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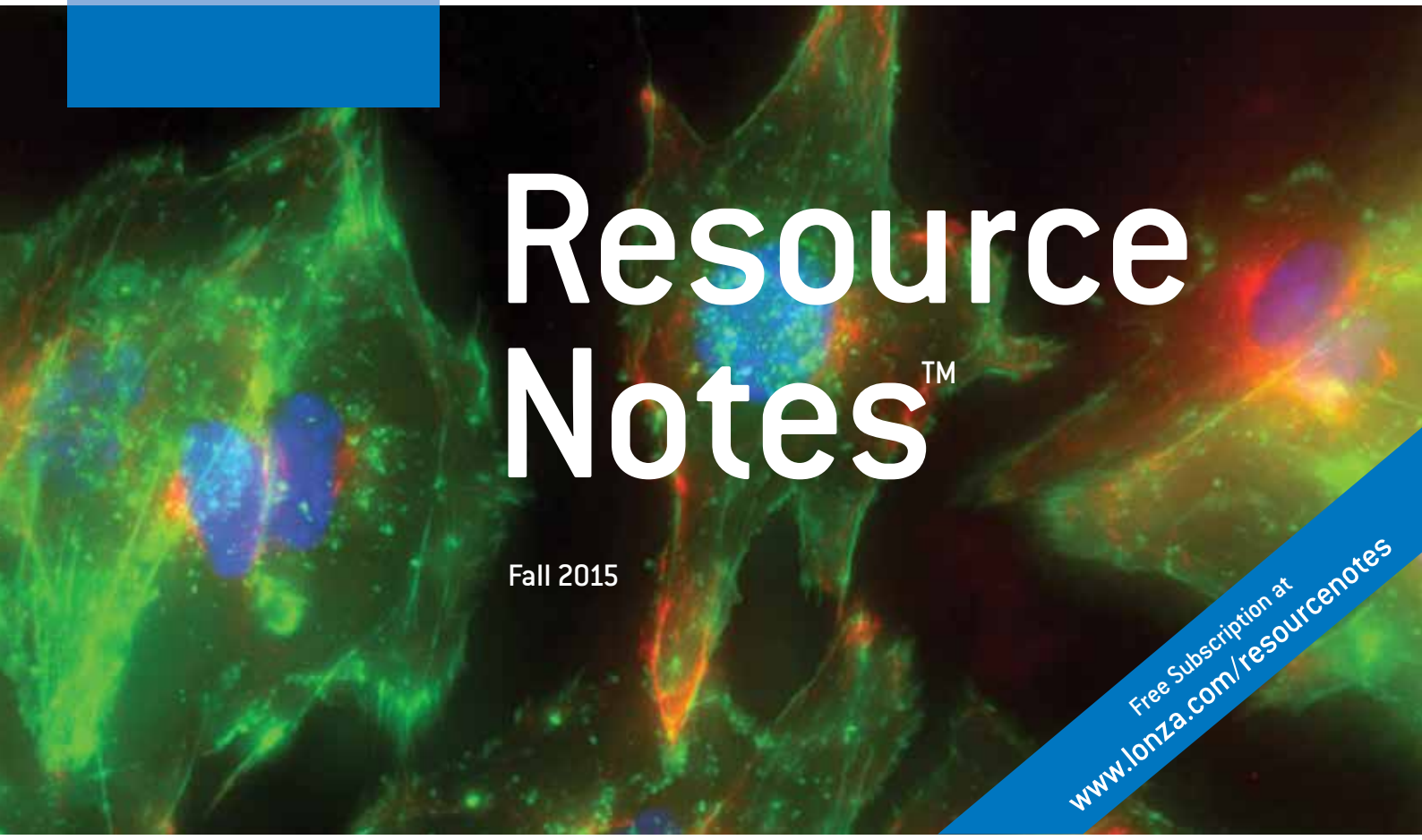

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The Newsletter for Life Science Researchers

BioResearch

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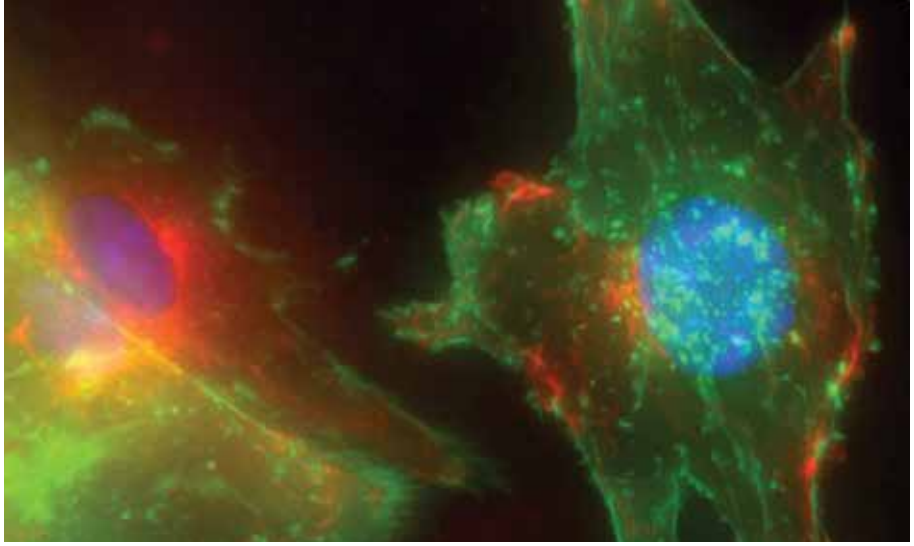

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Innovative Cell Culture

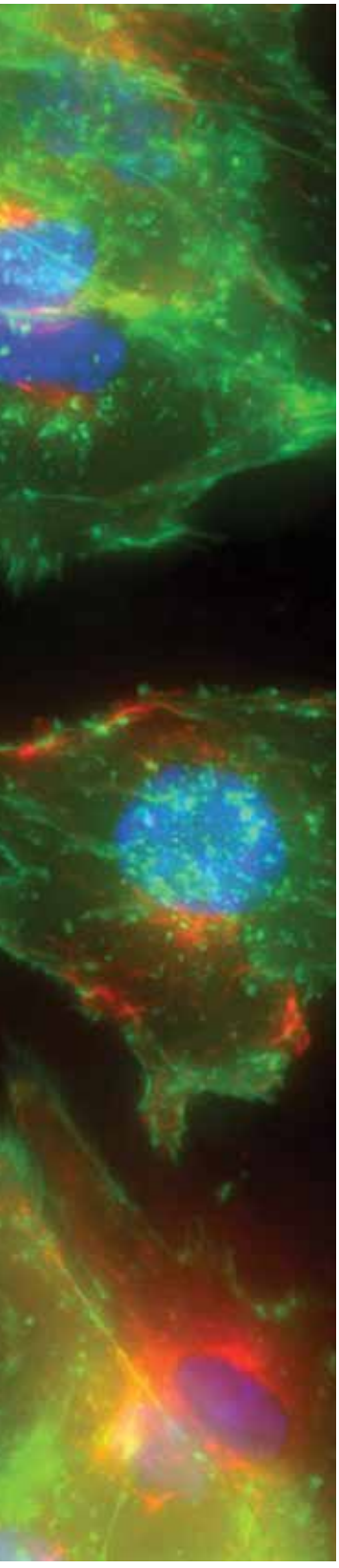
Over the past 40 years, researchers have relied on culturing and studying cells in two dimensions. However, the culture of cells in two dimensions has its limitations and does not reproduce the anatomy or physiology of a tissue for useful study. A third dimension for cell culture is now being considered more physiologically relevant with cells behaving and growing in a more *in vivo*-like environment. But these culture methods have also become more complex. When using 3D cell culture methods, scientists need to consider the design of scaffolds for supporting the organization of cells. As 3D culture systems become more mature and relevant to human and animal physiology, the ability to design and develop co-cultures and tissue models becomes possible. The applications of 3D cell culture systems vary widely by cell type, and range from engineering tissues for clinical delivery to the development of models for drug screening.

There is increasing awareness of the drawbacks of 2D cell culture and the related effect on the value of the research being performed. Studies have shown differences in 3D culture versus 2D culture in many research areas including differentiation, drug metabolism, gene and protein expression, general cell function, morphology, response to stimuli, proliferation, and viability.

There is a trending shift in both academia and industry to personalized research solutions and more *in vivo*-like models to understand cell behavior. In addition, animal welfare initiatives in many regions have also spurred the development of alternative and innovative *in vitro* testing methods. This is fueling the growing market need for better solutions in 3D cell culture, especially in areas such as cancer, stem cell, neurobiology and toxicology.

In this month's issue, we would like to introduce you to Lonza's new RAFT™ 3D Cell Culture System, which is a recent addition to our growing cell culture portfolio.

Your Lonza Team



News

Live Cell Monitoring – The Smart Way: Small, Easy and Affordable

How many weekends have you spent in the lab to check on your cells? With the CytoSMART™ System, you can watch your cell culture anytime, anywhere.* Watch the video to see how it works!

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Meet Cell. Cell is depressed. He has fully differentiated and reminisces about the opportunities of his youth. Then, Cell stumbles upon Lonza's L7™ hiPSC Reprogramming and hPSC Culture System and realizes it might not be too late to rediscover his full potential. Watch Cell's story!

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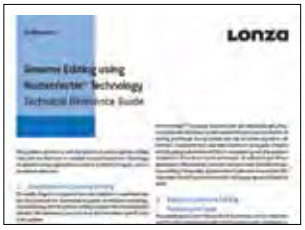
Genome Editing Using Nucleofector™ Technology



Get a brief background on various genome editing tools and learn how to establish Lonza's Nucleofector™ Technology for genome editing applications in hard-to-transfect cell types, such as pluripotent stem cells. This guide is now available for download on our website.

www.lonza.com/genome-editing

Conferences/Tradeshows

Visit us at:

Dates	Event	Booth No.	Location
18 September 2015	**Belgian Society for Stem Cell Research**	–	Brussels, Belgium
19–21 September 2015	**3rd Annual Helmholtz-Nature Medicine Diabetes Conference**	–	Munich, Germany
22–24 September 2015	**CHI Discovery on Target**	25	Boston, MA, USA
27–29 September 2015	**31st Ernst Klenk Symposium**	–	Cologne, Germany
18–21 October 2015	**Society for Neuroscience**	1483	Chicago, IL, USA
19–22 October 2015	**ISSCR China**	–	Suzhou, China

For more details and other events visit www.lonza.com/events

Webinars

Date	Webinar
22 September 2015	Cell Monitoring and Recording Remotely: The CytoSMART™ System
23 September 2015	Cell Monitoring and Recording Remotely: The CytoSMART™ System
27 October 2015	Enhance Your Cell Culture with the RAFT™ 3D Cell Culture System
28 October 2015	Enhance Your Cell Culture with the RAFT™ 3D Cell Culture System

For details and registration visit www.lonza.com/researchwebinars

*Internet access required

Fluorescence Microscopy-based Characterization of Cells Grown in RAFT™ 3D Culture

By Cecile Villemant[1], Grant Cameron[1], Lubna Hussain[2], Jenny Schroeder[3]

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Introduction

In vitro assays typically use cells grown on two-dimensional (2D) hard plastic or glass substrates, which are not representative of the true *in vivo* cell environment.[1] In tissue, cells interact with neighboring cells and with the extracellular matrix (ECM). In a simplified *in vitro* 2D environment, most of the tissue-specific architecture, cell-cell communication and cues are lost. Therefore, the need exists for advanced culture methods that better mimic cellular function within living tissue.

Three-dimensional (3D) cell culture methods, in comparison, provide a matrix that encourages cells to organize into structures more indicative of the *in vivo* environment, thereby developing normal cell-cell and cell-ECM interactions in an *in vitro* environment.

The RAFT™ 3D Culture System (Figure 1) uses a collagen matrix at physiologically relevant concentrations. Cells and neutralized collagen are mixed and dispensed into wells of standard cell culture plates, and subsequently incubated at 37°C to allow the formation of a cell-seeded hydrogel. Specialized RAFT™ Absorbers are placed on top of the hydrogels. The RAFT™ Absorbers gently remove the medium, thus concentrating the cell/collagen hydrogel to a layer approximately 120 µm thick, mimicking physiological conditions. The cultures are then ready for use. Optionally, additional collagen layers or epithelial or endothelial cell overlays may be added to study co-cultures or more complex cultures.

Assessing the viability of cells grown in a RAFT™ 3D Cell Culture, or performing immunofluorescence staining, may not seem as trivial as assessing the viability of a 2D cell culture. However, here we show that by adding some basic controls, the LIVE/DEAD® Assay is a straightforward and reliable method to assess the viability of cells inside a RAFT™ 3D Cell Culture. In addition, we show that immunofluorescence microscopy can be performed easily and routinely on RAFT™ 3D Cell Cultures.

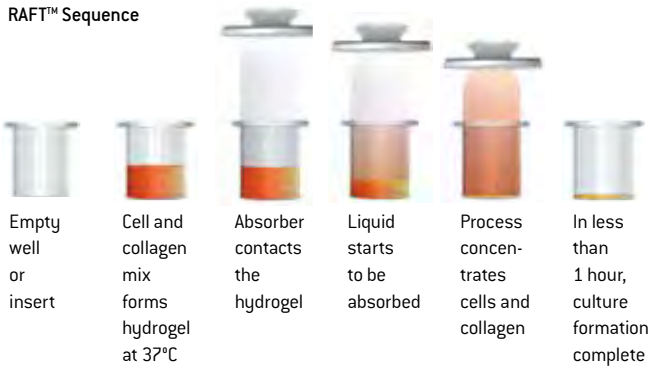

Figure 1
Creation of 3D cell/collagen hydrogel using the RAFT™ System in standard cell culture plates.

General Materials

- RAFT™ Absorbers and Reagent Kits, visit www.lonza.com/raft for a list of RAFT™ Products and RAFT™ Protocols
- For a list of recommended cell culture plates (either 96-well black wall or 24-well plate, not supplied with the kit), contact Lonza's Scientific Support Team
- Widefield fluorescence microscope with appropriate filters

Methods

Cell Culture

Early passage primary human neonatal dermal fibroblasts (HDFs) were cultured in standard growth medium that supports dermal fibroblasts.

Generation of RAFT™ Cultures

RAFT™ Cultures were made according to the protocol supplied with the RAFT™ Kits in black-walled, µClear® 96-well cell culture plates (Greiner, 655090). For the LIVE/DEAD® Assay, 3,000 HDFs per well were seeded and incubated at 37°C with 5% CO_2, either overnight (1 day) or for 7 days. In addition, two acellular cultures were made for use as a background control. For immunocytochemistry, either 5,000 or 50,000 HDFs per well were seeded and then cultured for either 11 days or 3 days, respectively, prior to being fixed and stained.

LIVE/DEAD® Assay

The LIVE/DEAD® Assay was carried out, using at least one dead control and two background controls (acellular cultures). The LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies) was used according to the "fluorescence microscopy protocol" provided up to point 3.5. However, with the following adjustments:

1. To get a thorough wash of the whole 3D cell culture, the medium on the culture is aspirated and replaced with 100 µL PBS and the plate left for 5–10 minutes on a rocker (while preparing the combined LIVE/DEAD® Assay Reagents).
2. To prepare the dead cell sample, we incubate the cells with 1% w/v saponin for at least 30 minutes. If you have added 100 µL of medium onto your RAFT™ Cultures, just add 25 µL of 5% w/v saponin 30 minutes before performing the assay.
3. Due to the presence of the collagen matrix, it is advised to include acellular RAFT™ 3D Cultures to act as a control for background noise.

Instead of point 4.1, onward, in the supplied LIVE/DEAD® Viability/Cytotoxicity Kit Protocol, we used the following protocol:
Aspirate the PBS, added previously from the wells, and replace it with 100 µL of the combined LIVE/DEAD® Assay Reagents. We have found that in the case of HDFs, final concentrations of 0.4 µM for Calcein AM and 4 µM for Ethidium homodimer 1 (EthD-1) were optimal.
Between 10 minutes and 1.5 hours after adding the reagents, z-series at 5 µm intervals were captured using a fluorescence microscope fitted with a z-focus drive. The numbers of live cells (stained with Calcein AM) and dead cells (stained with EthD-1) were counted from three separate images over two wells for the "live" samples. The background noise for EthD-1 was counted in two acellular cultures ($bckgd_{EthD\text{-}1}$① and $bckgd_{EthD\text{-}1}$②). The background noise for Calcein AM was assessed in the acellular cultures and in the dead control; however, there was no visible background detected. Therefore, for each image, the percentage of viability was calculated as follows:

$$\%viability_{d=1 \text{ or } d=7} = \frac{\text{Number of live cells}}{\text{Number of live + dead cells} - \text{average } (bckgd_{EthD\text{-}1}\ ①\&②)}$$

Immunocytochemistry

On the day of assay, the following protocol was used:

1. Each RAFT™ Culture was washed three times over a 15-minute period with 100 µL PBS.
2. The PBS was replaced with 100 µL of 3.7% formaldehyde solution to fix the cells, and the plate incubated at room temperature for 30 minutes.
3. The formaldehyde solution was replaced with 100 µL of quenching solution (1 mM Tris-HCl and 20 mM Glycine in PBS) to quench the formaldehyde cross-linking, and the plate incubated at room temperature for 10 minutes.
4. The RAFT™ Cultures were washed as in point 1.
5. The PBS was replaced with 100 µL of 0.1% Triton™ X-100 solution to permeabilize the cells, and the plate was incubated at room temperature for 4 minutes.
6. The RAFT™ Cultures were washed as in point 1.
7. The primary rat anti-tubulin antibody (YOL1/34; Abcam) was diluted 1:100 in 1% w/v bovine serum albumin in PBS (which was the same dilution that was optimal for cells cultured in 2D) and 50 µL of this solution was added in each well.
8. The plate was then incubated overnight at 4ºC.
9. The RAFT™ Cultures were washed as in point 1.
10. The secondary antibody Cy3-AffiniPure Goat Anti-Rat IgG (H+L) (Stratech Scientific), phalloidin (the recommended 1/40 dilution was used) and DAPI were diluted in 1% w/v bovine serum albumin in PBS and 50 µL of this solution was added in each well.
11. The plate was then incubated at room temperature for 2.5 hours.
12. The RAFT™ Cultures were washed as in point 1.
13. The wells were imaged on a fluorescence widefield microscope with 100–200 ms exposure times for the anti-tubulin antibody and the phalloidin. Confocal imaging or the use of a high content imaging device would also be possible.

Results

LIVE/DEAD® Assay

In Figure 2, we show the typical images that can be obtained after culturing HDFs in RAFT™ 3D Cell Cultures for 1 and 7 days and staining the cultures with the combined LIVE/DEAD® Assay Reagents. We also show, in Figure 2, some examples of the Calcein AM and EthD-1 stain on an acellular construct and a dead control.

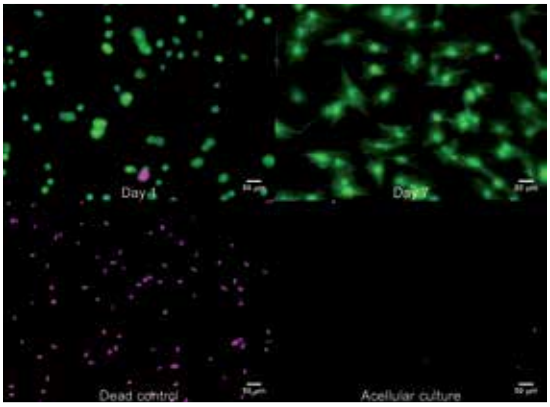

Figure 2
Examples of images that can be taken after staining RAFT™ Cultures with the combined LIVE/DEAD® Assay Reagents. HDF live cells are displaying Calcein AM staining (green) while dead cells display EthD-1 staining (magenta). Each image is a projection on the z-axis of a whole z-series.
Day 1 and Day 7: HDFs were cultured for 1 or 7 days, respectively, in a RAFT™ Plate before being treated with the combined LIVE/DEAD® Assay Reagents.
Dead control: HDFs cultured in RAFT™ Plate for 1 day were killed using 1% w/v saponin before being stained with the combined LIVE/DEAD® Assay Reagents.
Acellular culture: RAFT™ Acellular Culture stained with the combined LIVE/DEAD® Assay Reagents.

Figure 3 shows that the mean percentage viability of HDFs is 92% at Day 1 and 94% at Day 7 using the methods described above. For comparison, we have added the viability observed with the same cells cultured at the same time in a 2D planar environment, which is at 95% and 98% at Day 1 and Day 7, respectively.

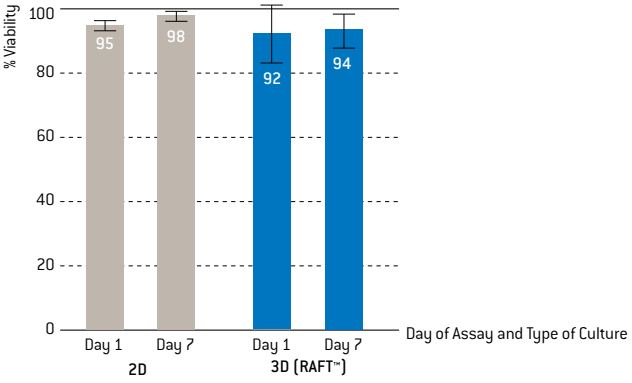

Figure 3
Comparison of the viability of HDFs after 1 and 7 days in a 2D or in a RAFT™ 3D Cell Culture. The percentage viability was determined as explained in the methods above and the average for two separate experiments is shown in this graph. The standard deviation is shown for each sample.

Immunocytochemistry

After 3 days in culture, HDFs have elongated within the collagen matrix and display a typical actin and microtubule cytoskeleton as can be seen in Figures 4 and 5. The presence of the collagen matrix has little impact on the background fluorescence of the culture, in particular, when the antibodies were diluted in a BSA-containing blocking buffer.

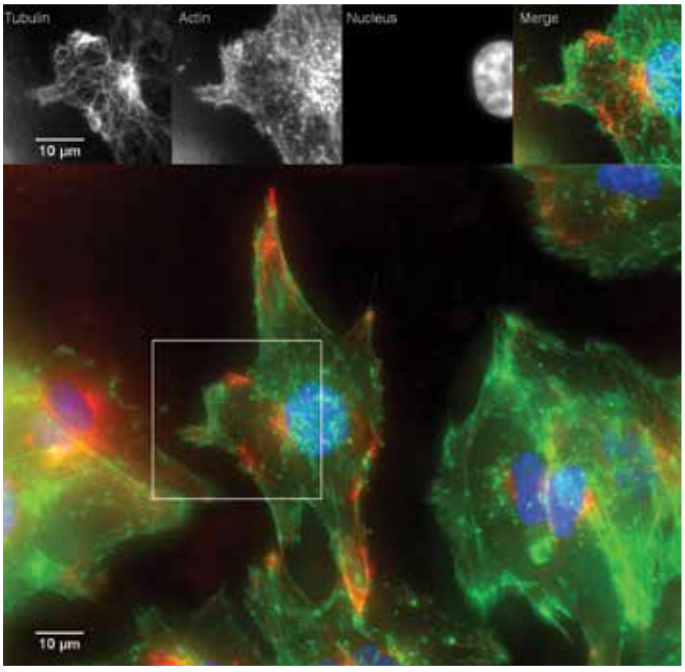

Figure 4
HDF cells fixed and stained for tubulin and actin after being cultured in the RAFT™ System for 3 days. A series of z-planes, taken at 0.5 µm intervals, was imaged on a widefield microscope after staining the RAFT™ Culture for tubulin (red), actin (green) and nucleus (blue). The z-stack from each channel was projected onto one plane using the maximum z-projection function of ImageJ software and the merge of all channels is shown in the large bottom panel. Across the top panel, one frame of the z-stack is shown with each individual channel represented separately in grayscale. This represents the area boxed in the large lower panel, to better show the detail of each staining.

As shown in Figure 5, using a simple type of image deconvolution, such as ImageJ 3D parallel spectral deconvolution (http://rsb.info.nih.gov/ij/plugins/), can help improve the sharpness of the signal and remove background noise from the cells surrounding the cell of interest (such as the blurry, out of focus cell observed above the fibroblast imaged in Figure 4). However, with our anti-tubulin antibody, images taken with our widefield microscope, and not subjected to deconvolution, were defined enough to observe microtubules within the cells in the RAFT™ 3D Culture (Figure 4). Actin fibers can also be seen clearly in cells embedded in the RAFT™ Collagen Matrix (Figure 4).

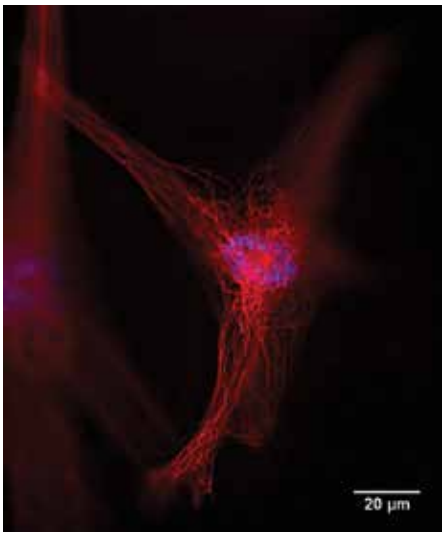

Figure 5
HDF cell fixed and stained for tubulin after being cultured in the RAFT™ System for 11 days. One z-plane location was imaged here on a widefield microscope, after staining the RAFT™ Culture for tubulin (red) and the nucleus (blue). The image from each channel was deconvolved using the 3D spectral deconvolution software from ImageJ (generalized Tikhonov) and the merge of the two channels is shown here.

Conclusion

The LIVE/DEAD® Viability/Cytotoxicity Kit is an easy and rapid assay that can be used to assess cell viability in a RAFT™ 3D Cell Culture, provided that controls are included in the test to be able to take into account the possible background noise. In this experiment, we show that HDFs display a mean viability between 92% and 94% when cultured in RAFT™ 3D Culture from Day 1 and for at least 7 days, which is comparable to the viability observed for cells cultured in a 2D environment.

Cells cultured in the RAFT™ 3D Cell Culture Collagen Matrix can be fixed and stained using standard immunofluorescence protocols. The presence of the collagen matrix has little impact on the background fluorescence of the culture, if an appropriate blocking solution is used. The resulting images were defined enough to observe microtubules and actin fibers within the cells in the RAFT™ 3D Culture. Image quality can be further enhanced by using simple types of image deconvolution software.

In conclusion, with its easy-to-follow protocols, the RAFT™ System allows researchers to set up biologically relevant 3D cell cultures quickly and reproducibly. Many 2D cell analysis methods can be easily applied to RAFT™ Cultures, often requiring no, or only minor modifications of existing protocols. This empowers researchers to generate more biologically meaningful data from their cell culture studies in multiple areas of basic research and drug discovery.

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Live Cell Monitoring with the CytoSMART™ System: Application Examples

By Claudia Schwartz and Katrin Höck, Lonza Cologne GmbH, Koeln, Germany

Introduction

While live cell imaging has been restricted to costly, high-end devices, the CytoSMART™ System offers an affordable and easy-to-use alternative for virtually any lab. The CytoSMART™ System can be set up in minutes, enabling untrained users to quickly perform their own time-lapse recordings. Images and videos can be easily accessed and retrieved from the CytoSMART™ Connect Cloud Project Page. Advanced functions, such as reporting of cell confluency via a graphical readout, and the option to use automatic email alerts, can be applied to inform the user when certain culture conditions are reached (for example, once the cell culture has reached the desired confluency). Hence, the CytoSMART™ System can be used in many different ways to facilitate cell culture work and research.

Hypoxic Culture of Cells with the CytoSMART™ Device As an Easy-to-use Monitoring System

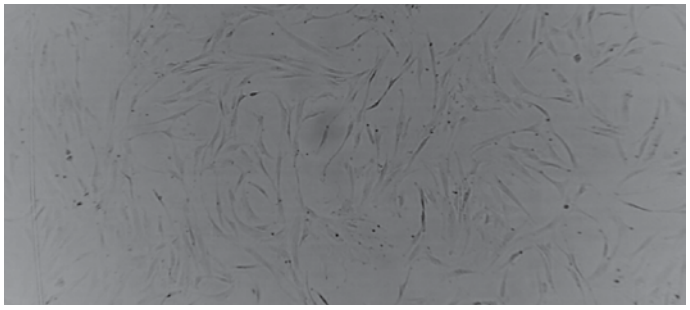

Figure 1
Image of human mesenchymal stem cells (PT-2501) cultured under normoxia taken with the CytoSMART™ Lux 10X System.

The use of hypoxic (i.e., low oxygen) culture conditions for cell cultures gains more and more interest in research as a means to mimic the *in vivo* situation more closely. Whereas physiological *in vivo* oxygen concentrations can range from 1% to 13%, most cell cultures are maintained at ambient 21% O_2. Many cellular responses to hypoxia depend on hypoxia-inducible factors (HIFs), which are stabilized under low oxygen conditions and bind the hypoxia response elements (HREs) in the genome, acting as transcription factors, inducing a wide variety of genes.[1]
For example, the HIF pathway has been shown to enhance vascular endothelial growth factor (VEGF) expression in cancer tissue to promote angiogenesis. Furthermore, it plays a major role in obesity-related type 2 diabetes, and has been demonstrated to initiate the metabolic switch from aerobic respiration to anaerobic glycolysis, a mechanism that is also taking place during the reprogramming of somatic cells into iPSCs.[1]
Therefore, hypoxic culture conditions are increasingly applied in stem cell culture and cancer research as well as in toxicological and metabolic studies.

Hypoxic Culture in Cancer Research

Hypoxia frequently occurs in the center of solid tumors where the presence and formation of blood vessels is often abnormal or limited. Hence, low oxygen conditions play a key role in regulating cancer cell metabolism and the deeper understanding of this correlation may advance our understanding of cancer formation and the development of potential new therapies.
Studies that fail to take account of the actual physiological levels of oxygen in tissues (approximately 5%) and tumors (approximately 1%) may fail to identify the real circumstances driving tumor response to treatment and/or malignant progression. This can be of particular importance in genetic *in vitro* studies when comparison to human tumor tissue is required.[2]
For example, the treatment of colorectal cancer cell lines with the cancer drug AW464 under normoxic vs. hypoxic culture conditions showed that hypoxia sensitizes colorectal cells to the drug. Furthermore, a comparison to normal cells, such as endothelial cells or fibroblasts (both from Lonza), revealed drug potency against endothelial cells but not fibroblasts, suggesting possible anti-angiogenic activity of the drug.[3]

Hypoxic Culture in Stem Cell Research

Hypoxic culture conditions are thought to enhance the generation of iPSCs, at least partially, as HIFs could be shown to increase pluripotency-related gene expression.[4] Recently, Mathieu *et al.* were able to show that the metabolic switch from aerobic respiration to anaerobic glycolysis takes place early during reprogramming. HIFs are necessary to initiate this metabolic switch, and the stabilization of HIFs during early phases of reprogramming is sufficient to induce the switch to glycolytic metabolism.[5]

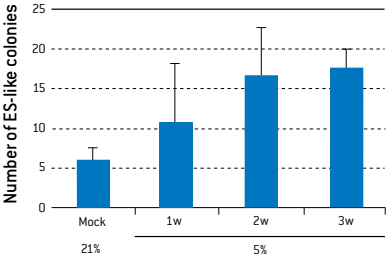

Figure 2
Hypoxia increases the efficiency of iPSC generation from human dermal fibroblasts. Four reprogramming transcription factors were introduced into adult human dermal fibroblasts. After 6 days, the cells were trypsinized and seeded onto the feeder layer of mitomycin C-treated STO cells. The cells were cultivated under 5% O_2 for 7 (1w), 14 (2w), or 21 (3w) days. The number of human ES-like colonies was determined on Day 32 after reprogramming.[4]

Culture and expansion of mesenchymal stem cells (MSCs) is typically performed under ambient O_2 concentration, regardless of the metabolic milieu of the niche in which they normally reside. Since scientists have started to study the impact of hypoxia on MSCs, the positive effect on self-renewal and differentiation potential has been clearly demonstrated. Various signaling pathways are involved in the hypoxia-induced cell functions, but HIFs are the key regulators of the cellular response to hypoxia.[6]

Cell Monitoring Under Hypoxia

While hypoxic conditions can be easily established using specialized incubators, the monitoring of cell cultures under hypoxic conditions has been extremely restricted until now. An online-monitoring system that allows direct monitoring of the cell culture in the incubator would be the preferred option. Thus far, however, this option has not been readily available to researchers. The CytoSMART™ System overcomes this issue in that it allows continuous online monitoring of cell cultures under hypoxia. With its small size (133 x 90 x 100 mm), the CytoSMART™ Device fits into virtually any specialized incubator. What is more, as the cell culture can be viewed via the CytoSMART™ Cloud Solution, the monitoring of hypoxic cultures becomes completely independent of the presence of the researcher in the lab.

Standardized Cell Culture Can Be Easily Achieved Using the CytoSMART™ System

A standardized cell culture is not easily obtained as biological systems do not always behave reproducibly, e.g., in particular primary cell systems, such as patient-derived materials, may widely vary in proliferation behavior. In addition, every cell biologist will vouch for the fact that cell culture performance is highly user-dependent and requires a certain degree of expertise to achieve consistency in cell cultures.

While not always easy to maintain, standardized cell culture is of great importance, as the condition of a cell culture may have a significant influence on the downstream results obtained with the cultured cells. One way to obtain a more standardized cell culture is to use cells for any downstream assay at consistent confluency. However, the determination of cell confluency is very user-dependent. The CytoSMART™ System overcomes this issue in offering a user-independent analysis of the cell confluency which is recorded in the CytoSMART™ Project Page (here referred to as cell coverage). In addition, automatic alerts can be set so that the researcher receives an email notification once the cell culture has reached a chosen confluency, offering a very simple yet effective tool to easily standardize cell culture.

Please visit Lonza's website to view the video of Normal Human Epidermal Keratinocytes – Adult (192627) recorded with the CytoSMART™ Lux 10X Device*

*To discover further CytoSMART™ Applications and view related cell videos, please visit www.lonza.com/cytosmart

Effects of Different Cell Culture Confluencies on Transfection Efficiency

The confluency of a cell culture can have a great effect on transfection efficiencies. In the example below, neonatal human epidermal keratinocytes were transfected using Lonza's Nucleofector™ Technology at 40% and 70% confluency (see Figure 3). As can be seen, the transfection efficiency with pmaxGFP™ shows a 20% difference in GFP expression.

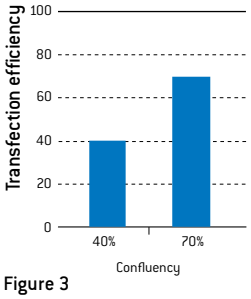

Figure 3
Neonatal human epidermal keratinocytes (Lonza, 192906) were cultured until they reached 40% and 70% confluency. Cells were transfected with pmaxGFP™ using the Nucleofector™ Technology under the same conditions. Transfection efficiencies were determined by flow cytometry.

This variation in transfection efficiency may affect the results of any downstream assay significantly, e.g., with higher standard deviations of the assay and, in consequence, a possible need for more repeated experiments to obtain an assay result with acceptable CV.

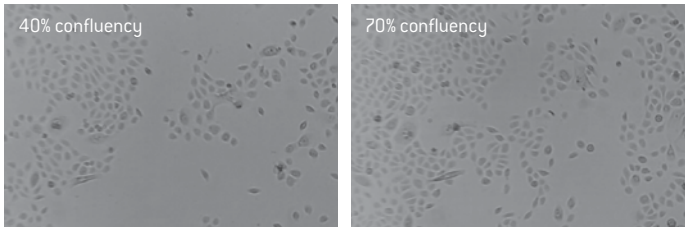

Figure 4
Images of neonatal human epidermal keratinocytes at 40% and 70% confluency taken with the CytoSMART™ Lux 10X System.

Summary

With the new CytoSMART™ System, live cell monitoring has become easy and affordable. Documentation and recording of a cell culture can be performed for a multitude of purposes, and at any given time and place, by using the CytoSMART™ Connect Cloud Service in conjunction with internet access.

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White Primary Human Preadipocytes Can Be Induced to Express UCP-1 and Transdifferentiate into Brown-like Adipocytes *In Vitro*

By Bodo Ortmann[1], Steffi Büsch[1], Tamara Grabeck[1], Michael Töller[1], Stephanie Nickles[2], Minh Hong[2]

[1]Lonza Cologne GmbH, Koeln, Germany; [2]Lonza Walkersville Inc., Walkersville, MD, USA

Introduction

White adipose tissue (WAT) in subcutaneous and visceral body compartments stores excess energy in the form of triglycerides. Excess accumulated fat, particularly in visceral adipose tissue, can lead to a number of disorders such as dyslipidemia, fatty liver disease, insulin resistance, Type 2 diabetes, hypertension, and cardiovascular diseases that are associated with other metabolic disturbances called metabolic syndrome. On the other hand, brown adipose tissue (BAT) aids to regulate body temperature in hibernating mammals and newborn humans. Within the brown adipocytes, accumulated fatty acids are degraded in the mitochondria and free energy is released as heat. This process, called thermogenesis, works by uncoupling the mitochondrial electron transport chain from the production of ATP. Expressed in the mitochondria's inner membrane, uncoupling protein 1 (UCP-1) bypasses the proton import path of the ATP synthase, thus releasing the energy of the proton gradient as heat. The discovery of active BAT in adult humans has raised great interest in the scientific community to expand research with BAT for new therapeutic strategies in the treatment of obesity and related disorders. Activating and expanding BAT *in vivo* may be one such strategy. BAT can be expanded through exposure to cold, or through pharmacological agents. For researchers and drug developers, an *in vitro* system of human brown adipocytes would be of great value to gain a better understanding of the key factors involved in transdifferentiation (see Figure 1 for an overview of the development of different types of adipocytes).[1]

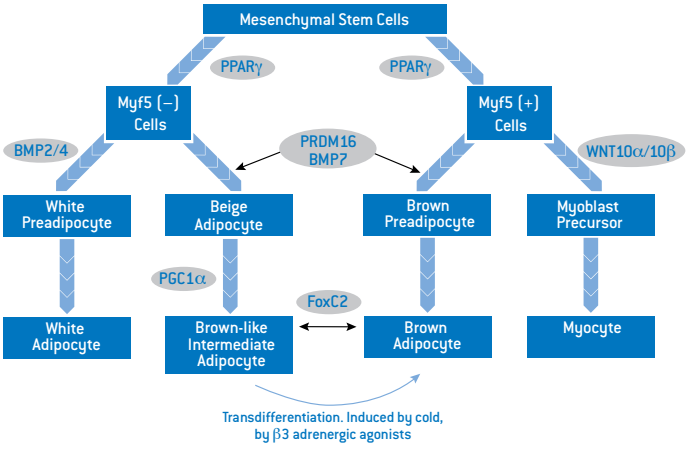

Figure 1. Origin and development of different types of adipocytes. Mesenchymal stem cells, triggered by the action of PPARγ, give rise to Myf5 negative and positive precursor cells. The latter ones give rise to brown adipocytes and muscle cells, the former to white and beige adipocytes (intermediates of brown and white adipocytes). Beige adipocytes differentiate to brown adipocytes upon treatment with β-adrenergic agonists. PRDM16 inhibits the induction of myoblast-specific genes and the expression of white adipocyte-specific genes.[1]
(PPARγ: Peroxisome Proliferator-activated Receptor γ, master regulator of adipogenesis; Myf5: Myogenic Factor 5; BMP: bone morphogenic protein; PRDM: PR domain containing protein; PGC1α: PPARγ coacitvator 1α)

As the supply of adult human BAT is limited, alternative robust sources would be advantageous. The aim of this study was to gain an understanding to which extent human preadipocytes can express a BAT phenotype. We demonstrate that primary human white preadipocytes of subcutaneous and visceral origin, treated with certain pharmacological factors *in vitro*, can give rise to a brown-like phenotype indicated by accumulated intracellular lipid droplets and expression of the marker UCP-1. In addition, we show that through treatment with forskolin, the UCP-1 marker can be induced in undifferentiated preadipocytes to various levels, depending on the donor. Taken together, such an *in vitro* system may provide results of greater relevance than those obtained with animal-derived cells.

Materials and Methods

Cell Culture

Human subcutaneous and visceral preadipocytes (Lonza) were thawed according to manufacturer's protocol and seeded in flat bottom well plates. Culture medium was DMEM (high glucose) with 10% FCS, 1% L-Glutamine, penicillin/streptomycin (all from Lonza). The cells were grown to confluence for 48 hours. Differentiation was started after confluence was reached.

Differentiating Preadipocytes to Brown-like Phenotype

The differentiation was performed according to two different published protocols which differed in the various factors added to the cell culture and the regimens of incubation times and media changes. Differentiation was started by replacing culture medium with differentiation medium containing the following factors (for actual concentrations see references listed at the end).

Protocol 1:
Differentiation medium was supplemented with IBMX (isobutyl-methyl-xanthine), dexamethasone, T3 (triiodo-L-thyronine), rosiglitazone, human insulin, and indomethacine. After 7 days, medium was replaced by medium containing only rosiglitazone and insulin for 10 days.[2]

Protocol 2:
Cells were seeded after thawing in proliferation medium (culture medium, see above, containing insulin, T3, isoprenaline). After reaching confluence, proliferation medium was replaced by differentiation medium containing additionally IBMX, dexamethasone, rosiglitazone, transferrin, pantothenate, biotin, human growth hormone, human insulin-like growth factor 1 for 7 days.[3]

Visualizing and Quantifying Intracellular Lipid Droplets

The lipid droplets were stained using the AdipoRed™ Assay (Lonza) according to supplier's protocol, subsequently followed by fluorescence-based imaging using a ZEISS microscope (AXIO Observer.Z1). Fluorescence was quantitated as relative fluorescence units (RFU) in a plate reader (FluoroSkan Ascent FL, Labsystems) after micrographs had been taken.

Quantitation of Cell Viability

The luminescence-based ViaLight™ Plus Assay (Lonza) was used according to manufacturer's protocol to quantify the cellular ATP concentration in individual wells as a measure of cell viability. Luminescence was quantified in a plate reader (FluoroSkan Ascent FL, Labsystems). The AdipoRed™ Assay and ViaLight™ Plus Assay could be multiplexed, i.e., subsequently be carried out in the same well.

Quantitative RT-PCR

Cells were lysed in the well using lysis buffer provided in the *Power* SYBR® Green Cells-to-C_T™ Kit (Life Technologies, Inc.). Cell lysates were used to generate cDNA from cellular mRNA using the specified reagents for reverse transcription supplied in the kit. For carrying out the PCR, again components of the kit were used. Primers for UCP-1 and for human β_2-microglobulin (β_2-m) were from Qiagen (RT² qPCR Primer Assay). The qPCR was run on a Bio-Rad C1000™ Thermal Cycler equipped with a CFX96™ Real-time System. Relative levels of UCP-1 mRNA were correlated with the expression level of the housekeeping gene β_2-m by the ΔC_T method, fold induction of UCP-1 normalized to a non-induced control by the $\Delta\,\Delta C_T$ method ($2^{-\Delta\,\Delta CT}$). Data shown represent means of two independent samples with standard deviations of 5% or below.

Immunofluorescent Staining

Cells were seeded in 24-well plates and treated with forskolin after reaching confluence. Forskolin was exchanged for culture medium after 24 hours and the cells incubated for another 24 hours. Cells were fixed with paraformaldehyde and permeabilized with Triton™ X-100. UCP-1 was stained with a rabbit polyclonal antibody (Novus Biologicals NBP2-20796) and with Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories). Nuclei were stained using Hoechst 33342. Micrographs were taken on a Zeiss AxioObserver.Z1 microscope. As a staining control, forskolin induced cells were incubated with the secondary antibody only. Micrographs were taken at the same exposure time as the properly stained images. No fluorescence was detected (not shown).

Results

1. Differentiation of Preadipocytes to a Brown-like Phenotype *In Vitro*

Preadipocytes derived from either subcutaneous or visceral WAT were put in culture and grown to confluence. Differentiation conditions were applied by changing media on Day 0. The two differentiation procedures were carried out according to published protocols (see Materials and Methods section). Cells were stained for accumulated lipid droplets using the Nile-red based AdipoRed™ Assay (Lonza). Staining of cells was documented by fluorescence microscopy (Figure 2). By semi-quantitative estimation, roughly 50% of the cells were differentiated, i.e., had accumulated intracellular lipid droplets.

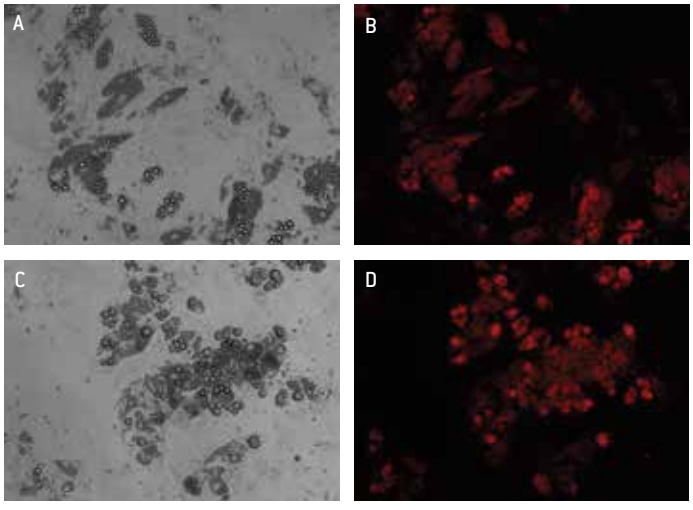

Figure 2. Differentiation according to Protocol 1: Accumulated intracellular lipid in adipocytes upon completion of differentiation. Micrographs (A, C brightfield; B, D AdipoRed™ Fluorescence) of differentiated adipocytes (A, B subcutaneous; C, D visceral) stained with AdipoRed™ Assay were taken at arbitrarily chosen positions in the wells on Day 18 after onset of differentiation.

The overall fluorescence was quantified in a plate reader. Viability of the cells was subsequently determined in the same wells through the luminescence-based ViaLight™ Plus Assay.

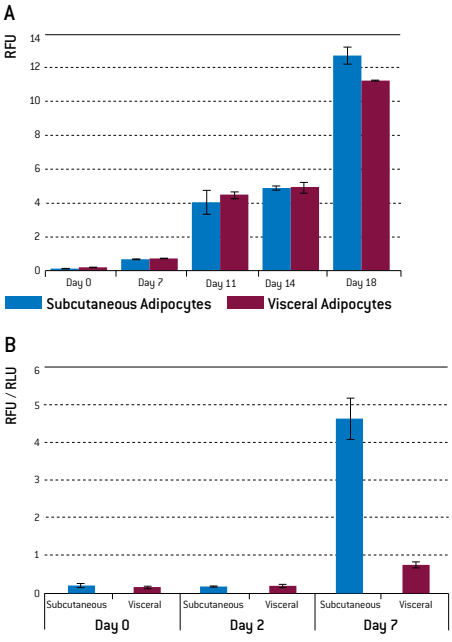

Figure 3. Differentiation of preadipocytes *in vitro* to brown-like adipocytes. Cells accumulate intracellular lipid steadily over the time course. The cells were grown and differentiated as described in Materials and Methods (A: Protocol 1, B: Protocol 2). Shown is the quantified fluorescence of cells stained with AdipoRed™ Assay. In B, cell viability, assessed through the ViaLight™ Plus Assay, was correlated with AdipoRed™ Fluorescence. Data represent means of three independent samples. Error bars are standard deviations.

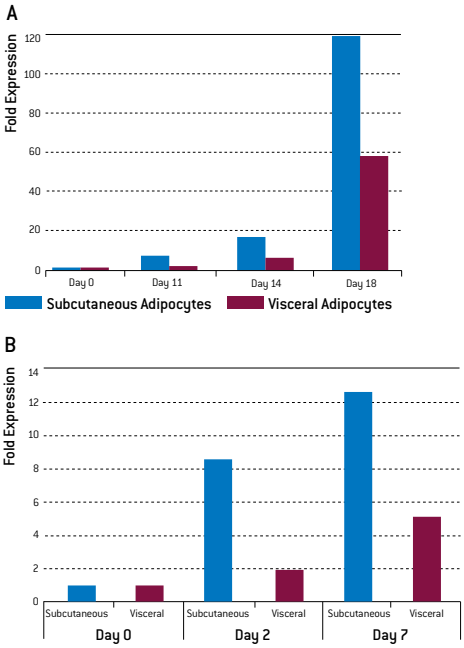

Figure 4. Expression level of UCP-1 mRNA over the time course of differentiation. Cells were grown and differentiated as described. On the respective day cells were lysed, cDNA was generated and stored at -80°C. qPCR was performed after all samples had been collected in one run. UCP-1 expression level, correlated with the expression level of the β2-m housekeeping gene, was normalized to the expression level on Day 0 (set to 1). Data represent means of two independent samples. Standard deviations were below 10%.

Both protocols led to an accumulation of intracellular lipid droplets shown in an example from Protocol 1 in Figure 2. The quantitation results shown in Figure 3A for Protocol 1 reveal a steady lipid accumulation over the time course where subcutaneous and visceral cells differentiate at about the same rate and intensity.

Protocol 2, in contrast, facilitated differentiation and lipid accumulation at a comparable level in a much shorter time interval. In these cases, subcutaneous and visceral preadipocytes responded very differently to the differentiation stimuli. Lipid storage in subcutaneous adipocytes was much higher than in visceral cells.

The differentiating cells expressed the brown adipocyte marker UCP-1 (quantified mRNA expression shown in Figure 4). The expression level of UCP-1 increases over the time course, roughly in correlation with the accumulation of intracellular lipids, except for the subcutaneous adipocytes in Protocol 2. Here, expression of UCP-1 mRNA was already significantly expressed on Day 2 in subcutaneous cells. This might be attributed to the fact that proliferation medium and differentiation medium of Protocol 2 is supplemented with isoprenaline, an adrenergic agonist also acting on β3 adrenergic receptors, thus enhancing transdifferentiation of intermediate to brown adipocytes (see Figure 1).

In this context, forskolin treatment of the cells was used to trigger UCP-1 expression at specific time points during the course of Protocol 1 differentiation. Forskolin is acting in the adrenergic signaling pathway by increasing intracellular levels of the second messenger cAMP through direct activation of the adenylyl cyclase. Figure 5 shows the fold increase of UCP-1 mRNA expression after forskolin treatment, based on the expression of non-induced samples. Induction under the chosen conditions is strongest on Days 11 and 14 for subcutaneous adipocytes, and at low levels on Days 0 and 18. For visceral adipocytes, there is one expression peak on Day 14.

Inducibility of UCP-1 is not very high at the latest time point as the cells express already high amounts of UCP-1 (compare Figure 4A).

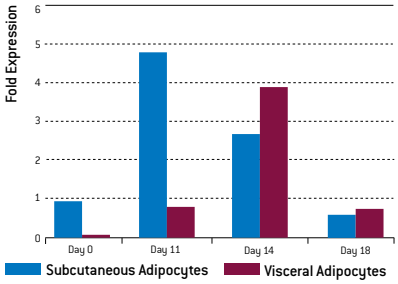

Figure 5. Induction of UCP-1 by treatment with forskolin in differentiated adipocytes. Cells were grown and differentiated according to Protocol 1 as described. Prior to induction with forskolin, cells were incubated in culture medium without factors for 24 hours, then induced with 100 µM forskolin for 5 hours. Cell lysis, cDNA synthesis and qPCR were performed as described in legend of Figure 4. The data were normalized to control samples with no forskolin induction (set to 1, not shown).

2. Inducibility of UCP-1 in Undifferentiated Preadipocytes

In order to assess the ability of preadipocytes to express UCP-1, cells were grown in plain culture medium to confluence for 72 hours, then treated with forskolin for 24 hours. Expression level of UCP-1 was determined by qPCR.

The expression of UCP-1 protein after induction with forskolin could be demonstrated by immunofluorescence staining (Figure 7). Exemplarily, the expression of UCP-1 protein in Lot F is shown, comparing cells stimulated by forskolin with unstimulated cells. The expression of UCP-1 is strongly induced by treatment with forskolin.

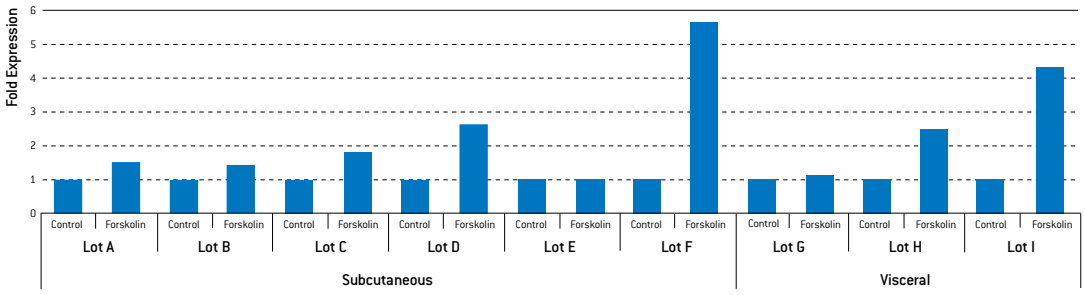

Figure 6. Induction of UCP-1 expression in undifferentiated preadipocytes by forskolin. Cells were grown in a 96-well plate to confluence for 72 hours. Treatment with 15 µM forskolin was performed for 24 hours. Preceding experiments had established this concentration as the optimal one (data not shown).

Figure 6 very clearly shows that in different preadipocyte lots, UCP-1 is expressed at varying levels, obviously depending on the tissue origin and the donor variability. This may reflect that only a portion of the cells is actually capable of giving rise to the brown or brown-like phenotype, so-called intermediate adipocytes, as depicted in Figure 1.

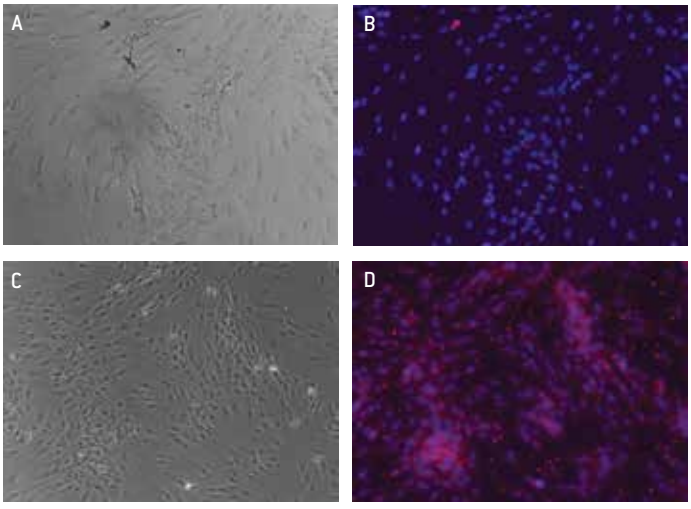

Figure 7. Expression of UCP-1 protein in forskolin treated preadipocytes (Lot F) detected by immunofluorescence microscopy. Cells were grown to confluence in a 24-well plate for 72 hours, treated with 15 µM forskolin (Panels C, D) for 24 hours (untreated control samples, Panels A, B), subsequently incubated in culture medium for another 24 hours. Staining the cells for immunofluorescence microscopy (using a 10x objective) was performed as described in the Materials and Methods section. Brightfield A, C. Overlays of anti-UCP-1 and nuclear staining B, D.

Conclusion

Subcutaneous and visceral human preadipocytes can be differentiated to brown-like adipocytes, accumulating intracellular lipid droplets and expressing the marker UCP-1. Expression of UCP-1 mRNA and protein could be induced by treatment with forskolin in undifferentiated preadipocytes. Taken together, these results suggest that primary white preadipocytes may be utilized to develop useful BAT model systems *in vitro*.

References

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Robust Generation and Maintenance of Human Pluripotent Stem Cells Under Defined and Xeno-free Conditions

By Xinghui Tian, Inbar Friedrich Ben-Nun, Ying Nie, Patrick Walsh, Jean S. Cadet, Amy M. Burkall, Huan Tran, Xu Yuan, Amy Walde, Kevan Shah, Laura Menendez, Carmine Carpenito, Boon Hwa Neo, Robert Keefe, Behnam Ahmadian Baghbaderani, Thomas Fellner, Lonza Walkersville, Inc., Walkersville, MD, USA

Abstract

In 2007, Dr. Shinya Yamanaka became the first to successfully convert adult human somatic cells to human induced pluripotent stem cells (hiPSCs). These cells have similar characteristics to embryonic stem cells (ESCs) and by definition have the ability to indefinitely self-renew and become any cell type in the body. Because of these attributes, hiPSCs have become an important scientific tool and are spurring advancements in basic research, disease modeling, drug development, and regenerative medicine.

However, the process to generate hiPSCs is inefficient and technically challenging, even when utilizing more efficient viral methods and mouse embryonic fibroblast feeder layers. Efficiencies fall even further when generating hiPSCs using non-integrating technologies under defined conditions. These realities led Lonza to focus on developing a robust system that could eventually be translated to current Good Manufacturing Practices (cGMP) and clinical grade manufacturing. The resulting system, named L7™ hiPSC Reprogramming and hPSC Culture System, is comprised of a culture platform (medium, matrix, passaging solution, and cryopreservation medium) that supports every-other-day (EOD) feeding of human pluripotent stem cells (hPSCs). In combination with an enhanced episomal reprogramming method, the resulting hiPSCs derived from blood cells share characteristics with human ESCs, including the expression of ESC-associated markers. In addition, these hiPSCs can efficiently differentiate into cells of all three germ layers and have a normal karyotype. Importantly, hiPSCs show no trace of exogenous DNA integration, confirming that cells have lost the episomal plasmids. To further test the system, we expanded multiple hESC and hiPSC lines beyond 40+ passages. In each case, the hPSCs maintained high expression of pluripotency markers and a normal karyotype.

Overview of Derivation of hiPSCs under cGMP-compatible Conditions

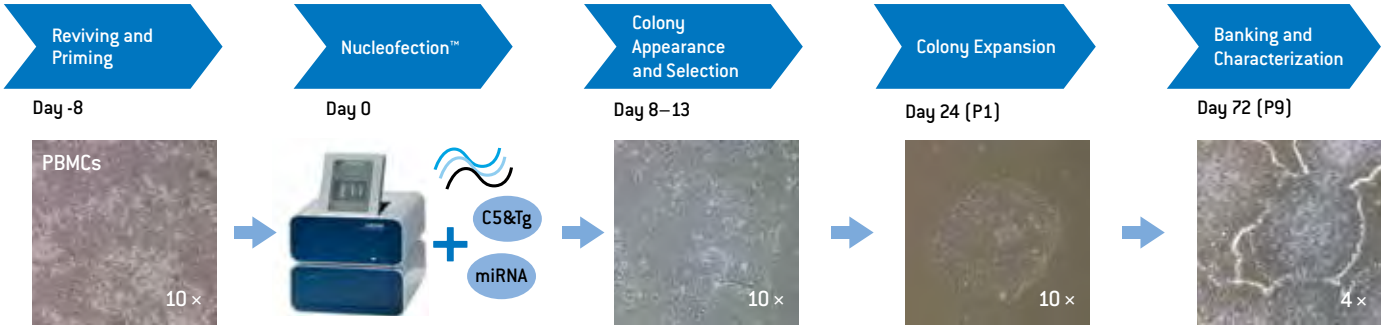


Figure 1. Derivation of hiPSCs under cGMP-compatible conditions. Combining Lonza's newly developed hPSC culture system (medium, matrix and passaging solution) with an optimized reprogramming technology results in vector-free hiPSC generation. This reprogramming technology allows robust and efficient derivation of hiPSCs from cord blood (CB)-derived $CD34^+$ cells and peripheral blood mononuclear cells (PBMCs).

Development of a cGMP-compatible Reprogramming and Pluripotent Stem Cell Culture System

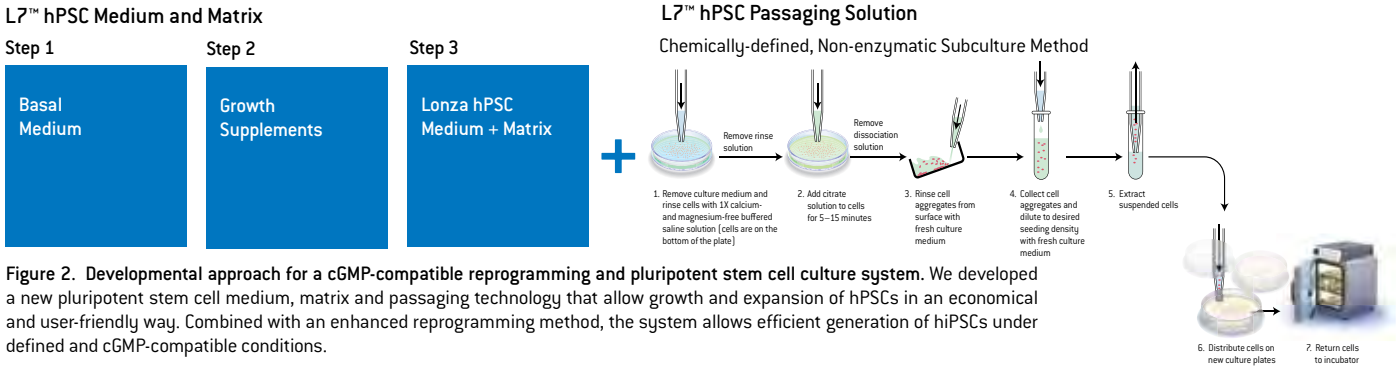
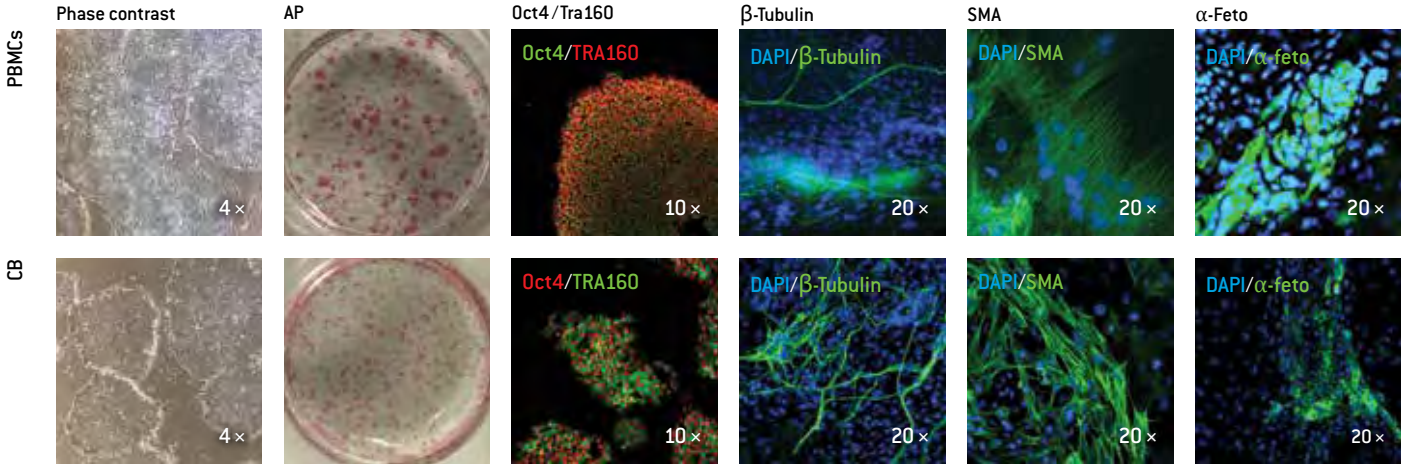
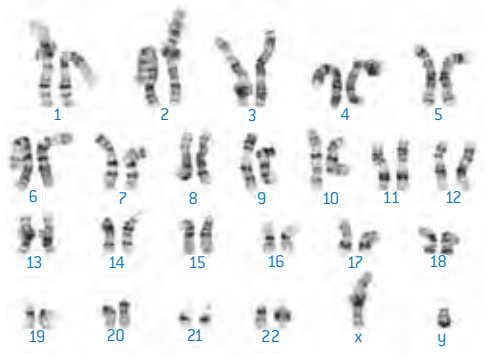


Figure 2. Developmental approach for a cGMP-compatible reprogramming and pluripotent stem cell culture system. We developed a new pluripotent stem cell medium, matrix and passaging technology that allow growth and expansion of hPSCs in an economical and user-friendly way. Combined with an enhanced reprogramming method, the system allows efficient generation of hiPSCs under defined and cGMP-compatible conditions.

Characterization of hiPSCs: Pluripotency Markers, Embryoid Body Differentiation, Karyotype, Cell Identity and Plasmid Clearance



A
Normal Karyotype (LiPSC-10F – P18)



B
STR Analysis (LiPSC-10F – P18)

Loci	Lonza LiPSC Donor 23316
AMEL	X, Y
CSF1PO	10
D13S317	12
D16S539	11
D21S11	30, 31.2
D5S818	11
D7S820	10, 11
Mus	
TH01	6, 8
TPOX	8
vWA	14, 16

Loci	CD34⁺ Cord Blood Donor 23316
AMEL	X, Y
CSF1PO	10
D13S317	12
D16S539	11
D21S11	30, 31.2
D5S818	11
D7S820	10, 11
Mus	
TH01	6, 8
TPOX	8
vWA	14, 16

C
Plasmid Clearance Confirmation (LiPSC-10F – P11)

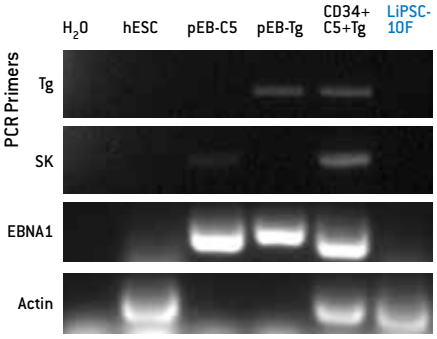


Figure 3. Characterization of hiPSCs generated with Lonza's cGMP-compatible reprogramming and cell culture system. These hiPSCs exhibit defining characteristics of pluripotent stem cells including expression of pluripotency markers, such as alkaline phosphotase (AP), POU5F1/OCT4 and TRA-1-60. In addition, they can be differentiated under appropriate conditions into the three germ layers, as demonstrated by β-tubulin (ectoderm), SMA (mesoderm), and AFP (endoderm) protein expression. Lonza hiPSCs maintain a normal karyotype (A), exhibit the unique cell identity of the donor cells (B), and show no trace of exogenous DNA integration (C).

hPSC Adaptation from MEF to L7™ Cell Culture System: Long-term Expansion and Characterization

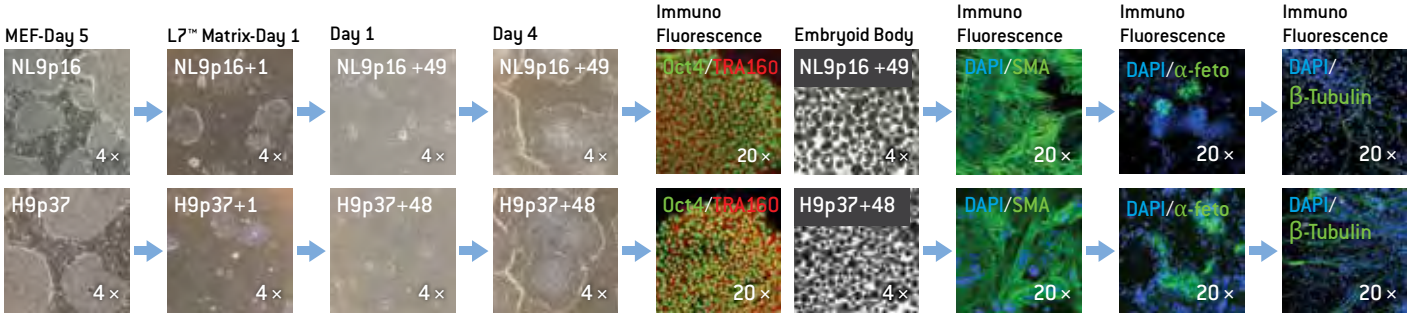


Figure 4. Development of L7™ hPSC Culture System. L7™ is comprised of a basal medium, supplemented with key cytokines, that was specifically developed for hPSCs. L7™ hPSC Matrix and L7™ hPSC Passaging Solution are both xeno-free, defined and, when used in conjunction with L7™ hPSC Medium, support long-term, EOD maintenance and downstream differentiation of hPSCs.

hPSC Cryopreservation with L7™ hPSC Cryosolution

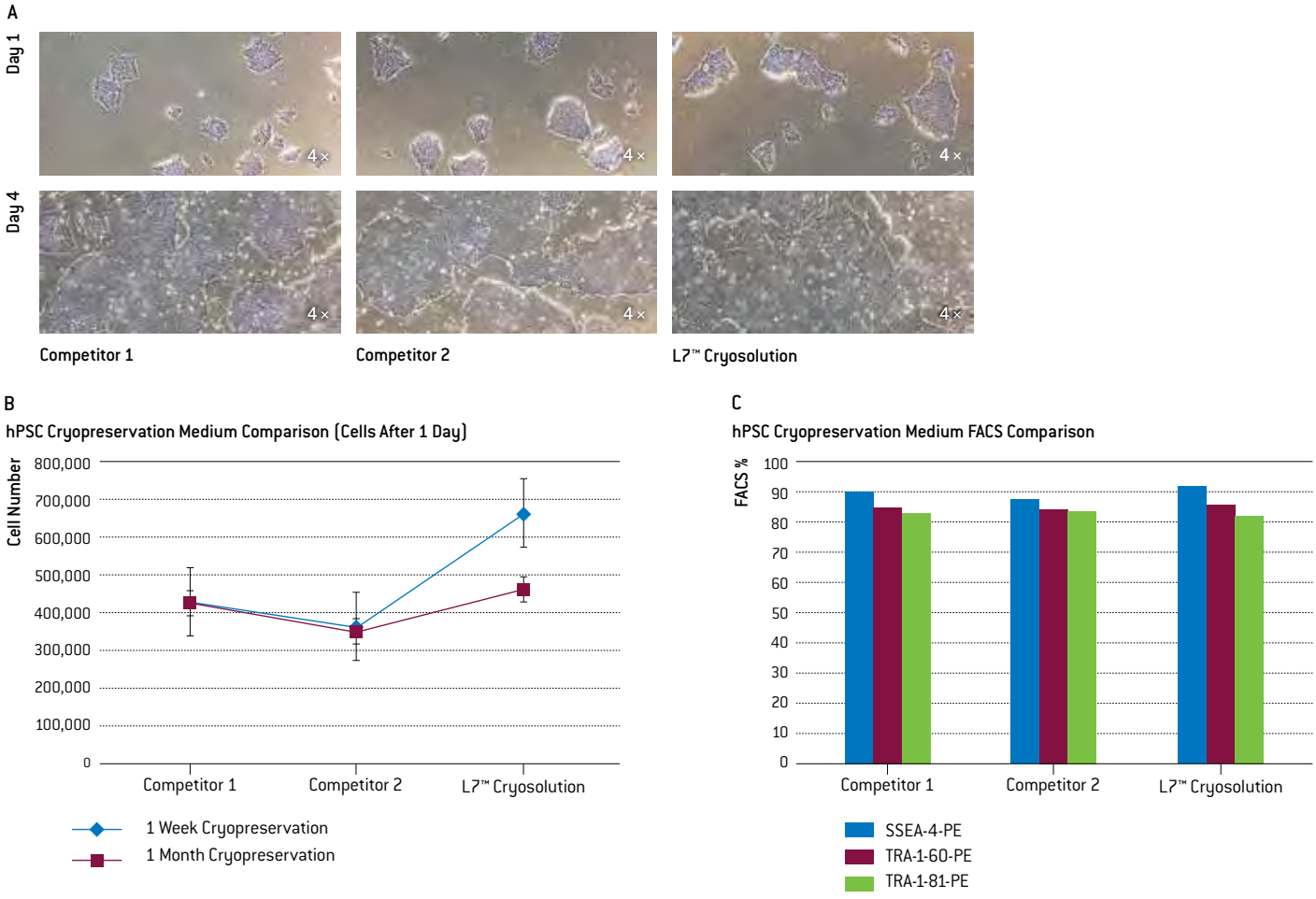


Figure 5. hPSC cryopreservation with L7™ hPSC Cryosolution. Approximately two million hPSCs were cryopreserved in L7™ hPSC Cryosolution and the recovered hPSCs were expanded (A) (B). The revived hPSCs maintained pluripotency as demonstrated by the high expression levels of stem cell surface antigens, such as SSEA-4 and TRA-1-60/81 analyzed by flow cytometry (C).

Lonza cGMP-compatible Pluripotent Stem Cell Products

Product	Attributes
L7™ hPSC Medium	Defined, xeno-free, cGMP-compatible, low FGF concentration, supports hiPSC generation, reduced spontaneous differentiation, enables EOD feeding
L7™ hPSC Passaging Solution	97 % cell viability, increased cell attachment, small homogeneous cell clusters, easy-to-use, xeno-free, cGMP-compatible
L7™ hPSC Matrix	Defined, xeno-free, reduced spontaneous differentiation, improved attachment, cGMP-compatible

References

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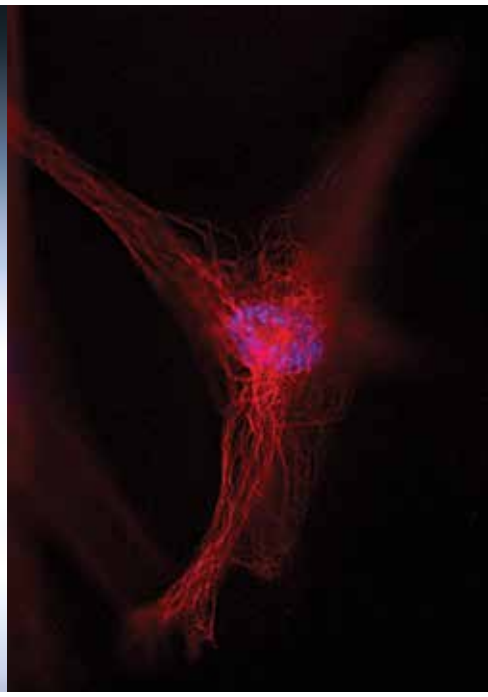
Chou *et al.* (2011) Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res.* 21(3): p. 518–29.



New RAFT™ 3D Cell Culture System

Advance Your Cells to the Next Generation

Lonza is supporting advances in cell culture by introducing the RAFT™ 3D Cell Culture System. This system has been designed with usability in mind, with easy-to-follow protocols that allow researchers to set up 3D cell cultures in under an hour. The patented RAFT™ Technology creates cell cultures, inside a high-density collagen scaffold, that mimics an *in vivo* environment. The RAFT™ Kit includes a collagen type I solution, a neutralizing solution, and biocompatible absorbers. RAFT™ Absorbers are designed to remove the medium from cell-seeded collagen hydrogels and allow researchers to control both cell concentration and matrix density. This enables researchers to generate more biologically meaningful data from their cell culture studies. The versatile RAFT™ Kit is available in 96-well or 24-well formats and is suitable for analysis using a wide variety of imaging, biochemical and histological techniques.

The RAFT™ System can be utilized with one cell type or multiple cell types in parallel. Cells can be cultured within the collagen scaffold, on top, or in both. The addition of permeable membrane cell culture inserts opens up enhanced options to the system, allowing the generation of barrier models, including air-lift models. Furthermore, the system is compatible with a variety of cell types and has already been used to successfully generate 3D cultures in a number of research areas including oncology, toxicology and neuroscience.

In combination with Lonza's human primary cells and media solutions, the RAFT™ 3D Cell Culture System empowers researchers to create physiologically relevant cell culture models for use across drug discovery and research applications.

RAFT™ Sequence

Empty well or insert

Cell and collagen mix forms hydrogel at 37°C

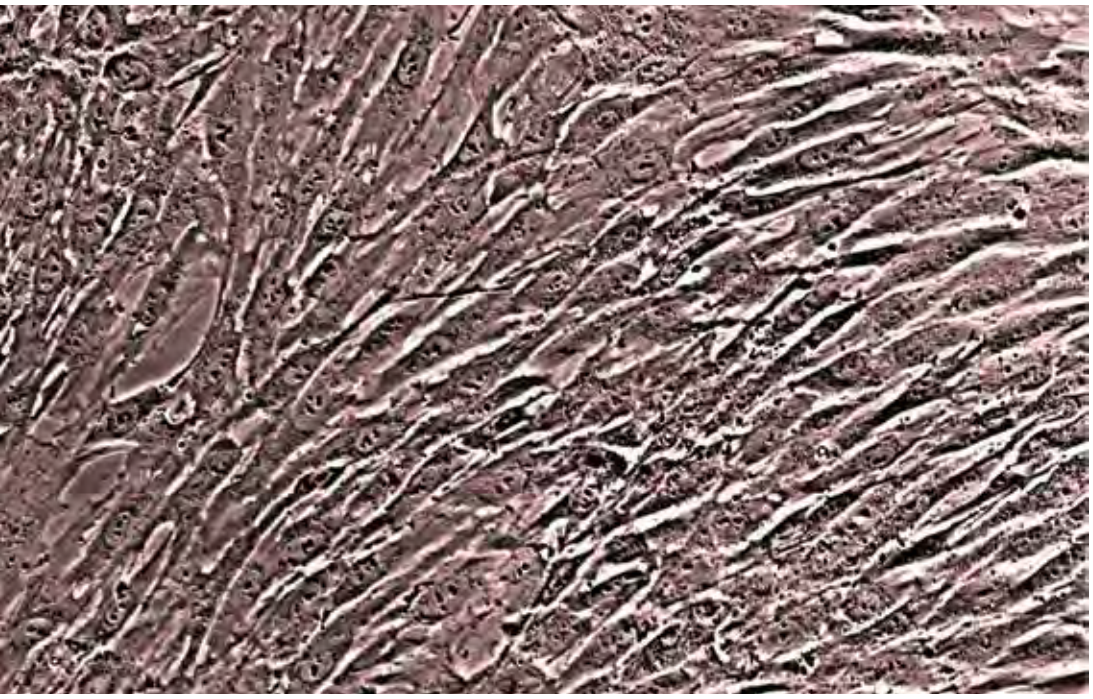
Absorber contacts the hydrogel

Liquid starts to be absorbed

Process concentrates cells and collagen

In less than 1 hour, culture formation complete

www.lonza.com/raft



Lonza Human Mesenchymal Stem Cells

Improved Characterization for More Relevant Results

Lonza's human bone marrow-derived mesenchymal stem cells (hMSCs) have expanded cell characterizations to meet industry guidelines for translational and cell therapy research applications. Multiple donors are available, including different donor ethnicities and ages. Use with Lonza medium (MSCGM™ Media BulletKit™ or TheraPEAK™ MSCGM-CD™ Media BulletKit™) for superior performance.

Lonza's hMSCs are cryopreserved at passage 2 and express CD73,* CD90,* CD29, CD105, CD166 and CD44, but not CD14, CD19,* CD34, CD45 and HLA-DR.* They are also tested for trilineage differentiation of the osteogenic (bone), chondrogenic (cartilage) and adipogenic (fat) lineages.

Key benefits include:

- Quality – Conforms to the International Society of Cellular Therapy (ISCT) standards for marker expression and trilineage differentiation
- Reliability – Fully tested to perform through passage 5 when using applicable protocol and medium
- Ease of Use – Just thaw cells and culture with recommended medium and protocol
- Convenience – Ready-to-use, cryopreserved cells offer flexibility with experimental schedules

*Designates the improved testing being performed on these cell types

www.lonza.com/hMSC

New L7™ PBMC Reprogramming Bundle

Simplify Your Reprogramming, Proliferation and Differentiation

Lonza has announced the release of the new L7™ PBMC Reprogramming Bundle for reprogramming human peripheral blood mononuclear cells (PBMCs) into human induced pluripotent stem cells (hiPSCs). This is the latest addition to Lonza's L7™ System, a comprehensive workflow for the reliable and efficient generation of hiPSCs and maintenance of human pluripotent stem cells (hPSCs). The L7™ System is a robust platform spanning basic stem cell research, disease modelling, drug development and regenerative medicine.

The entire L7™ System Features include:

- Robust performance, reduced workflow and increased flexibility
- Simple and reliable iPSC-generation tools and protocols
- Support for every-other-day feeding of long-term culture and maintenance
- Efficient differentiation into all three germ layers
- Xeno-free and fully defined for easy translation to clinical applications

www.lonza.com/L7

Product Highlights

New The CytoSMART™ System

Cell Monitoring and Cell Counting with One Device

Sized and priced for virtually any lab and budget, the new CytoSMART™ System has been developed for live cell imaging and monitoring. You can document and record your cell culture with the CytoSMART™ Device and monitor all data and experiments via innovative CytoSMART™ Cloud Technology – anytime and anywhere.*

In addition, the CytoSMART™ System offers a quick and easy-to-use cell counting feature using a standard hemocytometer. Within seconds, the CytoSMART™ App determines the exact number of living cells. Cell counts are reproducible and match the number achieved with manual cell counting.

	A. Cell Type 1			B. Cell Type 2		
	Count 1	Count 2	Count 3	Count 1	Count 2	Count 3
CytoSMART™ Cell Counting [10^6 cells/mL]	2.32	2.28	2.31	1.15	0.9	1.19
Manual Cell Counting** [10^6 cells/mL]	2.24			1.02		

Reproducibility of CytoSMART™ Cell Counting of living cells and comparison with manual cell counting. Cell counting performed with CytoSMART™ Device either counting the same area of the hemocytometer **(A)** or three different areas of a hemocytometer **(B)**.

Live Cell Imaging Applications

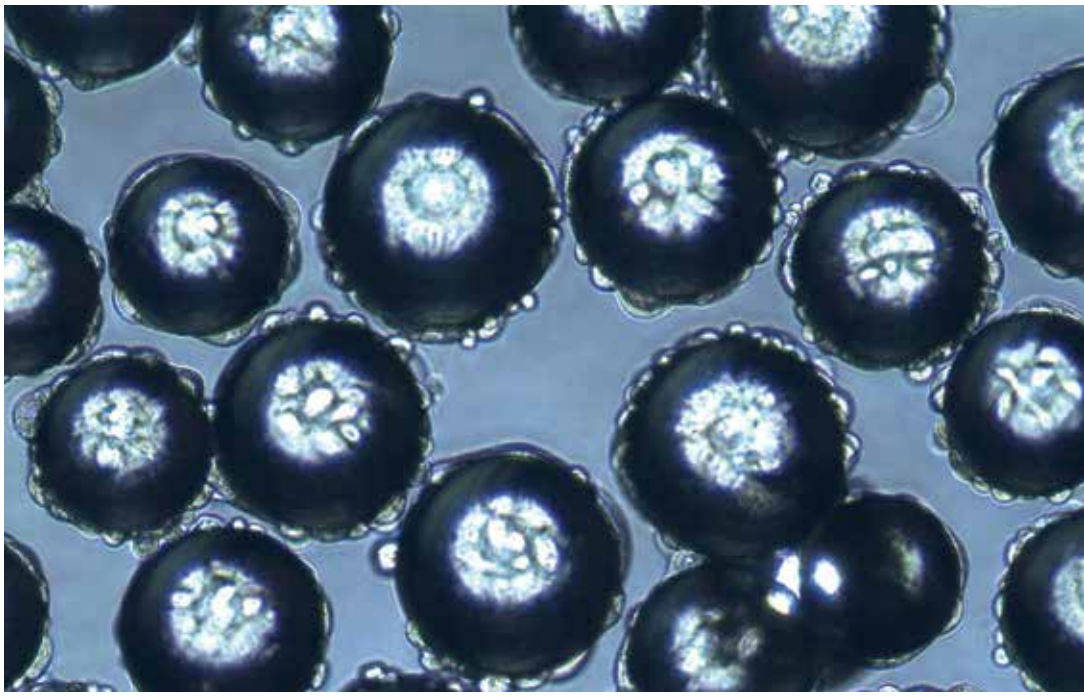
- Document your cell culture – record live cell images, cell confluency and temperature
- View your cell culture anytime* outside of the incubator

Cell Counting Applications

- Determine the cell concentration of your suspension cell culture
- Count the number of living cells in your trypan blue stained cell sample

*Requires internet access
**Performed according to standard laboratory practice

www.lonza.com/cytosmart



ProMDCK™ Media

High Performance Media for Vaccine Production

In July 2015, Lonza launched a new serum-free, non-animal origin media to support MDCK cell proliferation and virus infection. MDCK cells are adherent epithelial-like cells, now widely used in vaccine manufacturing as an alternative to the historical egg-based format, due to the rapid growth rate of the cells. Using MDCK cells allows vaccine manufacturers to target seasonal viruses instead of only pandemic viruses. Current media on the market for these cells is not truly serum-free and does not support 2D growth and transition to 3D growth where the virus infection occurs. Lonza's new ProMDCK™ 2D and ProMDCK™ 3D* Media Formulations allow seamless transition from culture flasks to microcarriers, and have been optimized for better virus infection on microcarriers.

*ProMDCK™ 3D Media exclusively available through Sartorius

Benefits of ProMDCK™ Media

- Completely defined, serum-free, non-animal origin media
- Supports the proliferation of MDCK cells in planar culture
- Easy transition from 2D to 3D MDCK cultures
- Optimal cell proliferation compared to competing media products

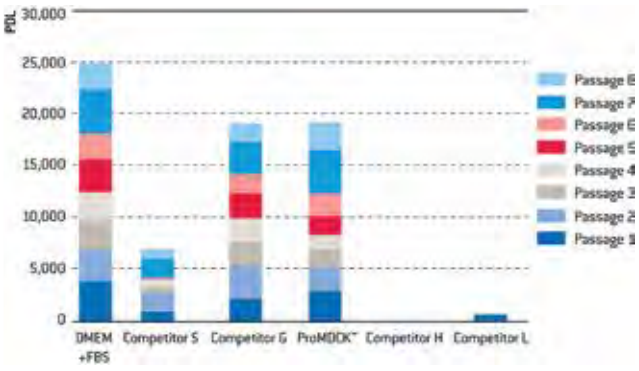
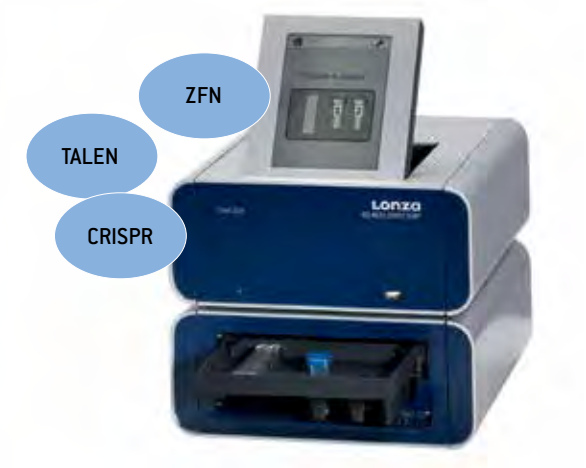


Figure 1. ProMDCK™ Media Supports Cell Proliferation Through Multiple Passages in 2D. Growth of MDCK cells was tested using classical media plus serum, various competitors' serum-free media and Lonza's new ProMDCK™ 2D Media. ProMDCK™ 2D has shown to perform better than most serum-free alternatives available on the market. Performance of ProMDCK™ 2D was only second when compared to serum use which is expected due to the number of undefined variables each lot of serum can contain.

www.lonza.com/promdck

Let's Talk About Genome Editing using Nucleofector™ Technology

In this issue of our **TechTalk**, we will provide you with guidelines for efficient genome editing using ZFN, TALEN or CRISPR/Cas9 in combination with our Nucleofector™ Technology.



Q. What is the advantage of genome editing?

A. Prior to genome editing, a stable, heritable DNA modification was accomplished either by random integration of plasmids, transposons, or viruses or via homologous recombination. The latter method results in site-specific integration, but is a very time-consuming and inefficient process. With the introduction of genome editing tools, site-specific stable modifications can now be performed easily. Zinc Finger Nuclease (ZFN) and Transcriptional Activator-like Effector Nuclease (TALEN) technologies were established over the last decade as useful tools for site-specific genomic modifications, but with the recent discovery of the CRISPR technology, another potent alternative has emerged.

Q. What is the CRISPR/Cas9 system?

A. Clustered regulatory interspaced short palindromic repeats (CRISPRs), discovered 1987 in *E. coli*, were recently shown to provide an even simpler genome editing tool (Jinek M *et al.*, *eLife* 2:e00471, 2013; Cho SW *et al.*, *Nat Biotechnol* 31:230–232, 2013; Cong L *et al.*, *Science* 339:819–823, 2013; Mali P *et al.*, *Science* 339:823–826, 2013). The CRISPR pathway is part of the bacterial immune system to defend against invading viruses. This system has been adapted for use in eukaryotic cells. The specificity is driven by a so-called "guide RNA" which typically binds to a complimentary stretch of 18–20 base pairs in the targeted DNA and has some additional sequence motifs that help in forming a complex with the Cas9 nuclease (CRISPR-associated nuclease). For successful binding of Cas9, the genomic target sequence must also contain a correct Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. The PAM is an NGG motif adjacent to the binding site. In contrast to ZFNs and TALENs, for CRISPR-based genome editing the DNA binding domain and the nuclease are not fused, since the DNA binding part is an RNA and not a protein. This feature makes it much easier to design a new guide RNA addressing a new target and also allows for multiplexed targeting.

Q. How does the CRISPR/Cas9 system differ from ZFNs or TALENs?

A. Briefly, ZFNs and TALENs require the generation of fusion proteins, thus making it more laborious to create a new engineered nuclease for another target site. For the CRISPR system, only a new guide RNA needs to be generated to target another sequence. In addition, with CRISPR, multiple targeting can be performed by combining the Cas9 nuclease with several guide RNAs.
Currently, however, ZFNs and TALENs are more specific than CRISPR and thus carry a lower risk for off-target effects. This is primarily due to their targeting of longer DNA stretches and the requirement for two partner molecules to form the final active nuclease dimer. To overcome this liability, some researchers have mutated the CRISPR/Cas9 nuclease to a "nickase", which can then be used in conjunction with paired sense and antisense gRNAs, thus providing enhanced specificity (Sander and Joung, *Nat Biotechnol* 1:1–9, 2014).

Q. What advantage does the Nucleofector™ Technology offer for genome editing?

A. One feature that is common to ZFN, TALEN or CRISPR/Cas9 is the need to co-transfer several substrates (plasmids, mRNAs, or oligonucleotides) into the cell type of interest for successful modification of genomic DNA (Figure 1). Co-transfection can be challenging, especially when it comes to hard-to-transfect cell types such as primary T cells, human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs). This challenge is overcome by Lonza's non-viral Nucleofector™ Technology, which has been shown to work as a reliable and efficient method for transferring the required DNA-, RNA-, or even protein-based components into various cell lines, primary cells, and stem cells. It has proven to work with any of the genome editing technologies described above.

Q. How do I establish the Nucleofector™ Technology for genome edting?

A. Lonza offers ready-to-use Optimized Protocols for a broad range of cell types (www.lonza.com/protocols) including hard-to-transfect cell lines and primary cells. Before performing a genome editing experiment, we highly recommend transfecting our pmaxGFP™ Positive Control Vector to verify that the optimal conditions we identified also work well in the user-specific setting.

If no ready-to-use protocol is available for a certain cell type, one can easily determine the optimal Nucleofection conditions using the pmaxGFP™ Vector by following the respective optimization protocol for a certain cell group or our general optimization protocols for primary cells or cell lines. For embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), for example, we recommend using our Basic Stem Cell Protocol, since each ESC or iPSC clone may require slightly different transfection conditions. Once the optimal conditions have been determined, they remain the same whether DNA- or RNA-based substrates (or both) are transfected.

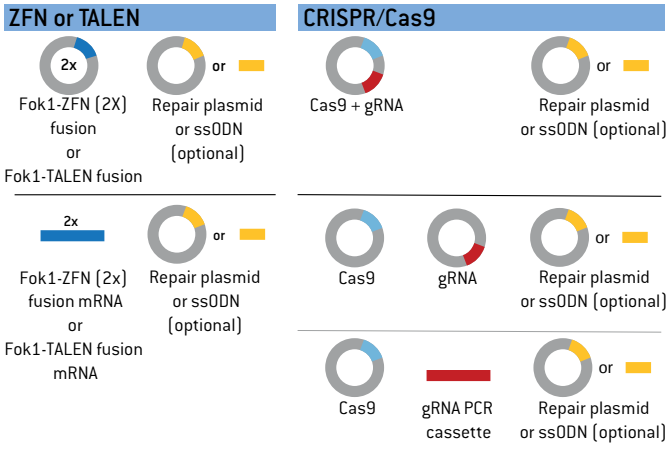


Figure 1. Possible co-transfection scenarios for ZFN, TALEN or CRISPR/Cas9. The scheme shows some substrate-type combinations (plasmids, mRNAs, or oligonucleotides) that have been described in the literature. However, additional scenarios may apply, e.g., transfection of proteins.

Q. What is the optimal substrate amount?

A. For successful genome editing, it is important to determine the optimal substrate amount. This may vary for different tools, types of substrates or cells. In our more comprehensive Technical Reference Guide (download at www.lonza.com/genome-editing), we provide some ranges published for ZFN, TALEN or CRISPR.

Q. If I want to transfect my genome editing tools as mRNA, is there anything special to consider?

A. Due to its shorter half-life, the use of mRNA instead of plasmids might be beneficial when aiming to minimize the exposure time of the nuclease and avoid multiple events. mRNA may also provide higher integration frequencies (Chen F *et al.*, *Nat Methods* 8:753–755, 2011; Hansen K *et al.*, *J Vis Exp* 64:e3304, 2012). When working with mRNA, the same protocol and program optimal for the transfection of DNA into the respective cell type can be used. However, there are a few additional things that should be considered:

- The mRNA should be capped and poly-adenylated.
- As with plasmids, the optimal mRNA amount has to be titrated, but it might be higher than for plasmid DNA.
- If higher amounts are required, the total volume added to the transfection reaction should not exceed 10% of the total sample volume.
- When collecting the cells for the transfection experiment, you may want to include an additional wash step with PBS to get rid of serum-derived RNAses (Hansen K *et al.*, *J Vis Exp* 64:e3304, 2012).
- Keep mRNA on ice prior to addition to the sample.
- To avoid any degradation, e.g., due to prolonged contact with cells, the mRNA might be transferred directly into the empty cuvettes before adding the cell-solution mix on top and transfection should be performed immediately.

Q. Can I also transfect CRISPR/Cas9 as protein?

A. Yes, the Nucleofector™ Technology is also suited to transfect peptides and proteins. As a starting condition, we would recommend using the established optimal conditions for nucleic acids, but some program fine-tuning might be required. Kim *et al.* (*Genome Res* 24:1012–1019; 2014) as well as Lin *et al.* (*eLife* 3:e04766; 2014) recently reported the transfection of Cas9-gRNA ribonucleoprotein using the 4D-Nucleofector™ System. They transfected various cell types with Cas9 protein premixed with *in vitro*-transcribed gRNA. For protein ranges used, please refer to these publications.

Q. At what time post Nucleofection should I start clonal selection?

A. Clonal selection can be started between 24 hours and 7 days post transfection. The optimal time point must be based on the individual experimental setting. One option to increase the number of clones is transfecting a vector that co-expresses a fluorescent protein, which would allow enrichment of transfected cells by FACS sorting. For cells that do not like to be grown as single cells (e.g., ESCs or iPSCs), FACS sorting might also be an alternative to the limiting dilution process.

Q. How do I typically analyze editing events?

A. Genome editing events can be analyzed by various means. Typically used methods comprise one or more of the following: PCR or RT-PCR, sequencing (e.g., deep sequencing, next generation sequencing), Southern blot, Northern blot or mutation frequency assays (mismatch assays such as Cel1 assay, T7 endonuclease I assay, SURVEYOR® Nuclease Assay, or RFLP analysis) or Western blot (to analyze protein knockout). For iPSCs, Yang *et al.* (NAR 41:9049–9061, 2014) have developed a robust and user-friendly system (genome editing assessment system) using next generation sequencing to screen for both HDR and NHEJ events.

www.lonza.com/research

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