BioResearch

Lonza

Resource Notes™

Spring 2013

The Newsletter for Life Science Researchers

Diabetes-related Differential Gene Expression in Primary ADSCs and HAECs

Generation of hiPSCs from Cord Blood Cells with the 4D-Nucleofector™ System

Evaluating an siRNA Screen Using the HT Nucleofector™ System

Accelerating Drug Development for Diabetes

Diabetes prevention has become a key focus area for the drug development market. The disease currently affects 366 million people worldwide and is expected to affect 552 million by 2030. According to Schulze Diabetes Institute, world renowned in islet isolation and transplantation, over $100 billion in costs are incurred annually by patients suffering from diabetes. With the prevalence of diabetes growing worldwide, the availability of primary human cells from diabetic donors is critical to increase research and knowledge about the disease at a cellular level.

On pages 5–7 of this issue, we present a study focusing on this topic. The researchers sought to identify genes differentially regulated in diabetic type 1 and type 2 adipose-derived stem cells (ADSCs) and human aortic endothelial cells (HAECs).

Pancreatic islets are the hormone-producing regions of the pancreas. Islets constitute approximately 1–2% of the pancreatic mass and contain a variety of cell types. The primary objective for islet research is to develop therapies to treat or cure diabetes. Please see page 21 to learn how to avoid roadblocks in diabetes research by using Lonza's Clonetics™ Fresh Human Pancreatic Islets.

Further topics covered in this issue include a scientific paper showing how the HT Nucleofector™ System can help to evaluate an siRNA screen in a mouse B cell line (pages 12–14) and a study on the generation of hiPSCs with the 4D-Nucleofector™ System (pages 8–11).
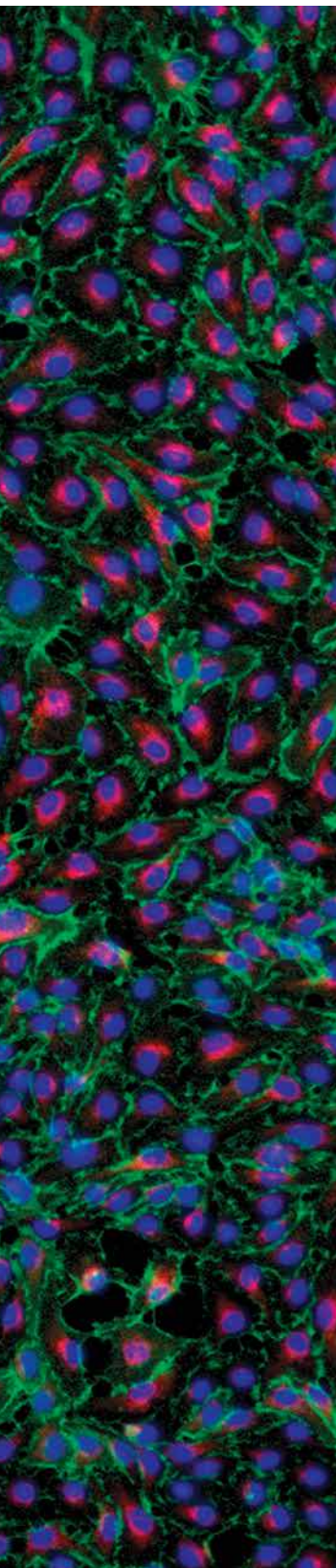
In addition, Nucleofection™ is the preferred technology used by Nobel Prize laureate Dr. Shinya Yamanaka for his studies on iPSC generation (see page 20).

We hope you enjoy this issue of Resource Notes™ and find it useful.

Your Lonza Team

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BioResearch Catalog 2013–2014

The BioResearch Catalog 2013–2014 is available. It includes the latest product information as well as a very useful section with technical tips and hints around primary cell culture, transfection and electrophoresis (see chapter 11). You can view or download it at:

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Lonza BioResearch Catalog 2013–2014

Conferences/Tradeshows

Visit us at:

Dates	Event	Booth No.	Location
06–10 April, 2013	AACR 2013	802	Washington, DC, USA
20–24 April, 2013	Experimental Biology 2013	146	Boston, MA, USA
04–07 June, 2013	Forum Labo & Biotech 2013	D60	Paris, France

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

Webinars

Date	Webinar
15 April, 2013	Cell Culture Techniques
16 April, 2013	Cell Culture Techniques
14 May, 2013	Pancreatic Islets
15 May, 2013	Pancreatic Islets
18 June, 2013	Dental Pulp Stem Cells
19 June, 2013	Dental Pulp Stem Cells
17 September, 2013	Cancer Applications for Primary Cells
18 September, 2013	Cancer Applications for Primary Cells

🌐 For details and registration visit www.lonza.com/webinars

Diabetes-related Differential Gene Expression in Primary Human Adipose-derived Stem Cells and Aortic Endothelial Cells

By Rochelle Myers[1], Lubna Hussain[1] and Ludger Altrogge[2]

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Introduction

With the prevalence of diabetes growing worldwide, the availability of primary human cells from diabetic donors is critical to increase research and knowledge about the disease at a cellular level. In this study, we sought to identify genes differentially regulated in diabetic type 1 and type 2 adipose-derived stem cells (ADSCs) and human aortic endothelial cells (HAECs). ADSCs are isolated from adipose tissue that can self-renew and are multipotent, immunoprivileged, and immunosuppressive. HAECs are endothelial cells that line macro blood vessels and play important roles in atherosclerosis, inflammation, barrier function, and angiogenesis. The primary cells are isolated from normal and diabetic type 1 and type 2 diagnosed human donors in accordance with all informed consent rules and regulations. The cells were cultured for several passages in optimized media. Gene expression analysis was performed using the Human Insulin Signaling 96 StellARray™ qPCR Array for ADSCs and the Human Endothelial Cell 96 StellARray™ qPCR Array for HAECs. The StellARray™ Gene Expression System is a quantitative polymerase chain reaction (qPCR)-based method and provides reliable profiling of biologically focused gene sets. This article summarizes a study comparing normal and diabetic type 1 and type 2 ADSCs and HAECs to explore differences in gene expression among the donor samples.

Materials and Methods

In this study, we grew primary aortic endothelial and adipose-derived stem cells from normal, diabetic type 1 and diabetic type 2 tissues in standard submerged culture (see Table 1) to assess gene expression changes associated with diabetic diseased states.

	HAEC Aortic EC—Normal	HAEC Aortic EC—Diabetes Type 1	HAEC Aortic EC—Diabetes Type 2	Adipose-derived Stem Cells—Normal	Adipose-derived Stem Cells—Diabetes Type 1	Adipose-derived Stem Cells—Diabetes Type 2
Cat. No.	CC-2535	CC-2919	CC-2920	PT-5006	PT-5007	PT-5008
Lot No.	0000227953	0000239326	0000235247	7F4089	1F4104	1F4103
Age	49 Years	29 Years	53 Years	47 Years	62 Years	83 Years
Sex	Male	Female	Male	Female	Female	Female
Race	Asian	Caucasian	Caucasian	Unknown	Caucasian	Caucasian

Table 1
Donor characteristics of primary cells used.

Cell Isolation

Endothelial cells were isolated from normal and diabetic aortae. Isolated cells were expanded in standard submerged culture in EGM™-2 Growth Medium (Lonza, cat. no. CC-3162) and then cryopreserved after the third passage. Adipose-derived stem cells from normal and diabetic donors were isolated and cryopreserved at the first passage in ADSC Growth Medium BulletKit™ (Lonza, cat. no. PT-4505). The vials were stored in liquid nitrogen until further use.

Cell Harvesting and Cell Lysis

Each lot of normal and diabetic endothelial cells was thawed and plated at a density of 5,000 cells/cm^2 in EGM™-2 Growth Medium (Lonza, cat. no. CC-3162). Each lot of normal and diabetic adipose-derived stem cells was thawed and plated at a density of 5,000 cells/cm^2 in ADSC Growth Medium (Lonza, cat. no. PT-4505). Growth media were changed after 24 hours and the cells were subcultured through multiple passages. At P7, cells were pelleted and cell lysates were obtained using the QIAshredder™ column (Qiagen, cat. no. 79654).

qPCR Experimental Design

To generate data with biologically relevant variance, three replicate samples were independently assayed for each cell type.

RNA Isolation and cDNA Synthesis

Each cell lysate was transferred to the Qiagen RNeasy® Mini Kit (cat. no. 74104) and RNA was extracted. cDNA was synthesized with 2 µg of RNA per sample using SuperScript® II Reverse Transcriptase and dNTP mix (Life Technologies, cat. no. 18064-014 and 10297-018, respectively). For primers, random decamers and oligo dT primers (Life Technologies, Inc., cat. no. AM5722G and 18418-012) were used. cDNA synthesis reactions were performed according to the specifications of the supplier.

For Each qPCR Plate, a Reaction Mix was Prepared in the Following Manner	
2x SYBR® Green Master Mix (Life Technologies: Fast SYBR® Green Master Mix)	1031 µl
H$_2$O	975 µl
cDNA template: 40 µl cDNA synthesis reaction mix + 310 µl H$_2$O	106 µl

Table 2
Real-time qPCR.

20 µl of the reaction mix was distributed into Human Insulin Signaling 96 StellARray™ qPCR Array Plate (Lonza, cat. no. 198061) for ADSCs and Human Endothelial Cell 96 StellARray™ qPCR Array Plate (Lonza, cat. no. 198043) for HAECs. The master mixes contained

AmpliTaq® Fast DNA Polymerase (Life Technologies, Inc.), designed to allow instant hot start. Arrays were run on the BioRad CFX 96 using a standard qPCR program. Post-run data collection involved the setting of a common threshold (Ct) across all arrays within an experiment, exportation and collation of the Ct values, and analysis via GPR.

GPR Algorithm

Data input for GPR consists of a list of Ct values, derived directly from real-time PCR instruments, for each sample (normal or diabetic). After designating the control and experimental sets of Ct values, GPR filters expression data to separate genes into two groups — genes considered for analysis (G) and genes that can be used as potential normalizers (N). Genes that are not expressed in either sample are not considered further. After filtering the data into two sets, GPR performs a proprietary global normalization and statistical analysis by comparing each G to each N. The magnitude of change in expression ("fold change") for each gene is subsequently determined using the ten best N genes, as defined within each experiment.

Results and Discussion

The Human Insulin Signaling 96 StellARray™ qPCR Array was used to analyze the human adipose-derived stem cell samples. This array was designed with genes to measure the effects of insulin signaling associated with processes like glycogen synthesis, glycolysis, and fatty acid synthesis. The Human Endothelial Cell 96 StellARray™ qPCR Array was used to analyze the human aortic endothelial cell samples. This array was designed to measure genes associated with differentiation, development, and tissue remodeling of endothelium. In each cell type, statistically relevant differences in gene expression were detected in the diabetic cells compared to the normal cells. Genes with P-values of <0.05 or better are reported. Out of the 94 genes on the insulin array, the expression of 18 genes changed by +25.7 to -3.3 fold for ADSC type 1 diabetes and 10 genes changed by +24 to -7.7 fold for type 2 diabetes (as compared to normal cells). Out of the 94 genes on the endothelial array, the expression of 16 genes changed by +17 to -8.7 fold for HAEC type 1 diabetes and 8 genes changed by +52557 to -54 fold for type 2 diabetes (as compared to normal cells). While this article is not meant to be a comprehensive analysis of the genes and their functions in diabetes, we will highlight several genes and their functions.

ADSC: Adipose-derived Stem Cells

Among the ADSC diabetic type 1 and type 2 cells, the two most upregulated genes (as compared to the normal) for both were INS (insulin) and G6PC2 (glucose-6-phosphatase, catalytic, 2). G6PC2 encodes an enzyme that belongs to the G6PC family. These enzymes are part of a larger unit that catalyzes the hydrolysis of G6PC, which releases glucose into the bloodstream. The enzyme family member specific to G6PC2 is found in pancreatic islets and is a main target of cell-mediated autoimmunity in diabetes[1].
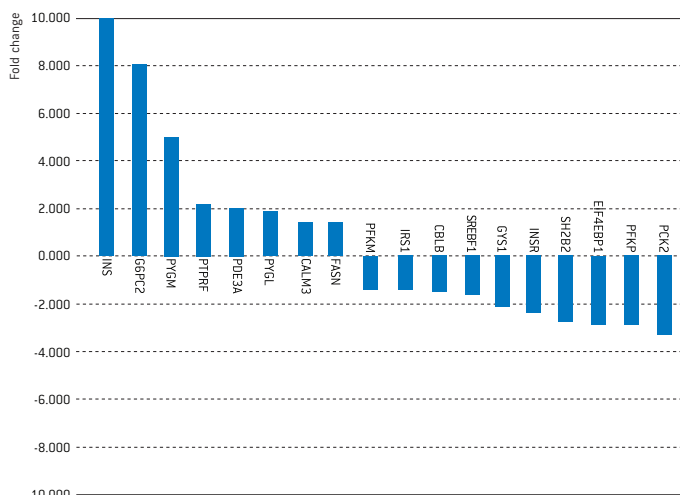
Figure 1
Genes up- or downregulated in ADSC diabetes type 1 sample vs. normal cells.

In the ADSC type 2 diabetic donors, PPARGC1A was downregulated 7 fold as compared to the normal donors. PPARGC1A encodes for a transcriptional coactivator protein that regulates genes involved in energy metabolism[2].
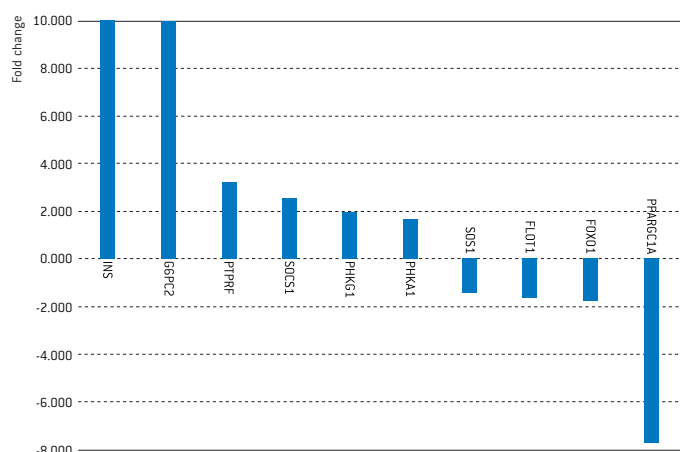
Figure 2
Genes up- or downregulated in ADSC diabetes type 2 sample vs. normal cells.

HAEC: Human Aortic Endothelial Cells

In the HAEC diabetic type 1 and type 2 cells, a common upregulated gene was SELE (14 fold in type 1 and 33.5 fold in type 2). E-selectin (SELE) is expressed by endothelial cells to recruit leukocytes during the inflammatory response, and increased levels of E-selectin are associated with type 2 diabetes. One study suggested that in high glucose conditions, macrophage stimulation of E-selectin may play a role in atherogenesis, and suggested this as a mechanism as to why arterial disease is accelerated in diabetes[3].
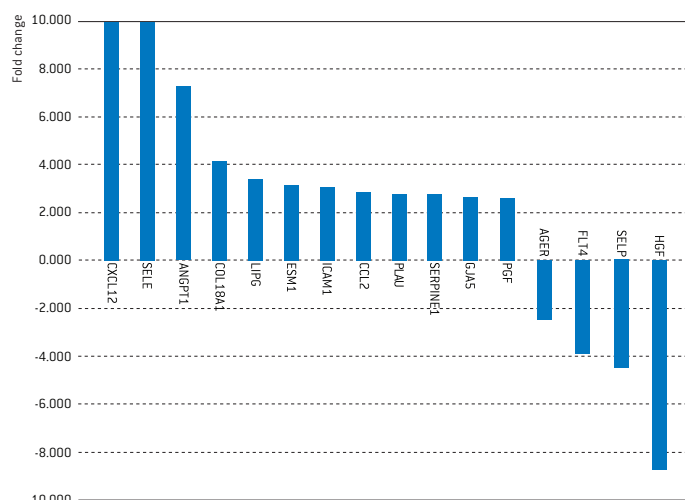
Figure 3
Genes up- or downregulated in HAEC diabetes type 1 sample vs. normal cells.

Hepatocyte growth factor (HGF) was downregulated in both type 1 (8.8 fold) and type 2 (54 fold) diabetic cells when compared to the normal donor cells. Increasing the concentration of HGF has been shown to induce therapeutic angiogenesis in high glucose environments in rats and to lessen apoptosis in human endothelial cells[4,5]. Injury to aortic endothelial cells has been suggested to be a trigger of the progression of atherosclerosis in diabetic patients[5].
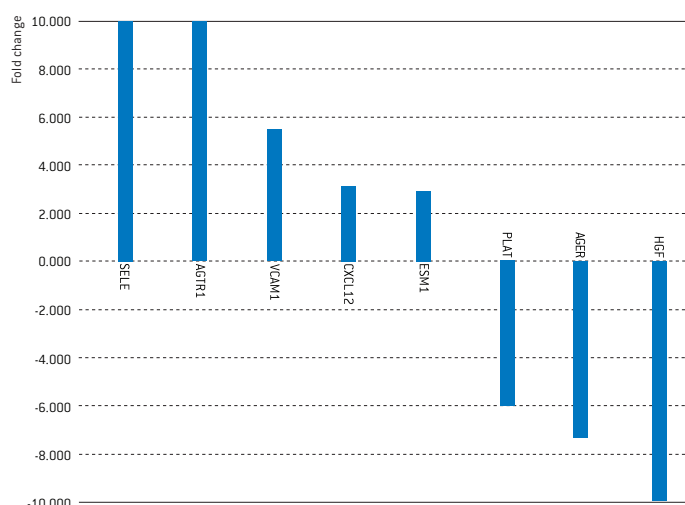
Figure 4
Genes up- or downregulated in HAEC diabetes type 2 sample vs. normal cells.

In the HAEC type 2 diabetic samples, AGER (advanced glycosylation end product-specific receptor) was downregulated 7 fold as compared to the normal donor samples. The receptor encoded by this gene interacts with molecules implicated in homeostasis, inflammation, and diabetes[6].

Summary

Data generated from this study shows up- and downregulation of genes in ADSC and HAEC cells from diabetic type 1 and type 2 donors as compared to normal donors using the Human Insulin Signaling 96 StellARray™ qPCR Array and the Human Endothelial Cell 96 StellARray™ qPCR Array. This data has indicated several differences in gene expression between the normal and diabetic cells from both cell types and is consistent with other published literature. The utility of primary cells has further strengthened the biological relevance of data generated as it relates more closely to the *in vivo* model. Results from this study encourage further exploration as to how diseases such as diabetes affect systems in the body and cause differences in gene expression and gene regulation pathways.

Lonza offers a variety of primary cells from diabetic type 1 and type 2 donors. The cells are tested and guaranteed to perform in Lonza's media which are customized for each cell type. Cells from both normal and diabetic donors can be cultured in the same media, or conditions can be modified to mirror different *in vivo* conditions. In addition to Lonza's normal and diabetic cryopreserved cell offerings, Lonza now offers fresh pancreatic cell islets from normal and diabetic donors for research use. Type 1 diabetes is characterized by the immune system destroying insulin-secreting beta cells of the pancreas. Islet-transplantation replenishes the beta cell supply, rendering better regulation of insulin levels. With Lonza's diabetic product portfolio, researchers will now have a more complete offering of biologically relevant research tools to aid in diabetes research. Additional information about any of Lonza's cell offerings can be obtained from Lonza's Scientific Support Team.

References

1. Pubmed GENE ID 57818 description, (10Nov2012).
2. Pubmed GENE ID 10891 description, (10Nov2012).
3. Chen T, *et al. J Biol Chem.* 2011. 286(29):25564–73.
4. Taniyama Y, *et al. Circulation.* 2001. 104(19):2344–50.
5. Nakagami H, *et al. Diabetes.* 2002. 51(8):2604–2611.
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Generation of hiPSCs from Cord Blood Cells with the 4D-Nucleofector™ System

By Inbar Friedrich Ben Nun, Xu Yuan, Patrick Walsh, Amy Burkall, Don Paul Kovarcik and Thomas Fellner
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Abstract

In 2007, Dr. Shinya Yamanaka and colleagues at Kyoto University became the first to successfully convert adult human cells to an embryonic stem cell(ESC)-like state[1]. To generate the first human induced pluripotent stem cells (hiPSCs), Dr. Yamanaka's team first utilized viruses to deliver transcription factors necessary for reprogramming cells. These viruses integrate, thus altering the host genome and potentially leading to aberrant gene expression. Since then, efforts have been made to generate hiPSCs using integration-free methods. Here, we successfully deliver non-viral vectors encoding the transcription factors using Lonza's 4D-Nucleofector™ System into cord blood CD34+ cells resulting in the generation of hiPSC colonies in less than 14 days.

Introduction

Pluripotent stem cells, such as hESCs or hiPSCs, have the ability to self-renew and generate any cell type in the human body. Therefore, they have the potential to produce an infinite quantity of cells for different applications, such as regenerative medicine, disease modeling, and drug development.

In many research laboratories, viruses are the preferred method used to deliver reprogramming factors into the cells. Although the process is robust and efficient, integration of exogenous viral DNA fragments may result in altered gene expression. To avoid genome modifications, several technologies were developed. One of the most promising methods utilizes episomal plasmids to generate integration-free hiPSCs.

Several laboratories have successfully reprogrammed multiple cell types using episomal vectors encoding the transcription factors[2,3]. hiPSCs generated using this method eventually lose the episomal vectors after several passages, leaving no trace of exogenous DNA. Here, we describe a reliable method for the introduction of episomal plasmids utilizing Lonza's 4D-Nucleofector™ System, resulting in the efficient generation of "zero footprint" hiPSCs.

Materials and Methods

Cell Culture

Cord blood CD34+ cells (Lonza, cat. no. 2C-101) were isolated from mononuclear cells using positive immunomagnetic selection. Cryopreserved cord blood CD34+ cells were thawed and grown in serum-free medium (SFM) containing 50% IMDM, 50% Ham's F12, 1x chemically defined lipid concentration, 1x Insulin-Transferrin-Selenium-X, 2 mM GlutaMAX™ I (Invitrogen) and 50 μg/ml ascorbic acid (Sigma). The medium is supplemented with cytokines: 100 ng/ml SCF, 100 ng/ml Flt3-ligand, 20 ng/ml TPO and 10 ng/ml IL-3 (PeproTech). CD34+ cells were cultured in SFM for 4–5 days before transfection with the 4D-Nucleofector™ System (Lonza, cat. no. AAF-1001B, AAF-1001X).

hiPSCs were maintained on mytomycin C-treated mouse embryonic fibroblast (MEF) feeders (Millipore) in hESC medium containing Knockout-DMEM/F12, 20% Knockout serum replacement, 1x NEAA, 2 mM GlutaMAX™ I, 0.11 mM 2-mercaptoethanol, and 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen). The cells were passaged with 1 mg/ml collagenase IV (Invitrogen).

Vectors

The episomal vectors pEB-C5 and pEB-Tg were used to generate hiPSCs[2]. The pEB-C5 plasmid encodes five mouse cDNAs, including *Oct3/4, Sox2, Klf4, c-Myc* and *Lin28*. The pEB-Tg plasmid encodes SV40 Large T antigen.

Nucleofection™

CD34+ cells were co-transfected with the two episomal vectors (pEB-C5 and pEB-Tg) using the 4D-Nucleofector™ System according to the manufacturer's instructions. We used 10^6 CD34+ cells per sample and suspended them in 100 μl P3 Primary Cell Nucleofector™ Solution containing 8 μg pEB-C5 and 2 μg pEB-Tg. Cells were nucleofected with program EO117. After transfection, 0.5 ml warmed SFM supplemented with cytokines was added to the cells in the cuvette. The transfected cells were gently suspended in the cuvette and transferred into 1.5 ml pre-warmed SFM supplemented with cytokines in one well of a 12-well plate.

Generation of hiPSCs

Generation of hiPSCs from CD34+ cells was performed as previously described[3]. After co-transfection of pEB-C5 and pEB-Tg, the transfected cells were cultured in SFM supplemented with cytokines for 2 days. The cells were collected and spun down (200 xg, 5 minutes). We used MEF medium (DMEM, 10% FBS, 1x NEAA) to suspend the cells and plate them into one well of a 6-well plate pre-coated with MEF feeders and gelatin (Millipore). After 24 hours, MEF medium was replaced with hESC medium containing 0.25 mM sodium butyrate (NaB) (Stemgent). Medium was replaced every other day. One week later, cells were fed with MEF-conditioned hESC medium, supplemented with 10 ng/ml bFGF and NaB. Colonies were observed as early as 6 days after transfection.

Characterization of Lonza hiPSCs

We performed immunocytochemistry to test pluripotent stem cell-associated marker expression in Lonza hiPSCs. Cells were fixed with 4% paraformaldehyde, permeabilized and stained with anti-SSEA4 antibody (Milipore, cat. no. MAB4304), anti-TRA-1-60 antibody (Millipore, cat. no. MAB4360), anti-OCT3/4 antibody (Abcam, cat. no. 19857) and anti-NANOG antibody (R&D Systems, cat. no. AF1997). Cells were tested for alkaline phosphatase activity using Alkaline Phosphatase Kit II (Stemgent, cat. no. 00-0055).

Flow cytometry was performed to study the surface markers of Lonza hiPSCs. The following antibodies were used in the flow cytometry analysis: mouse anti-human SSEA4-PE (BD, cat. no. 560128), mouse anti-human TRA-1-60-PE (BD, cat. no. 560193) and mouse anti-human TRA-1-81-PE (BD, cat. no. 560161).

Pluripotency was demonstrated by *in vitro* differentiation via embryoid body (EB) formation followed by immunocytochemistry. Undifferentiated hiPSCs were dissociated with 1mg/ml collagenase IV and cultured in suspension to form EBs. After culturing in suspension for 7 days in the presence of 20% FBS, EBs were plated onto a gelatin-coated plate for further differentiation. The differentiating cells were maintained in DMEM medium containing 20% FBS and stained for markers of the three germ layers with polyclonal rabbit anti-human alpha-1-fetoprotein (AFP) antibody (Dako, cat. no. A000829), anti-tubulin beta III monoclonal antibody (TUJ1) (Millipore, cat. no. MAB1637), and mouse anti-actin smooth muscle (SMA) antibody (Millipore, cat. no. CBL171).

To confirm the absence of the exogenous plasmid DNA in Lonza hiPSCs, PCR with three pairs of specific primers for the episomal vectors was performed using hiPSC genomic DNA. Genomic DNA was purified using DNeasy® Blood & Tissue Kit (Qiagen, cat. no. 69540Q). The primer sequences are in Table 1. The PCR program is: 95°C, 3 minutes; 95°C, 30 seconds; annealing 30 seconds; 72°C, 30 seconds; repeat step 2 to step 4 for 30 times; 72°C, 5 minutes; 4°C hold.

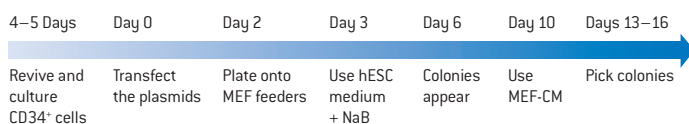| Primers	Sequences	Annealing Temperature
EBNA1-D	TTTAATACGATTGAGGGCGTCT	51.2°C
EBNA1-U	GGTTTTGAAGGATGCGATTAAG	51.2°C
Tg-F	GCCAGGTGGGTTAAAGGAGC	54.4°C
Tg-R	GGTACTTATAGTGGCTGGGCTGT	54.4°C
SK-F	CCATTAACGGCACACTGCCCCTGT	62.6°C
SK-R	AGGACGGGAGCAGAGCGTCGCTGA	62.6°C
Actin F	TGAAACAACATACAATTCCATCATGAAGTGTGAC	60°C
Actin R	AGGAGCGATAATCTTGATCTTCATGGTGCT	60°C

Table 1
PCR primer sequences.

Karyotyping analysis was performed on Lonza hiPSCs by Cell Line Genetics, Inc. (Madison, WI).

Results

Efficient Reprogramming of Human Cord Blood CD34+ Cells Using Episomal Vectors and the 4D-Nucleofector™ System

Cryopreserved human cord blood CD34+ cells were thawed and cultured in SFM for 4–5 days before transfection with the episomal plasmids using the 4D-Nucleofector™ System. CD34+ cells were reprogrammed as described in the Materials and Methods. After plating the transfected cells on the MEF feeders, small colonies could be identified as early as 6 days after transfection (Figure 1). On days 13–16, colonies exhibiting flat hESC-like morphology were manually picked and plated onto fresh MEF feeders for further expansion and characterization (Figure 1).


4–5 Days	Day 0	Day 2	Day 3	Day 6	Day 10	Days 13–16
Revive and culture CD34+ cells	Transfect the plasmids	Plate onto MEF feeders	Use hESC medium + NaB	Colonies appear	Use MEF-CM	Pick colonies

Figure 1
Timeline of cord blood CD34+ cell reprogramming with two episomal vectors. Cord blood CD34+ cells were co-transfected with pEB-C5 and pEB-Tg using Lonza's 4D-Nucleofector™ System. After plating onto MEF feeders, small colonies appeared on day 6. Some colonies were large enough for picking on day 13.

Lonza hiPSCs Are Capable of Self-renewal and Exhibit hESC-like Characteristics

Select Lonza hiPSC colonies were successfully expanded and further characterized. They are similar to hESCs in their self-renewal ability and exhibit flat colony morphology (Figure 2A). These cells are positive for alkaline phosphatase activity (Figure 2B). More importantly, Lonza hiPSCs express the core regulators of pluripotency: OCT3/4 and NANOG (Figure 3). The activation of endogenous NANOG expression in generated hiPSCs is a critical milestone indicating successful reprogramming of CD34+ cells. Furthermore, both flow cytometry analysis and immunocytochemistry demonstrated the presence of typical hESC-associated surface markers SSEA4, TRA-1-60 and TRA-1-81 (Figure 3 and Table 2).
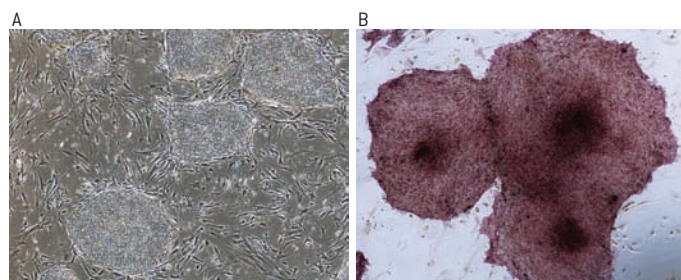
Figure 2
Lonza hiPSCs show typical hESC-like colony morphology (A) and express alkaline phosphatase activity (B).
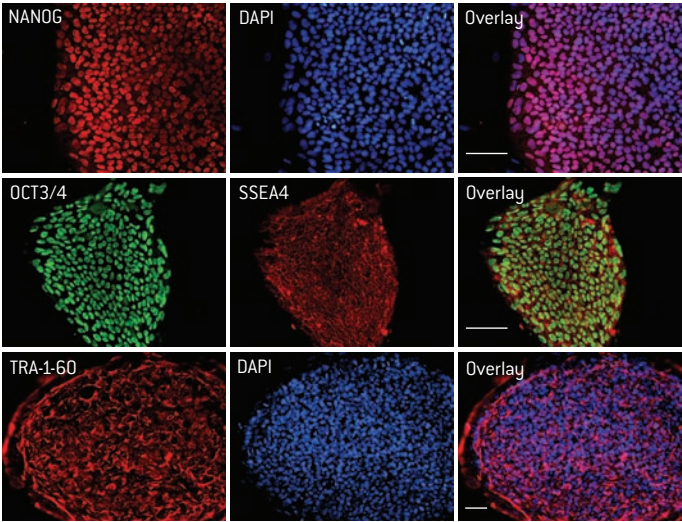
Figure 3
Lonza hiPSCs express transcription factors NANOG and OCT3/4, and exhibit surface markers SSEA4 and TRA-1-60. Cells were fixed and stained for NANOG (top, red), OCT3/4 (middle, green), SSEA4 (middle, red) and TRA-1-60 (bottom, red). DAPI: blue; Scale bar 100 μm.

Surface Markers	SSEA4	TRA-1-60	TRA-1-81
hiPSC line 1	90%	85%	85%
hiPSC line 2	96%	94%	92%
hiPSC line 3	97%	92%	91%
hiPSC line 4	95%	91%	89%

Table 2
Flow cytometry analysis reveals the presence of surface markers SSEA4, TRA-1-60 and TRA-1-81 in Lonza hiPSCs. Flow cytometry analysis was performed on four hiPSC lines generated in two different experiments. Live cells were dissociated and stained with PE-conjugated antibodies for SSEA4, TRA-1-60 and TRA-1-81. PE-conjugated mouse IgG3 and IgM were used to set up the corresponding controls. For each line, >80% of cell population is positive for the three surface markers tested.

Lonza hiPSCs can differentiate into cells representing three germ layers

Pluripotency was demonstrated through *in vitro* differentiation. After 14 days of differentiation in the presence of FBS, differentiated cells were tested for the expression of neuron-specific beta-tubulin III, alpha-fetoprotein and smooth muscle actin. These proteins are expressed in representative cell types of the three germ layers. As shown in Figure 4, Lonza hiPSCs are able to differentiate into neurons (ectoderm), alpha-fetoprotein positive cells (endoderm) and smooth muscle cells (mesoderm), thus displaying differentiation potential similar to hESCs.
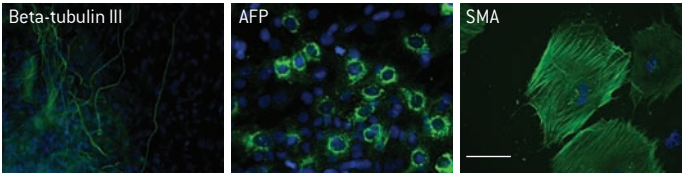
Figure 4
Lonza hiPSCs can differentiate into cells of all three germ layers. We show differentiated cells derived from Lonza hiPSCs through EB formation. From left to right: Beta-tubulin III positive neurons (green), AFP positive cells (green) and SMA positive cells (green). DAPI: blue; Scale bar 100 μm.

Lonza hiPSCs Maintain Normal Karyotype and Contain No Trace of Exogenous Episomal DNA

We cultured Lonza hiPSCs on MEF feeders and enzymatically passaged those cells using collagenase IV. Karyotype analysis (Cell Line Genetics, Inc.) was performed on four hiPSC lines at passages 7, 16 and 18. All four hiPSC lines have a normal karyotype and maintain genome stability. Figure 5 shows a representative picture of a normal hiPSC karyotype.
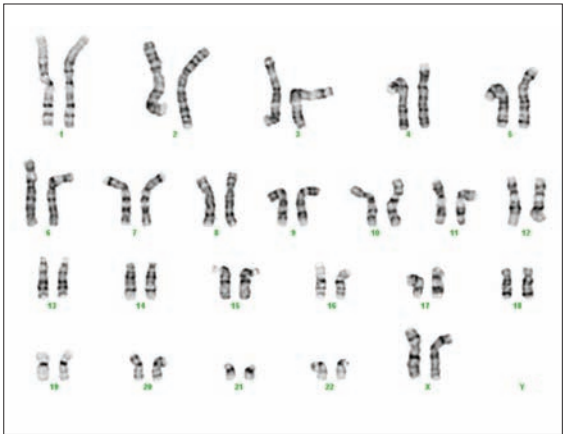
Figure 5
Lonza hiPSCs maintain a normal karyotype. Shown: Normal karyotype of one hiPSC line at passage 7, generated from a female donor.

The episomal vectors initially delivered into the CD34$^+$ cells did not integrate into the cell genome and were gradually lost after serial passaging. Three pairs of PCR primers targeting elements of the episomal plasmids were designed to detect the presence of episomal DNA in the hiPSC genome. Genomic DNA (gDNA) was prepared from both Lonza hiPSCs and the transfected CD34$^+$ cells, the latter serving as a positive control. We collected gDNA from different hiPSC lines of different passages (Passages 5–7). PCR analysis failed to amplify any episomal DNA fragments from hiPSC gDNA, confirming the absence of the episomal vectors in established hiPSC lines. However, the same primers did amplify episomal DNA fragments from the transfected CD34$^+$ cells (Figure 6).
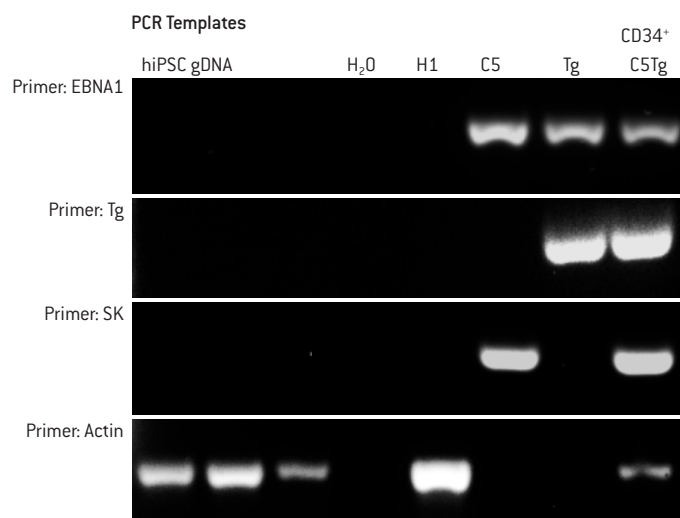
Figure 6
Absence of episomal vector DNA in Lonza hiPSCs. Genomic DNA was purified from three hiPSC lines and the pEB-C5/Tg-transfected CD34+ cells were collected 2 days after transfection. PCR amplification was performed using three pairs of primers (EBNA1, Tg and SK) targeting episomal vectors and actin primers as a control. No DNA fragments were amplified from the gDNA of hiPSCs, confirming the loss of episomal plasmids. PCR templates from left to right: hiPSC gDNA; H_2O is the PCR negative control without DNA template; gDNA purified from hESC line H1; pEB-C5; pEB-Tg; gDNA purified from CD34+ cells co-transfected with pEB-C5 and Tg.

Conclusion

We successfully reprogrammed cord blood CD34+ cells into hiPSCs by delivering episomal vectors expressing five transcription factors and SV40 Large T antigen. The two episomal vectors were delivered using Lonza's 4D-Nucleofector™ System. The Lonza hiPSCs share similar characteristics with hESCs, including self-renewal ability, hESC-associated surface marker expression, alkaline phosphatase activity, and key pluripotency transcription factor expression. These lines maintain a normal karyotype and can efficiently differentiate into cells of all three germ layers. Notably, Lonza hiPSCs show no trace of exogenous DNA integration, confirming that cells were reprogrammed with a "zero footprint" technology. Recently, using the same technology, we were able to generate hiPSCs under feeder-independent conditions with a defined medium and matrix.

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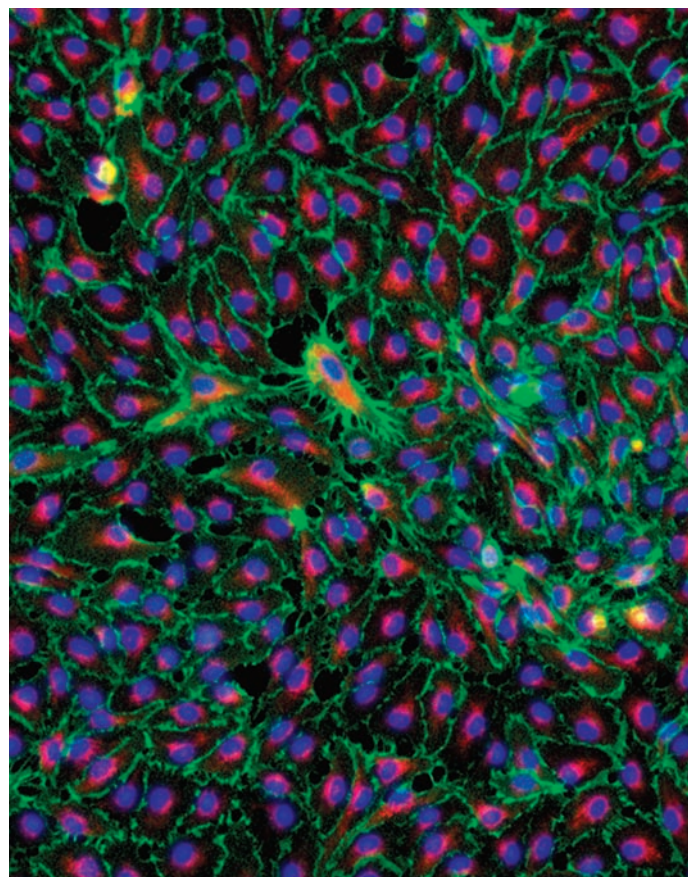
Evaluating an siRNA Screen in a Mouse B Cell Line Using the Lonza 384-well Nucleofector™ System

By Scott Martin, NIH, National Center for Advancing Translational Science,
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HT Nucleofector™ System

Introduction

RNAi screening has become a widespread tool for illuminating gene function. Screening is primarily conducted with commercially available siRNA resources. These reagents are designed to target mRNAs with corresponding sequence complementarity, resulting in their down-regulation. In this way, RNAi screening provides an excellent way to interrogate gene function in a high-throughput manner. However, RNAi screening suffers from a number of limitations, including the ability to efficiently transfect cells with nucleic acid. Lipid-based reagents are typically adequate for most adherent, immortalized cell lines (e.g., HeLa), but many cells, especially primary cells, are refractory to this approach. Nucleofection™ offers a possible solution to this problem by enabling the efficient transfection of physiologically-relevant primary cells and difficult-to-transfect cell lines with DNAs and siRNAs. In this study, we sought to evaluate the feasibility of conducting moderate-scale siRNA screens using 384-well Nucleofection™ of siRNA in primary mouse B cells that are deficient in DNA repair.

Results

For any screening methodology to be successful, it must be reproducible. Nucleofection™ based screening offers a few additional challenges compared to lipid-based screens, especially in terms of liquid handling. For example, cells first need to be transfected in specialized Nucleocuvette™ Plates before transfer to destination assay plates. Since many assays are highly dependent on accurate knowledge of the starting cell number, this transfer must be reproducible. To test this, we transferred B cells suspended in Nucleofector™ Solution to 384-well Nucleocuvette™ Plates using a Multidrop® Combi (Thermo Fisher Scientific) and transfected them with negative control siRNA (preprinted to the 384-well Nucleocuvette™ Plates).

Post transfection, we transferred cells to 384-well assay plates using a Biomek® FX liquid handling system (Beckman Coulter), and assessed viability 72 hours post-transfer by luminescence assay. As shown in Figure 1A, transfection and subsequent transfer of cells yielded a consistent viability across the interior wells of a 384-well plate (less than 10% CV). After optimizing conditions for robust cell transfer, we next examined transfection of nucleic acid in terms of efficiency and reproducibility. Initial conditions were optimized by testing various Nucleofector™ Programs with GFP plasmid, and a number were identified that yielded high GFP expression in B cells. A few of these top programs were then tested in terms of reproducibility. As shown in Figure 1B, transfection yielded a consistent number of GFP-positive primary B cells across 16 sample wells with an efficiency of ≈ 80%.

A

105.6	101.8	106.8	102.3	100.4	100.5	126.1	94.6	108.7	100.9	106.2	102.0	98.0	95.7	96.5	99.9	100.1	97.5	113.0	96.2	116.9	97.5
104.5	103.8	100.4	95.7	105.7	112.2	114.8	92.6	104.4	112.6	96.2	104.2	110.9	105.0	104.9	117.0	112.4	104.6	117.5	121.8	119.2	121.7
117.0	112.6	106.4	108.2	125.5	119.9	116.1	111.7	112.0	108.6	116.4	118.5	113.9	114.4	113.8	112.4	107.2	115.8	108.3	113.7	113.6	111.2
100.3	111.2	99.2	116.2	99.1	99.1	107.2	97.7	98.8	100.3	114.3	100.9	99.3	100.4	94.2	92.5	86.6	97.2	90.5	96.3	98.4	104.1
98.3	111.0	125.2	129.4	121.2	106.7	117.0	101.6	96.0	98.7	91.8	94.7	96.0	90.6	92.1	93.8	92.4	92.9	93.3	90.8	98.9	95.5
93.9	93.0	100.0	97.1	96.8	102.8	95.8	99.2	104.4	106.8	104.6	95.5	93.5	102.1	109.1	102.6	97.1	92.4	95.2	92.7	93.5	98.5
91.1	113.3	115.6	102.6	123.0	94.4	114.5	93.3	120.4	110.9	86.5	87.2	96.7	104.3	84.3	89.2	89.0	90.2	85.6	90.7	91.0	92.5
118.7	109.3	102.4	93.9	104.1	98.7	94.9	100.5	93.8	88.1	86.9	101.0	85.8	112.5	93.3	94.8	86.7	94.4	91.4	96.4	113.7	127.7
89.1	94.7	91.2	103.9	89.8	89.5	129.4	124.1	96.0	87.2	85.2	90.6	89.9	84.5	87.6	88.5	85.4	98.7	102.3	103.3	110.6	92.4
102.1	96.5	96.3	103.1	95.1	97.9	103.2	100.3	94.4	88.9	91.6	94.4	96.2	91.8	110.3	104.5	93.3	92.0	94.8	91.3	95.0	98.0
104.5	99.5	115.2	91.7	93.8	94.4	92.8	97.3	93.6	89.5	110.5	96.8	100.1	89.9	89.3	91.7	92.3	93.4	90.8	94.0	91.8	98.1
92.8	96.3	95.8	98.5	95.5	92.7	98.0	101.8	95.3	92.2	96.0	103.5	94.9	91.0	91.2	90.5	94.9	97.0	92.1	95.8	112.5	94.0
95.2	96.6	91.5	90.7	91.2	93.4	95.0	91.6	93.7	93.0	93.7	94.7	96.1	91.5	86.7	94.2	100.0	93.0	90.3	95.3	90.7	97.0
111.9	99.6	99.3	98.1	104.6	101.3	92.2	96.7	101.9	94.8	98.8	101.9	109.0	93.8	92.6	89.2	112.6	106.1	98.8	93.7	85.0	94.4

B

B Cells Transfected with GFP

Figure 1. Testing 384-well transfection reproducibility in terms of liquid handling and transfection efficiency. **A.** B cells transfected with negative control siRNA in 384-well plates are evenly transferred to destination 384-well assay plates as determined by viability measured 72 hours post-transfer (CellTiter-Glo®, Promega). Each well is normalized to the plate median value and a color scale was set using 0% and 200% as min. and max. values to help visualize homogeneity. **B.** Optimized protocols yield highly efficient and reproducible transfection of B cells with GFP plasmid. Cells were stained with Hoechst 33342 and imaged on an ImageXpress® Micro (Molecular Devices). Sample population scored 80% positive for GFP.

After demonstrating robust liquid handling and transfection conditions with the system, we next moved towards evaluating this approach for moderate-scale siRNA screening. We initially focused on NF-κB-related siRNAs. NF-κB is an important transcription factor and much is known about the NF-κB signaling pathway. We have previously conducted a lipid-based "druggable genome" siRNA screen for genes that modulate TNFα-stimulated NF-κB in a beta-lactamase reporter cell line (CellSensor® NF-κB-bla ME-180 Cell Line, Invitrogen). We assembled a 384-well plate comprising a number of active siRNAs from the primary screen and tested their activity via Nucleofection™. As shown in Figure 2, 384-well Nucleofection™ of corresponding siRNAs verified a number of actives, including known NF-κB components such as IKKα, RELA, and various proteasome constituents, showing that 384-based Nucleofection™ yields expected phenotypic results. We next moved towards a larger, unbiased screen with the B cell system. These cells are deficient in certain DNA repair pathways and we sought siRNAs that either mitigate or enhance this sensitivity. To do so, we conducted a moderate-scale siRNA screen comprising over 5,000 siRNAs. This scale was manageable with standard liquid handling devices. Screening yielded a number of statistically significant siRNAs, which remain of interest.

Conclusion

Overall, the 384-well Nucleofector™ System enables medium-throughput siRNA screening in systems not amenable to lipid-based siRNA screening such as human primary cells, which are likely to offer more physiologically-relevant results compared to cell lines. Robust transfection and liquid handling protocols appear attainable and, under optimized conditions, transfections yield expected phenotypes. Curiously, counter screening with the 384-well Nucleofector™ System may also provide a way to eliminate false positives arising as a function of lipid-based effects. As RNAi screens suffer significantly from off-target effects, any such methodology may enhance one's ability to arrive at true actives.

Materials and Methods

For all screening, siRNAs (1 μl of a 5 μM stock) were preprinted to 384-well transfection plates using a Beckman Coulter Biomek® FX liquid handling system. Cells were added in 10 μl of Nucleofector™ Solution using a Multidrop® Combi (Thermo Fisher Scientific). Transfections were carried out on the 384-well Nucleofector™ System and 65 μl of media was then added to each well. 25 μl of the cell suspension was then transferred to 384-well plates and incubated at 37°C prior to endpoint assays. B cell viability was measured by CellTiter-Glo® (Promega). NF-κB reporter activity was measured 5 hours after stimulation with TNFα (2ng/ml).

The NF-κB reporter assay was conducted using a LiveBLAzer™-FRET B/G loading kit (Invitrogen). See Table 1 for more information.
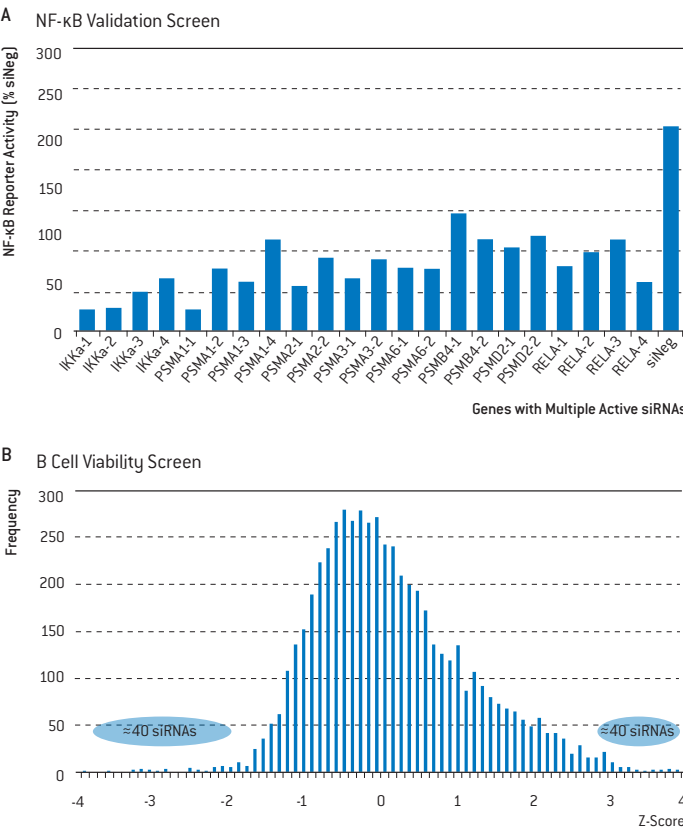

Figure 2. siRNA screening with the 384-well system. **A.** 384-well Nucleofection™ of siRNAs selected for their effect on TNFα-stimulated NF-κB activity in a primary lipid-based screen verified a number of actives, including known NF-κB components such as IKKα, RELA, and various proteasome constituents. **B.** A moderate-scale siRNA screen comprising over 5,000 siRNAs yielded a number of statistically significant siRNAs that affect the viability of B cells deficient in DNA repair.
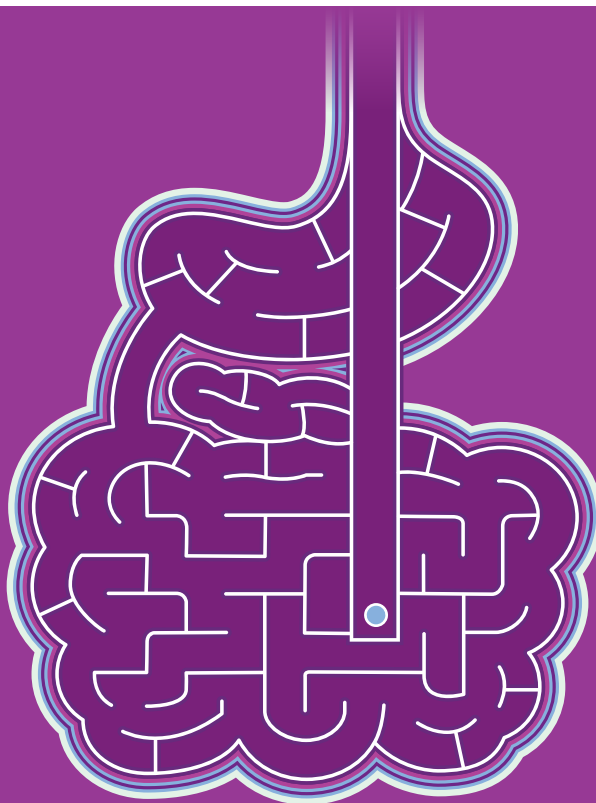
Table 1

Cell	Media	Cells Per Transfection	Solution	Program Code
B cells, mouse	RPMI, 10% FBS	75,000	P3	CA-113-AA
ME180 NF-κB	DMEM, 10% FBS	30,000	SE	CA-139-AA

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A Short Introduction Into Endotoxin Detection

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Introduction

For the pharmaceutical manufacturer of parenteral (injectable) drugs, endotoxin detection is a primary concern. Endotoxin is a pyrogen originating from gram-negative bacteria. It is heat stable, associated with injectable products and not removed via sterilization. When injected into humans and animals, endotoxin can cause fever, shock, vascular collapse or even death. Endotoxin testing of all parenteral medicines is required by US law. All parenteral products, including vaccines, intravenous drugs, intravenous fluids and implantable medical devices, are tested for endotoxin.

In addition to being an injectable concern, endotoxin can also have an *in vitro* effect on cell culture and has been shown to significantly affect cell growth and function. Examples of this phenomenon include altered recombinant protein production in CHO cells, induced contractile dysfunction of cardiac myocytes and modified clonal efficiencies in epithelial cells. Ensuring that cell culture vessels and media are pyrogen-free greatly reduces the likelihood of endotoxin contamination in your cell culture.

LAL History

The earliest accepted endotoxin detection method was the rabbit test, which involved monitoring the temperature of the animal pre- and post-injection of the sample. The sample was considered positive for endotoxin if a temperature spike (fever) was seen 180 minutes after injection. Although the test was widely utilized, the non-quantitative nature of the results, coupled with the logistics required to maintain the animals, made this method suboptimal.

In the 1950's, scientists Fred Bang and Jack Levin discovered that horseshoe crab blood clots in the presence of gram-negative bacteria. Using information published by Bang and Levin, scientists developed the first non-rabbit endotoxin detection assay. The Limulus Amebocyte Lysate assay, otherwise known as LAL, subsequently replaced the rabbit test as the primary test utilized to detect endotoxin in parenteral pharmaceutical preparations. The LAL assay is the test method specified by all of the major Pharmacopeias—USP, EP, JP, etc.

LAL assays measure endotoxin in terms of potency - not by endotoxin weight. The labeling universally applied is the Endotoxin Unit or EU. Frequently, endotoxin levels are noted as "ng/ml", but this can be misleading as 1 ng/ml of a given endotoxin does not always have the same potency.

Assay Descriptions

In the pharmaceutical community, researchers will find it necessary to measure endotoxin levels associated with commonly used laboratory items, such as water, glassware, media, etc. Testing can be performed using one of Lonza's many easy-to-use endotoxin detection assays. Prior to selecting the endotoxin assay most suited to your needs, a variety of factors should be considered. First, how many samples will you be testing and how frequently? If you will be testing one or two samples, one time only, then you may want to consider using Lonza's Endotoxin Testing Service. If you plan to test on a regular basis, then you first need to evaluate the available equipment and the type of results you wish to obtain. Assay sensitivity is another important factor and varies from test to test. Finally, if you will be testing many samples on a frequent basis, then you may want to contemplate purchasing equipment and software. Lonza offers both kinetic and fluorescent microplate readers, as well as our WinKQCL™ Software specifically developed for endotoxin detection testing. Below you will find detailed descriptions of Lonza's endotoxin assays.

PYROGENT™ Gel Clot Assay

PYROGENT™ Gel Clot Assays are a popular choice for researchers and companies who wish to perform endotoxin testing without the need for quantitative results. The test is semi-quantitative and requires a 37°C non-circulating water bath or heat block. After a one-hour incubation period, gelation or clot formation will occur in response to endotoxin. We offer kits with the following sensitivities: 0.25, 0.125, 0.06, and 0.03 EU/ml.

QCL-1000™ Endpoint Chromogenic LAL Assay

The QCL-1000™ Endpoint Chromogenic LAL Assay is appropriate for any laboratory looking for a rapid turnaround. This 16-minute quantitative endpoint assay measures endotoxin levels photometrically and has a sensitivity range of 0.1 EU/ml to 1.0 EU/ml. A sample is mixed with the LAL supplied in the test kit and incubated at 37°C ($\pm$1°C) for 10 minutes. A substrate solution is then mixed with the LAL sample and incubated at 37°C ($\pm$1°C) for an additional 6 minutes. The reaction is terminated with the addition of stop solution. If endotoxin is present in the sample, a yellow color will develop. The absorbance of the sample can be determined spectrophotometrically at 405−410 nm. Since this absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin can be calculated from a standard curve. This assay can be run using either tubes or microplates.

PYROGENT™-5000 Kinetic Turbidimetric LAL Assay

The PYROGENT™-5000 is a cost-effective, quantitative, kinetic method for testing water and large volume parenterals. The sample is mixed with the

reconstituted LAL reagent, placed in the photometer, and monitored over time for the appearance of turbidity. The time required before the appearance of turbidity (reaction time) is inversely proportional to the amount of endotoxin present. That is, in the presence of a large amount of endotoxin the reaction occurs rapidly; in the presence of a smaller amount of endotoxin the reaction time is increased. The concentration of endotoxin in unknown samples can be calculated from a standard curve. This assay requires a kinetic plate reader to incubate at 37°C (±1°C) and to read at 340 nm. The detection range of this kit is 0.01 EU/ml–100.0 EU/ml.

Kinetic-QCL™ Kinetic Chromogenic LAL Assay

Kinetic-QCL™ is our most sensitive kinetic assay with a broad detection range of 0.005 EU/ml to 50 EU/ml. This assay is ideal for testing biological products such as vaccines and antibiotics. The sample is mixed with the LAL reagent, placed in an incubating plate reader, and monitored over time for the appearance of a yellow color. As with the kinetic turbidimetric assay, the reaction time is inversely proportional to the amount of endotoxin present. The concentration of endotoxin in unknown samples can be calculated from a standard curve. This assay also requires an incubating kinetic microplate reader as well as kinetic software.

PyroGene™ Recombinant Factor C Assay

Lonza offers an alternative method to the LAL assay that does not contain horseshoe crab amebocyte lysate as a raw material. This assay utilizes a recombinant form of the endotoxin sensitive protein, Factor C. It works in combination with a fluorogenic substrate, an incubating fluorescence microplate reader and appropriate software to detect endotoxin. Studies have demonstrated the ability of Factor C to selectively recognize endotoxin and activate the protease cascade. To create an endotoxin-specific assay, the gene encoding Factor C has been cloned and a recombinant protein produced. When activated by endotoxin binding, recombinant Factor C cleaves a fluorogenic substrate in the assay mixture to produce a fluorescent signal in proportion to the endotoxin concentration in the sample. The assay is carried out in a 96-well plate and fluorescence is measured at time zero and after a one-hour incubation at 37°C (±1°C) in a microplate reader using excitation/emission wavelengths of 380/440 nm. The difference between the one-hour reading and the time zero reading (ΔRFU) is blank corrected. The log net fluorescence is proportional to the log endotoxin concentration and is linear in the 0.005 EU/ml to 5 EU/ml range. Endotoxin in a sample is calculated relative to a standard curve.

Since Lonza's PyroGene™ Assay is not a biological product, it has several advantages over the other assays. The recombinant Factor C (rFC) assay has a better specificity for the detection of endotoxin and will not detect beta (1,3)-glucan activity. Using recombinant production methods makes the assay less variable from lot-to-lot and there is no dependence on the horseshoe crab. Also, this assay method has been shown to be comparable to the quantitative LAL methods.

Sample Testing/Interference

One of the most time consuming aspects of endotoxin testing using LAL is pretreating samples to overcome assay inhibition and enhancement. All assays, independent of methodology, are standardized using endotoxin in water. Therefore, unless your sample is water, some components of the solution may interfere with the LAL test such that the recovery of endotoxin is affected. If the product being tested causes the endotoxin recovery to be less than expected, the product is inhibitory to the LAL test. Products which cause higher than expected values are enhancing. Inhibition/enhancement testing is required by the FDA to ensure the validity of the test result for each product.

Inhibition and enhancement testing is an integral part of the validation process for the use of LAL in the final release of products. The United States Pharmacopeia (USP) Chapter 85, Bacterial Endotoxins Test, allows for pretreatment of products to render them testable. As long as it can be demonstrated that the pretreatment allows for the recovery of added (spiked) endotoxin, within the acceptable limits of the LAL method being used, the pretreatment will be acceptable. Once a pretreatment that will successfully overcome the inhibition or enhancement of three lots of the product is found, the product can be validated for release with LAL. For routine testing of products, the product must be prepared in the manner in which it was treated to pass the inhibition/enhancement test. Otherwise, a negative result may be mistaken as indicating a lack of endotoxin in the product, when in reality the negative is a result of inhibition.

The basic sample treatment in order to overcome LAL interference is dilution in LAL reagent water, within the maximum valid dilution (MVD). However, in some cases simple dilution is not enough to overcome the interference and additional pretreatment of the sample is necessary. Here are a few recommendations for overcoming common interferences:

Adjust the pH with Tris Buffer

Although there are some products which will cause LAL assay enhancement, the majority of LAL interference is due to inhibition. The LAL assay functions best in the 6.0 to 8.0 pH range and extremes of pH will actually inhibit gel formation. If a product is outside the pH range, pH adjustment may be necessary for best results. For example, highly basic solutions, such as NaOH, could cause non-enzymatic chromogenic substrate cleavage, thus resulting in an artificially high reading. Adjusting the pH of an unbuffered solution with endotoxin-free acid or base is not recommended. Quite often, the desired pH is overshot several times during the adjustment, thus altering the ionic strength of the solution just enough to inhibit LAL functionality. Instead, a buffer in the correct pH range is recommended as a diluent for acidic and basic product solutions. We recommend Lonza Tris Buffer, for use with LAL, which is a 50 mM solution with an appropriately neutral pH.

Comparison of Amplification Methods in Bacterial Endotoxin Detection Assays
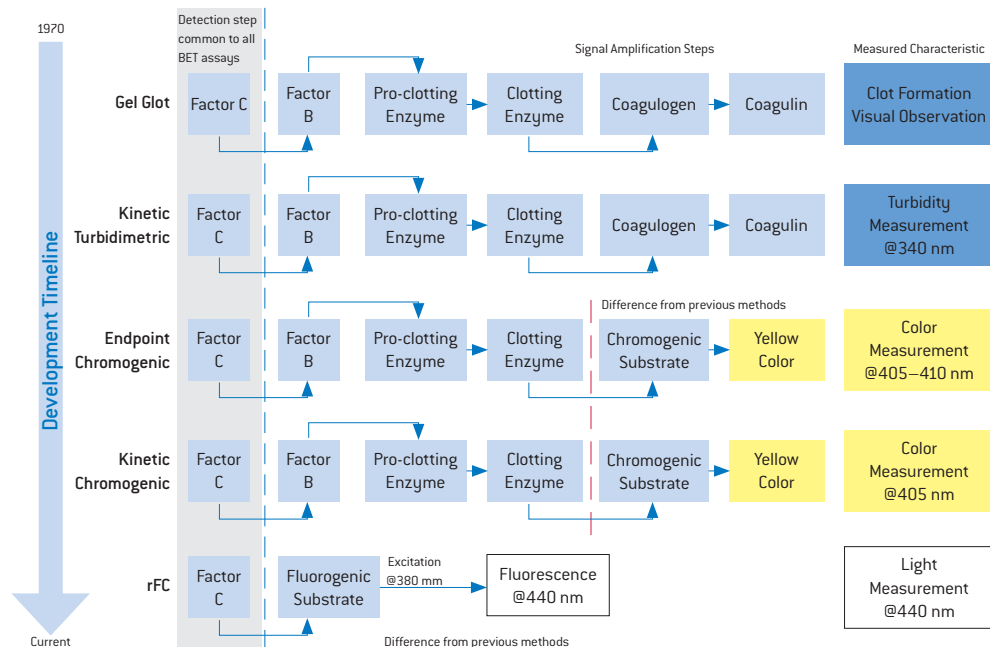
Figure 1. The recombinant Factor C (rFC) is the same binding protein operating in the LAL assay. The activated rFC enzyme cleaves a substrate directly instead of activating another enzyme in a series (the LAL cascade). The substrate has a fluorescent tag, which gives a wide dynamic range with better resolution.

Use MgCl$_2$ to Overcome Chelation Effect

Products which have a high affinity for divalent cations will chelate or sequester them from the lysate formulation. Without the proper concentration of divalent cations, the enzymes in the lysate cannot function properly and will not indicate the endotoxin content. Reconstituting and/or diluting the sample in a solution containing divalent cations, such as MgCl$_2$, will help overcome the inhibitory nature of the chelator. Using 10 mM MgCl$_2$ to dilute heparin or other cation-chelating products will help your product easily pass the LAL inhibition/enhancement test.

Try PYROSPERSE™ to Solve Aggregation Problems

In some instances, the endotoxin can be "hidden" from the LAL by components of, or the very nature of, the test solution. If the LAL does not "see" the endotoxin, the gel will not form or the substrate will not be cleaved. Endotoxin is a molecule with a split personality with the polysaccharide portion of endotoxin preferring an aqueous environment. The lipid portion, however, is hydrophobic. As a result of their nature, endotoxin monomers will aggregate such that the lipid portion is hidden from the aqueous solution. It is the lipid portion of the endotoxin molecule that activates the LAL reagent. Some products will actually attract and hide endotoxin. In order to detect endotoxin in these products, the endotoxin must be coaxed from its comfortable environment. Detergents or surfactants can be used to dissociate endotoxin from itself and products; much like dishwashing liquid disperses grease in a water-filled frying pan. PYROSPERSE™ is a metallo-modified polyanionic dispersing agent and dilution of a sample with the appropriate concentration of PYROSPERSE™, plus a vigorous vortexing, can help to dissociate endotoxin aggregates. PYROSPERSE™ will change the environment of the solution just enough that the endotoxin is now more "comfortable" and it will expose itself to the LAL.

Testing Serum and Plasma

Testing of human and animal blood and serum for endotoxin can be a bit tricky since blood/serum is viscous and high in proteins - which will make the sample inhibitory to the endotoxin detection assay. The following is a description of a procedure that can be used to prepare serum or plasma for LAL-based endotoxin testing. Plasma or serum can be tested for endotoxins after some preliminary preparation. If testing plasma, one should use "platelet-rich plasma" which can be obtained from whole blood by centrifuging at low speed to remove the white and red blood cells. Blood serum products, whole blood processed to remove fibrogens, coagulants, whole blood cells, etc., can also be tested in the same manner as blood plasma.

1. Dilute plasma or serum 1:10 with LAL reagent water (LRW).
2. Heat-inactivate the 1:10 dilution of plasma or serum by placing in a water bath or heat block at a temperature of 70°C at a minimum of 15 minutes.
3. Next, make a series of 1:2 dilutions with LRW of the 1:10 diluted and heat-treated sample.
4. Run the LAL endotoxin test according to the directions supplied in the appropriate package insert. Run each dilution of plasma or serum in duplicate and include a positive product control for each dilution.

🌐 www.lonza.com/LAL

Protein Separation Differences on SDS PAGE Dependent on the Buffer System

By Liz Horton and Foner Curtis, Lonza Walkersville, Inc., Walkersville, MD, USA

Introduction

When choosing an SDS PAGE gel to run, in order to get optimal separation of the proteins of interest it is important to consider both the gel percentage as well as the buffer system. Here, we present separation data using protein markers on the new PAGEr™ EX Gels run using ProSieve™ EX Running Buffer compared to similar standard gels with well-known or alternate buffer systems, along with Lonza's PAGEr™ Gold run with traditional Tris-glycine SDS.

In Figure 1, we have a comparison of several gel buffer chemistries on 10% gels and separation range similarities and/or differences of protein sizes relative to the PAGEr™ EX Low/Mid Gels. Each gel was run using the running buffer and voltage that is noted under the image according to manufacturer's recommended protocol. The run time is also noted, which is the time it took for the ion front to reach the bottom of the gel. The marker used is Lonza's ProSieve™ Unstained Marker II. The gels were stained post-run using ProSieve™ EX Safe Stain. With the Tris-glycine SDS on PAGEr™ Gold and Company B gels, and the Company L Bis Tris gels run with MOPS, the 10 kDa is lost in the ion front. The PAGEr™ EX Gel shows the lowest band and a greater separation up to the 50–75 kDa range relative to the others.
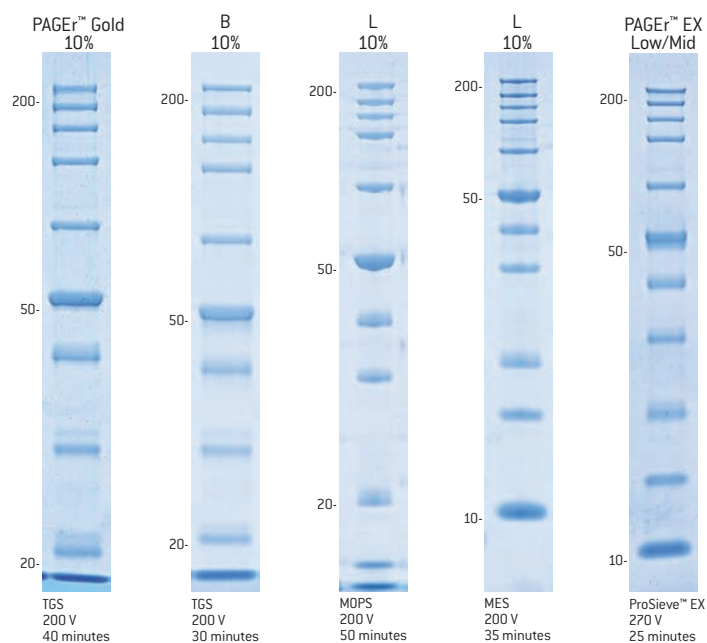
In Figure 2, we look at a comparison of several gel buffer chemistries on gradient gels and separation range similarities and/or differences of protein sizes relative to the PAGEr™ EX Mid/High Gels. Each gel was run using the running buffer and voltage that is noted under the image according to manufacturer's recommended protocol. The run time is also noted, which is the time it took for the ion front to reach the bottom of the gel. The marker used is Lonza's ProSieve™ Unstained Marker II. The gels were stained post-run using ProSieve™ EX Safe Stain. The PAGEr™ EX Mid/High Gel range gives optimal separation in the 50–200 kDa range yet still has greater separation from 50 kDa down to 10 kDa, making this a quite versatile gel for analysis of a broad range of sizes.

Figure 2

PAGEr™ Gold 4–20%	B 4–20%	L 4–12%	L 4–12%	PAGEr™ EX Mid/High
TGS 200 V 40 minutes	TGS 200 V 30 minutes	MOPS 200 V 50 minutes	MES 200 V 35 minutes	ProSieve™ EX 270 V 25 minutes
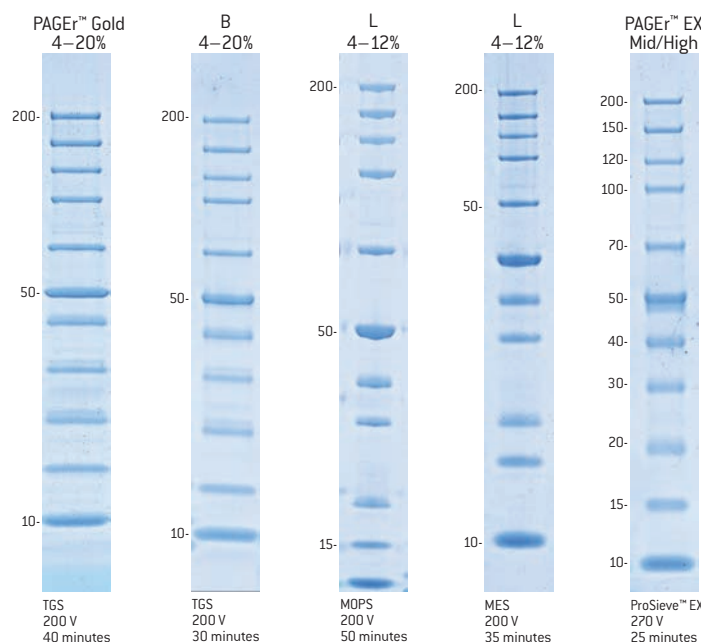
Conclusion

PAGEr™ EX Gels cover a broad range with optimal separation in only two gel formats and an extended one year shelf life. When combined with ProSieve™ EX Running Buffer, Transfer Buffer and Stains, the process is reduced from 5 hours down to 1 hour in total with no compromise.

Figure 1

PAGEr™ Gold 10%	B 10%	L 10%	L 10%	PAGEr™ EX Low/Mid
TGS 200 V 40 minutes	TGS 200 V 30 minutes	MOPS 200 V 50 minutes	MES 200 V 35 minutes	ProSieve™ EX 270 V 25 minutes

4D-Nucleofector™ System

Innovative Applications of Nucleofector™ Technology
Nobel Prize Laureate Uses Nucleofection™ for iPSC Generation

Human induced pluripotent stem cells (iPSCs) have the capability to self-renew in culture and to differentiate into any cell type in the body. Therefore, iPSC technology has the potential to be a powerful tool to develop cell transplantation therapies for the treatment of degenerative diseases, including diabetes, Parkinson's and a number of cardiovascular diseases. iPSCs can be generated from adult somatic cells, e.g. fibroblasts or CD34^{+} hematopoietic progenitor cells, by overexpressing a set of defined factors. For human cells, this cellular reprogramming process was first described in 2007 by two independent research groups[1,2]. These reprogramming factors were initially transferred into the somatic cell by retroviral transduction, thereby diminishing their applicability to the clinic. Lonza's Nucleofector™ Technology has been demonstrated to be an efficient, convenient and cost-effective, non-viral alternative for iPSC generation.[3-8] It is now being used by 2012 Nobel Prize winner Dr. Shinya Yamanaka (Kyoto University) and other leading scientists around the world.

Benefits of Nucleofector™ Technology for iPSC Generation

– Simple, single-step, non-viral procedure to introduce DNA/RNA
– Efficient delivery of reprogramming factors into various cell types including fibroblasts and blood cells
– Ability to deliver multiple plasmids in one transfection step
– iPSC colonies ready for picking in less than two weeks
– Availability of Nucleofector™ Kits with cGMP Solutions

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🌐 www.lonza.com/ipsc
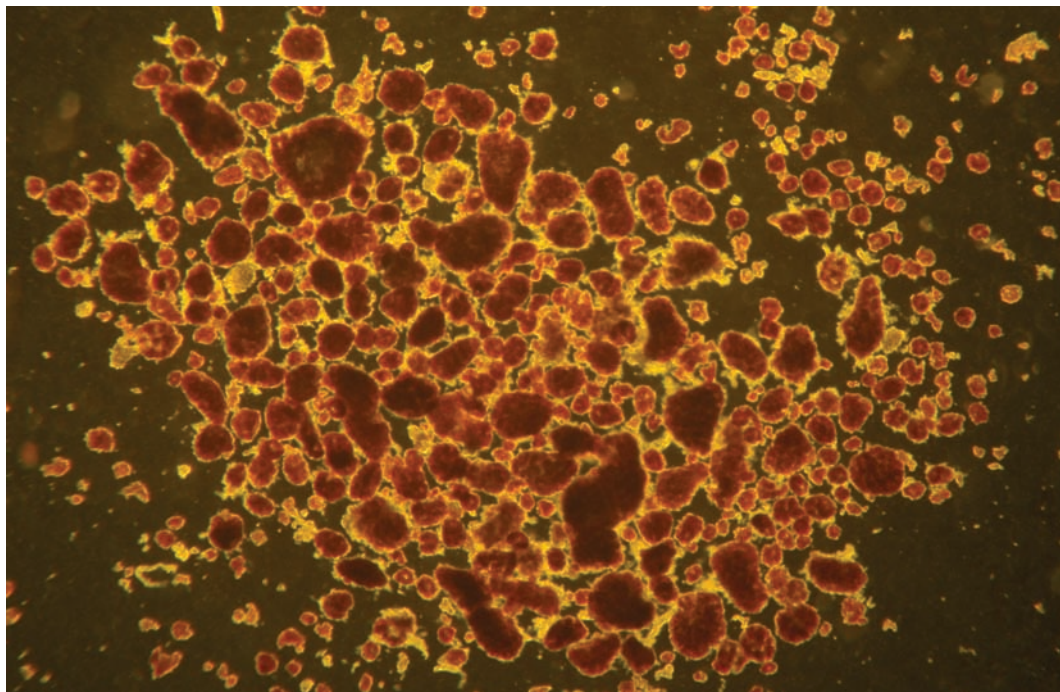
Avoid Roadblocks in Diabetes Research

Clonetics™ Fresh Human Pancreatic Islets
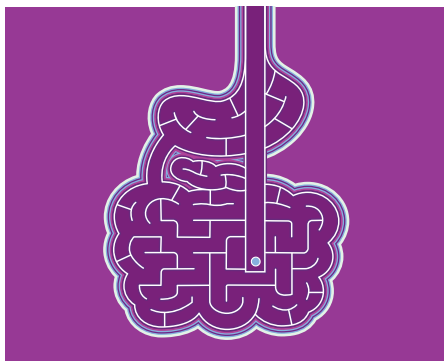
Lonza introduces fresh human pancreatic islets to support advances in your diabetes research. Lonza's pancreatic islets provide a guaranteed quality with each batch tested for quantity, viability, purity, and sterility.

Some of the major challenges within diabetes research are the acquisition and availability of high-quality islets. Lonza now bridges the gap between high market demand and limited accessibility of these cells. Researchers will have easy access to islets to support research in long-term islet grafting survival, prevention of islet rejection, and prevention of adverse effects from immunosuppressive drugs. Within a growing diabetes research market, this extension to Lonza's existing diabetes product portfolio helps to further advance research and drug discovery in this area.

Clonetics™ Human Pancreatic Islets Are:
- **Guaranteed for quality** with each batch tested for quantity (total IEQ), viability, purity, and sterility
- **Cost-effective** *in vitro* **tools** that include various cell types: alpha, beta, delta, PP
- **Made to order** and offered in 5K, 10K, 20K, and 100K product sizes

- Available upon request for **specific donor characteristics** and **additional testing**
- Also available from **type 2 diabetic tissue** upon request
- Shipped in a gas permeable vessel for **optimum viability** upon arrival

Research Applications for Clonetics™ Fresh Human Pancreatic Islets:
- Islet transplantation research to support advances in treatment
- Insulin production and regulation
- Cellular metabolism and pathways
- Immunosuppressive drug development post-islet transplantation
- Metabolic disorders: Type 1 and 2 diabetes, hypoglycemia, and insulin resistance

🌐 www.lonza.com/islets

■ TechTalk

In this issue of **TechTalk**, we will focus on answering some commonly asked questions concerning Lonza's Clonetics™ Primary Cell Products.

Q. Why should I use primary cells?

A. When performing *in vitro* research, the goal is always to replicate the *in vivo* environment as closely as possible. Lonza primary cells help you do this by providing high quality, non-transformed, non-immortalized primary human and animal cells. We offer more than 150 Clonetics™ Cell Types from different tissues. Normal and diseased cells are available from a variety of donors, individual or pooled. Our systems come complete with cells, media and supplements and are optimized to provide unparalleled performance.

Q. What are the specifications for Lonza's Clonetics™ Cells?

A. Clonetics™ Cells must meet the following specifications:

– At least 70% viability upon recovery from cryopreservation
– A minimum cell count of 500,000 cells per ml
– At least 20% seeding efficiency
– A doubling time of 15–48 hours in the first passage out of cryopreservation
– Cells must achieve at least 10–15 total population doublings without significant deterioration
– Negative for bacterial, fungal and mycoplasma contamination
– Negative for HIV-1, Hep-B and Hep-C
– Morphological specifications depending on cell type
– Staining specifications depending on cell type

Q. What types of Clonetics™ Cell Formats are available?

A. Cells are available cryopreserved or proliferating, in either flasks or plates, or as pellets in RNAlater®.

Q. Are the cryopreserved cells you provide equivalent to their fresh counterparts?

A. Yes, the cells perform equivalently by all measures we have tested. Some cells do die in the cryopreservation and thawing processes, but it does not seem to be selective to a particular cell type and we place additional cells in each vial to account for this loss.

Q. How many donors are represented in pooled products?

A. Pooled cell products contain cells from at least three different donors. Both single donor and pooled donor cell products undergo the same QC testing.

Q. What type of guarantee does Lonza provide for Clonetics™ Cells?

A. Lonza guarantees that its cells will perform as stated in the previously noted specifications providing Clonetics™ Media and ReagentPack™ Subculture Reagents are used, and cell-specific Clonetics™ Instructions for use are followed.

Q. How should I store my ampule of cryopreserved cells?

A. Cryopreserved cells should always be stored in liquid Nitrogen. Storing the cells at lower temperatures can cause irreversible damage.

Q. Should I centrifuge my cells right out of cryopreservation?

A. We don't recommend centrifugation due to the fragile state of the cells coming out of cryopreservation. The damage from centrifugation outweighs the potential damage from culturing the cells in a low concentration of DMSO for the first 24 hours. Utilizing the recommended seeding density and medium volume will dilute the DMSO sufficiently so it is not harmful to the cells. Please note that centrifuging the cells after thawing voids Lonza's performance guarantee for the cells.

Q. Can I seed my ampule of cells into one flask/dish?

A. Each cell type has a recommended seeding density optimized for cell attachment and proliferation. Using information from the Certificate of Analysis shipped with each cryopreserved vial of cells, you can use the following equations to calculate the number of flasks or dishes that can be set up:

$$\frac{\text{Number of cells per amp x percent viability}}{\text{Recommended seeding density}} = \text{Maximum number of } cm^2 \text{ that can be plated}$$

$$\frac{\text{Maximum number of } cm^2 \text{ that can be plated}}{\text{Effective growth area of flask/dish}} = \text{Maximum number of culture vessels that can be set up}$$

Q. What reagents should I use when I subculture my Clonetics™ Cells?

A. Lonza offers a ReagentPack™ (CC-5034) which must be used when Clonetics™ Cells are subcultured. The ReagentPack™ consists of HEPES-BSS, Trypsin/EDTA and Trypsin Neutralizing Solution. Please note that the Trypsin/EDTA in the ReagentPack™ is formulated specifically for use with primary cells which are not able to withstand Trypsin concentrations typically used for other cell types.

Q. Why do I obtain slightly different results with cells while using the same assay?

A. Each lot of cells can have different results due to donor variation. To ensure uniformity across assays, we suggest you specify the same lot when you place your next order. The best way to ensure a specific lot of cells is always available is to place a number of amps on reserve. Please contact your Sales Representative for more information.

Q. I thawed and plated my cells yesterday and most of the cells are floating. What happened?

A. Clonetics™ Cells will not be confluent on day one. After 24 hours, you will see few attached cells per field under the microscope. Aspirate off the media and the floating cells, and replace with fresh, pre-warmed media. Those attached cells will begin to proliferate and a confluent monolayer should be established in 5−10 days.

Q. I've had my cells in culture for a few months. They're no longer growing and don't look like healthy cells. What's wrong?

A. All Clonetics™ Cells are primary cells with a finite life span. Most Clonetics™ Cells are guaranteed for 10−15 population doublings, which equates to about 2−3 passages. As cell death begins to occur, morphology and cell performance will begin to change and growth will slow down drastically.

Q. Can I use Clonetics™ Media for cells from other species?

A. While most of our media systems have been optimized for the growth of human cells, many researchers have had success using our media systems for other species. However, we only test and guarantee the media with the human cells stated in the product documentation.

Q. Can I transfect my Clonetics™ Primary Cells?

A. Transfection of primary cells is possible with up to 90% efficiency using our Nucleofector™ Technology, optimized protocols, publications and customer data. There are several reasons to choose the Nucleofector™ Technology for your gene transfer experiments. One is the direct transfer of DNA to the cell nucleus. This makes gene expression independent from cell division. Therefore, the technique is the ideal tool for primary cells. In many cases, transfection efficiencies of over 50% can be detected in as little as 2−4 hours. We have Nucleofection™ Data on over 50 of the Clonetics™ Primary Cells we offer. For more information, see http://bio.lonza.com/go/literature/52.pdf

Q. I don't see the specific cell type I want in your catalog or on your website. Do you offer custom isolations?

A. Yes, Lonza offers custom isolations and formats for various types of non-catalog cell products. Please contact your Sales Representative or visit the "Cells on Demand" section of our website to request your custom isolation (http://www.lonza.com/products-services/pharma-biotech/drug-discovery/cells-on-demand-cell-culture-services.aspx?item={57076940-EDF6-4040-8ABC-1C8A 8042DEB3}).

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