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Fall 2012

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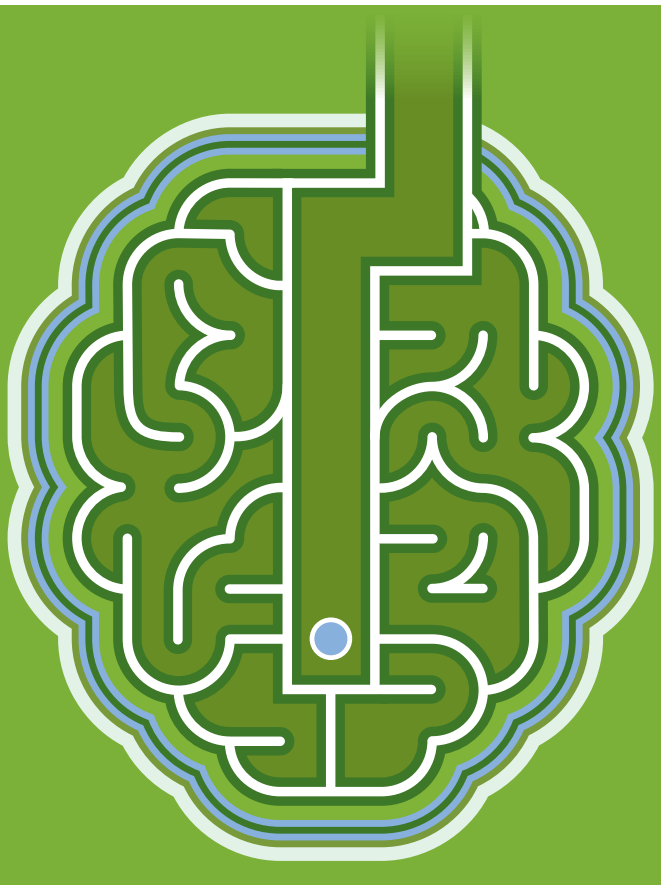

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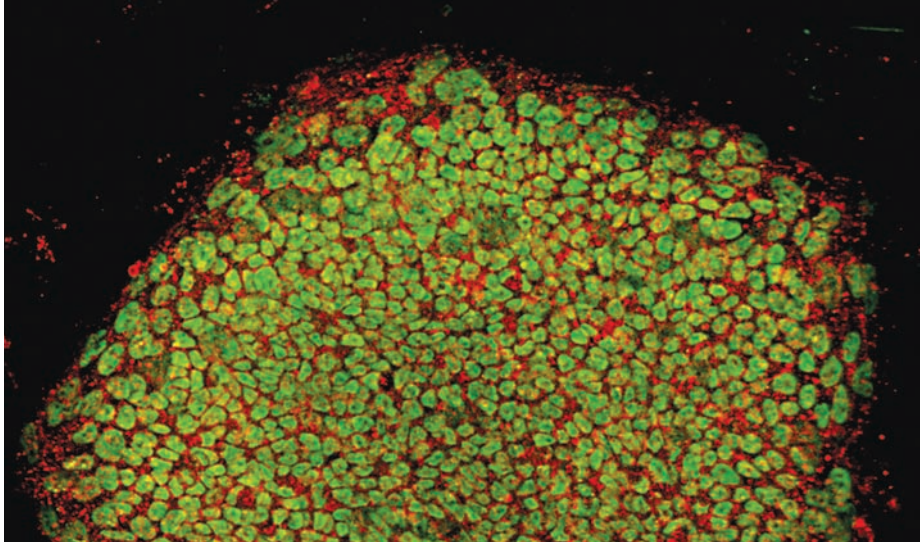

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Neurological Disease Research Continues to Expand

Neurodegenerative diseases are becoming increasingly more prevalent due to the aging population. Implications of these diseases are grave, both for quality of life and healthcare costs. Recent findings have revealed potential commonalities in genetic and cellular mechanisms across neurodegenerative diseases. Consequently, basic and therapeutic research in neurological degenerative diseases continues to expand with the majority of the focus on multiple sclerosis, Parkinson's Disease, and Alzheimer's Disease.

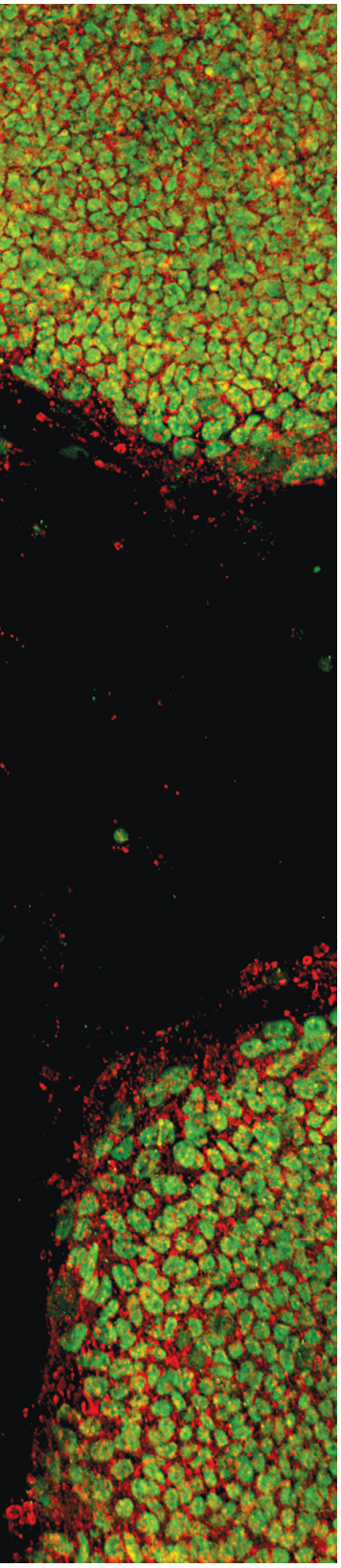
Transplantation of stem cells or stem cell-derived progenitors has long been seen as a therapeutic solution to repair the damaged brain. With the advent of the induced pluripotent stem cells, new techniques are becoming available for iPSCs as an attractive source in rejection-tolerance personalized replacement therapy. In recent years, patient-derived iPSCs have been used to replicate the phenotypes of neurological diseases and broaden our understanding of the pathogenesis of many neurological diseases. It is now expected that iPSCs will serve as an unlimited source of disease-specific neural cells for use in disease modeling.

Other research areas include using *in vitro* models of neurodegenerative disease, as well as primary astrocyte and primary neuronal cultures from all major areas of the brain, to discover novel, effective treatment agents in well-established disease models.

Lonza offers a broad range of *in vitro* research products to facilitate this type of research, including primary neurons and media for cell culture, Nucleofection™ Instruments and Reagents for iPSC generation, and protein separation products for gene identification and targeting.

Find out more in this issue of Resource Notes™.

Your Lonza Team



News

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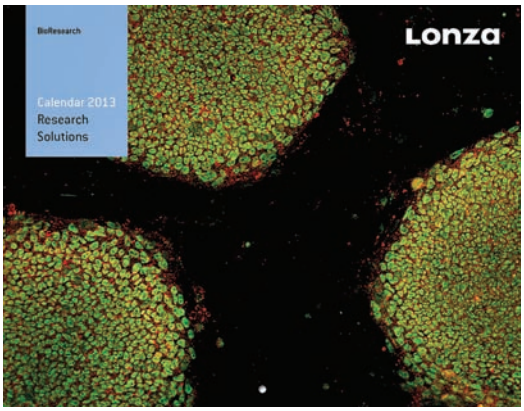

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Conferences/Tradeshows

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Dates	Event	Booth No.	Location
01–05 September, 2012	**European Respiratory Society 2012**	A3.04	Vienna, Austria
25–27 September, 2012	**Scanlab 2012**	C13-001B	Copenhagen, Denmark
13–17 October, 2012	**Neuroscience 2012**	1813	New Orleans, LA, USA

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Webinars

Date	Webinar
25 September, 2012	Innovative Applications with Nucleofector™ Technology
26 September, 2012	Innovative Applications with Nucleofector™ Technology
16 October, 2012	Alternative Solutions for Mycoplasma Testing
17 October, 2012	Alternative Solutions for Mycoplasma Testing
23 October, 2012	A New, Faster Protein Solution for Separation, Transfer/Western Blot and Staining
24 October, 2012	A New, Faster Protein Solution for Separation, Transfer/Western Blot and Staining

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Cryopreserved Dissociated Rat Brain Neuronal Cells Are Easily Transfected Using the 4D-Nucleofector™ Y Unit: A Convenient and Practical Substitute for Freshly Prepared Neuronal Cells

By Anthony Krantis[1], Susan VanderHoek[1], Barbro Tinner-Staines[1], Paul Shock[1] and Scott D'Andrea[2]
[1]QBM Cell Science, Inc., Ottawa, Canada; [2]Lonza Walkersville, Inc., Walkersville, MD, USA

Ready-to-use, batch tested, cryopreserved dissociated neuronal cells combined with high quality, high-throughput adherent neuronal transfection of the 4D-Nucleofector™ Y Unit is an effective and time saving approach for researchers employing genetically modified strategies for their neurobiology programs.

Cryopreserved primary neuronal cells dissociated from the rodent brain and periphery have been commercially developed by QBM Cell Science as ready-to-use primary cells and are ideal for a spectrum of studies in basic research and drug development fields. To date, more than 100 peer-reviewed publications prove these cryopreserved dissociated rodent neuronal cells (available exclusively from Lonza) to be an excellent research tool in neurobiology. The scope of applications of these cells is easily surveyed from the general literature as well as industrial monographs (see publications at www.qbmcellscience.com) where they are cited as used to develop cell assay, screening and interrogation technologies. When thawed and cultured, these batch tested, cryopreserved neuronal cells display all of the properties of freshly dissociated neuronal cells with the added advantage that they are guaranteed bacteria and mycoplasma free and show extraordinary inter- and intra-batch consistency. Collaborators employing different techniques can be testing cells from the same batch and experiments can also be re-visited on the same batch of cells that can be archived in liquid nitrogen. Our testing shows that when thawed and cultured, neuronal cells cryopreserved and frozen since 2000 display no deterioration.

The transfection of nucleic acids into cells is now considered crucial for the study of many aspects of neurobiology. However, primary neurons are difficult to transfect, making genetic modification of neuronal cells by delivery of either siRNA or plasmid DNA challenging. The cryopreserved brain neuronal cells are amenable to transfection by a variety of manual and automated techniques where the efficiency of transfection was comparable to freshly prepared neuronal cells with no obvious signs of toxicity post transfection. These studies include the use of virus-based, electroporation, chemical- and photo-transfection methods (Dityateva et al., 2003; Gartner *et al*., 2006; Gehl, 2003).

A relatively new form of electroporation-based transfection, Nucleofection™ – where plasmids are transfected directly into the nucleus (Iversen *et al*., 2005; Karra and Dahm, 2010) – has gained prominence across many research fields. The advantage of this technique is better transfection rates than classical electroporation or chemical methods, combined with good cell survival and quality of the culture post transfection due to specific programming of electrical parameters and use of specific transfection solutions to optimize the physiological environment and viability of the cells (Zeitelhofer *et al.*, 2007).

The advent of a Nucleofector™ System with multi-well plate 'adherent' formats, i.e. the 4D-Nucleofector™ Y Unit, means that the immature neuronal cells can now be first cultured to allow for normal development of neuronal processes, network connectivity and neurochemistry, then transfected. Here we report the application of adherent Nucleofection™ based transfection in cultures of cryopreserved dissociated rat brain neuronal cells.

Methods

Cell Culture

Primary neuronal cells are labor intensive and less convenient than cell lines since the researcher is faced with the tedium, caprice and waste associated with tissue dissection/dissociation first hand. These difficulties are now overcome by ready-to-use, cryopreserved primary neuronal cells, available from Lonza. Shipped frozen, they can be simply thawed and cultured in a variety of single- or multi-well formats to obtain high quality and high yield cultures of dissociated primary neuronal cells.

For the studies described herein, vials of frozen neuronal cells dissociated from rat brain cortex (R-Cx-500) and hippocampus (R-Hi-501) were thawed and plated into 24-well culture plates using a standard protocol. Briefly, 24-well plates were coated with poly-D-lysine/laminin (50:50) overnight at 37°C. Rat hippocampal or cortical neuronal cells were thawed and plated onto the multi-well plates. For comparison, freshly prepared hippocampal cells (from the same dissection batch) were plated and processed in the same way. Freshly isolated hippocampal cells were plated at 150,000/ml. Cryopreserved cells were plated at a higher cellular density of 200,000/ml (hippocampal cells) and 400,000/ml (cortical cells) to account for the reduction in viability due to cryopreservation. After 4 hours, the cultures were changed to PNGM™ Media and then again at 4 days *in vitro* (DIV). Cultures were subjected to an 80% media change every 3–4 DIV thereafter.

Transfection

We used the 4D-Nucleofector™ Y Unit – configured for 24-well adherence culture formats – using the AD1 4D-Nucleofector™ Y Kit with pmaxGFP™ Vector. Expression of GFP fluorescent protein was evaluated at 4 hours, 24 hours to 19 days post Nucleofection™ by inverted fluorescence microscopy. The Nucleofector™ Solutions were prepared following the protocol provided. Typically, for 5 ml of supplemented AD1 solution, 250 µl of 1 µg/µl pmaxGFP™ Vector was added. Media was carefully removed from the wells and 350 µl of the solution-DNA mix added. The 24-well dipping electrode array was carefully placed into the wells making sure there were no air bubbles under the electrodes. The plate was positioned into the 4D-Nucleofector™ Y Unit for Nucleofection™. Post transfection, the Nucleofector™ Solutions from the wells were removed and replaced with fresh 37°C PNGM™ Media. This was followed by a 50% media change at 4 hours post transfection. The cells were fixed at various time points post transfection using our standard procedure.

Morphological Assessment

Fluorescent micrographs were used to qualitatively assess viability, transfection efficiency, and general morphology of cryopreserved neuronal cells following Nucleofection™, compared to non-transfected cryopreserved neuronal cells and freshly prepared neuronal cells prior to and post Nucleofection™. Cultures were fixed and stained for immunohistochemical examination directly in the wells and examined using inverted microscopy. Immunohistochemical identification of cell types within the cell cultures was performed using commercially available antibodies. The same general procedures were followed; primary antibody diluted to working concentration was applied to the wells overnight at 4°C. After rinsing in 10 mM phosphate-buffered saline (PBS), secondary antibody at its appropriate working dilution was applied for 30 minutes. Wells were again rinsed in PBS. Neuronal cells were examined using the general neuronal marker anti-PGP9.5 (1:1600, Rb; Abcam). Astrocytes were identified using anti-GFAP (Abcam). Alexa Fluor® 594 anti-Rb antibodies (Millipore) were used as secondary antibody.

Results and Discussion

The frozen cryopreserved neuronal cells can be thawed and cultured with ease on coated multi-well plates. When thawed and cultured using the optimized protocol provided, these batch tested cells display the properties of freshly dissociated neuronal cells. The time course for morphological differentiation of cryopreserved dissociated neuronal cells following plating on coated 24-well plates was characterized over 7 days in culture (Figure 1). They displayed typical neuronal neurite networks, cell morphology and neurochemistry. Neurons could be easily distinguished from astrocytes.

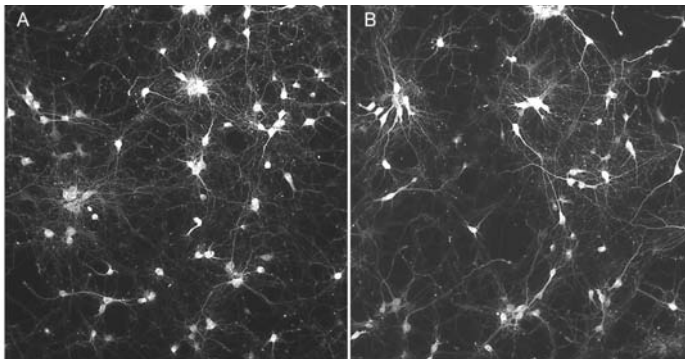

Figure 1
Neural networks in non-transfected cultures prepared from **(A)** fresh rat hippocampal neuronal cells and **(B)** cryopreserved rat hippocampal neuronal cells in 24-well plates. After 7 DIV they were immunostained with the specific neuron marker anti-PGP9.5.

We found the quality of transfection between cultures prepared from freshly dissociated cortical and hippocampal neuronal cells versus neuronal cells cryopreserved from the same batch then cultured, were comparable. Both neurons (Figure 2) and glia (Figure 3) are transfected. For both fresh and frozen cells, good quality transfection could be achieved from 3–11 DIV. However, best results were achieved using cultures at 5 DIV, with fixation 24 hours post Nucleofection™ (Figure 4). Nucleofector™ Programming Conditions were critical as shown in Figure 5. Programs ED-158 and EH-166 were equivalent and gave the optimal results.

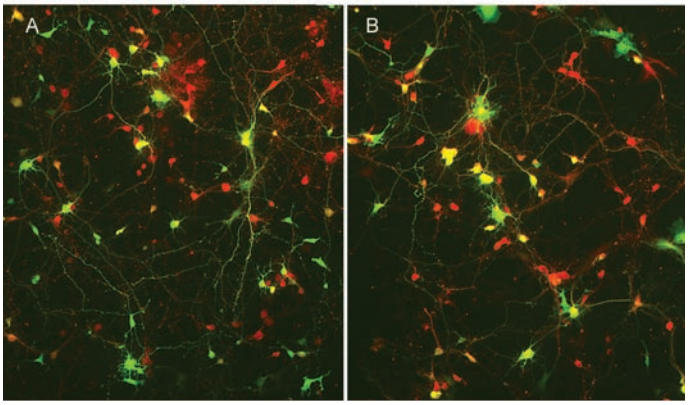

Figure 2
Comparison of transfection in cultures of cryopreserved neuronal cells dissociated from different rat brain regions (5 DIV, 24 hours post Nucleofection™). (A) Cryopreserved cortical cells (program EH-166). **(B)** Cryopreserved hippocampal cells (program ED-158). The overlayed images of typical cultures display rich neuropil with comparable numbers of maxGFP™ positive cells (green) relative to PGP9.5 positive neurons (red).

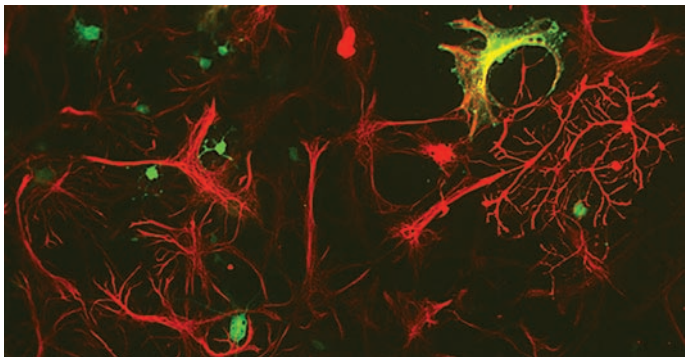

Figure 3
Cultures of cryopreserved rat cortical cells transfected 11 DIV (program ED-158). In this overlayed image, glial cells (red) can be seen together with maxGFP™ positive neurons (green). Some transfected glia can also be seen (yellow-green).

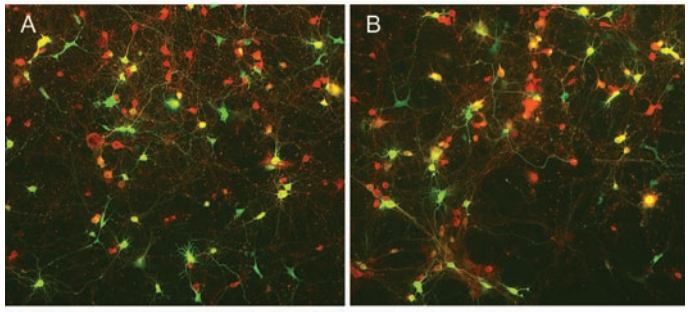

Figure 4
Comparison of transfection of freshly prepared and cryopreserved rat hippocampal cells. Neuronal cell cultures were transfected at 5 DIV and fixed 24 hours post Nucleofection™ (program EH-166). Transfection of freshly prepared cells **(A)** can be directly compared with **(B)** showing neuronal cells from the same dissection-dissociation batch that were first cryopreserved, frozen, then thawed and cultured.

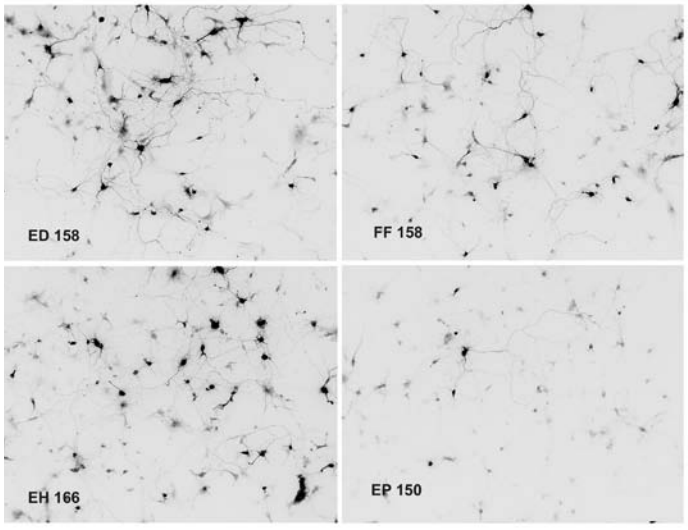

Figure 5
The effects of different Nucleofector™ Programs on transfection of cryopreserved rat hippocampal neuronal cells in culture at 5 DIV, examined 24 hours post Nucleofection™. The montage shows inverted GFP fluorescence images from wells of plates subjected to different Nucleofector™ Programs.

The cultures transfected in adherence with the 4D-Nucleofector™ Y Unit displayed neural networks and cell morphology comparable to the non-transfected controls. Close examination of the transfected cultures showed that neuropil disruption appeared equivalent between cultures of fresh versus cryopreserved cells. Cultures which were transfected >5 DIV displayed highly differentiated networks, with high quality transfection. However, the number of transfected cells was less. Cultures transfected at 5 DIV but examined 4 hours post Nucleofection™, showed few or weakly transfected cells.

Taken together, these data show optimal conditions for transfection by Nucleofection™ of cryopreserved neuronal cells require cultures to be 5 DIV with 24 hours for expression of signal. Nucleofector™ Programs ED-158 and EH-166 were found to be best for both cryopreserved rat hippocampal and cortical neuronal cells, and for freshly cultured neuronal cells. The quality of transfection using these Nucleofector™ Programs with cultures of 5 DIV or 11 DIV was striking. The GFP expression was intense in the cell soma and in the outermost extensions of dendrites and axons and it was straightforward to visualize a high amount of spines on transfected neurons (Figure 6).

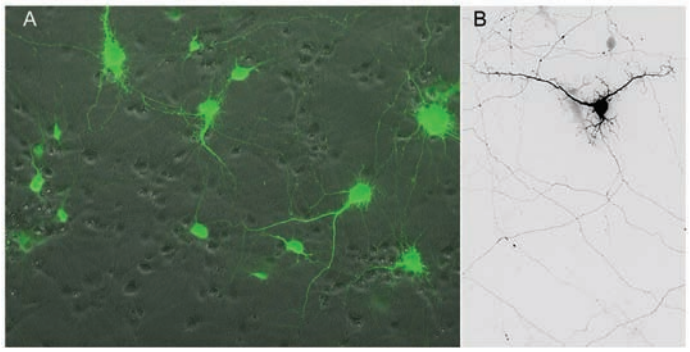

Figure 6
High quality transfection by Nucleofection™. maxGFP™ Expression was intense in the cell soma and in the outermost extensions of dendrites and axons shown here in phase contrast (A) and inverted fluorescence (B) images from different cultures of cryopreserved hippocampal neuronal cells transfected 11 DIV and photographed 24 hours post Nucleofection™. Spines and processes on transfected neurons are easily observed and axons can be followed long distances.

The ready-to-use advantage of batch tested cryopreserved dissociated rodent neuronal cells, combined with the high quality, high-throughput transfection of these cells in culture using the 4D-Nucleofector™ Y Unit, offers a convenient, effective and time saving approach for researchers employing genetically modified strategies for their neurobiology R&D programs.

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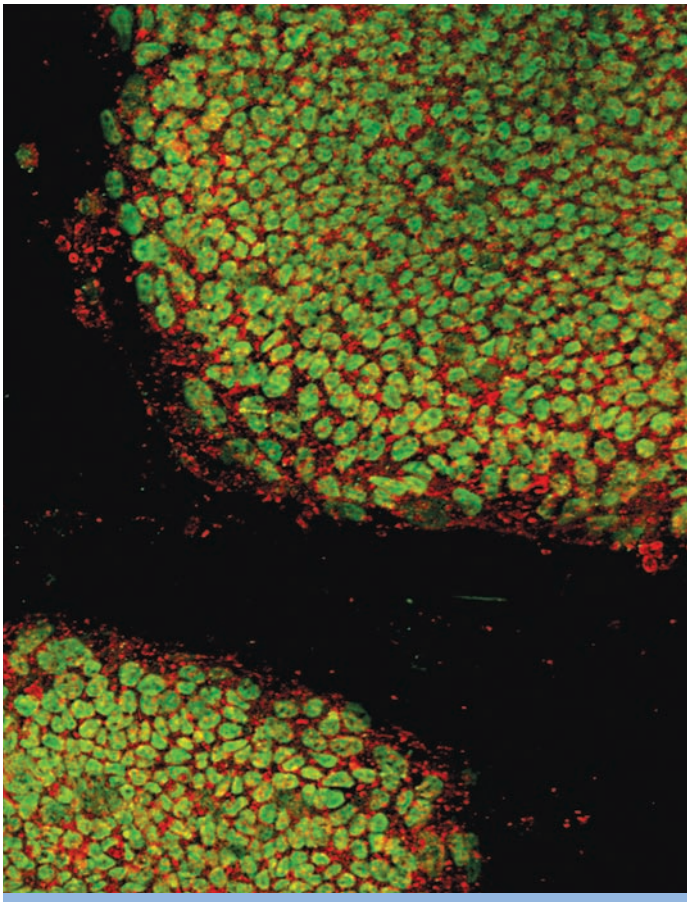

Generation of Induced Pluripotent Stem Cells from Primary Human Fibroblasts by Nucleofection™ of Episomal Plasmids

By Erik Olsen[1], Matthew F. Kuhar[1], Christopher N. Mayhew[1] and Gregory Alberts[2]

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[2]Lonza Walkersville, Inc., Walkersville, MD, USA

Introduction

Induced pluripotent stem cells (iPSCs), generated by reprogramming somatic cells via the ectopic expression of defined factors, appear to be phenotypically and functionally equivalent to human embryonic stem cells (hESCs). The ability of hESCs and iPSCs to self-renew in culture and their potential to differentiate into all human cell types makes them a powerful tool for identifying the mechanisms of human development, for generating new models to study human disease pathogenesis, and for the production of specific cell types for *in vitro* drug screening and toxicity studies. These cells also have great potential as a renewable source of material for producing therapeutic cells for transplantation in regenerative medicine. Furthermore, because they can be derived from any individual, iPSCs may be useful for generating autologous therapeutic cells for transplantation.

The initial reports that described human iPSC generation used integrating viral vectors to express the required reprogramming factors[1,2]. Although the use of integrating viral vectors is a relatively efficient method to reprogram somatic cells, this approach results in random integration of reprogramming vectors into the genome of iPSCs, potentially resulting in insertional mutagenesis and/or disruption of differentiation into desired cell types[3]. Furthermore, in chimeric mice harboring iPSC-derived tissues, reactivation of silenced cMYC-expressing retroviral vectors during differentiation was associated with tumorigenesis[4]. Thus, the use of integrating viral vectors is not suitable for generating clinically relevant cell types from iPSCs. Consequently, there has been a considerable effort towards generating efficient integration-free approaches for human iPSC derivation. Several methods for generating integration-free human iPSCs have been described, including repeated transient transfection of mammalian expression plasmids[5,6], DNA minicircles[7], synthetic mRNAs[8] or microRNAs[9]; single transfection of EBNA-based episomal plasmids [10–12]; the use of the piggyBac transposon/transposase system to excise integrated reprogramming factors[13]; using non-integrating recombinant viruses, such as adenovirus[14] and Sendai virus[15] to express reprogramming factors; and the repeated delivery of membrane-soluble recombinant reprogramming factor proteins[16]. Each of these diverse approaches to generating integration-free human iPSCs has potential drawbacks, such as exposure of somatic cells to recombinant viral particles, extremely low reprogramming efficiencies, technically demanding methodologies, and/or expensive reagent costs. Ultimately, for widespread use in regenerative medicine, it will be important to identify simple and cost-effective vector-free iPSC-derivation methods that are amenable to efficient generation of high quality iPSCs from multiple human somatic cell types under Good Manufacturing Practice (GMP) conditions.

In this study, we tested a protocol adapted from methods previously reported by Yu *et al*.[10] and Okita *et al*.[11] for generating integration-free human iPSCs from primary human fibroblasts using non-integrating EBNA1/OriP-based episomal plasmids.

Methods and Materials

Cell Culture

Primary human foreskin fibroblasts (HFFs) used in this study were cultured from neonatal human foreskin tissue obtained from two donors through the Department of Dermatology, University of Cincinnati, and were a kind gift from Susanne Wells, PhD. HFFs were cultured in fibroblast media consisting of DMEM (Life Technologies) supplemented with 10% FCS (Hyclone) and used for reprogramming between passages 5 and 8.

Episomal Reprogramming Plasmids

EBNA1/OriP-based episomal plasmids pCLXE-hOct3/4-shp53, pCLXE-hSox2-Klf4, pCLXE-hLmyc-Lin28, and pCLXE-GFP used for this study were originally described by Okita *et al*.[11] and obtained from Addgene (ID #s: 27077, 27078, 27080, and 27082 respectively).

Nucleofection™

The optimized Human Dermal Fibroblast Nucleofector™ Kit (VPD-1001; Lonza) was used for transfection of HFFs with episomal plasmids. Briefly, for each transfection 1 x 10^6 HFFs were pelleted by centrifugation at 200 xg for 10 minutes at room temperature and resuspended in 100 µl room temperature Nucleofector™ Solution. Plasmid DNA was combined (1.25 µg each of pCLXE-hOct3/4-shp53, pCLXE- hSox2-Klf4, pCLXE- hLmyc-Lin28, and pCLXE-GFP for a total of 5 ug), mixed with the HFF cell suspension, and transferred to a certified cuvette. The Nucleofector™ I Device (Program U20) was used to transfect HFFs. Cells from two transfections (2 x 10^6 total cells) were replated in a 10 cm tissue culture plate in fibroblast media, and cultured at 37°C/5% CO_2.

iPSC Generation and Culture

Six days post transfection, 4.5 x 10^5 HFFs were replated in fibroblast media in a gelatin-coated 10 cm dish containing 1.07 x 10^6 irradiated mouse embryonic fibroblasts (MEFs). Starting on day 7 post transfection, cells were fed daily with DMEM/F12 media supplemented with 20% knockout serum replacement, 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol, 0.1 mM non-essential amino acids, and 4 ng/ml basic FGF (all from Invitrogen).

Approximately 2 weeks later, discrete colonies with hESC-like morphology were manually excised and replated in mTeSR1 media (Stem Cell Technologies) in tissue culture dishes coated with hESC-qualified matrigel (Becton Dickinson). Following adaptation to mTeSR1/matrigel culture, iPSCs that maintained robust proliferation and hESC-like morphology with minimal spontaneous differentiation were expanded for cryopreservation and characterization.

iPSC Characterization

Immunofluorescence analysis and alkaline phosphatase activity: For immunofluorescence, undifferentiated cultures or cultures exposed to specific differentiation protocols were fixed for 10 minutes at room temperature with 3.7% formaldehyde in PBS. Cells were then permeabilized for 10 minutes with PBS containing 0.5% Triton® X-100 and incubated for 30 minutes at room temperature in blocking buffer (PBS + 10% normal donkey serum). Antibodies to human Oct4 (sc-5279; Santa Cruz), Nanog (ab21624; Abcam), FoxA2 (H00003170-M01; Novus), Sox17 (AF1924; R&D Systems), or Pax6 (PRB-278P; Covance) were diluted in blocking buffer at 1:500 and incubated with cells overnight at 4°C. After incubation with appropriate fluorescent-labeled secondary antibodies, cultures were visualized using fluorescent microscopy. For analysis of alkaline phosphatase (AP) activity, cultures were fixed for 1–2 minutes with 3.7% formaldehyde in PBS followed by AP staining using the Alkaline Phosphatase Detection Kit (Millipore).

Analysis of *in vitro* differentiation capacity: Definitive endoderm (DE) and neuroectoderm (NE) induction was performed using methods modified from those described by D'Amour *et al.*[17] and Morizane *et al.*[18]. Briefly, a single cell suspension of iPSCs was prepared using Tryple Express (Invitrogen), and replated at $2x10^5$ per well of a matrigel-coated 4-well plate in mTeSR1 supplemented with 10 µM Y27632 (Sigma). When cells were ~95% confluent, media was changed to differentiation media. For DE induction, cells were incubated for 3 days with RPMI supplemented with 100 ng/ml Activin A (R&D Systems) containing 0, 0.1 and 1% defined FCS (Hyclone) on days 1, 2 and 3 respectively. For NE induction, cells were exposed to hESC media without bFGF supplemented with 10 µM SB542431 (Cayman Chemical) and 2 µM dorsomorphin (Sigma). Media was changed daily for 10 days. Immunofluorescence for definitive endoderm (Sox17 and FoxA2) and neuroectoderm (Pax6) markers was performed as described above. For generation of beating cardiomyocytes, iPSCs were pre-differentiated with EB media (DMEM supplemented with 20% defined-FCS (Hyclone) and 0.1 mM non-essential amino acids) for 3 days. Cells were then exposed to dispase before being manually scored, scraped and replated as clumps in EB media in an ultra-low attachment cell culture dish. After 7 days in suspension culture, cellular aggregates were replated in gelatin-coated cell culture dishes in EB media. Within 10 days in adherent culture, spontaneously beating foci were routinely identified.

Analysis of *in vivo* differentiation capacity: For teratoma formation, iPSCs from 3 wells of a 6-well dish were combined and gently resuspended in ice-cold DMEM/F12. Immediately before injection, matrigel was added to a final concentration of ~33% and cells were injected subcutaneously into immune-compromised NOD/SCID GAMMA C-/- mice. Tumors formed within 6–12 weeks. Excised teratomas were fixed, embedded in paraffin, and sections were stained with hematoxylin and eosin for histological examination.

Analysis of episomal DNA by Q-PCR: Total DNA was extracted from human iPSCs and HFFs using the QIAamp® DNA Mini Kit (Qiagen). DNA from HFFs transfected with reprogramming plasmids harvested 6 days post transfection and from non-transfected HFFs were used as positive and negative controls, respectively. Quantitative PCR reactions were carried out using ~0.1 µg genomic DNA combined with 5 ng of each sense and antisense primer and 1 x IQ SYBR® Green Master Mix (BioRad). Primer sequences were as follows: pCEP4-FWD: 5'-ggagctgagtgacgtgacaa-3'; pCEP4-REV: 5'-gatggagatgagggtgagga-3'; Sox1-FWD: 5'-caagttgctaaccgatgtga-3'; Sox1-REV: 5'-ggaaacctaaggctgctga-3'. Relative episomal DNA (pCEP4) levels were calculated following normalization to levels of Sox1, representing total genomic DNA.

Results and Discussion

iPSC generation and initial quality assessment: HFFs obtained from two donors were separately transfected with episomal plasmids expressing the reprogramming factors Oct4, Klf4, Sox2, L-myc, LIN28 and sh-p53, and an episomal plasmid expressing GFP by Nucleofection™ on day 0. HFFs robustly expressed GFP within 18 hours, indicating efficient episomal plasmid transfection (Figure 1A). Six days post transfection, HFFs were replated on MEFs and fed daily with hESC media containing bFGF. Within 2 weeks, large colonies with hESC-like morphology were evident (Figure 1B). These colonies were largely GFP-negative. Several discrete colonies were then manually excised and replated in feeder-free culture conditions for expansion. AP activity analysis of remaining colonies demonstrated the presence of numerous AP+ colonies, indicating relatively efficient HFF reprogramming (~0.3%) by Nucleofection™ of episomal plasmids (Figure 1C). Excised colonies plated in feeder-free conditions rapidly acquired growth characteristics and colony/cellular morphology typical of pluripotent cells cultured in these conditions (Figure 1D). Three putative iPSC lines from each donor were then chosen for larger-scale expansion for characterization and cryopreservation.

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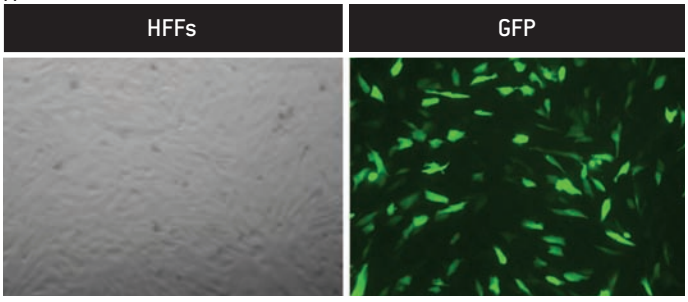

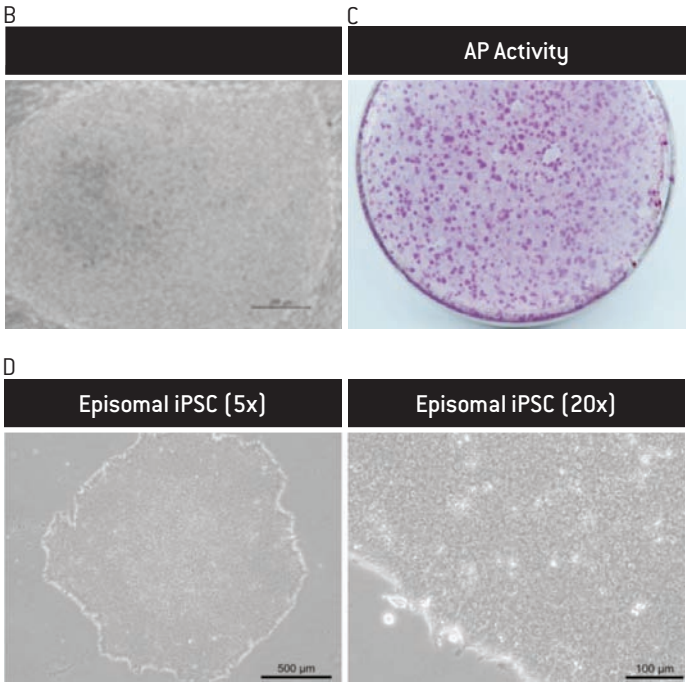

Figure 1
iPSC generation by episomal-based HFF reprogramming. (A) GFP expression 18 hours post transfection of HFFs with episomal plasmids expressing reprogramming factors and GFP. (B) Morphology of a putative iPSC colony and (C) alkaline phosphatase activity ~2 weeks after replating transfected HFFs in hESC culture conditions. (D) Typical hESC-like morphology of a representative episomal iPSC line cultured in feeder-free culture conditions for 15 passages. All six expanded episomal iPSC lines had identical morphology.

Quantitative PCR analysis of episomal DNA content in three expanded episomal iPSC lines from one donor revealed that episomal plasmid DNA was undetectable in iPSCs harvested at passage 17, in contrast to d6 transfected HFFs (Figure 2A). These data suggest that episomal DNA had been spontaneously lost in these iPSCs. Furthermore, all six episomal iPSC lines that were expanded were karyotypically normal (Figure 2B) and exhibited appropriate nuclear expression of the pluripotency markers Oct4 and Nanog (Figure 2C).

Analysis of functional pluripotency: The most stringent pluripotency assays for assessment of mouse iPSC differentiation capacity, such as tetraploid complementation assays, are not possible for human iPSCs for ethical reasons. Therefore, the ability of human iPSCs to differentiate *in vitro* and *in vivo* into cells derived from each of the three embryonic germ layers, endoderm, mesoderm and ectoderm, is employed as a surrogate to assess functional pluripotency. For analysis of *in vitro* differentiation, we subjected three episome-derived iPSCs from one donor and H1 hESCs to protocols for inducing neuroectoderm (NE), definitive endoderm (DE). Additionally, episomal iPSCs were differentiated to mesoderm-derived beating cardiomyocytes.

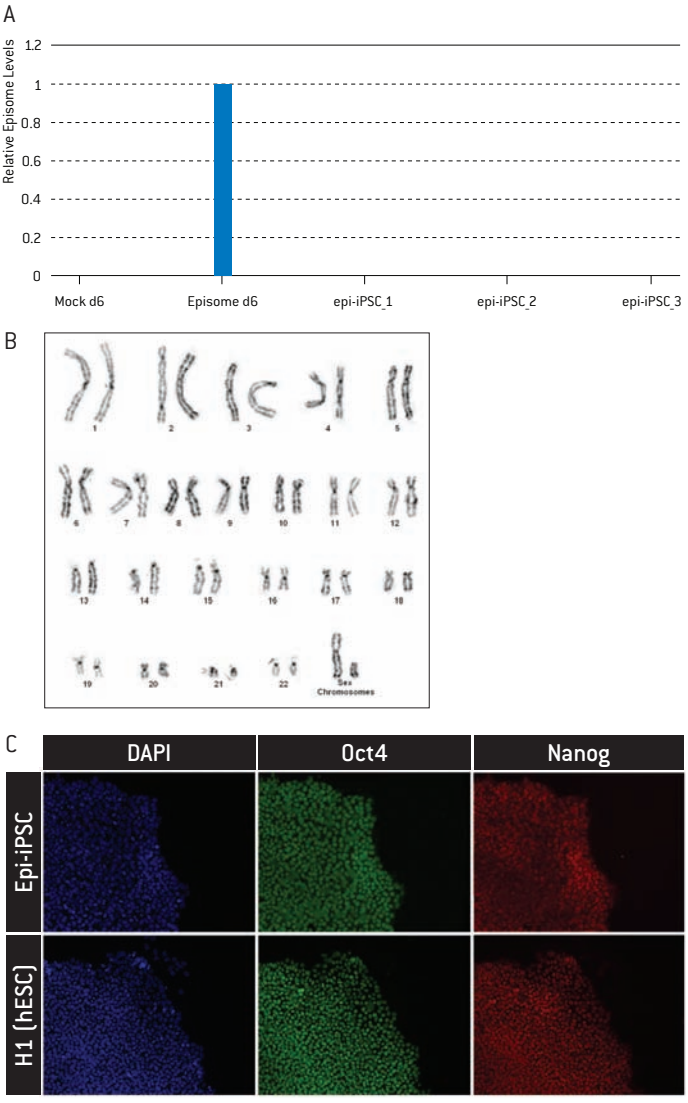

Figure 2
Episomal iPSCs have undetectable episomal DNA, are karyotypically normal and express pluripotency markers. (A) Quantitative PCR analysis of relative episomal DNA levels in three expanded iPSC lines from a single donor. Episomal DNA levels were normalized to the levels of genomic Sox1 DNA and are expressed relative to normalized episomal levels in HFFs 6 days post transfection. Analysis was performed on cells at passage 17. (B) Representative G-banded karyotype analysis of an episomal iPSC line. (C) Immunofluorescence staining for pluripotency markers Oct4 and Nanog in a single episomal iPSC line. Staining is representative of all six expanded episomal iPSC lines.

Following *in vitro* differentiation, immunofluorescence analysis demonstrated that all three episomal iPSCs were competent to generate both NE and DE (Figures 3A and B). Additionally, all iPSC lines generated beating cardiomyocytes *in vitro* (Figure 3C). Finally, all episomal iPSC lines formed teratomas *in vivo* following subcutaneous injection into immunodeficient mice. Histological analysis of excised tumors demonstrated that each of these teratomas harbored differentiated tissues derived from all three germ layers (Figure 4). Together, these data indicate that episome-derived iPSCs are functionally pluripotent.

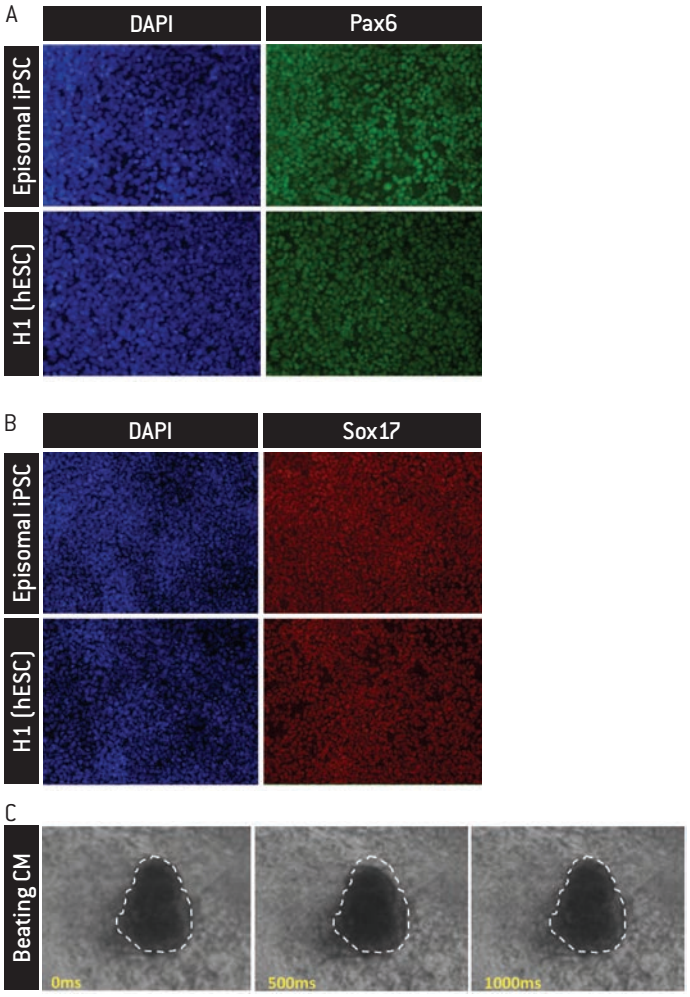

Figure 3
Tri-lineage differentiation of episomal iPSCs *in vitro*. (**A**) Immunofluorescence analysis of expression of the neuroectoderm marker Pax6 in an episomal iPSC line and H1 hESCs exposed to dorsomorphin and SB542431 for 10 days. (**B**) Immunofluorescence analysis of Sox17 expression in an episomal iPSC line and H1 hESCs exposed to activin A for 3 days. NB: Sox17+ cells were also FoxA2+ (data not shown), unequivocally identifying these cells as DE. The response of each line to dorsomorphin/SB542431 or activin A was variable, but in each case was significantly elevated compared to controls exposed to vehicle alone. (**C**) Generation of spontaneously beating foci of cardiomyocytes. Rhythmically beating foci were identified and a video recorded. Three stills (each separated by 500 ms) taken from the video of a single beating focus are shown. The white hashed line represents the original position of the focus (0 ms). Note the changed position of the focus relative to the original position at 500 ms and the return to the original position at 1000 ms, indicating contraction and relaxation.

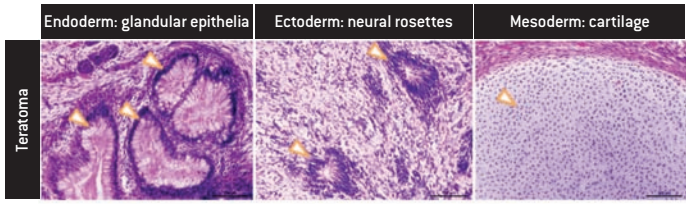

Figure 4
Tri-lineage differentiation of episomal iPSCs *in vivo*. A hematoxylin- and eosin-stained section from a representative episomal iPSC-derived teratoma is shown. The presence of the indicated tissue derived from each embryonic germ layer is identified by arrows.

Summary and Conclusions

Similar to published reports[10, 11], these data indicate that introduction of episomal reprogramming factors to human fibroblasts generates iPSCs that appear to be free from exogenous DNA and that meet the standard quality control criteria currently applied to human iPSCs. Furthermore, Nucleofection™ is an effective approach to introduce episomal plasmids to human fibroblasts, and represents a simple, efficient, and cost-effective method for reprogramming these cells. For widespread applicability of this methodology to reprogramming patient-specific cells for use in regenerative medicine, it will be critical to optimize protocols for efficient episomal transfection of additional cell types, such as peripheral blood (e.g.,[12]) that are easily obtained from patients in a minimally invasive manner. Furthermore, it will be important to identify robust quantitative quality control metrics to identify those iPSC lines that will be the safest and most effective for generating mature, functional cell types suitable for transplantation.

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New, Faster Protein Solution for Separations, Blotting Transfer and Staining

By Hugh White[1], Liz Horton[2], Mary Riley[1] and Denise Trevarrow[1]

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Introduction

The Laemmli method, based on Tris-glycine SDS, has been a standard method for evaluating protein size by polyacrylamide gel electrophoresis (PAGE) for decades. Efforts have been made to improve the time and efficiency of the Laemmli gel methods, but none have resulted in a complete solution for scientists' needs.

Lonza's novel, fast protein solution addresses the need for faster, more process-efficient and reliable quality solutions for protein electrophoresis. Ultimately, our new products, when combined, reduce protein separation, western blot transfer/blotting and staining from 5+ hours down to 1 hour.

This complete solution consists of new gels, buffers and stains.

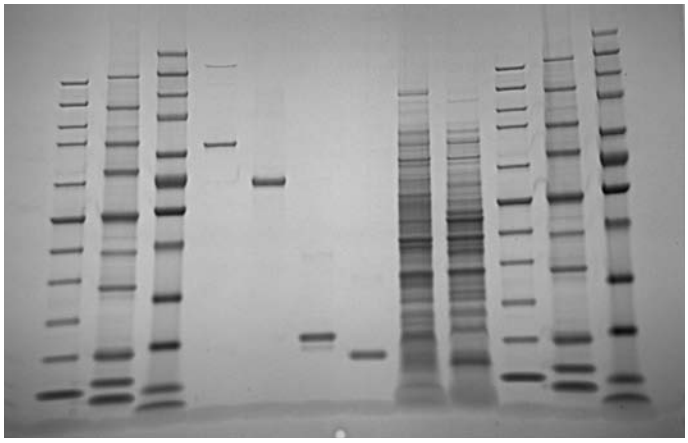
- The new PAGEr™ EX Gels are designed for fast 20-minute separation when used with the ProSieve™ EX Running Buffer.
- ProSieve™ EX Safe Stain provides an ultimate, fast and safe stain solution in one step of 10 minutes or less. ProSieve™ EX Stain Enhancer offers a cost-effective option for Coomassie® Stain users to speed and maximize their staining process.
- ProSieve™ EX Western Blot Transfer Buffer can accelerate western blot/transfer from hours to minutes without compromising resolution of separation, or efficiency of transfer.

Data

In order to evaluate the time savings and efficacy of our complete solution in the case of protein separation and staining of gels, mid- to high-range 9 cm PAGEr™ EX Gels were loaded with 4 µl of the ProSieve™ Marker II, 3 µl of ProSieve™ Marker I, 5 µl ProSieve™ QuadColor Marker, 4 µl loads of the individual proteins Phosphorylase B, BSA, Myoglobin, alpha-lactalbumin (approximately 200 ng/band), and 4–6 µl loads of the *E. coli* lysates (not quantitated). The gels were run using 0.67X ProSieve™ EX Running Buffer at 250 V according to the protocol. The run was completed in 20 minutes.

For staining using ProSieve™ EX Safe Stain, the gel was submerged in 50 ml of stain with microwave treatment to warm to approximately 65°C. Incubation was then carried out in covered containers on a shaker. Staining was completed in 7–10 minutes with good contrast. By performing a water wash using 150 ml of water (with a small piece of paper towel), with warming in microwave prior to incubation for 5 minutes, contrast could be further enhanced.

20-minute gel run, 10-minute stain (30 minutes total)



20-minute gel run, 10-minute stain, 5-minute water wash added (35 minutes total)

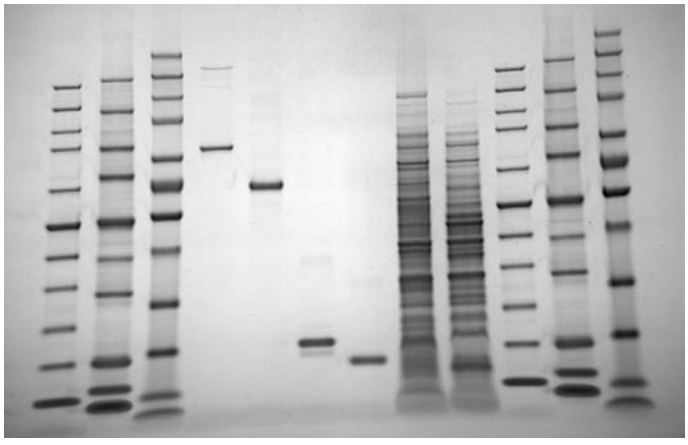
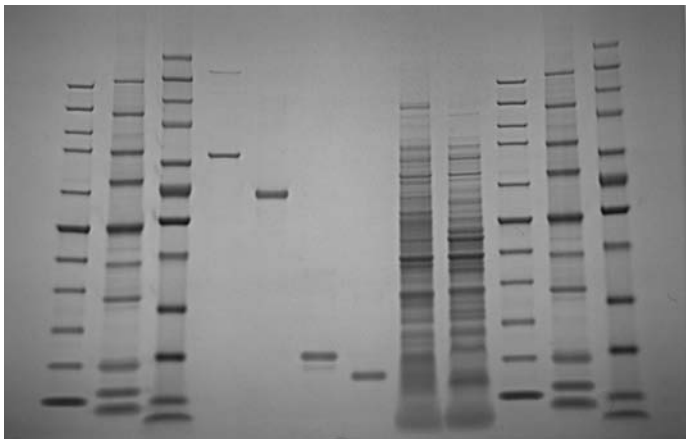


Figure 1
PAGEr™ EX Gels stained using ProSieve™ EX Safe Stain.

20-minute gel run, 5-minute ProSieve™ EX Stain Enhancer, 5-minute Coomassie Brilliant Blue® Stain (30 minutes total)



20-minute gel run, 5-minute ProSieve™ EX Stain Enhancer, 5-minute Coomassie Brilliant Blue® Stain (30 minutes total), 5-minute water wash added (35 minutes total)

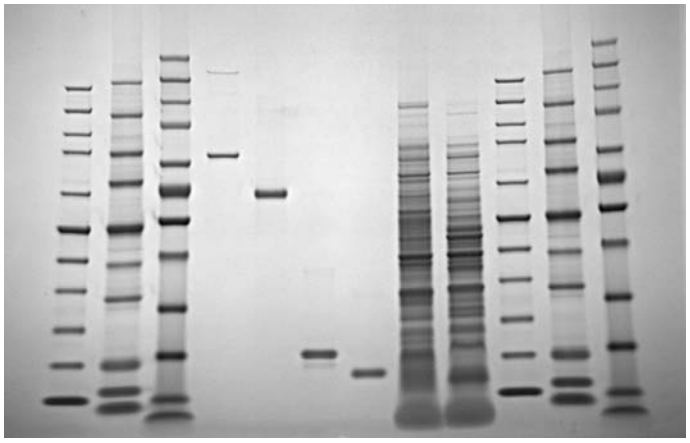


Figure 2
PAGEr™ EX Gels stained using ProSieve™ EX Stain Enhancer and 0.0075% Coomassie Brilliant Blue® G in water.

For staining using ProSieve™ EX Stain Enhancer in conjunction with Coomassie® Blue, the gel was submerged in 50 ml of ProSieve™ EX Stain Enhancer with microwave treatment to warm to approximately 65°C. Incubation was then carried out in covered containers on a shaker for 5 minutes. The gel was then submerged in 50 ml of 0.0075% Coomassie Brilliant Blue® G in water for an additional 5 minutes. By performing a water wash using 150 ml of water (with a small piece of paper towel), with warming in microwave prior to incubation for 5 minutes, contrast could be further enhanced.

20-minute run followed by 2-minute water wash 60°C, 2-minute 1X ProSieve™ EX Western Transfer Buffer equilibration at 60°C and transfer via semi-dry transfer device using ProSieve™ EX Western Transfer Buffer for 10 minutes at 400 mA (32 minutes total)

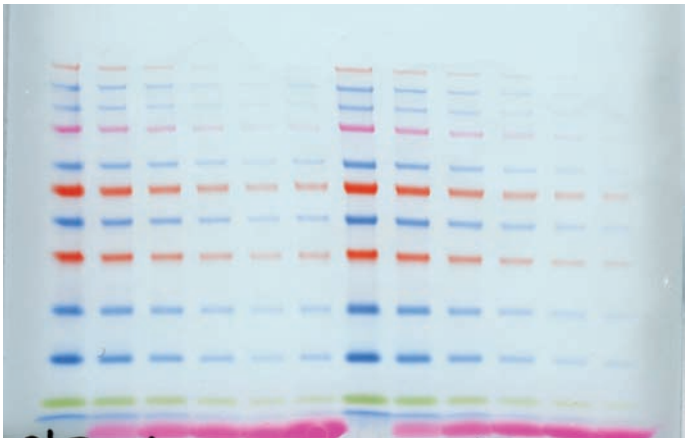


Figure 3
PAGEr™ EX Gels transferred to a nitrocellulose membrane.

In order to evaluate the time savings and efficacy of our complete solution in the case of protein separation and transfer of gels, again mid- to high-range 9 cm PAGEr™ EX Gels were loaded with a dilution series of ProSieve™ QuadColor Marker. The gels were run using ProSieve™ EX Running Buffer according to the protocol. The run was completed in 20 minutes.

Gels were then processed by washing for 2 minutes in water and equilibrating 2 minutes in 1X ProSieve™ EX Transfer Buffer at 60°C prior to transfer. Using the heated buffer was a modification to the protocol found to cut the time of equilibration prior to transfer with equal results. All transfers were for 10 minutes at 400 mA with semi-dry blotting unit.

Conclusion

Using Lonza's novel, fast protein solution results in robust separation with clear, simple staining and effective transfer in a fraction of the time of standard methods. Each product is sold separately or packaged together in Lonza's new Fast Protein Staining Kit and Fast Protein Transfer Kit.

Scientists can also use any of these products separately to help accelerate protein separation, transfer and staining in their current electrophoresis platforms just by using the novel buffers with standard Tris-glycine or PAGEr™ EX Gels and Staining Reagents with their gel system of choice.

Nucleofection™ Innovation Continues

New 4D-Nucleofector™ Protocols

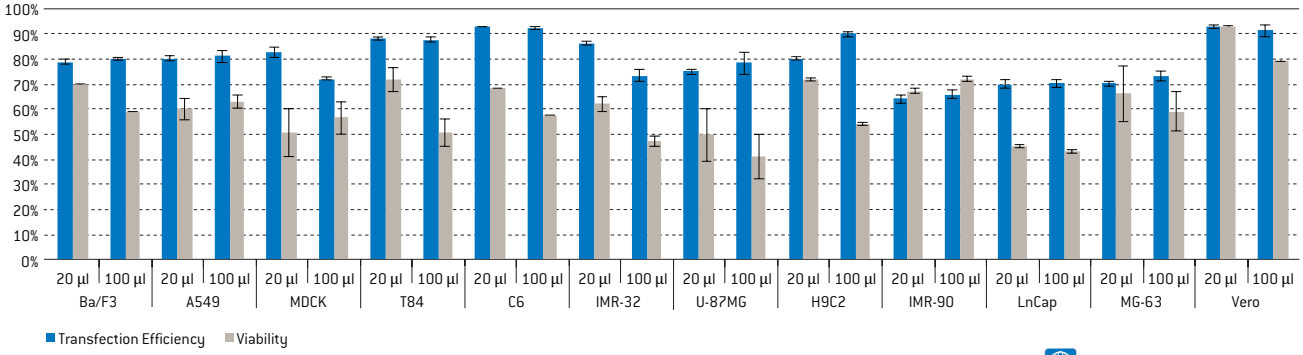
Since the launch of the 4D-Nucleofector™ System in summer 2010, we have been continuously expanding our list of ready-to-use optimized protocols for the 4D-Nucleofector™ X Unit. During the past months, nine additional protocols have been developed for hard-to-transfect cell lines relevant to various research fields:

Research Field	Cell Type
Immunology	Ba/F3
Neurobiology	C6 IMR-32 U-87MG
Metabolic diseases	MDCK T84
Respiratory diseases	A549
Other	H9C2 IMR-90 LnCap MG-63 Vero

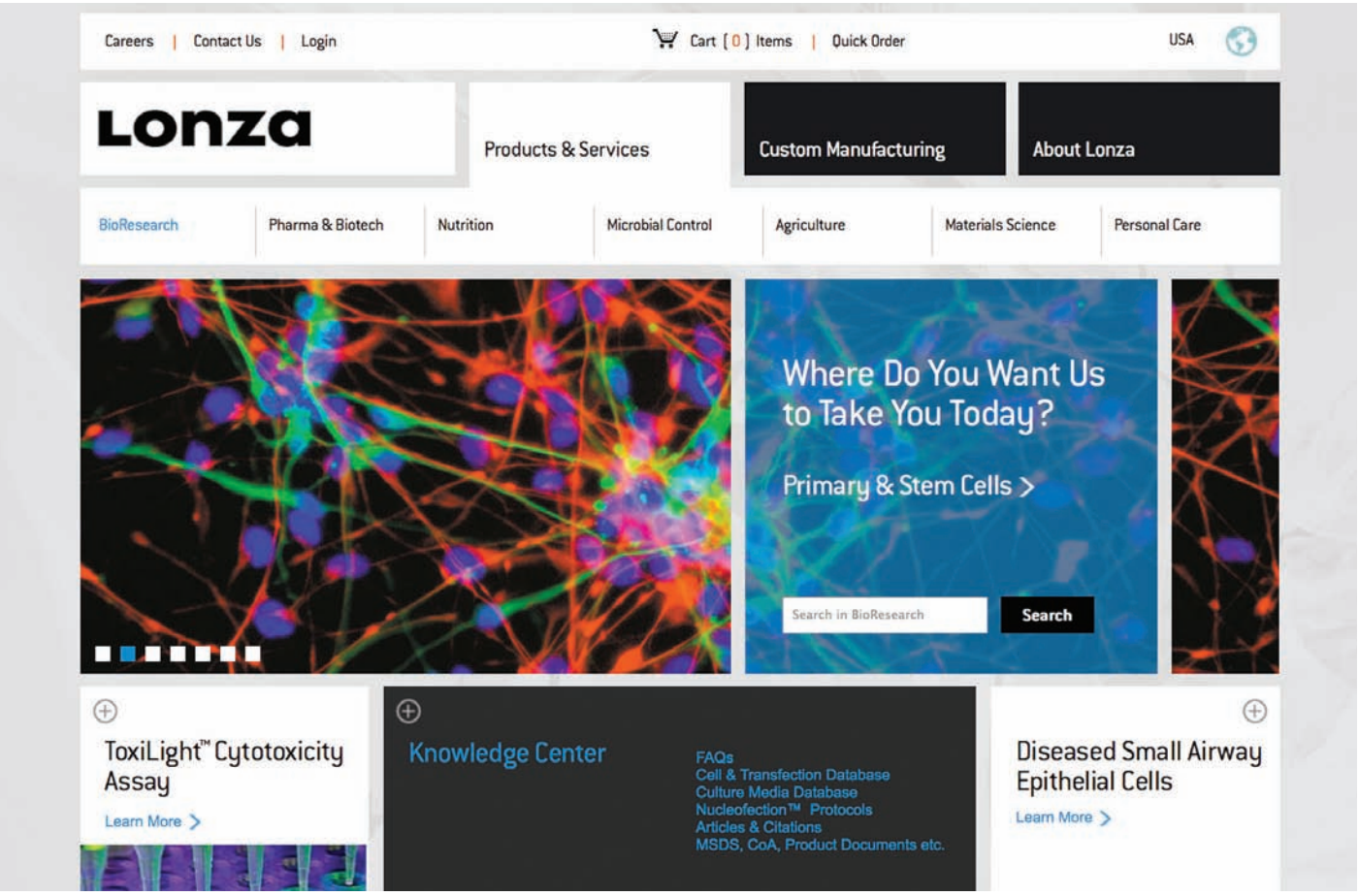
Benefit from transferable conditions between protocols suited for:

- High cell numbers using 100 µl Nucleocuvette™ Vessel
- Low cell numbers using 20 µl Nucleocuvette™ Strips

Comparison of 20 µl and 100 µl Nucleocuvette™ Vessels



www.lonza.com/4d-nucleofector



Welcome to the New Lonza Website

To support your everyday needs better than before, we have redesigned our website based on your input and wishes. Our new website offers quicker and easier access to our entire life science offering, grouped into the seven markets that Lonza serves.

Benefit from:

- New streamlined navigation to explore our products and service offerings
- Fully revised product pages offering detailed product information, including technical references and related products
- Customer friendly search capabilities for quick and efficient identification of technical data, e.g., MSDS, product sheets, CoA, and direct contact information
- A new knowledge center comprising access to all technical information
- Integration of online purchasing in our product pages for a seamless shopping experience
- Customer-specific pricing display if logged in with valid customer number
- Easy linkage to social media

Quick Link

www.lonza.com/research-contact

Use the above link to request:

- A Nucleofection™ Demo
- Cell line optimization
- Protocol for a new cell type
- BioResearch literature

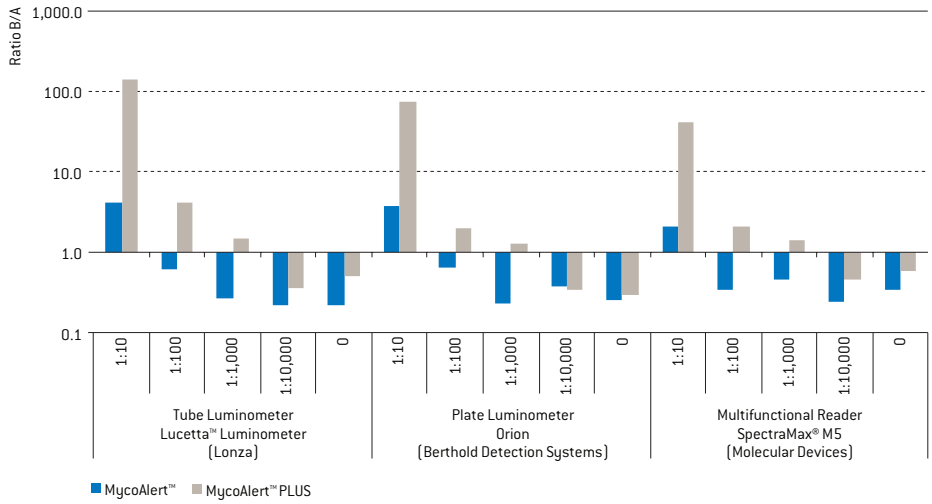
Expanded Mycoplasma Testing

Introducing MycoAlert™ PLUS Mycoplasma Detection Assay

Lonza's new MycoAlert™ PLUS Mycoplasma Detection Assay provides a broader compatibility with plate luminometers and multifunctional readers and allows the testing of fresh, cell-free media or supplements. The MycoAlert™ Assay is a selective biochemical test that exploits the activity of mycoplasmal enzymes which are found in the vast majority of 180 mycoplasma species, but are not present in eukaryotic cells. It has been established in the research market since 2003 as a convenient and rapid testing tool which provides reliable results within 20 minutes.

Benefits of MycoAlert™ PLUS Assay

- More than 20 times higher light output compared to the first generation MycoAlert™ Assay
- Broader compatibility with plate luminometers and multifunctional readers
- Suited for testing of fresh media and supplements



www.lonza.com/mycoplasma

In this issue of **TechTalk**, we will provide you with guidelines to optimize Nucleofection™ Conditions for your 4D-Nucleofector™ X Unit as well as answer frequently asked questions on this topic.

Q. When should I optimize the Nucleofection™ Conditions for my cells using the 4D-Nucleofector™ X Unit?

A. An optimization helps to determine the best conditions for a successful Nucleofection™. This is necessary when there is no Lonza recommended protocol for the 4D-Nucleofector™ X Unit for your cells of interest. To determine if an optimized protocol is available, search for "Optimized Protocols" at the Lonza website or in our Cell Transfection Database (www.lonza.com/celldatabase) or contact our Scientific Support Team.

Note that the 96-well Shuttle™ System Conditions are compatible with the 4D-Nucleofector™ X Unit, as a 96-well Nucleofection™ Reaction is equivalent to a 20 µl reaction in Nucleocuvette™ Strips with the 4D-Nucleofector™.

Q. Are Nucleofector™ I or II Conditions compatible with the 4D-Nucleofector™ X Unit?

A. The 4D-Nucleofector™ System is based on an upgraded Nucleofector™ Technology and features these main improvements:

- Ability to use two Nucleofection™ Vessel Formats: 100 µl Nucleocuvette™ for high cell numbers (2 x 10^5 to 2 x 10^7) and 20 µl Nucleocuvette™ Strips for low cell numbers (2 x 10^4 to 10^6). Nucleofection™ Conditions are transferable between these two formats.
- Improved cellular viability after Nucleofection™ due to the Nucleocuvette™ being composed of conductive polymer rather than aluminum.
- Streamlined offering of 4D-Nucleofector™ Solutions that make it easy to find the correct Nucleofector™ Solution for your cells.
- Broader spectrum of programs offering more opportunities to fine tune your optimization.

Due to these differences, solutions and programs recommended for the Nucleofector™ I or II Devices are not valid for the 4D-Nucleofector™ System. However, running an optimization on the 4D-Nucleofector™ X Unit is a great opportunity to potentially improve your Nucleofection™ Results.

Q. What parameters can I optimize?

A. In a Nucleofection™ Optimization, the most important parameters are the Nucleofector™ Solution (provided in the Nucleofector™ Kits) and the program (electrical pulse delivered by the Nucleofector™ Device). When no indications are available, we recommend starting with 200,000 cells and 0.4 µg of plasmid for a 20 µl Nucleofection™ Reaction. Cell number and substrate amount could be further optimized[1].

Q. What substrate should I use for my optimization?

A. Independent of your substrate of interest (plasmid, siRNA, protein or chemical compound), we always recommend using our pmaxGFP™ Vector for the optimization. Once the Nucleofection™ Conditions are determined with the pmaxGFP™ Vector, they can be used with your substrate. Using the pmaxGFP™ Vector for the optimization eliminates a number of variables, such as the nature and quality of preparation of your substrate and the timing of analysis.

Q. In what Nucleofection™ Format should I perform my optimization?

A. The 4D-Nucleofector™ X Unit has been designed to simplify the optimization step. We recommend using the 20 µl Nucleocuvette™ Strips for the optimization, reducing the consumption of precious cells. Our Primary Cell or Cell Line Optimization Nucleofector™ Kits are tailored to this format. Once you determine the optimal conditions, you can simply switch to the 100 µl Nucleocuvette™ by multiplying the cell number and substrate amount by 5, using the same solution and program.

Q. How do I optimize Nucleofection™ Conditions on the 4D-Nucleofector™ X Unit?

A. When no recommended protocol is available for your cells, then one of the three scenarios would apply (see Decision Guiding Scheme):

- If no recommendations are available, then start a first round of optimization. Each of the several Nucleofector™ Solutions (P1 to P5 for primary cells and SE, SF and SG for cell lines) is to be tested with 15 different programs[2].
- Follow a basic 4D-Nucleofector™ X Unit Protocol if available. This applies only for certain primary cell categories. Test one or two Nucleofector™ Solutions with a reduced number of programs (6–7).
- Limited Nucleofection™ Conditions and data for a 4D-Nucleofector™ X Unit are described. You then have the choice of testing them or going back to one of the first two options.

Q. What readouts should I use for my Nucleofection™ Optimization?

A. Record the transfection efficiency and the cellular viability at 24 hours post Nucleofection™ and at any other time point of interest. Transfection efficiency is expressed as the percentage of living cells expressing GFP, evaluated by fluorescence microscopy or by FACS analysis. Depending on your application, you might consider recording by FACS the intensity of the GFP signal, as X-mean. Cell viability could be estimated using any cellular viability assay. We recommend testing the

no pulse control with each Nucleofector™ Solution, as it indicates the basal level of cellular viability independently from the electrical program.

Q. How can I further improve my Nucleofection™ Conditions?

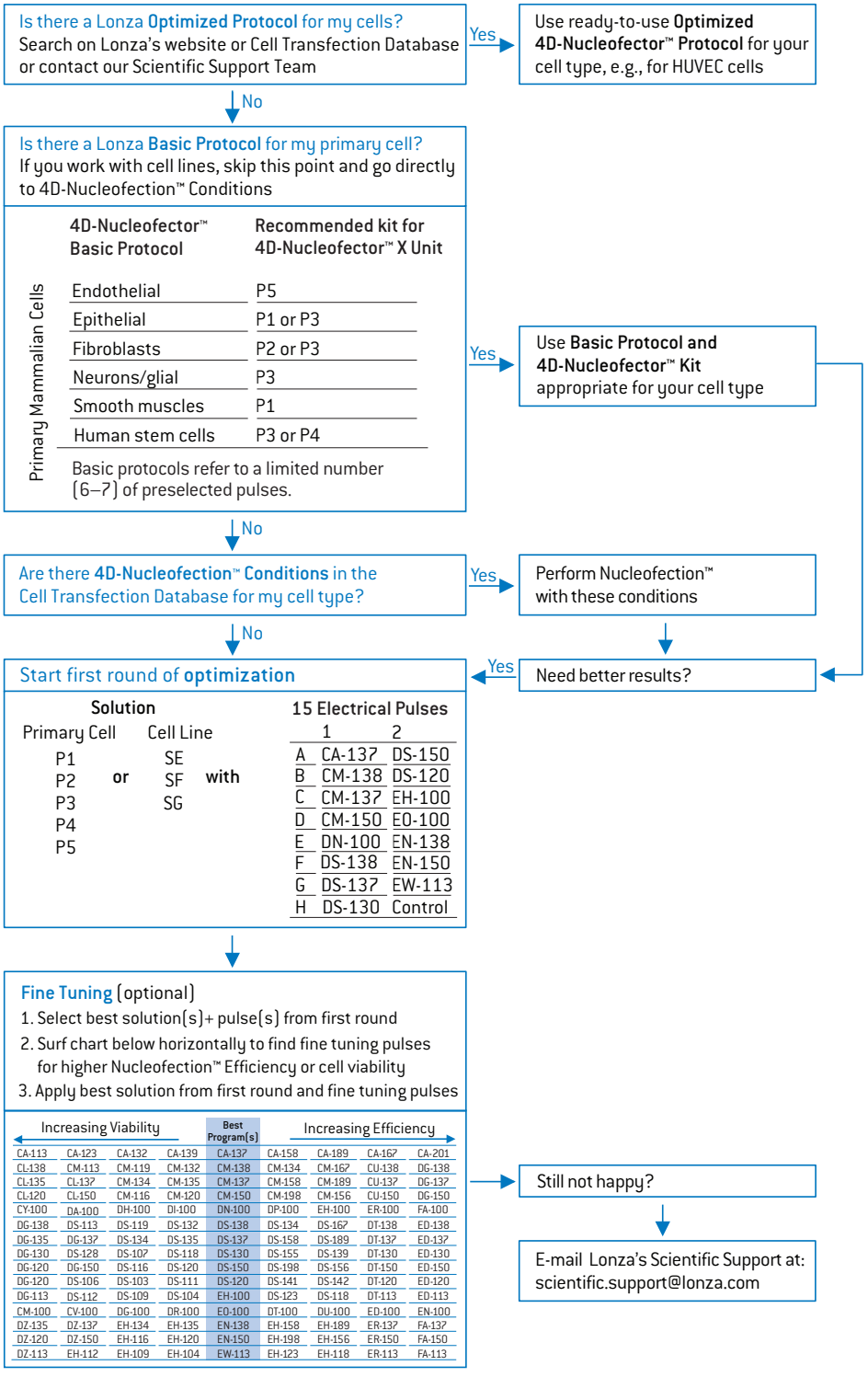
A. Once the results of Nucleofection™ are obtained from a first round of optimization, you may request our feedback using our web-based optimization form or e-mail your results to: scientific.support@lonza.com

It is possible to perform a fine tuning optimization that may improve your Nucleofection™ Results[2], using the fine tuning table shown in the Decision Guiding Scheme:

- Select the initial program that worked best (e.g., DN-100) within the fine tuning table. The 15 programs described in the first optimization round are listed vertically in the middle of the table.
- Use programs to the left of the selected program to increase cellular viability (e.g., DH-100). Use programs to the right to increase transfection efficiency (e.g., ER-100). The further to the left or right from the initial program, the higher the probability of improvement of viability or efficiency, respectively.

Decision Guiding Scheme

Is an Optimization of 4D-Nucleofector™ Conditions Needed for My Cells?



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Ireland	1 800 654 253 (toll free)
Italy	800 789 888 (toll free)
Japan	+81 3 6264 0660
Luxemburg	+32 87 321 611
Poland	+48 781 120 300
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