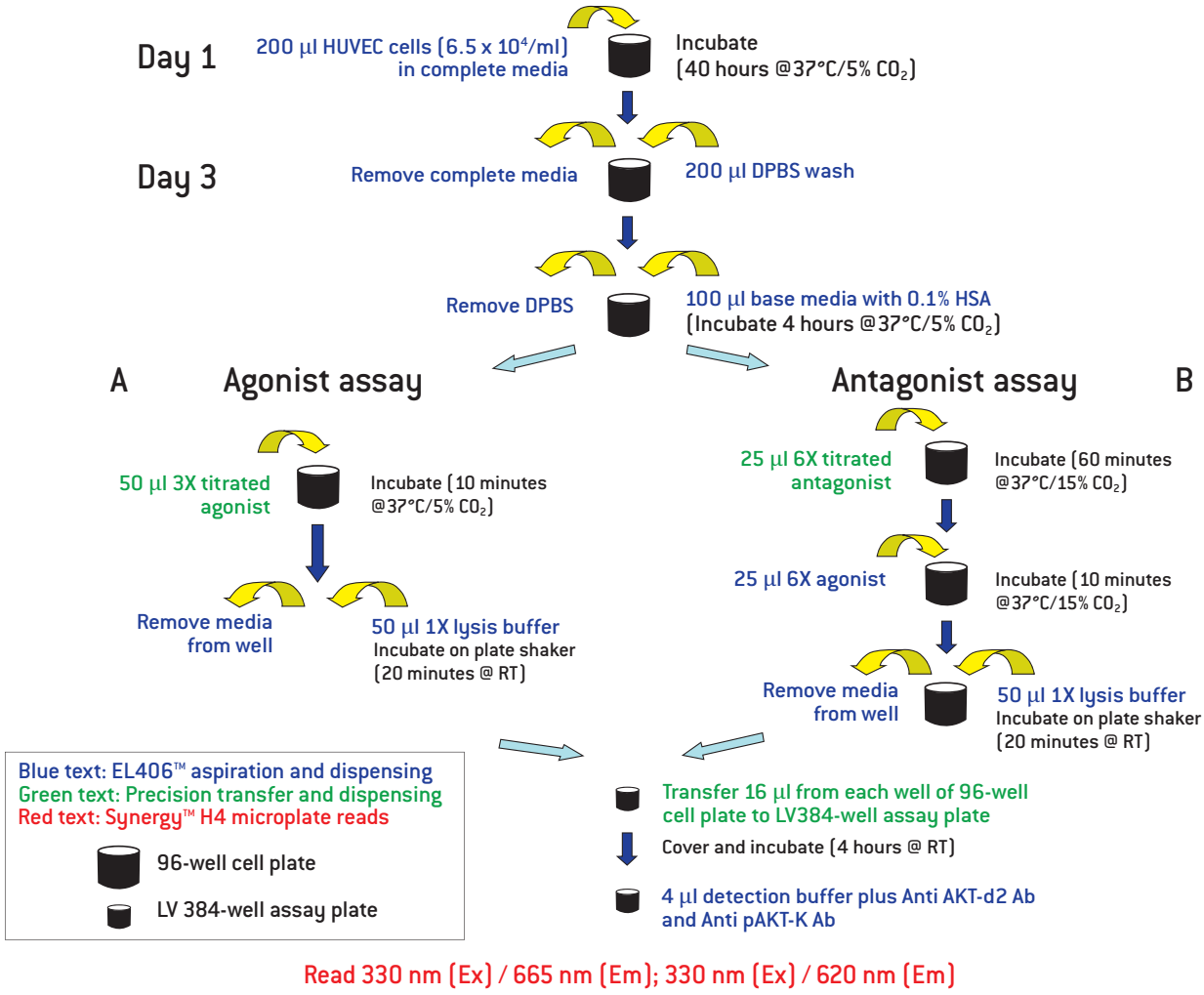


Figure 2

Schematic of the optimized automated assay procedure. The plates (96- or 384-well) used are indicated by well cartoon size, and the instruments used are color coded.

HTRF® data reduction
The HTRF® ratio for each cell containing well was calculated using the following formula:
Ratio = ((665 em/620 em)*10,000)
A negative control was also run on the plate containing 1X lysis buffer and the two HTRF® antibodies, only. The data was normalized to eliminate plate-to-plate variations by determining the Delta F (ΔF) calculation. This value was determined using the following formula:
$\Delta F = ((Ratio_{(cell\ containing\ well)} - Ratio_{(negative\ control)})/Ratio_{(negative\ control)})$

Results and discussion

Assay optimization conditions

HUVEC were stimulated with VEGF, using concentrations of 0, 1, 10, and 50 ng/ml. Stimulation times used were 3, 10, 30, and 60 minutes. Per the HTRF® and ΔF ratio values, and as seen in Figure 3A, it is apparent that the best stimulation of AKT phosphorylation was obtained after the 10-minute incubation with 10 ng/ml of VEGF in this non-automated assay optimization experiment.

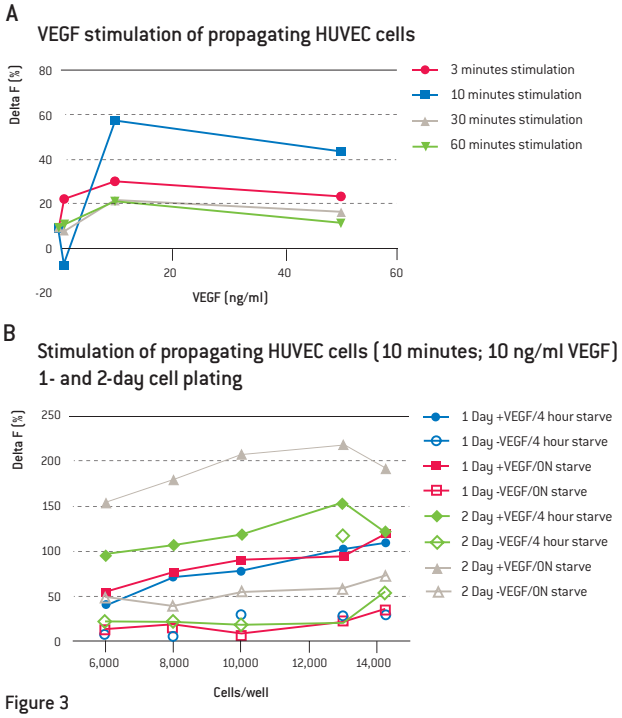


Figure 3

HTRF® phospho-AKT assay optimization results. A. Different concentrations of VEGF were added to HUVECs for times between 3 and 60 minutes, and phospo-AKT was measured as described in the Methods section. **B.** 13,000 HUVECs were plated in each well and allowed to proliferate for 16 (1 day) or 40 (2 days) hours followed by a change to serum starvation media for 4-hour or overnight incubation prior to assay of AKT phosphorylation.

In order to further optimize the assay conditions, the process was repeated using 16- and 40-hour cell incubations in complete media (EGM™), and 4-hour and overnight incubations using serum starvation media (EBM™ with 0.1% HSA). The optimal 10 ng/ml VEGF concentration and 10-minute stimulation incubation were applied, and HTRF® and ΔF ratio values were again calculated. It is apparent that the ΔF ratio for wells containing VEGF-stimulated cells increases with longer plating times and higher cell concentrations (Figure 3B). Only at the highest cell concentrations and using a 2-day plating, do the ΔF ratios decrease. The data also show that unstimulated well ΔF ratios are stable for the first three conditions tested. Additionally, the ΔF ratios increase 2 - 3-fold using a 2-day plating and overnight serum starvation media incubation.

Since stimulated cell ΔF ratios increase with longer plating times, and ΔF ratios do not increase for a 2-day plating with 4-hour serum starvation media incubation, the optimal conditions for greatest fold stimulation of AKT phosphorylation are plating 13,000 cells/well, incubating them in complete media for 40 hours followed by a serum starved media incubation of 4 hours (Figure 3B, filled green diamonds). Despite the high ΔF ratios obtained for VEGF-stimulated cells following 2-day plating and overnight incubation in starvation media (Figure 3B, filled red triangles), the corresponding unstimulated control cells also displayed an increased ΔF ratio relative to the other control conditions, which results in an overall lower apparent VEGF stimulation of AKT phosphorylation.

The conditions determined in the assay optimization were carried forth in the remaining validation experiments. A schematic showing the steps of the automated assay and the instruments used for each step is shown in Figure 2.

Automated assay validation

A Z'-factor assay was performed to validate the automated procedure. The assay was run in antagonist mode, with 25 µl PI-103 (6X) added to each well as the control antagonist. After a 60-minute incubation at 37°C/5% CO_2, 25 µl of 6X VEGF was added to a final concentration of 10 ng/ml in each well as the control agonist, and the microplate was incubated for an additional 10 minutes. 40 replicates of 0 µm or 10 µm PI-103 (1X) were used as the positive and negative control, respectively. The remainder of the antagonist procedure was carried out as previously described.

Automated HTRF® assay Z'-factor validation

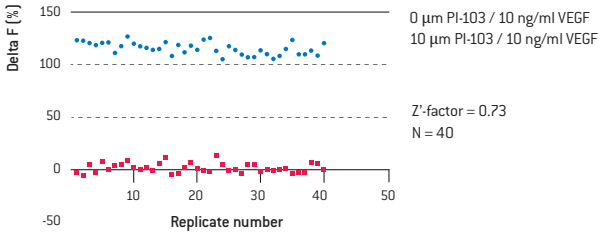


Figure 4

Automated Z'-factor assay validation data. VEGF (10 ng/ml) was used as the control agonist, and PI-103 was used as the antagonist. The automated assay was performed as described above using BioTek instruments for sample handling, processing and reading. Z' was calculated as described[1].

The data in Figure 4 indicate that the calculated Z'-factor equals 0.73, which correlates to excellent assay robustness as values greater than 0.5 are indicative of an excellent assay[1].

Assay pharmacology validation

The automated assay was further validated by creating an agonist dose response curve with VEGF. The assay was performed with 50 µl of VEGF concentrations ranging from 40 - 0 ng/ml added to the wells, and the remainder of the agonist procedure being performed as previously described.

Automated HTRF® assay agonist dose response

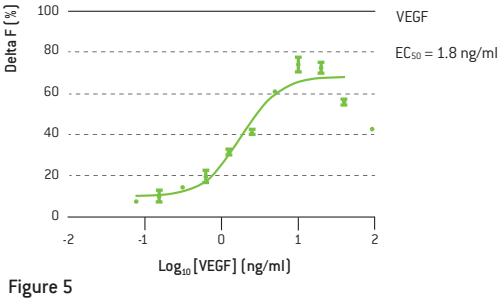


Figure 5

VEGF agonist validation. A dose response curve for VEGF activation of AKT phosphorylation was generated using the automated assay format. The data were fit to a Sigmoidal dose response (variable slope) curve using GraphPad Prism V. 5.01 to generate an EC_{50} value.

An EC_{50} value of 1.8 ng/ml was obtained from the data in Figure 5, and compares favorably to published EC_{50} values ranging from 1 - 6 ng/ml[2].

Antagonist dose response curves were also generated using PI-103 and LY294002 with concentrations of 10 - 0 µm and 100 - 0 µm, respectively. VEGF, at a 6X EC_{80} concentration of 60 ng/ml, was used as the agonist for the experiment.

The calculated IC_{50} values were 5.2 nm and 332.6 nm for PI-103 and LY294002, respectively (Figure 6). These values compare favorably to published IC_{50} values of 11 nm[3] for PI-103 and 310 and 730 nm for LY294002[4,5].

Automated 2-plate assay compound inhibition curves

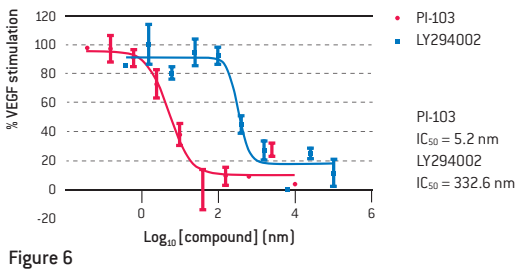


Figure 6

Antagonist validation. PI-103 (red circles) and LY294002 (blue squares) were used to generate antagonist dose response curves in the automated assay of AKT phosphorylation. The data were fit to a Sigmoidal dose response (variable slope) curve using GraphPad Prism V. 5.01 to generate IC_{50} values.

Summary

Primary HUVECs are an excellent cell type in which to measure the effects of pharmacologically-important compounds on vital signaling pathways, and they provide increased data relevancy compared to cancer cell lines. The Cisbio HTRF® phospho-AKT (Ser473) assay effectively monitors PI-3 kinase and VEGF receptor cell signaling pathways in these cells. The assay may be automated using a 2-microplate protocol where pathway stimulation or inhibition is carried out in a 96-well microplate, and then cell lysates are transferred to a 384-well microplate for assaying. The Synergy™ H4 reader is sensitive enough to accurately detect signal changes caused by endogenous kinase phosphorylation events. Automation of the assay provides excellent robustness, and appropriate inhibition pharmacology is evident at the PI-3 kinase level. Finally, the combination of cells, assay and instrument provide a unique and automatable method to measure kinase signaling pathways.

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4D-Nucleofector™ Y unit – enabling adherent Nucleofection™ in 24-well culture plates

By Andrea Toell, Gerhard Muster, Katrin Hoeck, Lonza Cologne GmbH, Cologne, Germany

Adherent Nucleofection™

Adherent Nucleofection™ was introduced in 2010 with the invention of the 16-well Nucleocuvette™ AD strips which can be used in the 4D-Nucleofector™ X unit or the 96-well Shuttle™ add-on. This overcame a general obstacle of electroporation-based methods, i.e., the requirement of cells to be in suspension for transfection, by allowing Nucleofection™ of cells in adherence. Primary neuronal cells, which typically grow in adherence in cell culture, can now be kept in their physiological state and transfected by Nucleofection™ at a later developmental stage. This new feature also facilitates the use of the Nucleofector™ technology for cryopreserved Clonetics™ primary neurons which are quite sensitive and do not "like" to be transfected directly after thawing. Now they can be thawed and cultured for a certain time frame before transfection.

NEW: 4D-Nucleofector™ Y unit with 24-well dipping electrodes

The 4D-Nucleofector™ system is now extended by a new functional module, the Y unit (Figure 1, bottom) which enables adherent Nucleofection™ in 24-well culture plates. It can be used in addition or alternatively to the X unit.



Figure 1
4D-Nucleofector™ system with
- Core unit (top) controlling the system
- X unit (middle) for 100 µl single Nucleocuvette™ or 20 µl Nucleocuvette™ strips
- Y unit (bottom) for adherent Nucleofection™ in 24-well culture plates

The 4D-Nucleofector™ Y unit works with disposable conductive polymer **24-well dipping electrode arrays** (Figure 2). This array, together with a Y unit specific Nucleofector™ solution, supplement and pmaxGFP™ control vector, is part of the 4D-Nucleofector™ Y unit kits.
A first available kit, the **AD1 4D-Nucleofector™ Y kit**, is suited for adherent Nucleofection™ of primary neural cells. In combination with a "basic" neuron protocol, one can easily determine the optimal conditions for a broad range of primary neurons, glial cells or neural stem cells. Optimal Nucleofection™ conditions provide transfection efficiencies between 20% and 70% depending on the neuron type, the quality of the culture and the culture duration.



Figure 2
24-well dipping electrode array for insertion into a 24-well culture plate.

Application - Adherent Nucleofection™ of primary neurons in 24-well culture plates

Prior to Nucleofection™, neural cells are cultured in standard 24-well culture plates with or without cover slips. For Nucleofection™, a lid with an array of 24 dipping electrodes is inserted into the 24-well culture plate. The plate-lid sandwich is then transferred into the Y unit. Each well can be addressed individually.

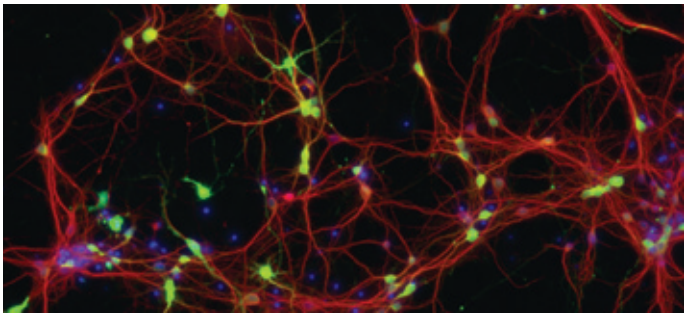


Figure 3
Efficient adherent Nucleofection™ of neurons in 24-well culture plates. Mouse cortical neurons were isolated and seeded into poly-D-lysine coated 24-well plates at a density of 1 x 10^5 cells/well. After 6 days, cells were transfected with 17.5 µg pmaxGFP™- vector using the AD1 4D-Nucleofector™ Y kit and program ER-137. One day post Nucleofection™, cells were stained by MAP2 antibody (red) and analyzed by fluorescence microscopy for maxGFP™ protein expression.

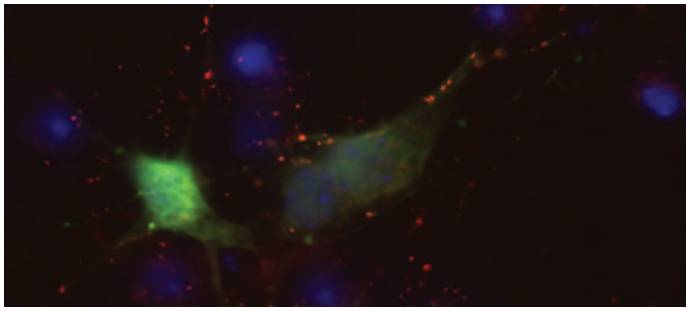


Figure 4
Preserved functionality after adherent Nucleofection™ of neurons. Mouse cortical neurons were isolated and seeded into poly-D-lysine coated 24-well plates at a density of 1 x 10^5 cells/well. After 6 days, cells were transfected with 17.5 µg pmaxFP™-yellow-C vector using the AD1 4D-Nucleofector™ Y kit and program EH-166. 4 days post Nucleofection™, cells were stained for maxFP™-yellow expression (green), synapses (red) and nuclei (DAPI, blue) and analyzed by fluorescence microscopy. Transfected neurons show normal synapse formation.

Neurons transfected with this new Nucleofection™ format show high transfection efficiencies (Figure 3) and keep their phenotypic properties (Figure 4).

Summary

Adherent Nucleofection™ can now be performed in two different ways (Table 1):

- The new 4D-Nucleofector™ Y unit which works with disposable conductive polymer dipping electrode arrays that can be inserted into standard 24-well culture plates for the Nucleofection™ process. This format is recommended when cells are analyzed by confocal microscopy or when high cell numbers are required.
- The 4D-Nucleofector™ X unit (or the 96-well Shuttle™ add-on) that utilizes specialized 16-well Nucleocuvette™ AD strips (20 µl) in which neural cells can be cultured for up to 14 days and transfected at any time during this period. This format is suited for applications requiring low cell numbers analyzed by fluorescence microscopy, absorption, fluorescence or luminescence assays.

	Nucleocuvette™ AD strips (20 µl)	Dipping electrode arrays
Required functional 4D-Nucleofector™ unit	X unit or 96-well Shuttle™	Y unit
Pre- and post-Nucleofection™ culture	Nucleocuvette™ wells	24-well culture plate
Nucleofection™ of cells plated on glass cover slips		■
High transfection efficiencies and viabilities	■	■
Cell analysis by		
– Transmission light or fluorescence microscopy	■	■
– Absorption, luminescence or fluorescence assays	■	■
– Confocal microscopy		■
– Patch clamping		■
Nucleofection™ of cryopreserved Clonetics™ primary animal neurons	■	■

Table 1
Overview of adherent Nucleofection™ platforms.

Benefit from

- Culturing of neural cells in standard 24-well culture plates prior to Nucleofection™
- Nucleofection™ of primary neural cells including cryopreserved Clonetics™ neural cells in adherence after several days of culture, i.e., at a later developmental stage
- Non-viral transfection of primary neural cells with up to 70% efficiency
- Preservation of functionality

Ordering information

https://shop.lonza.com

Cat. No.	Description	Size
AAF-1001B	4D-Nucleofector™ core unit	
AAF-1001Y	4D-Nucleofector™ Y unit	
AAF-1001X	4D-Nucleofector™ X unit (for suspension Nucleofection™ or adherent Nucleofection™ in 16-well strips)	
V4YP-1A24	AD1 4D-Nucleofector™ Y kit	24 reactions

www.lonza.com/adherent-nucleofection
www.lonza.com/celldatabase - Find data for different platforms and neuron-specific protocols.

The StellARray® system supports adherence to MIQE guidelines

By Martina Reiter, Lonza Cologne GmbH, Cologne, Germany

Introduction

The increasing number of citations of the MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments, Bustin *et al.,* 2009) demonstrates a growing emphasis on standardized experimental practice for qPCR.

Lonza's SYBR-based StellARray® qPCR array system offers a simple and reliable system for gene expression analysis that meets the MIQE standards and makes it easier for qPCR users to compare and publish their results.

Simple and reliable achievement of MIQE standards

qPCR target and oligonucleotide information

The amplicon context sequence is available for each well of both custom and pre-designed StellARray® plates, so MIQE requirements like gene symbol, accession number and amplicon length are provided.

qPCR validation

Each and every primer used in a StellARray® plate, both standard and custom, undergo rigorous wet-lab quality control, so the melt curve of every primer has been checked. No NTC is needed. Additionally, the limit of detection, calibration curve and PCR efficiency can all be calculated with the information and free software provided with the StellARray® system.

Data analysis

The latest version of Lonza's data analysis software, GPR 2.0, provides features such as: A report on normalizer genes selected for analysis, information on Cq values for every gene on plate, gDNA contamination scores and conventional ΔΔCT analysis using 18S rRNA as a normalizer or reference gene. StellARray® users can also analyze data using the Global Pattern Recognition™ software, which provides a true statistical analysis of results based on consistency in the data. Global Pattern Recognition™ software globally positions the expression level of each gene with respect to all genes within an experiment. This can be done without prior assumption that a gene (normalizer) has an invariant expression level. The Global Pattern Recognition™ software is unbiased in that it enables the experimental data to define the invariant normalizer genes, not the experimenter. The use of any gene as a potential normalizer also maximizes the use of the limited real-estate on a StellARray® plate by eliminating the loss of wells used to contain predefined normalizers.

Essential MIQE guideline:	StellARray® provides:
qPCR target information	
Gene symbol	Provided online
Sequence accession number	Amplicon context sequence provided
Amplicon length	Amplicon context sequence provided
In silico specificity screen	Performed for every primer
Location of each primer	Amplicon context sequence provided
Which splice variants are targeted?	In amplicon context sequence
qPCR oligonucleotides	
Primer or amplicon context sequence	Amplicon context sequence provided
Manufacturer of oligos	Integrated DNA technologies
Purification method	Salt free
qPCR validation	
Specificity (gel, sequence, melt)	Pre-validation qPCR run for each array and melt curve checked
Cq of NTC	No NTC necessary on plate because primers are pre-validated
Calibration curve with slope and y intercept r^2 of calibration curve linear dynamic range	Gene regulations are calculated via relative quantification; no calibration curve on the array is needed
PCR efficiency calculated from slope	Slope can be generated with analysis tools provided
Cq variation at LOD	Not more than 5% within replicates
Evidence for LOD	Can be calculated with data provided
Data analysis	
qPCR analysis program	GPR data analysis tool
Method of Cq determination	Provided by instrument manufacturer
Outlier identification and disposition	Predefined cycle cutoff (Cq = 37.5)
Results for NTC	Can be calculated with data provided
Justification of number and choice of reference genes	Ten most stable reference genes are proofed via ANOVA-based normalizer calculation (via GPR)
Description of normalization method	GPR algorithm (Akilesh *et al.,* 2003)
Number and stage of technical replicates	Dependent on your experiment setup
Repeatability (intraassay variation)	Can be calculated with data provided; SD for Cq variance or copy number variance
Statistical method for results significance	Standard T-test
Software (source, version)	Provided by instrument manufacturer

Conclusion

Since its publication, the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" or MIQE has become an accepted standard by the scientific community for performing qPCR, and the use of the technique is becoming standard practice among molecular biology labs everywhere. Lonza is committed to helping researchers meet these standards. Request a free sample and see what MIQE-compliant StellARray® can do for you:

- **High amplification specificity** due to extensive wet-lab testing and proprietary immobilization of PCR primers
- **Accurate and convenient data analysis** by conventional ΔΔCT analysis or by using GPR 2.0, Lonza's new, free online analysis tool
- **Target the genes you want** with Lonza's broad panel of >150 research area-specific qPCR arrays in 96- and 384-well formats

Find the gene panels most relevant for your research area from more than 150 standard pathway or disease area-specific StellARray® qPCR arrays in 96-well and 384-well plate formats.

- Allergy
- Angiogenesis
- Blood disease
- Cancer
- Cardiovascular disease
- CNS disorder
- Developmental biology
- Immune disorder
- Immunology
- Infectious disease
- Mental disorder
- Metabolism
- Obesity
- Signal transduction
- Stem cells
- Toxicology
- Wound healing

array.lonza.com – Find more information or request your free sample unit of StellARray® plates.

BioResearch

Lonza

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Wednesday, 14 September 2011
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Future webinars:

27, 28 September	Clonetics™ primary sensors - Measuring calcium and cAMP signaling from endogenous targets in primary cells in high-throughput formats
18, 19 October	Tools for biologically relevant results in diabetes research
15, 16 November	Tools for biologically relevant results in respiratory disease research

A serum-free chemically defined medium for the expansion of adult normal human dermal fibroblasts

By Theresa D'Souza, Karen Roderick, Amy Walde and Alexis Bossie
Therapeutic Cell Solutions, Lonza Walkersville, Inc., Walkersville, MD, USA

Dermal fibroblasts are a vital component of the skin, producing various cytokines and chemokines that promote the growth of keratinocytes, affecting formation of extracellular matrices and promoting wound repair. *In vitro* expanded dermal fibroblasts are used clinically for acute and chronic wound repair and as a source of collagen for cosmetic surgery. Traditionally, *in vitro* cell culture medium contains animal-derived products like serum, tissue extracts or hydrosylates. They are also a source of undesirable factors, such as endotoxin, and pose potential BSE risks. The undefined and variable nature of these components is a challenge in basic research and applied biomedical sciences. Lonza has developed a serum-free chemically defined, fibroblast growth medium (TheraPEAK™ FGM-CD™ medium), to propagate primary adult normal human dermal fibroblasts (NHDF). The characteristics of NHDFs from several donors that were cultured in FGM-CD™ medium were compared with cells grown in serum-containing medium. The properties of NHDFs in FGM-CD™ medium grown on different plastic surfaces were also examined. Key factors of wound repair, such as cell migration and extracellular matrix production by dermal fibroblasts in FGM-CD™ medium, were also examined. Replacing serum-containing medium with TheraPEAK™ FGM-CD™ medium for the *in vitro* expansion of fibroblasts provides a more defined environment for pharmacological manipulations for research studies and for clinical applications using fibroblasts.

Materials and methods

Cell culture

Clonetics™ NHDFs (Lonza) were thawed and processed according to the published protocol. Cells were seeded directly in TheraPEAK™ FGM-CD™ medium or DMEM with 10% FBS (Lonza). NHDFs were plated either in T25 flasks, 6-well or 48-well plates, depending on the assays.

Cell proliferation

Population doublings were assayed from cells grown in flasks by trypan blue exclusion.

Immunofluorescence (IF) and flow cytometry (FACS)

For both direct IF and FACS, control antibodies used were mouse isotype IgG1κ (Sigma) and phycoerythrin (PE) mouse IgG1κ (BD Biosciences). The primary antibodies used were mouse monoclonal (5B5) to fibroblast and collagen I (Abcam) and PE mouse anti-human CD90 (BD Biosciences). The secondary antibody, goat anti-mouse Alexa Fluor® 555 (Invitrogen), was used to label 5B5 and collagen I. Briefly, for IF, cultured NHDFs in 48-well plates were fixed with methanol, washed with PBS and stained with primary and secondary antibodies. NHDFs collected from T25 flasks were fixed and permeabilized with paraformaldehyde and methanol, followed by staining with the antibodies. The expression of 5B5 and CD90 was measured using a FACSort™ flow cytometer and analyzed using CellQuestPro™ software.

Wound scratch and collagen assays

For the scratch assay, a confluent NHDF monolayer was wounded using a sterile 200 µl pipette tip. For baseline, the scratch was imaged immediately (0 hours) and after 24 hours at a reference point, using a Zeiss inverted light microscope. The wound healing was determined by measuring the gap of the scratch at the two time points using the Image J program. Collagen assays were performed using the picric Sirius Red method. Briefly, NHDFs grown to confluence were washed with PBS and pelleted. Cell pellets were incubated with 0.5 N acetic acid overnight at 4˚C. 1 ml of dye reagent composed of picric acid and Sirius Red (Sigma) was added to the pellet or collagen standard, and mixed at room temperature for 30 minutes. After centrifugation for 5 minutes, supernatants were discarded. The bound dye was released with 0.5 M NaOH. The 200 µl solution was transferred to a 96-well plate and absorbance at 540 nm was read using SpectraMax® M5 microplate reader (Molecular Devices).

Results

NHDF expansion in TheraPEAK™ FGM-CD™ medium

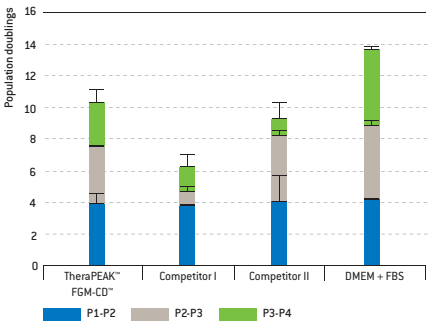


Figure 1A
NHDFs grown in TheraPEAK™ FGM-CD™ medium outperform the cells grown in competitors' serum-free dermal fibroblast media. The total population doubling yield was 75% to serum-containing medium (and significantly better than both competitors at passage 3, $p < 0.05$). The fibroblasts were fed every 3 days and passaged at 80% confluence. Data shown is an average of three donors.

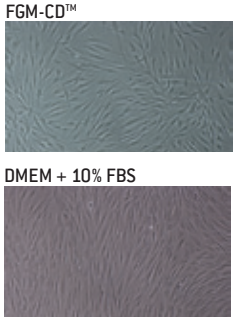


Figure 1B
Morphology of NHDFs in TheraPEAK™ FGM-CD™ medium. Cells grown in FGM-CD™ medium (shown here at passage 2, day 7) are spindle-shaped, a characteristic trait of fibroblasts.

Growth of NHDF in FGM-CD™ medium on different flask surfaces

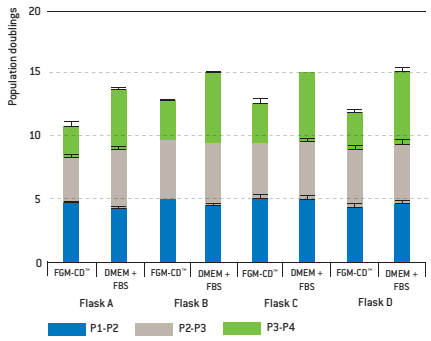


Figure 2
Population doublings of NHDFs grown in (A) TheraPEAK™ FGM-CD™ medium and (B) DMEM + 10% FBS on different T25 flask surfaces were assessed for 3 passages. Growth and morphology were similar across all surfaces.

Collagen synthesis by NHDF in FGM-CD™ medium

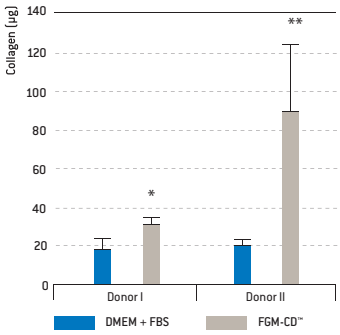


Figure 5
Collagen detection by the picric Sirius Red method. The collagen production in the NHDFs grown in FGM-CD™ medium is significantly greater than for cells grown in serum-containing medium (* $p < 0.05$, ** $p < 0.001$).

Immunofluorescence staining of NHDF in FGM-CD™ medium

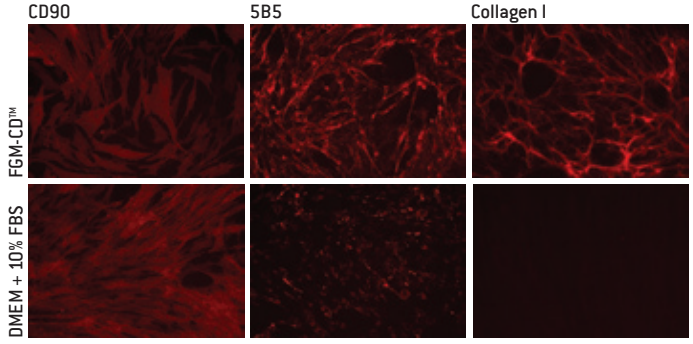


Figure 3
Representative immunofluorescence micrographs show similar expression of CD90 when grown in TheraPEAK™ FGM-CD™ medium or serum-supplemented medium. NHDFs show immunoreactivity to fibroblast marker 5B5 in both media. NHDFs in FGM-CD™ medium show marked increase in type I collagen.

Migration of NHDFs in a scratch assay

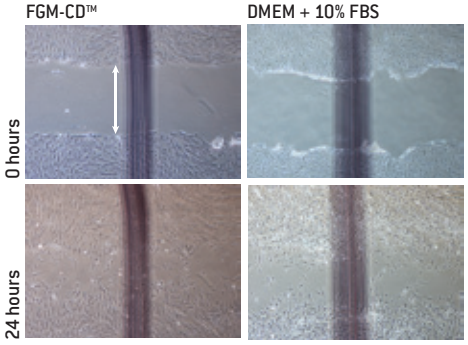


Figure 6A
Scratch assay micrographs: the wounded monolayer was imaged at time 0 hours and 24 hours and the distance measured at the reference point (arrow, dark lines).

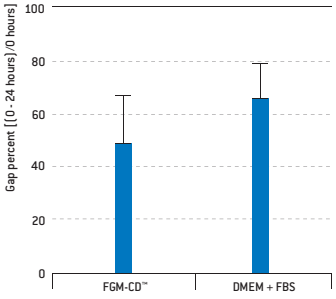


Figure 6B
Cell migration is shown by the gap percent calculated by the difference (0 - 24 hours)/distance at 0 hours. There is no significant difference in migration between cells in FGM-CD™ medium and DMEM with FBS (n=3).

Expression of CD90 and 5B5 by NHDF in FGM-CD™ medium

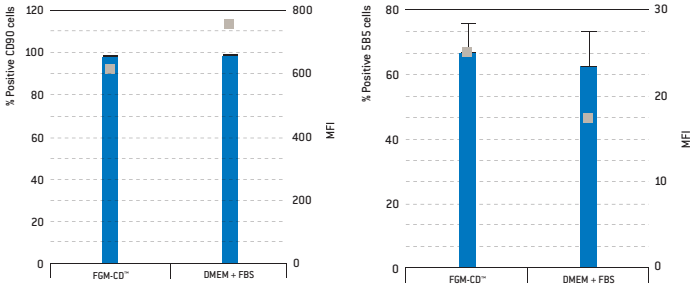


Figure 4
FACS analysis demonstrates that CD90 is positively expressed on NHDF in both serum-free and serum-containing media. The mean fluorescence intensity (MFI) of CD90 was slightly higher in DMEM + FBS. Expression of 5B5 was similar in both media, but MFI was slightly higher in FGM-CD™ medium.

Conclusion

- TheraPEAK™ FGM-CD™ medium is a serum-free chemically defined medium that supports expansion of adult human dermal fibroblasts.
- The growth performance of NHDF in FGM-CD™ medium is 75% to DMEM with serum averaging 11 population doublings over 3 passages.
- NHDFs in FGM-CD™ medium express fibroblast markers similar to cells grown in DMEM-FBS.
- The fibroblasts grown in the chemically defined FGM-CD™ medium secrete collagen and maintain migration capability.
- Replacement of serum-containing medium with TheraPEAK™ FGM-CD™ medium for the *in vitro* expansion of fibroblasts provides a more defined environment for pharmacological manipulations for research studies and clinical applications using fibroblasts.

New PAGE buffers yield faster migration, improved resolution and decreased blotting time

By Hugh White, Denise Trevarrow and Foner Curtis, Lonza Rockland, Inc., Rockland, ME, USA

Introduction

The Laemmli gel system, based on Tris glycine SDS buffer, is the recognized gold standard for analyzing proteins by PAGE. For over 40 years, efforts to improve the time and efficiency of this method have been limited... until now. Lonza's new buffer systems yield faster protein migration, improved resolution and decreased blotting transfer time. Overall, the standard 2-hour method for protein separation and transfer is reduced to less than 30 minutes.

Materials and methods

Initial testing compares the standard Laemmli method to Lonza's new buffer system. Figure 1 shows a comparison between a normal run time of 40 minutes using Tris glycine SDS buffer (A) versus a comparable run of 15 minutes at a higher voltage using the new running buffer (B). The new buffer method yields razor sharp separation and improved resolution of smaller MW proteins.

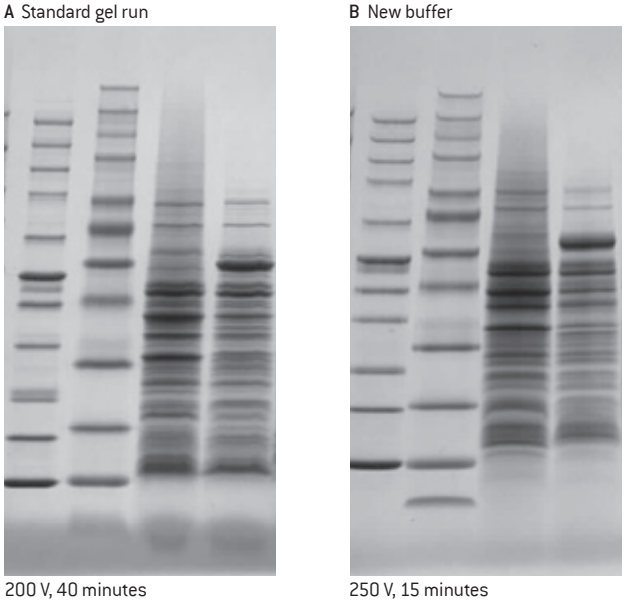


Figure 1
PAGEr™ gold gels were run to compare performance using standard Tris glycine SDS running buffer and Lonza's new buffer system. Gels were loaded with identical sample sets and run under indicated voltage/time conditions. Gels were stained using ProSieve™ blue stain. Samples used were: ProSieve™ unstained protein marker II, ProSieve™ QuadColor™ protein marker, and *E. coli* cellular lysates.

Further investigation analyzes the performance in semi-dry blotting for time and conditions using color markers to assess protein transfer from PAGEr™ gels. Figure 2 shows a comparison of gels run and blotted with Tris glycine running buffer and Towbin transfer buffer (A) versus Lonza's new running and transfer buffers (B). In addition to the improved resolution from the new running buffer, the data demonstrate a significant reduction in blotting time with the new transfer buffer.

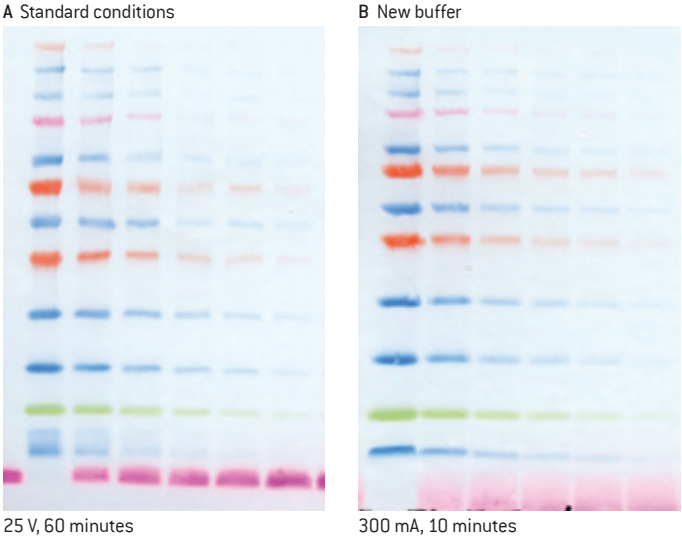


Figure 2
Comparison of transfer performance using Lonza's new buffer system. Dilution series of ProSieve™ QuadColor™ protein markers was separated on 4 - 20% PAGEr™ gold gels using run conditions described in Figure 1. Following separation, gels were equilibrated in transfer buffer. Proteins were then transferred to supported nitrocellulose membranes by semi-dry transfer under conditions indicated.

Conclusion

The results confirm that Lonza's new buffer system obtained a total 25-minute run/transfer time versus the 115-minute run/transfer time required using standard buffer methods. Such performance and efficiency improvements represent a significant step forward for the PAGE method and protein researchers.

www.lonza.com/protein

An air-liquid interface culture system for small airway epithelial cells

By Sorin Damian, Marjorie R. Smithhisler and George J. Klarmann, Lonza Walkersville, Inc., Walkersville, MD, USA

Introduction

Availability of primary normal human small airway epithelial cells facilitates the *in vitro* research of complex aspects of the airway morphology, functionality and physiology. Here we present a functional system consisting of small airway epithelial cells established in a 3-D model of air-liquid interface. The model reproduces the epithelial inner lining of small airways where the cells are attached by their basal pole, and faces the lumen with the apical pole. Our model is highly functional *in vitro* after an induced process of differentiation that necessitates the usage of particular media formulations, a special filter membrane attachment support and an optimized differentiating protocol. The existence of such a model opens the door to a very large number of relevant experimental approaches, like study of disease of small airways resulting from exposure to tobacco smoke, mineral dust, air-pollutants, viral infections etc., and study of inflammatory mediators as well as physiological changes of bronchioles, small bronchi or alveolar area. The model can be used in a more relevant way than standard submerged culture for the research of nearly any aspect of the biology of the small airway epithelium. Macrophage cell lines or normal human macrophages can be added and the interactions with small airway epithelial cells can be addressed experimentally from a new perspective in 3-D airlifted cultures.

This 3-D model system more directly resembles the human airway compared to traditional bronchial epithelial cell culture methods, which should facilitate better experimental design and results[1].

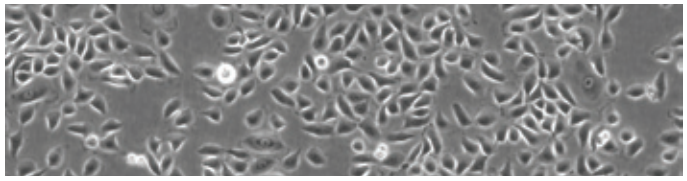
Methods and materials

In this study we grew small airway epithelial cells (Clonetics™ SAEC, Cat. No. CC-2547) from normal, human donor lung tissues in an air-liquid interface (ALI) model to assess potential morphological and functional changes *in vitro* toward the differentiated phenotype.

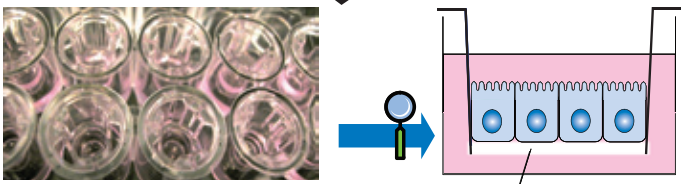
Cell isolation: Small airway epithelial cells (SAEC) were isolated through an enzymatic digestion protocol from normal cadaveric lung tissue samples located at the lower end of either left or right lung (small airway sections). Isolated cells were expanded in standard submerged culture in SAGM™ small airway growth medium then passaged once and cryopreserved at 500,000 cells/vial with a specific computer cryopreservation program. Vials were stored in liquid nitrogen until further use.

Cell culture: For airlifted cultures, normal SAEC were expanded for 3 days in S-ALI™ growth media (Lonza, Cat. No. CC-3281) then seeded in collagen-coated Corning PET Transwells® 0.4 µm filter inserts at a density of 60,000 live cells. Media was removed from the apical chamber at day 4 and S-ALI™ differentiation media (Lonza, Cat. No. CC-3282) was added to the basal chamber to initiate the air-liquid interface (ALI) culture. Trans-epithelial electrical resistance (TEER) was measured with EVOM equipped with STX2 electrodes. Tight junction proteins were assessed through immunostaining with ZO-1 (Life Technologies, Cat. No. 33-9100) and detected with ALEXA Fluor 488 (Life Technologies, Cat. No. A11017).Cytokeratin 19 was detected with mouse anti-human CK-19 (Sigma) and developed with a goat anti-mouse-IgG FITC conjugate (Sigma). Secreted mucin was directly stained with Alcian Blue, and cilia formation was assessed through immunostaining with anti-ß-tubulin FITC conjugate (Sigma clone TUB 2.1, Cat. No. F-2043).

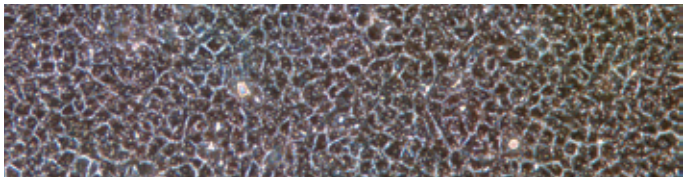
Figure 1
SAEC differentiation process using S-ALI™ small airway ALI media kit.



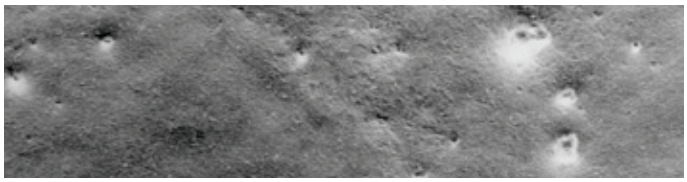
Monolayer culture expansion in S-ALI™ growth medium.



Seed on Transwells® insert and grow for 4 days in S-ALI™ growth medium.



Remove media from apical chamber at day 4.

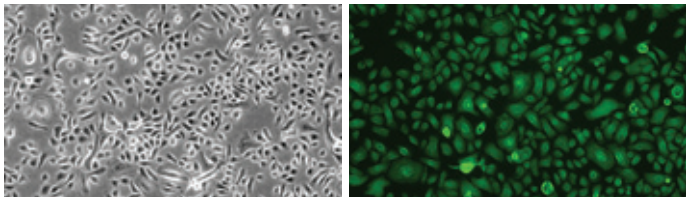


Apical surface of the confluent monolayer at the beginning of the airlift phase (3-D aspect).

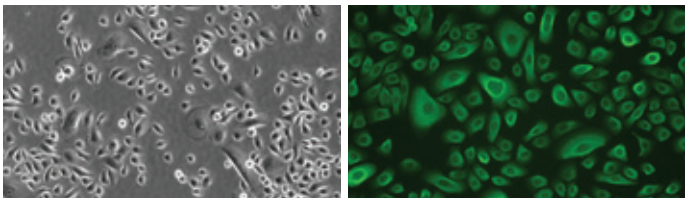
Maintain differentiated SAEC in air-liquid interface for ≥3 weeks. Use cells for various experiments: viral infection, drug screening, transport studies, gene expression analysis, etc.

Figure 2
In submerged culture SAEC express a typical proliferative epithelial phenotype (left column) and express cytokeratin 19, a specific marker (right column).

Donor 1



Donor 2



Donor 3

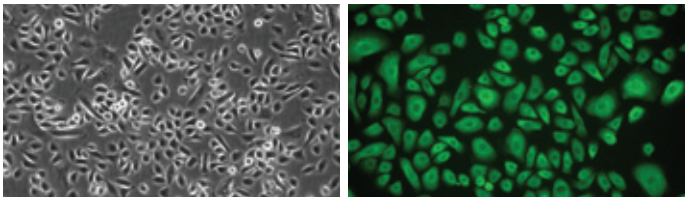
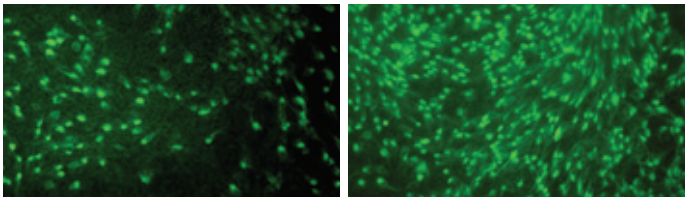
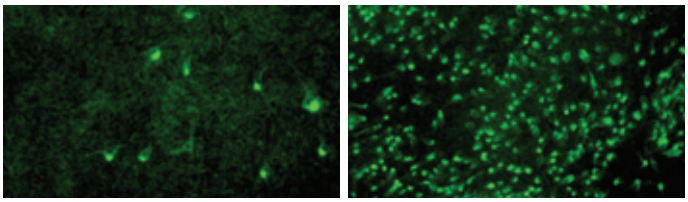


Figure 4
SAEC in air-liquid interface culture exhibit a ciliated phenotype (ß-tubulin staining by day 20). Control is day 0 in airlifted conditions.

Donor 1 | Donor 4



Donor 2 | Donor 5



Donor 3 | Sample control

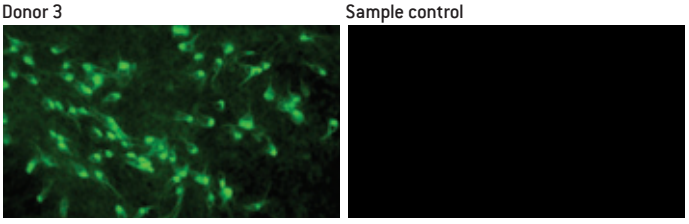
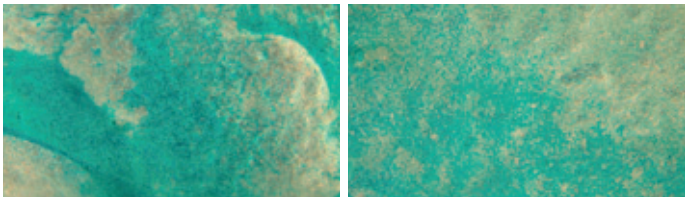
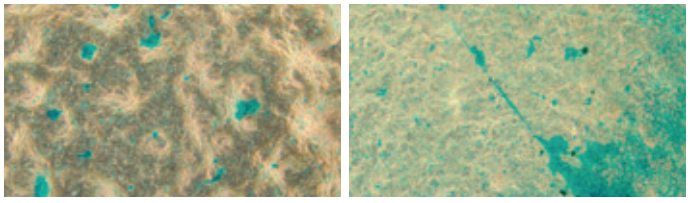


Figure 3
SAEC in air-liquid interface culture exhibit a secretory phenotype (mucin secretion by day 20). Control is day 0 in airlifted conditions.

Donor 1 | Donor 4



Donor 2 | Donor 5



Donor 3 | Sample control

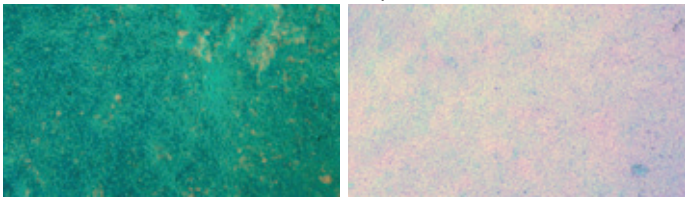
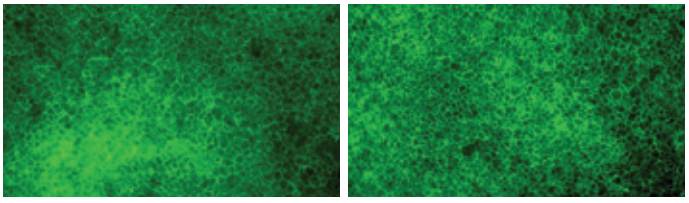
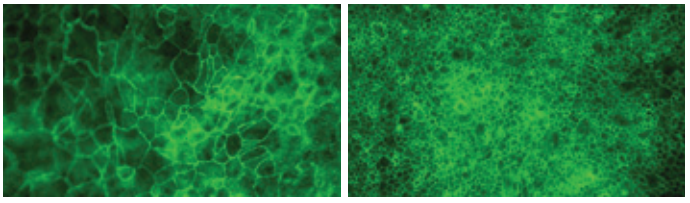


Figure 5
SAEC in air-liquid interface culture have tight junctions. On day 20, cells were stained for ZO-1 directly into Transwells® inserts. Control is day 0 in airlifted conditions.

Donor 1 | Donor 4



Donor 2 | Donor 5



Donor 3 | Sample control

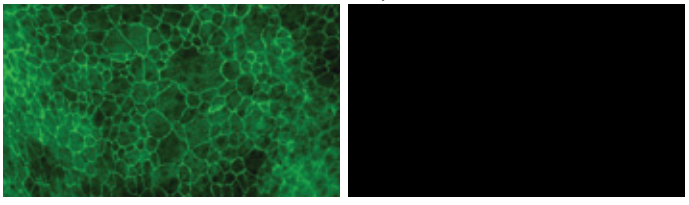
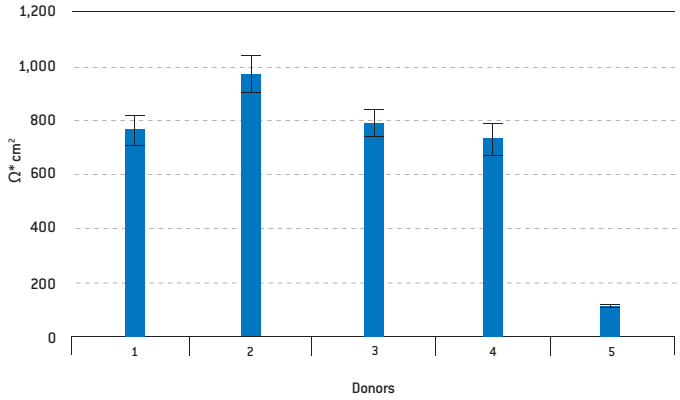


Figure 6
SAEC in air-liquid interface culture exhibit a strong barrier, measured by TEER.



Results

TEER measurements, which exhibited donor-dependent response, were as high as 600Ω* cm^2 between 10 - 20 days in airlifted status, which indicates a solid barrier function. In most SAEC lots, ASL is visible by 10 days after airlift, but compared with similar 3-D cultures of tracheobronchial epithelial cells, SAEC secrete less mucin. Cilia were detected at 20 days after airlift, and indicated a donor dependency. Cells were positive for ZO-1 expression and exhibited a donor-dependent variation in the extent of tight junction networks.

- We successfully isolated highly pure cultures of small airway epithelial cells (SAEC) from normal donors. Those cells can be easily used in standard classical 2-D cultures for any study that requires a proliferative phenotype.
- All lots have a standard epithelial phenotype in submerged culture and express a specific epithelial marker, cytokeratin 19 (Figure 2; only three donors shown).
- We were able to differentiate SAEC in a 3-D environment by airlifting and differentiating the cells on PET filters and culturing in S-ALI™ media kit supplemented media.
- We screened multiple SAEC lots (from different donors) and found nearly 50% of the lots will be suitable for the airlifted model.
- The availability of guaranteed SAEC lots suitable for airlifted cultures, as well as specific media for the growth and differentiation of SAEC in filter plates, offers a biologically relevant *in vitro* model to the *in vivo*-like differentiated model.
- The differentiation process is characterized by consistent reproducibility and is based on the use of an original protocol and media formulation.
- The differentiated phenotype was validated through the most commonly recognized functional markers for small airway lining epithelium.
- Additional markers for cell junctions, like ß-catenin, desmoplakin, and occludin were also assessed at a later stage in the differentiation process. Distribution and abundance of all those markers were donor-dependent, but always present (data not shown).

Conclusion

Our data demonstrate that SAEC can be differentiated in an ALI culture system that is capable of forming tight junctions, displaying cilia and secreting ASL. This system should facilitate more physiologically relevant studies of both normal SAEC and those from diseases, such as COPD and asthma. The availability of SAEC for ALI culture from different donors with known health history increases our ability to perform more in-depth studies of apoptosis, cytokine secretion/stimulation. This 3-D model is extremely relevant for the *in vitro* study of airborne toxicants or epithelial injury by lethal toxicants (e.g., anthrax toxin) or bacterial/fungal/viral insults.

Research application examples

- Gene therapy studies i.e., delivery of genetically engineered cells or gene segments by way of specifically targeted adenoviral vector delivery
- Host defense mechanisms
- Gene expression analysis
- Preclinical drug development, disease models
- HTS using 96-well filter plates
- Airborne toxicant studies
- DOD bio-defense models
- *Bacillus anthracis* utilizing human lung epithelial cells[2]
- Thus, the cells and ALI culture system provide researchers with valuable tools in which to aid in their quest to understand the mechanisms of diseases.

References

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2. Russell BH, Vasan R, Keene DR, Koehler TM, Xu Y., Potential dissemination of *Bacillus anthracis* utilizing human lung epithelial cells, *Cellular Microbiology, 2008, 10(4):945–57*.

BioResearch
Resource Notes™
Fall 2011

Cell-based assays using biosensors in human mesenchymal stem cells

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Abstract

Primary cells provide model systems closely resembling the *in-vivo* situation and allowing for a higher predictability of the situation in humans. In spite of this, they are rarely used for drug screenings. This is mainly due to lack of assay formats suited for primary cells and the limited availability of reliable high quality primary cells. Here we show that Poietics™ primary hMSCs transiently expressing either a calcium biosensor (i-Photina®) or a cAMP biosensor (GloSensor™) can be stored in a frozen state without loss of functionality. After a brief recovery period overnight, they can be used in high-throughput formats (i.e., 96-well or 384-well plate) to monitor changes of intracellular Ca^{2+} and cAMP concentrations in response to external stimuli. This was demonstrated in separate samples for either Ca^{2+} or cAMP by dose-dependent responses to agonists triggering second messenger increase via signaling through G-protein coupled receptors. When both biosensors were co-expressed in hMSC, calcium and cAMP could be monitored in the same sample due to the consecutive appearance of i-Photina® and GloSensor™ luminescence. This ready-to-use cell-based assay system represents an excellent versatile tool to study agonist and antagonist effects on calcium and cAMP signaling in primary and stem cells and will help to open new roads for more predictive drug screenings.

Introduction

In drug discovery research, cell-based assays are a widely accepted tool for high-throughput screening of potential drug candidates. Typically, immortalized cell lines are used in cell-based assays. They can be easily cultivated and modified *in-vitro*, but they bear some limitations as they are (1) often not originated from the actual tissue or native cell of interest, (2) sometimes non-human (e.g., hamster derived CHO cell line), and (3) often express the transfected targets at non-physiological levels. For these reasons, there is a growing demand for primary human cells, such as human mesenchymal stem cells and endothelial cells in drug screening and drug discovery research. Primary cells genuinely express the relevant drug targets, e.g., cell surface receptors at physiological levels, and carry the endogenous cofactors necessary for efficient and specific signal transduction. We combine Lonza's expertise in providing reliable high quality primary cells and the Amaxa™ Nucleofector™ technology for highly efficient non-viral transfection of these cells without affecting their functionality. This enables expression of biosensors like Axxam's i-Photina® for second messenger Ca^{2+} and Promega's GloSensor™ for second messenger cAMP. Biosensors could therefore be used to monitor specific signal transduction events, such as protein kinase A (PKA) activation during osteogenic differentiation of hMSC. Here we demonstrate that Poietics™ hMSC and Clonetics™ primary cells expressing biosensors can be provided in a ready-to-use frozen state. These cells, after a brief recovery, are employed for studies on ligands of different G-protein coupled receptors triggering changes in intracellular Ca^{2+} and cAMP concentrations. The EC_{50} values obtained nicely confirm published data. HMSC co-expressing both biosensors allowed for following both signaling pathways in the same sample due to the time-staggered appearance of i-Photina® and GloSensor™ luminescence.

Materials, methods and results

Analysis of intracellular cAMP levels in hMSC using the pGloSensor™-22F cAMP plasmid

Poietics™ human mesenchymal stem cells (hMSC, Lonza PT-2501) isolated from bone marrow were cultured and expanded according to manufacturer's instructions. HMSC were transfected with the pGloSensor™-22F cAMP plasmid (Promega) using the appropriate optimized Nucleofection™ protocol. 17 - 20 hours after Nucleofection™, cells were equilibrated for 2 hours with GloSensor™ cAMP reagent (Promega) according to manufacturer's protocol. For the dose-dependent responses, the cells were stimulated in 96-well plates with various concentrations of forskolin, formoterol, and prostaglandin E1. Luminescence was analyzed at 25°C in a microplate reader every 5 minutes for up to 30 minutes. Results of the dose-response curves and EC_{50} values are consistent with published data (shown in Figure 1).

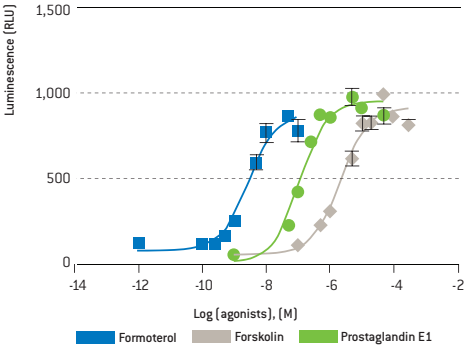


Figure 1
Changes of intracellular cAMP concentrations in hMSC can be detected through transiently expressed GloSensor™. Cells were transfected, frozen, recovered, and treated for stimulation as described in "Materials, methods and results". EC_{50} values: Forskolin 2.2 µM, prostaglandin E1 120 nM, formoterol 3 nM.

Monitoring intracellular calcium levels in primary endothelial and smooth muscle cells transfected with a calcium biosensor

Human umbilical vein endothelial cells (HUVEC), human lung microvascular endothelial cells (HMVEC-L) and aortic smooth muscle cells (AoSMC) were transiently transfected with an expression plasmid encoding i-Photina® using the Nucleofector™ technology. After Nucleofection™, cells were incubated for 6 hours and loaded with 10 µM native coelenterazine for the last 2 hours right before freezing. The cells were frozen in vials in cryoprotective agent. For performing the Ca^{2+} assay, cryopreserved cells were thawed, seeded on a 96-well plate, and were allowed to recover for 20 - 24 hours in a humidified incubator at 37°C, 5% CO_2. 4 hours after thawing, medium was exchanged to remove the cryoprotective agent. For stimulation, histamine was injected in a microplate reader equipped with an automatic dispenser. Luminescence signals were recorded at 25°C every second for a total of 35 seconds. The results shown in Figure 2 clearly illustrate that dose-dependent responses to histamine can be measured in different primary cell types using the calcium biosensor i-Photina®, resulting in almost identical EC_{50} values (Figure 2).

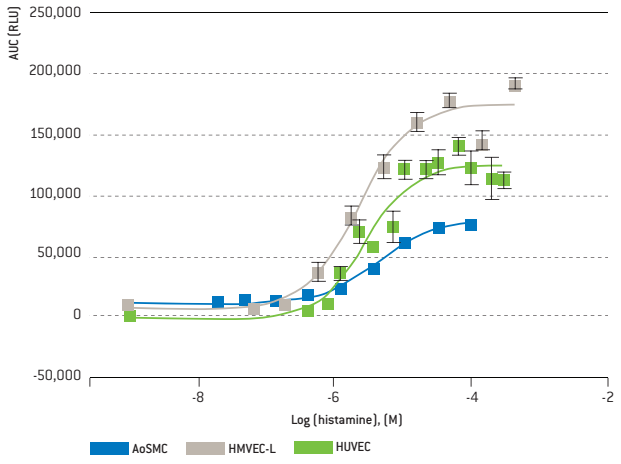


Figure 2

Intracellular calcium flux in primary smooth muscle (AoSMC) and endothelial (HUVEC, HMVEC-L) cells due to stimulation with histamine can be investigated by i-Photina®. Cells were transfected, frozen, recovered, and treated for stimulation as described in "Materials, methods and results". EC_{50} values: AoSMC 5 µM, HUVEC 3 µM, HMVEC-L 2.4 µM.

Monitoring intracellular calcium and cAMP levels using hMSC co-expressing both biosensors

HMSC were co-transfected with the GloSensor™ and i-Photina® expression vectors as described above. Incubation after Nucleofection™, freezing the cells, reactivating frozen cells for performing the Ca^{2+} and cAMP assay was carried out exactly as described above for transfection with the calcium biosensor. Cells were equilibrated with GloSensor™ cAMP reagent (Promega) for 2 hours right before the assay. Cells were stimulated and analyzed as described above. The two assays were performed separately in individual wells.

These results (shown in Figure 3) clearly demonstrate that calcium biosensors and cAMP biosensors do not interfere when co-expressed in hMSC. Kinetics of the luminescence signal are the same as if expressed in separate samples (see Lonza Resource Notes™, Spring 2010 for GloSensor™, and Lonza Resource Notes™, Fall 2010 for i-Photina®).

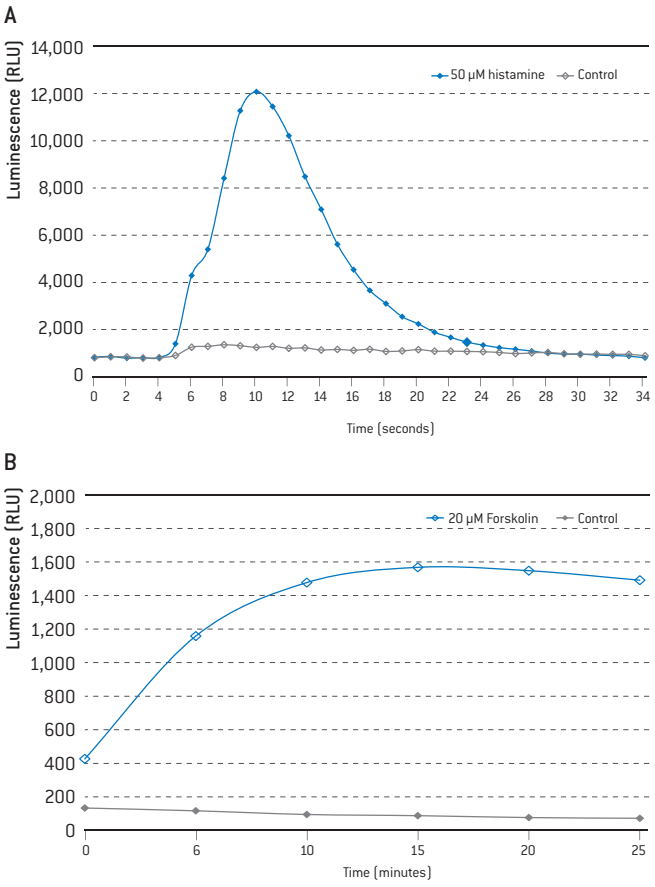


Figure 3

Luminescence signal kinetic of i-Photina® (A) and GloSensor™ (B) co-expressed in hMSC can be independently recorded. Co-transfection of hMSC, freezing, recovery, and treatment for stimulation was carried out as described in "Materials, methods and results". For stimulation, final concentrations were 50 µM histamine (A) or 20 µM forskolin (B), respectively. Control samples were treated with water (A) or medium (B). The two assays were performed separately in individual wells.

Human MSC co-expressing calcium and cAMP biosensors can be used to consecutively monitor both signaling pathways in one sample well

For the experiments where both assays were performed in the same well, cells were treated exactly as described in the previous section. Histamine + isoproterenol, histamine or isoproterenol were injected into the samples. For detection of intracellular calcium, luminescence signals were recorded at 25°C every second for a total of 35 seconds starting immediately after injection. Afterwards, for detection of intracellular cAMP, luminescence was recorded at 25°C every 5 minutes for up to 30 minutes.

If both biosensors are assayed in the same sample, both luminescence signals from i-Photina® and GloSensor™ can be detected independently and this will allow for monitoring both signaling pathways in one cell. Thus, with fewer samples, signaling pathways of compounds could be identified almost simultaneously. This can be particularly useful for elucidating the signaling of so far uncharacterized compounds, and determining signaling mechanisms of GPCRs, especially when they switch pathways. Potential ligands for orphan GPCRs (about one third of all known GPCRs), as well as their signaling mechanisms, could be determined for the endogenously expressed receptor in the correct tissue type.

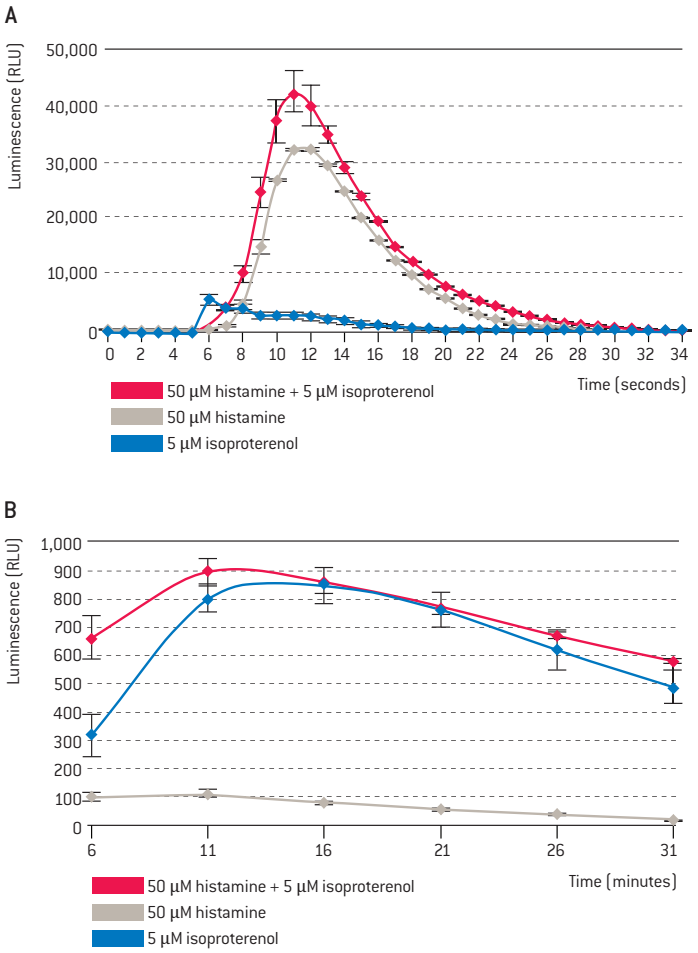


Figure 4
The biosensors i-Photina® (A) and GloSensor™ (B) co-expressed in hMSC can simultaneously be stimulated and recorded in the same sample. Co-transfection of hMSC, freezing, recovery, and treatment for stimulation was carried out as described in Materials, methods and results. For stimulation, final concentrations were 50 µM histamine + 5 µM isoproterenol, 50 µM histamine, 5 µM isoproterenol. These agonists were injected into the samples. The rapidly appearing luminescence of i-Photina® was detected right after injection; and subsequently the GloSensor™ luminescence.

Conclusion

We have shown here that cAMP and Ca^{2+} signaling can be reliably monitored in stem cells after transient transfection of Promega's GloSensor™ and Axxam's i-Photina® using the Amaxa™ Nucleofector™ technology. For the cAMP and calcium biosensors, we obtained pharmacologically relevant dose-response data for beta-2 adrenergic receptor agonists and an H receptor agonist, respectively. Both assays can be co-transfected and performed with cells cryopreserved after transfection. Intracellular calcium and cAMP levels can be monitored in the same sample. Taken together, our findings provide a basis for using transiently transfected stem cells in high-throughput screenings.



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