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Spring 2014

The Newsletter for Life Science Researchers

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Efficient Transfection of Cancer Cell Lines Using the 4D-Nucleofector™ System

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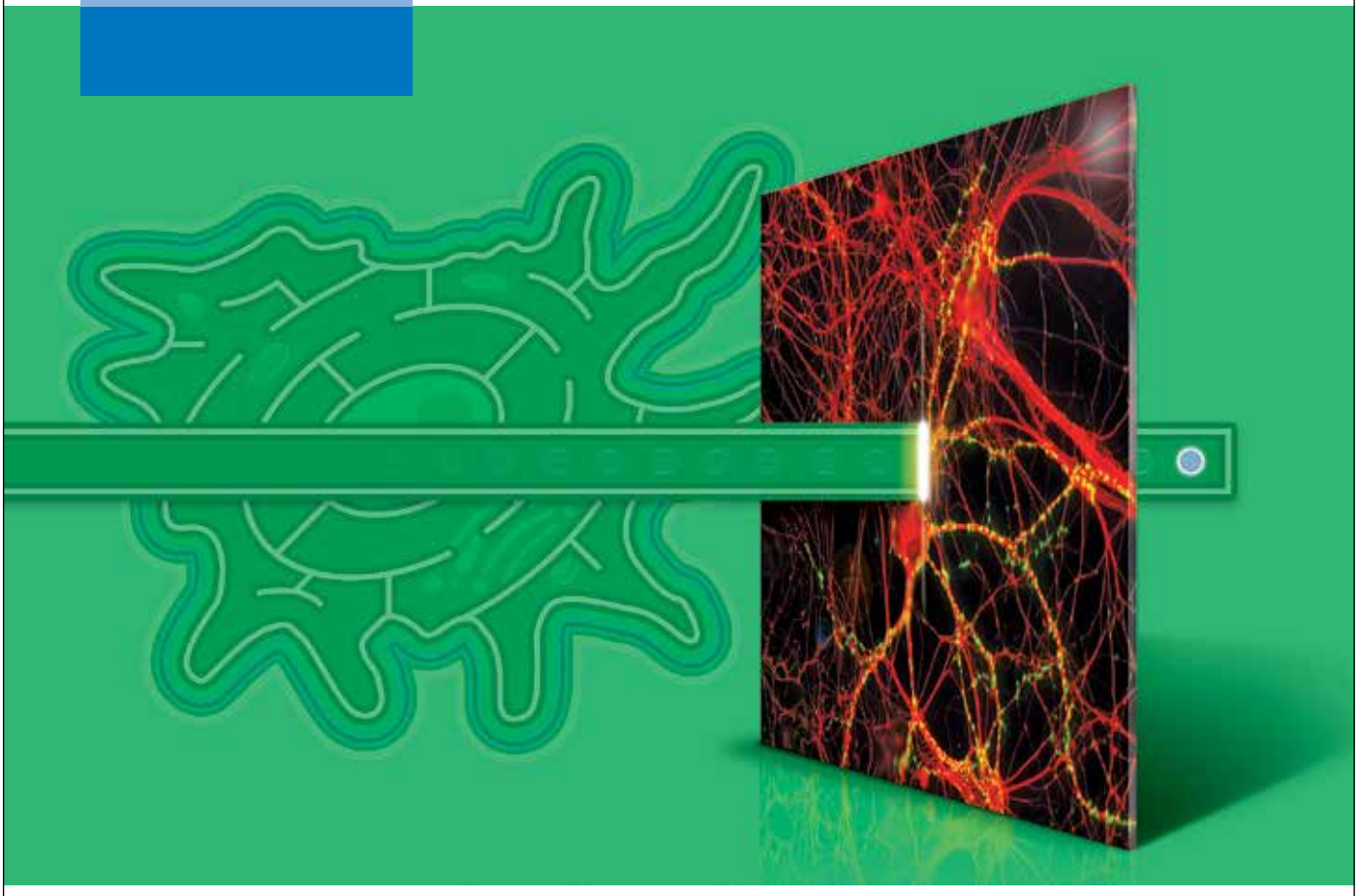

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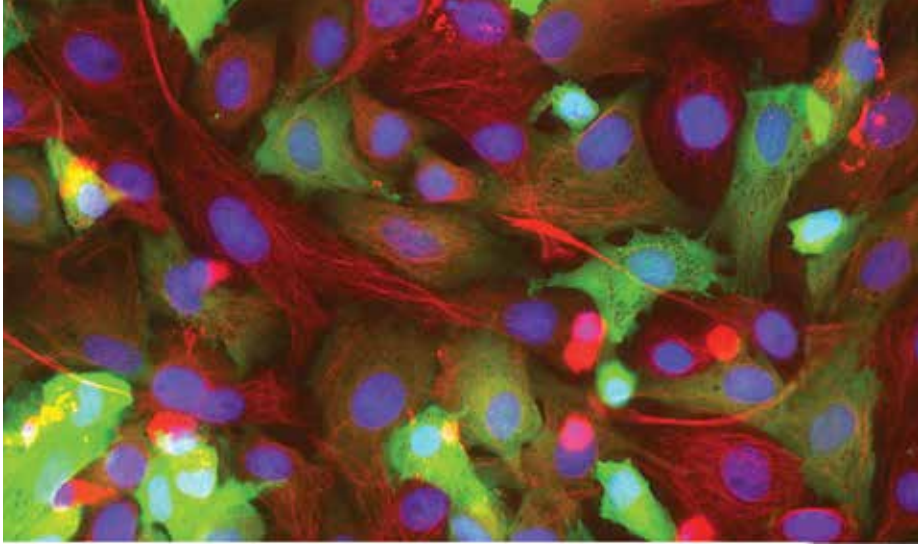

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Cancer Research Tools for Biological Relevance

As the battle against cancer remains one of the major endeavors in clinical research, we have decided to dedicate our spring issue of Resource Notes™ to Cancer Research.

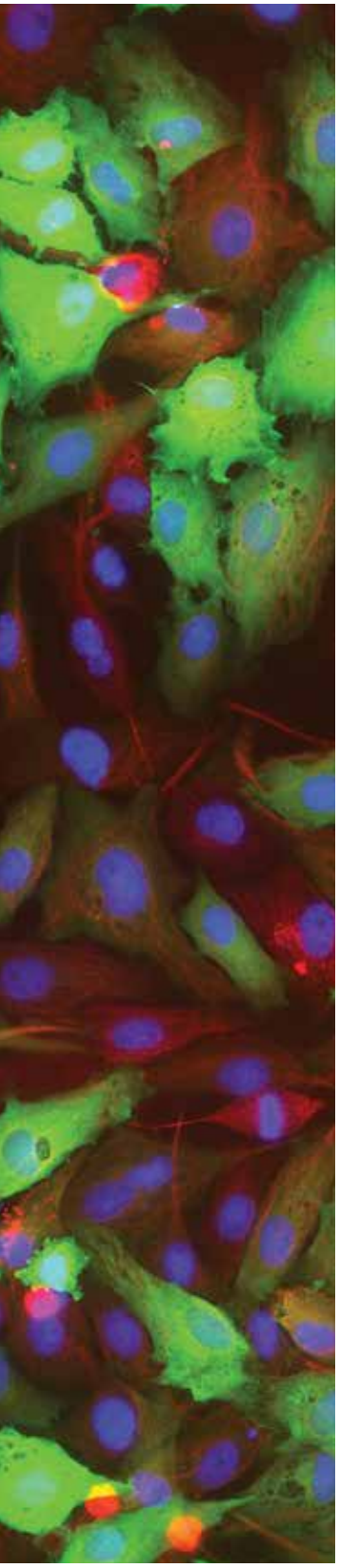
With products that are geared to deliver biologically relevant results, our focus is to support you in your fight against cancer. The Nucleofector™ Technology is ideally suited to help you understand the formation of cancers. With its reliable performance and easy optimization scheme, it enables you to transfect virtually any cancer cell line (for details, see pages 13–15).

If you want to study the transformation of cancers in a more biologically relevant system, with Lonza's optimized primary cell solutions you can overexpress your cancer genes in more than 100 different primary cell types that we offer. Our optimized Nucleofection™ Protocols and guaranteed-to-perform primary cells and cell culture systems ensure that you spend your time on your experiments and not on lab preparation. Primary cells can also help accelerate your study of biomarkers since only one or two primary cell types, vs. three to ten cell lines, are needed to build a complete biomarker profile. Or you may also consider doing your anticancer drug screening directly in primary cells to achieve a higher preclinical relevance.

Just browse through this issue to learn about new ways of obtaining quicker, clinically relevant results. You may also consider subscribing to our new e-newsletters for cell culture and transfection. These are released bi-monthly and feature product application highlights, selected citations, the latest laboratory tutorials and tech tips. Subscribe today at **www.lonza.com/enews-transfection** or **www.lonza.com/enews-cellculture** and be sure to participate in our photo contest to win prizes!

We hope you enjoy this issue and that it helps you move one step further in uncovering the remaining mysteries in cancer research.

Your Lonza Team



News

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The MycoAlert™ Assay is a selective biochemical test which provides reliable results within 20 minutes. This video shows how it works.

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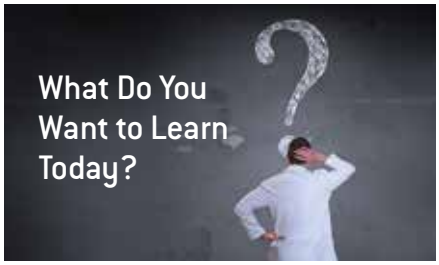

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26–30 April 2014	**Experimental Biology**	422	San Diego, CA, USA
16–24 May 2014	**American Thoracic Society (ATS)**	4217	San Diego, CA, USA
18–21 June 2014	**ISSCR**	1000	Vancouver, Canada

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Date	Webinar
01 April 2014	Clonetics™ Cells in Pancreatic Cancer Research
02 April 2014	Clonetics™ Cells in Pancreatic Cancer Research
13 May 2014	Efficient Transfection of Biologically Relevant Cells in Immunology Research
14 May 2014	Efficient Transfection of Biologically Relevant Cells in Immunology Research
24 June 2014	Convenient Generation and Culture of Induced Pluripotent Stem Cells
25 June 2014	Convenient Generation and Culture of Induced Pluripotent Stem Cells

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Advancing Bone Disease Research – Dissociated Rat Calvariae Osteoblasts Display Optimal Mineralization and Are Easily Transfected Using Nucleofection™

By Anthony Krantis[1], Karine Vaillancourt[2], Susan VandenHoek[2], Barbro Tinner-Staines[2] and Paul Schock[2]

[1]University of Ottawa, Centre for Research in Biopharmaceuticals and Biotechnology, Ottawa, Canada;

[2]QBM Cell Science Inc., Ottawa, Canada

Abstract

High-quality, batch-tested dissociated rat calvariae osteoblasts, cryopreserved and ready-to-use, offer a standardized primary cell test bed approach for evaluating proliferation-mineralization *in vitro*. Combined with the Nucleofector™ System, which is an effective and time-saving transfection tool, this provides a potent *in vitro* system for investigating bone biology.

Introduction

The predominant research interest in bone biology has been osteoporosis, which is increasing in prevalence. Osteoporosis-related fractures are more common than the combined incidences of cardiac arrest, stroke and breast cancer. In North America, more than 10 million people have this disease, and during their lifetime, approximately 30% of women and 20% of men will suffer an osteoporotic fracture[1].

Toward more effective treatments, researchers are seeking to develop strategies for slowing down bone remodeling and thereby restore bone strength and integrity. Much of the osteoporosis research has been undertaken using primary bone cell cultures. Employing dissociated osteoblasts offers advantages for studying cell growth control mechanisms and mineralization, since osteoblasts are also responsible for the regulation of the differentiation and activity of osteoclasts. A key issue for studies employing primary cultures is having the dissociated bone cells retain the specific function of bone forming[2].

The most extensively studied osteoblasts are those dissociated from calvariae and maintained in primary cultures[3,4,5]. These studies have now been made easier with the availability of batch-tested and hence "standardized" dissociated calvariae osteoblasts from Lonza, cryopreserved in their native state. This provides the researcher with the ideal tool for speeding the research workflow. The dissociated bone cells can be simply thawed and cultured and display excellent viability. We demonstrate herein the quality of the cryopreserved rat calvariae osteoblasts (developed by QBM Cell Science Inc.) in long-term culture, and show the flexibility in their application for evaluating osteogenesis-mineralization, and the sensitivity of the cells to transfection employing the 4D-Nucleofector™ System. Nucleofection™ allows plasmids to be transfected directly into the nucleus[6,7] with the advantage of better transfection rates, cell survival and quality of the cell culture post transfection, due to the specificity of the electroporation together with use of physiological buffers to optimize cell viability[8,9]. This non-viral transfection system can be used to transfect cells and assist gene delivery to prescribed populations of cells.

Materials and Methods

Cells: Vials of frozen cells (Lonza, cat. no. R-Ost-583) were thawed and prepared for cell culture studies and for transfection using the 4D-Nucleofector™ X Unit. Dissociated from the calvariae of Sprague Dawley rat embryos (E20,21), the osteoblasts are harvested and cryopreserved in the native state. For application in culture-based mineralization studies, the cells cannot be passaged since this affects the mineralization capacity of the cells. For proliferation studies, this is not a restrictive factor and the cells can be thawed and cultured through 10 population doublings. Viability of the cryopreserved calvariae osteoblasts is guaranteed based upon batch sampling for morphology and mineralization assay using Alizarin Red, a biochemical marker to determine the presence of calcific deposition by cells, and staining for Alkaline Phosphatase (ALP), a biochemical and histochemical marker for primary osteoblasts in proliferative phase.

Morphological assessment/imaging: Fluorescent micrographs were used to assess viability, transfection efficiency, and morphology of the osteoblasts. Expression of GFP-fluorescent protein was examined together with immunohistochemical identification of the cell cultures directly in the wells using the commercially available antibody for vimentin (abcam, cat. no. ab24525), α-actin (Millipore, cat. no. CBL171) and phalloidin (Sigma, cat. no. P1951). Cell nuclei were stained using Hoechst (Sigma, cat. no. H6024). Expression of the T lymphocyte differentiation antigen, Thy-1, was examined using anti-Thy-1 (Chemicon MAB 1406).

All immuno- and bright field staining was viewed directly in the wells using a Zeiss inverted microscope, except for Thy-1 labeling where calvariae osteoblasts were cultured on cover slips for evaluation using a Zeiss transmission fluorescence microscope.

Mineralization assays: Osteoblasts were prepared in 6-well plates and then cultured for up to 35 days. Rat osteoblast media for mineralization was prepared using 87 ml Dulbecco's modified Eagle Medium (DMEM) supplemented with 10 ml FBS (heat inactivated) and 1 ml penicillin/streptomycin. The medium was filtered and stored sterile. Prior to use, 50 µg/ml ascorbic acid (Sigma, cat. no. A-4034), 10 mM ß-glycerophosphate (Sigma, cat. no. G-6376) and 0.1 µM dexamethasone were added. The mineralization cultures were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After fixation, the cultures were washed with 10 nM PBS. The PBS was then removed and each well rinsed with 1–2 ml of Millipore H_2O. Cultures were then stained for ALP and for Alizarin Red S (Sigma, cat. no. A3757).

For Alizarin Red staining, H_2O was removed and enough Alizarin Red stain was added to completely cover the cell layer, and the cultures were incubated at room temperature for 30 minutes. Wells were washed with Millipore H_2O to remove most of the dye, followed by another 2–3 washes at 5–10-minute intervals. Cultures were stored in 10 mM PBS + azide.

For the ALP assay, the PBS was removed from the culture well and BCIP/NBT Liquid Substrate (Sigma, cat. no. B-1911) was added to completely cover the culture. The substrate stains cells blue-violet when ALP is present. The culture was incubated for 30 minutes at room temperature and then 10 mM PBS was added to the well to stop the reaction. The cultures were then washed with 10 mM PBS.

Transfection: The cells were thawed and immediately used for Nucleofection™. As the first step, optimal Nucleofection™ Conditions were determined in the 16-well 20 µl Nucleocuvette™ Strip following the recommendations of the Primary Cell Optimization Protocol using 4D-Nucleofector™ Solutions P1–P5 and 15 different programs. The optimal conditions were transferable to the 100 µl single Nucleocuvette™ Vessel. Per sample, 1 x 10^5 cells (in 20 µl strips) or 1 x 10^6 cells (in 100 µl cuvettes) were transfected with 2 µg/100 µl pmaxGFP™ Vector. After Nucleofection™, prewarmed media was added and 100 µl of cells from each cuvette were transferred to a microtube containing 800 µl of warm media. Cells were plated in 96-well plates (0.8 or 1.6 x 10^5/well), cultured 24 hours and then processed for morphological assessment.

Results and Discussion

Morphological Assessment of Osteoblast Cultures

Primary osteoblast cultures offer an excellent model system for the study of bone cell growth and differentiation related to a mineralizing matrix. Employing our optimized protocols, the cryopreserved dissociated calvariae osteoblasts were easy to culture and displayed normal morphology, growth and mineralization properties.

The time course for morphological differentiation of the cryopreserved calvariae osteoblasts, following plating, was characterized for up to 21 days in culture. These can be compared with freshly prepared and cultured calvariae osteoblasts (Figure 1). All of the osteoblasts were positive for vimentin, a Type III intermediate filament (IF) protein. Osteoblasts showed a distinct morphology that could be easily distinguished from osteoclasts (which were rare) based upon size, shape and a single nucleus versus multiple nuclei (typical of osteoclasts).

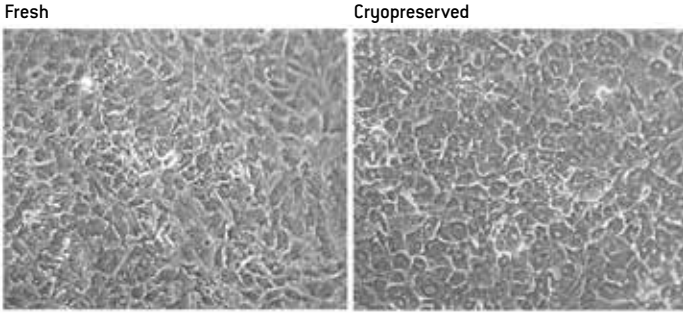

Figure 1
Comparison of cell morphology of freshly prepared versus cryopreserved calvariae osteoblasts in culture. The phase contrast images show osteoblasts dissociated and immediately cultured for 7 days in a 96-well plate (left). Cryopreserved osteoblasts from the same dissociation batch were cryopreserved, then thawed and cultured for 7 days in a 96-well plate (right).

The cytoskeleton filaments of osteoblasts are critical components for bone formation and normal signaling in response to mechanical stimulation. The actin filament cytoskeleton arrangement in the cultured cryopreserved calvariae osteoblasts can be seen in Figure 2. The calvariae osteoblast cultures displayed distinct populations of cells: those with predominant bundles of α-actin filament traversing the cytoplasm, or cells with a meshwork of thinner F-actin (phalloidin-positive) filaments. This is consistent with osteo-differentiation and maturation where there is remodeling of the actin cytoskeleton.

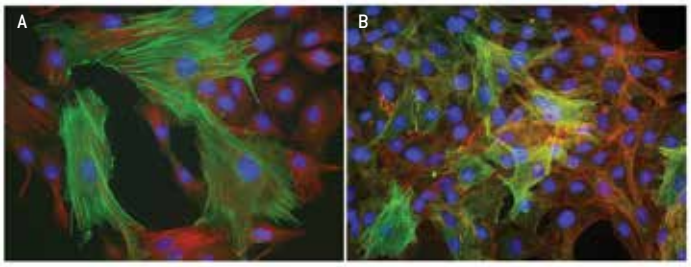

Figure 2
Fluorescence micrographs of cytoskeleton filaments. Cryopreserved calvariae osteoblasts thawed and cultured for 3 days were immunostained for (A) α-actin (green) and vimentin (red) or (B) α-actin (green) and phalloidin (red). Nuclei (blue) were stained with Hoechst.

Osteoblasts express Thy-1 antigen, and this expression has been proposed to be a useful differentiation marker for osteogenesis[10]. The cultures of cryopreserved calvariae osteoblasts displayed extensive Thy-1 immunoreactivity from day 3 in culture (Figure 3). Almost all of the cells displayed some typical punctate Thy-1 labeling with many cells showing intense levels.

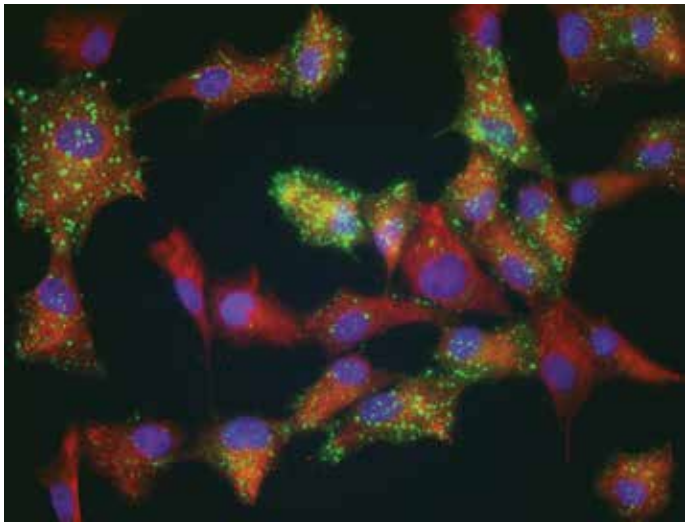

Figure 3
Fluorescence micrographs of immunohistological staining for Thy-1 antigen. Cryopreserved calvariae osteoblasts thawed and cultured for 3 days showed extensive staining for Thy-1 (green) which can be easily seen against vimentin (red) immunostaining. Blue = Hoechst-stained nuclei.

Furthermore, ALP (an enzyme that has a role in the mineralization of bone) activity is evident as early as day 3 in our calvariae osteoblast cultures (Figure 4). Together, these are hallmarks of osteogenesis and the ability of these cells to mineralize is significant at 21 days in culture. By day 35 there is profound mineralization and ALP activity (Figure 5).

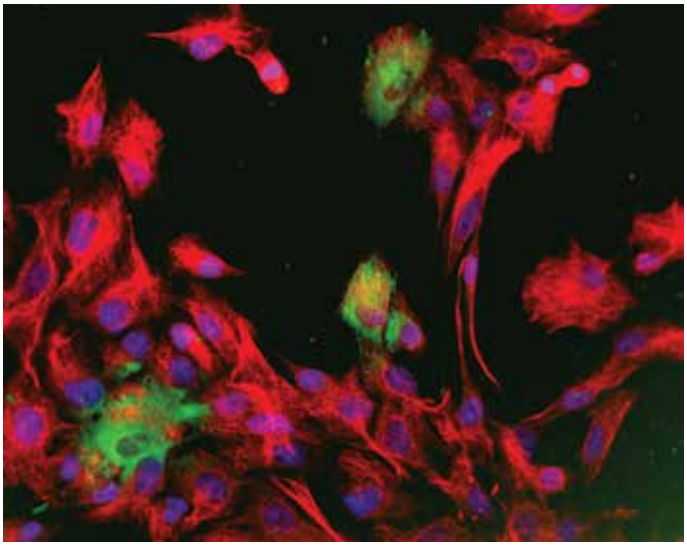

Figure 4
ALP localization in cryopreserved calvariae osteoblasts. Cells were thawed and cultured for 3 days. As shown by fluorescence microscopy, ALP activity (green) is evident in some osteoblasts easily identified in the culture stained with anti-vimentin.

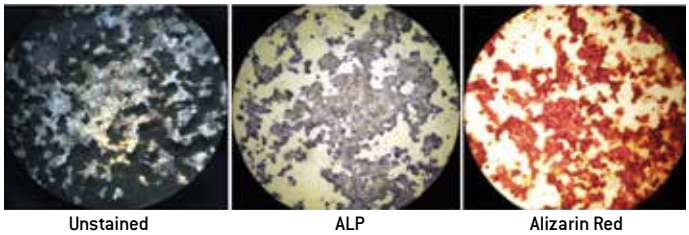

Figure 5
Mineralization of cryopreserved rat calvariae osteoblasts. Mineralization was examined using Alizarin Red staining together with staining for ALP activity. Both were profound in cryopreserved calvariae osteoblasts thawed and cultured for 35 days.

Nucleofection™ of Rat Osteoblasts

Our results showed that good quality transfection of the cryopreserved dissociated osteoblasts could be obtained in suspension using the 4D-Nucleofector™ X Unit. Following the recommended optimization protocol for primary cells in the 20 µl Nucleocuvette™ Strips, we identified all of the 15 recommended programs to be effective for transfecting the osteoblasts. However, programs DS-150 and DS-137, employed together with P1 Primary Cell Solution, gave the optimal results. The results of the cuvette-based transfection under these conditions are shown in Figures 6 and 7. Following Nucleofection™ and plating, and culture in the 96-well format, the cells displayed typical osteoblast morphology. This was consistent across different plating densities (Figure 7). Based upon observer cell counts, Nucleofection™ using the 4D-Nucleofector™ X Unit gave a transfection rate of >50%.

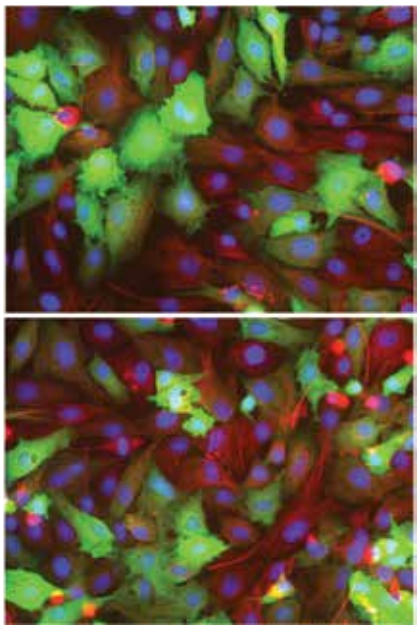

Figure 6
Transfection of cryopreserved calvariae osteoblasts using the 4D-Nucleofector™ X Unit. Cells were thawed and directly subjected to Nucleofection™ (1 x 10^6 cells/100 µl cuvette) using P1 Primary Cell Solution and program DS-150 (top) or DS-137 (bottom). Post Nucleofection™, cells were plated in 96-well plates with a plating density of 1.6 x 10^5 cells/well and fixed after 24 hours for fluorescence microscopy analysis. The cells were easily and efficiently transfected (green = maxGFP™ Protein) and show comparable morphology to the non-transfected cells (not shown here) which can be appreciated through immunostaining for vimentin (red).

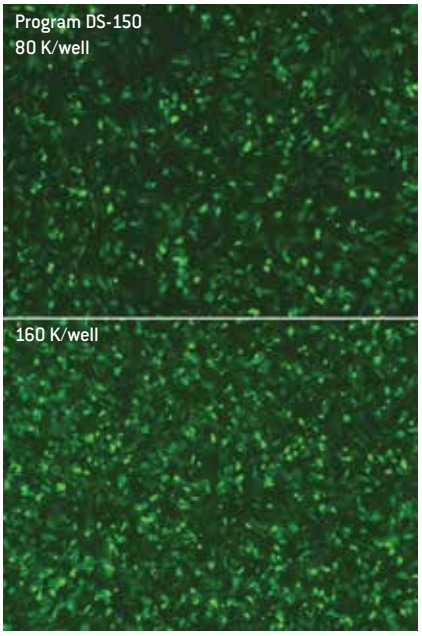

Figure 7
High quality transfection of rat calvariae osteoblasts using the 4D-Nucleofector™ X Unit. Cells were transfected using P1 Primary Cell Solution and program DS-150. Post Nucleofection™, cells were plated at a density of 0.8 x 10^5 cells/well (top) or 1.6 x 10^5 cells/well (bottom) in 96-well plates. Expression of maxGFP™ Protein was analyzed after 24 hours and showed high quality transfection across cultures.

Conclusion

Together, these data show the quality and sensitivity of cryopreserved dissociated rat calvariae osteoblasts for *in vitro* studies. Furthermore, application of the 4D-Nucleofector™ System for efficient transfection of dissociated osteoblasts represents a significant advantage for investigating bone biology.

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Tube Formation Assay with Primary Human Umbilical Vein Endothelial Cells

By Renu Kapoor, Vijay Reddy and Preeti Kapoor, Lonza India PVT Ltd., Hyderabad, India

Abstract

The tube formation assay is a simple *in vitro* angiogenesis assay which is routinely used to screen the pro-angiogenic or anti-angiogenic potential of substances and compounds. It is, thus, of great utility in diseases like cancer where inhibition of angiogenesis is one of the key research areas to combat the disease. It is also applicable in limb ischemia and cardiovascular disease research where ischemia is a key component and induction of angiogenesis could be one of the means that could be tried to alleviate these diseases.

In the current study, we have optimized this assay using Lonza's Clonetics™ Primary Human Umbilical Vein Endothelial Cells (HUVECs) and EGM™-2 Media, and Corning's Phenol Red Free Matrigel® Matrix. Cell numbers, reagent volumes and Suramin concentrations have been optimized using two different HUVEC lots. Complete tube formation is observed within 16 hours of cell seeding and tubes can be easily visualized after staining with Calcein AM. This assay is compatible with both 48- and 96-well plate formats and is highly reproducible using our cells with EGM™-2 Media.

Introduction

Angiogenesis is a multi-step process involving the generation of new blood vessels from pre-existing vasculature and is mediated primarily by endothelial cells. It plays a critical role not only in normal tissue repair and wound healing, but also in tumor development and cancer metastasis. Inhibition of angiogenesis is thus a key target in a number of cancers including breast, prostate, ovary, lung, colon, rectum, and brain (glioma)[1–6]. In comparison, induction of angiogenesis is required in diseases such as arteriosclerosis, myocardial infarction, limb ischemia, tissue ischemia, etc.[7,8].

Angiogenesis involves multiple steps: basement membrane disruption, endothelial cell migration, invasion, proliferation and differentiation into capillaries. One of the key steps of this process is the assembly of endothelial cells into tubes – this is known as tube formation. Tube formation can be modeled *in vitro* by plating endothelial cells onto or within extracellular matrix components like Matrigel® (isolated from Engelbreth-Holm-Swarm mouse sarcoma cells) or Type I collagen[9]. The most widely used tube formation assay involves plating HUVECs onto Matrigel® and examining the branching structures within 12–24 hours of cell plating[9,10].

Traditionally, HUVECs have been used extensively in this assay. However, the tube formation assay can also be performed with various types of endothelial cells, with endothelial progenitor cells and with transformed or immortalized endothelial cells.

While there are other assays used to measure angiogenesis *in vitro* and *in vivo*[10], the tube formation assay is simple, rapid, relatively inexpensive and accurate. Thus, it is commonly used to screen drugs or compounds for anti- or pro-angiogenic activity *in vitro*. It is therefore useful in discovering new compounds to counter diseases such as cancer or vascular ischemia, to define mechanisms and pathways involved in angiogenesis, and to identify endothelial-like cell populations or cells capable of inducing angiogenesis. Due to the high baseline levels of morphogenesis with this assay, it is more suitable for screening anti-angiogenic agents; though pro-angiogenic effects can also be measured. The effect of anti-angiogenic agents on tube formation is generally measured at later time points (12–16 hours) while the effect of pro-angiogenic factors is generally measured at earlier time points (4–8 hours).

One key advantage of using this assay is that it has various phases like cell migration and alignment, followed by the development of capillary tubes, sprouting of new capillaries, and finally the formation of the cellular networks that take place in a timed manner. Thus, varying the time point of measurement may give some information regarding the mechanism of action of an anti-angiogenic agent and the steps in the pathway it affects.

A number of inhibitors have been referenced in the literature as positive inhibitor controls for this assay. One of these inhibitors is Suramin. Suramin is a specific and competitive inhibitor of G-protein-coupled receptor (GPCR) activity and impacts multiple outputs of tubule formation, i.e. number of junctions, number of tubules and the total tubule length[11]. We have used this compound as a positive inhibitor control for the current set of studies.

Once tube formation is complete, it can be observed using an inverted microscope either in bright field, or after staining with a live cell staining dye like Calcein AM. Staining with Calcein AM enables better visualization of the tubules. Image acquisition can then be performed either manually or using an automated software.

Assessment of tube formation is basically qualitative, but some quantitation may be possible by using any one of several commercially available software systems. These systems measure different parameters such as tubule characteristics (number of tubules, number and mean

number of junctions, tubule area (%), total, mean and standard deviation of tubule length and number of independent tubules) and/or net characteristics (number of loops, mean perimeter loop and number of nets)[11]. In the current study, we have performed qualitative assessment of tube formation.

There are many references in the literature using the tube formation assay as a method for screening anti-angiogenic agents. These methods vary with regard to the extracellular matrix used, the cell type seeded, the media used for the assay, etc. In the current study, we optimized this assay using primary HUVECs and EGM™-2 Media from Lonza to provide a well standardized, optimized and reproducible procedure for researchers to use in the screening of anti-angiogenic agents.

Materials

Cells

HUVECs (Lonza, cat. no. C2517A) of two different cell lots (lot 8F3178 and lot 0000094182) were used in this study. Cells were thawed and expanded in EGM™-2 Growth Medium (Lonza, cat. no. CC-3162) as per instructions given in the tech sheet and were allowed to undergo at least one passage after thawing prior to use in the experiments. Cells were always used within passage 5 for best results in this assay. For donor details see Table 1.

Lot No.	Age	Sex	Race	Seeding Efficiency	Cell Viability	Doubling Time	Cell Passage	Total Populations
0000094182	Newborn	Male	H	71	76	21	1	17
8F3178	Newborn	Female	B	59	87	17	1	19

Table 1
Details of the donors.

Reagents

EGM™-2 Endothelial Growth Medium-2 BulletKit™ Kit (cat. no. CC-3162), HBSS (cat. no. BE10-547F), Trypsin/EDTA (cat. no. BE17-161E) and Trypsin neutralizing solution (TNS, cat. no. CC-5002) were from Lonza. BSA (cat. no. A-3294), Suramin (cat. no. S-2671) and DMSO were procured from Sigma. Matrigel® Basement Membrane Matrix Phenol Red Free was purchased from Corning (cat. no. 356237). Calcein AM (cat. no. C3100MP) and Pluronic acid (cat. no. P3000MP) were from Life Technologies. Tissue culture plasticware and serological pipettes were purchased from Corning.

Methods

Reagent Preparation

EGM™-2 Endothelial Growth Medium-2 BulletKit™ Kit: The complete media was reconstituted by adding the SingleQuots™ (hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamicin/amphotericin-B) to the basal media. The complete media was kept protected from light, stored at 4°C and used within one month of reconstitution.

BSA (20 mg/ml): The stock solution was prepared by dissolving 0.5 gm BSA in 25 ml HBSS. It was stored at 4°C and used within 15 days of preparation.

Calcein AM stock (1 mg/ml): The stock solution was prepared by adding 50 µl DMSO to the vial containing the 50 µg solid. It was stored in aliquots at -20°C and kept protected from light.

Calcein AM staining dye: The following components were mixed to make the dye: 20 µl Calcein AM stock (1 mg/ml stock), 20 µl Pluronic acid and 2 ml BSA stock solution (20 mg/ml stock). The Calcein AM staining dye was prepared just before use and kept protected from light. The Calcein AM dye was diluted in HBSS and not in media since culture medium results in the auto-hydrolysis of the label giving high fluorescence background.

Suramin (300 µM stock): The stock solution was prepared by dissolving 0.42 gms of Suramin (Mol. wt. - 1429.17) in 1 ml of HBSS. It was prepared just before use and kept protected from light.

Thawing the Matrigel®

Matrigel® with a minimum protein concentration of 10 mg/ml is required to obtain optimal results in this assay[12, 13]. Thus, we used Corning Matrigel® for the current assay. The Phenol Red Free variant of Matrigel® was used in our assays. The Phenol Red Free variant was selected since phenol red has estrogenic activity.

The Matrigel® was thawed by placing it overnight on ice and keeping the ice bucket in the refrigerator at 4°C, since Matrigel® may gel at the slightly elevated temperatures in a refrigerator. Immediately after thawing, the Matrigel® was aliquoted out into smaller volumes and stored at -20°C. Since Matrigel® gels rapidly at 22°C to 35°C, pre-cooled pipettes were used and Matrigel® was kept on ice during the handling process.

Coating Culture Plates with Matrigel®

The culture plates and tips were pre-cooled by placing in the refrigerator overnight and also kept on ice during the coating process. The required number of wells was coated with Matrigel® using pre-cooled pipette tips; 150 µl per well was used for coating 48-well culture plates and 75 µl per well was used for coating 96-well culture plates. While coating the wells with Matrigel®, care was taken to avoid introducing bubbles in the well. However, if air bubbles did get trapped in the wells, the plate was centrifuged at 300 g for 10 minutes in a pre-cooled centrifuge at 4°C. Plates were coated just before the experiment. The plate was left undisturbed in the biosafety cabinet for 10 minutes at room temperature. After 10 minutes, the Matrigel®-coated plate was gently shifted to a CO_2 incubator at 37°C. It was left in the incubator for 30 minutes to allow for gelation of the Matrigel®. During this incubation process, the cell cultures were trypsinized and made ready for the assay.

Cell Growth and Trypsinization

Cells were grown to 70–80% confluence in complete EGM™-2 Media. Once they reached confluence, they were detached from the cell surface by washing once with HBSS, trypsinizing and resuspending in TNS as per the recommended protocol. TNS was used to stop the action of Trypsin-EDTA since EGM™-2 Media has a low concentration of FBS (2%). Cells were spun down by centrifugation at 1,200 rpm for 10 minutes at room temperature. The supernatant was discarded and cells were resuspended in complete EGM™-2 Media. The cell viability was determined using Trypan blue. Cells were used for the assay only if the cell viability was greater than 80%. Cells were used in early passages (up to passage 4) and they were passaged at least once after being removed from liquid nitrogen before being used for this assay.

Experimental Set Up

Cells were diluted in complete EGM™-2 Media at a concentration of 0.4×10^6 cells/ml, i.e., 100,000 cells in 250 µl complete media. Requisite dilutions of the above cell suspension were then made in Eppendorf tubes. Angiogenesis inhibitors were also added to the Eppendorf tubes, along with the cells, such that 250 µl of final cell suspension contained the required number of cells for the assay and the requisite concentrations of the angiogenesis inhibitor. The above cell suspension was then added on to the Matrigel®-coated wells of a 48-well (250 µl per well) or a 96-well (125 µl per well) plate. Suramin was added in parallel as a positive inhibitor control in each assay.

In the initial set of experiments, a cell-seeding optimization experiment was performed where a range of cells from 25,000–75,000 cells per well were added in a 48-well format and 12,500–37,500 cells per well were added in a 96-well plate format.

Once the cell number was optimized (50,000 cells per well in a 48-well plate format or 25,000 cells in a 96-well plate format) for optimal tube formation (Figures 1 and 2), the optimized cell number was used for the subsequent Suramin experiments.

Staining with Calcein AM

Once the incubation was over, the wells were washed gently with HBSS, taking care not to disrupt the tubules. Calcein AM staining dye was added to the wells (250 µl per well in a 48-well plate or 125 µl per well in a 96-well plate) and the cells were incubated in the dark for 30 minutes at 37°C in a CO_2 incubator. The extra dye was removed carefully by giving two washes with HBSS, taking care not to disturb the tubules. The tubules were observed under an epifluorescence microscope using a standard FITC filter.

Results and Discussion

The current assay has been standardized and optimized using Lonza's HUVEC primary cells, EGM™-2 Media and Corning's Phenol Red Free Matrigel® Matrix (extracellular matrix gel prepared from Engelbreth-Holm-Swarm tumor cells). The assay results have been shown for only one cell lot (lot no. 8F3178) since both lots gave similar results.

It is known, based on the literature, that tube formation starts within 4 hours after plating the cells, complete tube formation takes about 12–16 hours, tubules start getting disrupted after 16–18 hours and the cells begin to undergo apoptosis after 24 hours using a different extracellular matrix. We repeated this assay using our HUVECs and EGM™-2 Media at some of the above time points and found that tubules start getting disrupted after 16 hours of cell plating, and they appear to detach from the cell surface at 24 hours after cell plating (data not shown). As a result, we chose 16 hours as the ideal time point for the assessment of tube formation.

Figures 1 and 2 depict bright field and fluorescence images of HUVECs (lot no. 8F3178) plated at varying cell densities of Matrigel® in EGM™-2 Media and their tube formation at 16 hours after cell plating in a 48-well plate and a 96-well plate format respectively. These cell optimization experiments demonstrated that the number of cells plated is critical: too few cells yield incomplete tubes (i.e., 25,000 cells per well using a 48-well plate format), while too many yield large areas of cell clusters or monolayers (i.e., 75,000 cells per well using a 48-well plate format). The optimum cell number for this assay was found to be 50,000 cells per well for a 48-well plate format and 25,000 cells per well for a 96-well plate format. Similar results were obtained with lot no. 0000094182 (data not shown), demonstrating the reproducibility of the assay across cell lots and across 48- and 96-well plate formats.

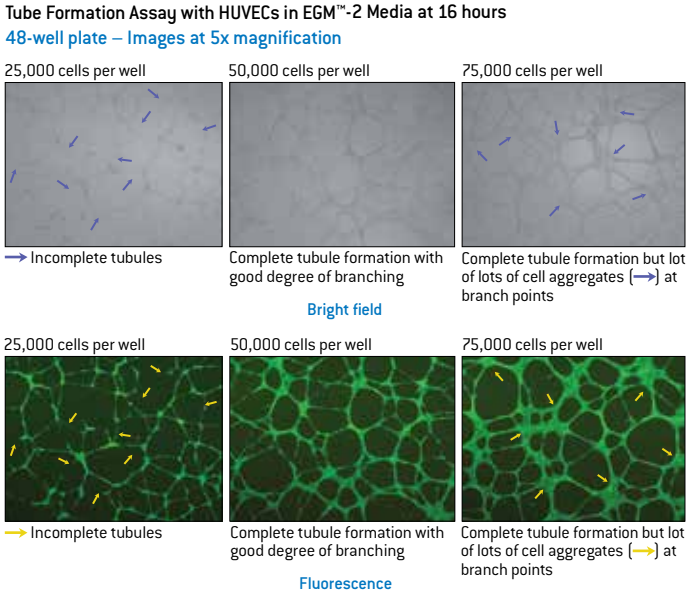

Figure 1. Figure 1 depicts cell number optimization for the tube formation assay using HUVECs (lot no. 8F3178) seeded on Matrigel® in EGM™-2 Media at a 16-hour assay point. This assay was conducted in a 48-well plate format. Both bright field and fluorescence images have been depicted here and indicate that 50,000 cells per well is the optimal cell seeding concentration for this plate format.

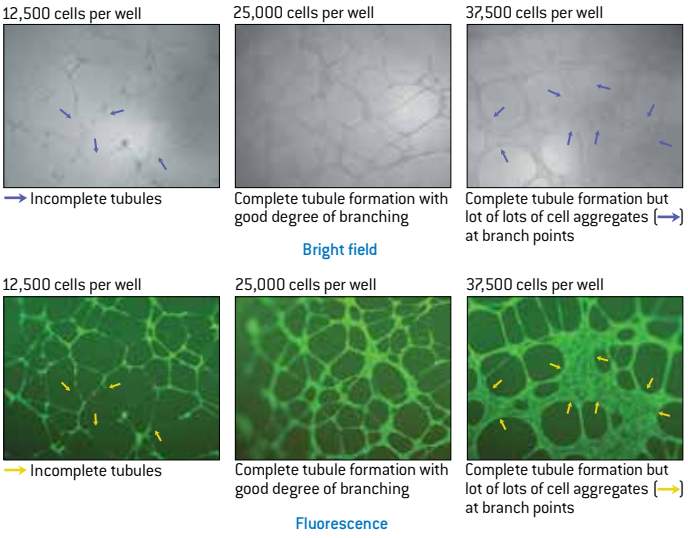

Figure 2. Figure 2 depicts cell number optimization for the tube formation assay using HUVECs (lot no. 8F3178) seeded on Matrigel® in EGM™-2 Media at a 16-hour assay point. This assay was conducted in a 96-well plate format. Both bright field and fluorescence images have been depicted here and indicate that 25,000 cells per well is the optimal cell seeding concentration for this plate format.

Suramin, when used at a concentration of 7.5–60 µM, was found to inhibit the tubule branches in lot no. 8F3178 cells in a dose-dependent manner (Figure 3), in concurrence with the literature. Suramin effect was tested again in lot no. 0000094182 cells at 15 µM and 30 µM concentrations (data not shown), and it appeared that Suramin effect was lot-specific and that the degree of inhibition of tube formation across tested lots appeared to vary slightly.

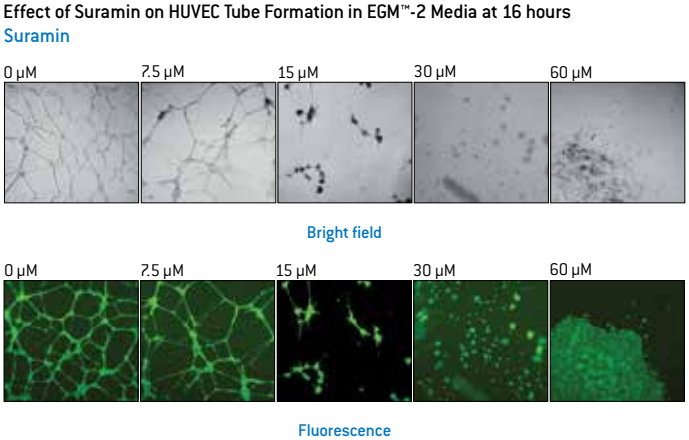

Figure 3. Figure 3 depicts the effect of the inhibitor Suramin on tube formation assay of HUVECs (lot no. 8F3178) seeded on Matrigel® in EGM™-2 Media at a 16-hour assay point. This assay was conducted in both 48- and 96-well plate formats and both gave similar results. Only the 48-well plate data has been presented here. Both bright field and fluorescence images are depicted here and they indicate that Suramin, at a concentration of 15–30 µM, can be ably used to demonstrate the inhibitory effect of Suramin on the tube formation assay.

Conclusion

In conclusion, we found that the current assay using Lonza's HUVEC primary cells and EGM™-2 Media offers a well standardized, optimized and reproducible platform for researchers wishing to use the tube formation assay for screening anti-angiogenic agents using either 48-well or 96-well formats.

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Efficient Transfection of Cancer Cell Lines Using the 4D-Nucleofector™ System

By Jenny Schroeder[1], Ludger Altrogge[1], Elke Lorbach[1], Srinivasan Kokatam[2], Sabine Schaepermeier[1], Meike Weigel[1], Gina Andretta-Beu[1], Stefanie Buesch[1], Tamara Grabeck[1], Alexandra Krumnow[1], Sonja Spicker[1], Sampada Kallol[2], Preeti Kapoor[2] and Andrea Toell[1]

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Introduction

Cell lines isolated from tumors are an important tool for studying cancer *in vitro*. They can be used for drug development as well as for understanding the basic mechanisms underlying cancer. Transfection of cancer cell lines with different molecules such as plasmid DNA, siRNA or mRNA is often an integral part of this kind of research.

Lonza's 4D-Nucleofector™ System (Figure 1A) is a modular system for the efficient transfection of primary cells and cell lines with a variety of substrates including plasmid DNA and siRNA. The 4D-Nucleofector™ X Unit supports Nucleofection™ in two different formats. The aluminum-free 20 µl Nucleocuvette™ Strip (Figure 1B) allows the transfection of low cell numbers down to 2 x 10^4 cells per reaction. As 16 reactions can be performed in parallel, it is well suited for optimizing Nucleofection™ Conditions for cells lacking a ready-to-use Optimized Protocol. For higher cell numbers of up to 2 x 10^7 cells per reaction, the same Nucleofection™ Conditions can be applied in the 100 µl single Nucleocuvette™ Vessel (Figure 1C). For higher throughput needs, the 96-well Shuttle™ Add-on can be connected to the 4D-Nucleofector™ System (Figure 1A). With this add-on, six 20 µl Nucleocuvette™ Strips can be processed in parallel, allowing for screening applications or accelerating the optimization of transfection parameters for many cell types.

Figure 1
4D-Nucleofector™ System. (A) 4D-Nucleofector™ Core- and X Unit with 96-well Shuttle™ Add-on. (B) 20 µl Nucleocuvette™ Strip. (C) 100 µl single Nucleocuvette™.

In this study, we used the 4D-Nucleofector™ System in combination with the 96-well Shuttle™ Add-on for optimizing transfection conditions for more than 30 cancer cell lines. An exemplary optimization process is depicted for the human prostate carcinoma cell line DU 145 and the human colorectal adenocarcinoma cell line COLO 205.

Materials and Methods

Initial Optimization of Nucleofection™ Conditions

All cell lines were cultured according to standard protocols. Adherent cell lines were harvested using trypsin (Lonza, cat. no. 17-161E). Dependent on the cell type, 1–5 x 10^5 cells per sample were resuspended in 20 µl of the respective Nucleofector™ Solution SE, SF or SG containing 0.4 µg of pmaxGFP™ Vector. Samples were transferred into a 96-well Nucleocuvette™ Plate and processed in parallel with 31 different programs and a no program control in the 96-well Shuttle™ Add-on. After transfection, 80 µl of equilibrated medium was added to each sample. Dependent on cell type, 2.5–10 x 10^4 cells were seeded into a standard 96-well cell culture plate.

Further Fine-tuning of Nucleofection™ Conditions (Optional)

A) 10 minutes post-incubation: 2 x 10^5 cells per sample were resuspended in 20 µl of the selected Nucleofector™ Solution containing 0.4 µg pmaxGFP™ Vector and pulsed with the selected Nucleofector™ Program. Post Nucleofection™, samples were incubated for 10 minutes in Nucleofector™ Solution prior to adding 80 µl of equilibrated cell culture medium.

B) DNA Titration: 2 x 10^5 cells per sample were resuspended in 20 µl of the selected Nucleofector™ Solution containing 0.4 µg, 1 µg or 5 µg of pmaxGFP™ Vector. After transfection – if indicated by fine-tuning step A – samples were incubated for 10 minutes in Nucleofector™ Solution prior to adding 80 µl of equilibrated cell culture medium.

Transfer to 100 µl Single Nucleocuvette™ Vessels

Based on the cell number used in the 20 µl format, a 5 fold increased cell number per sample was resuspended in 100 µl of the respective Nucleofector™ Solution SE, SF or SG containing five times the amount of pmaxGFP™ Vector. Samples were transferred into the 100 µl single Nucleocuvette™ Vessel and processed with the Nucleofector™ Program identified in the 20 µl Nucleocuvette™ Strip. After transfection, 400 µl of equilibrated medium was added to each sample. Dependent on cell type, 2.5–10 x 10^4 cells were seeded into a standard 96-well cell culture plate.

Analysis

24 hours after transfection, the percentage of maxGFP™-positive, propidium-iodide negative cells was determined using flow cytometry (FACSCalibur™, Becton Dickinson) according to standard procedures. Cell viability was determined using the ViaLight™ Plus BioAssay Kit (Lonza, cat. no. LT07-321) according to the protocol. Cell viability is expressed as % viability compared to the non-transfected no program controls.

Results

Using the 4D-Nucleofector™ System in combination with the 96-well Shuttle™ Add-on, we found that the human prostate carcinoma cell line DU 145 can be easily transfected. Best results were obtained with Nucleofector™ Solution SE and Program CA-137, resulting in a transfection efficiency of 78% (Figure 2A) with a cell viability of 55% (Figure 2B). These results show that efficient Nucleofection™ Conditions for cancer cell lines can often be easily determined in a single experiment.

For the human colorectal adenocarcinoma cell line COLO 205, the initial optimization experiment achieved maximal transfection efficiencies of ~30% (Figure 3A) with program-dependent cell viabilities (Figure 3B). Thus, we performed two further program fine-tuning rounds and identified Nucleofector™ Program DP-113, in combination with Nucleofector™ Solution SG and a cell seeding density of 1 x 10^5 cells in 96-well, as the best selections under the given experimental conditions (data not shown). However, transfection efficiency still remained below 30% with a cell viability of 50%.

In order to improve transfection efficiency and viability, further optimization experiments were performed. Incubation of COLO 205 cells for 10 minutes in Nucleofector™ Solution post transfection increased transfection efficiency and cell viability (Figure 4A). An additional increase of transfection efficiency was observed when increasing the amount of plasmid DNA per reaction. Cell viability remained above 60% compared to the no program control with DNA amounts up to 1 µg per reaction. With a higher DNA concentration, a drop in cell viability was observed (Figure 4B).

One important feature of the 4D-Nucleofector™ X Unit is the transferability of conditions from the 20 µl Nucleocuvette™ Strips (for low cell numbers and higher throughput) to the 100 µl single Nucleocuvette™ Vessels (for higher cell numbers). For the latter, the same Nucleofection™ Conditions can be applied using a five times higher cell number and substrate amount. This is shown for 32 different human cancer cell lines in Figure 5.

Summary

Excellent transfection efficiencies of up to 99% combined with high cell viability can be obtained with the 4D-Nucleofector™ System for different adherent and suspension cancer cell lines. With the 96-well Shuttle™ Add-on, six 20 µl Nucleocuvette™ Strips can be processed in parallel enabling screening applications as well as offering a convenient, effective and time-saving approach when optimizing Nucleofection™ Conditions for multiple cells lacking a ready-to-use Optimized Protocol. In many cases, the optimal Nucleofection™ Conditions can already be determined during the first optimization round. The same protocol can be applied for 100 µl and 20 µl transfection volumes, allowing the transfection of variable cell numbers.

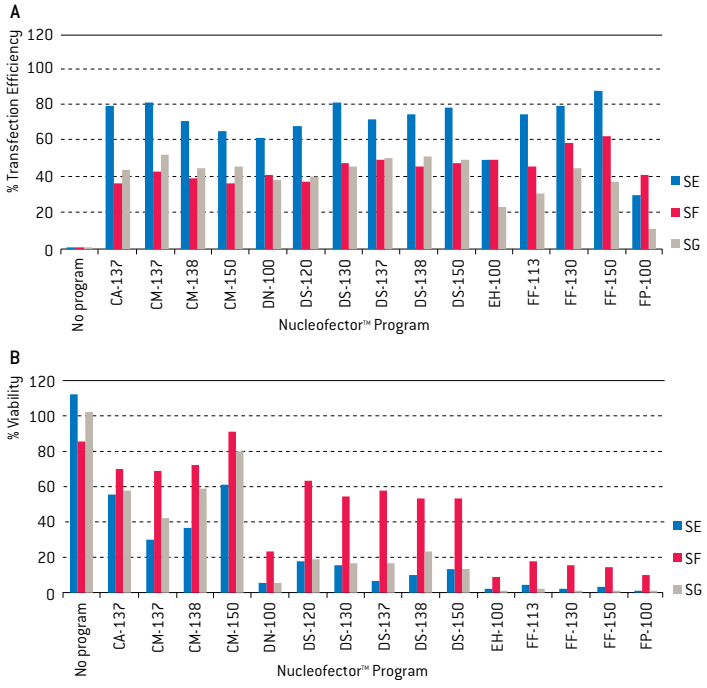

Figure 2
First optimization round for the human prostate carcinoma cell line DU 145 using the 4D-Nucleofector™ System in combination with the 96-well Shuttle™ Add-on. 2 x 10^5 cells were transfected with 0.4 µg pmaxGFP™ Vector using Nucleofector™ Solution SE, SF or SG and 31 different programs (15 of which are shown). 5 x 10^4 transfected cells were seeded and transfection efficiency was analyzed 24 hours post transfection by flow cytometry (A). Viability was determined with the ViaLight™ Plus BioAssay Kit (B). Cell viability is expressed as % viability compared to the non-transfected no program controls.

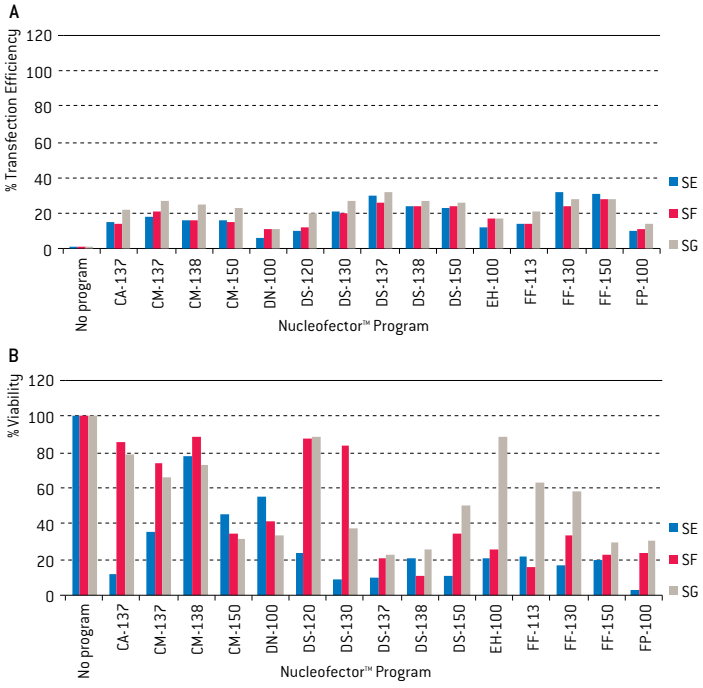

Figure 3
First optimization round for the human colorectal adenocarcinoma cell line COLO 205 using the 4D-Nucleofector™ in combination with the 96-well Shuttle™ Add-on. 2 x 10^5 cells were transfected with 0.4 µg pmaxGFP™ Vector using Nucleofector™ Solution SE, SF or SG and 31 different programs (15 of which are shown). 5 x 10^4 transfected cells were seeded and transfection efficiency was analyzed 24 hours post transfection by flow cytometry (A). Viability was determined with the ViaLight™ Plus BioAssay Kit (B). Cell viability is expressed as % viability compared to the non-transfected no program controls.

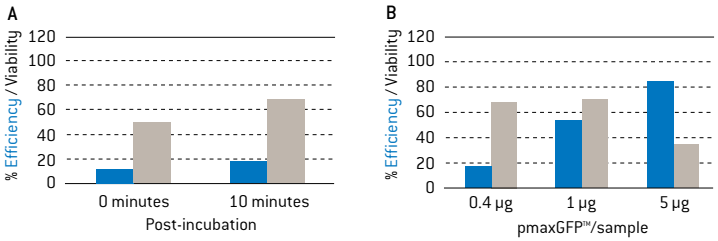

Figure 4

Further optimization of transfection conditions for the human colorectal adenocarcinoma cell line COLO 205. Cells were transfected using Nucleofector™ Solution SG and Nucleofector™ Program DP-113. (A) After transfection with 0.4 µg pmaxGFP™ Vector, 80 µl of equilibrated culture medium was added either directly to the samples (0 minutes) or after a 10-minute incubation in Nucleofector™ Solution. (B) Various amounts of pmaxGFP™ Vector (0.4 µg, 1 µg or 5 µg) were used per 20 µl sample for the transfection of COLO 205. Samples were incubated for 10 minutes in Nucleofector™ Solution prior to adding 80 µl of equilibrated cell culture medium.

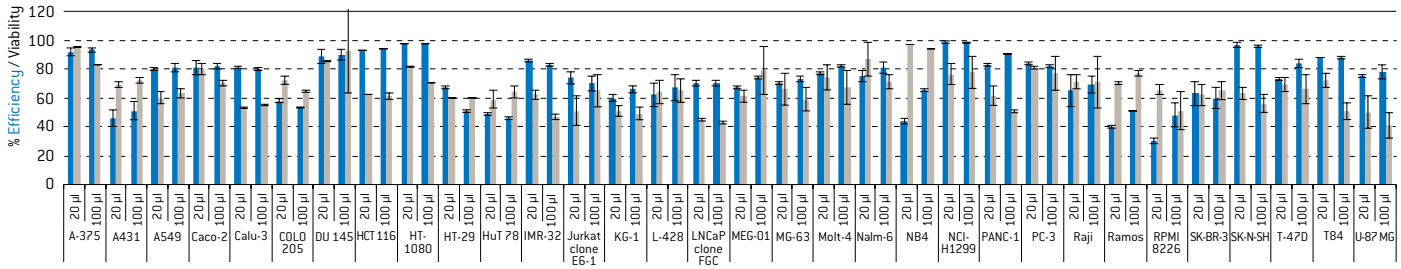

Figure 5

Transferability of Nucleofection™ Conditions from the 20 µl Nucleocuvette™ Strip to the 100 µl single Nucleocuvette™ Vessel for 32 cancer cell lines. Transfection parameters that have been optimized in the 20 µl Nucleocuvette™ Strip are directly applicable to the 100 µl single Nucleocuvette™ Format [n=3].

Spotlight on Lonza Products Used in Cancer Research

By Liz Horton, Lonza Walkersville, Inc., Walkersville, MD, USA

Scientific Support at Lonza likes to keep an eye on how customers are using our products in research. Here we shine the spotlight on several JoVE video articles highlighting the use of Lonza's primary cells or the Nucleofector™ Transfection Technology for studying cancer formation and potential cancer-related therapies. We focused on JoVE as it presents scientific research in a visual format, making it easier for researchers to learn and reproduce experiments.

A Real-time Electrical Impedance-based Technique to Measure Invasion of Endothelial Cell Monolayer by Cancer Cells

S Rahim and A Üren (2011), *J. Vis. Exp.* (50), e2792

The authors provide a detailed description for creating an *in vitro* invasion assay for studying metastasis. Creating an endothelial cell monolayer using Lonza's Primary Human Umbilical Vein Endothelial Cells (HUVECs) and EGM™ -2 Media, and the Roche xCELLigence instrument, the researchers were able to monitor invasion with real-time results. They say this procedure can be useful in studying inhibitors or stimulators of metastasis.

www.lonza.com/jove1

Genome-wide Screen for miRNA Targets Using the MISSION Target ID Library

MJ Coussens *et al.* (2012), *J. Vis. Exp.* (62), e3303

MicroRNAs (miRNAs) are being studied in relationship to malignancies and possible treatments (*Clinical Pharmacology & Therapeutics* (2013); 93 1, 98–104). In this JoVE article, Sigma describes their tool for finding miRNA targets which can be difficult as they are not always fully complementary to the miRNAs. Nucleofection™ is used for the stable transfection of a plasmid-based cDNA library into cells. Subsequently the library cells are transfected by Nucleofection™ with a plasmid expressing the miRNA of interest. Cells that express a cDNA, including the miRNA target, survive the selection scheme and the cDNA can be isolated and the target sequenced.

www.lonza.com/jove2

Clinical Application of Sleeping Beauty and Artificial Antigen-presenting Cells to Genetically Modify T Cells from Peripheral and Umbilical Cord Blood

MH Huls *et al.* (2013), *J. Vis. Exp.* (72), e50070

Here, Nucleofection™ is used to stably co-transfect human T cells using the Sleeping Beauty transposon and transposase technology to create therapy-scale numbers of artificial antigen-presenting cells that could be used in the fight against cancer.

www.lonza.com/jove3

Generation of Multivirus-specific T Cells to Prevent/Treat Viral Infections after Allogeneic Hematopoietic Stem Cell Transplant

U Gerdemann *et al.* (2011); *J. Vis. Exp.* (51), e2736

The authors present a method for activating human T cells to fight viruses which can be deadly to immune-compromised patients such as those undergoing stem cell transplantation. Nucleofection™ is used to transfect human dendritic cells which are subsequently used to activate cytotoxic T lymphocytes against multiple viruses. These cytotoxic T lymphocytes could then potentially be used to protect against or fight off viral infection in patients.

www.lonza.com/jove4

Heterokaryon Technique for Analysis of Cell Type-specific Localization
R Gammal *et al.* (2011), *J. Vis. Exp.* (49), e2488

In this article, a method is presented for the study of the difference of protein trafficking and shuttling activity between primary and transformed mammalian cells. The authors use Nucleofection™ to transfect and express a GFP-tagged Chicken Anemia Virus-VP3 protein into normal primary human fibroblasts. They then transfect and express the same protein with a different fluorescent label, RFP, into a transformed cell line. The localization of the protein differs in the normal cell versus the cancerous cell. By then making a cell fusion between a single normal cell and a cancerous cell, they are able to demonstrate the change in localization of the GFP-labeled protein to match that of the cancerous cell. They point out that the ability to study differences in localization and to understand these pathways can be important in the study of oncogenesis and the development of possible therapies.

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BioResearch

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- Compliance with Title 21 CFR part 11/annex 11
- User administration
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- Animal origin-free formulation
- Solutions produced under cGMP conditions
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- Validation of ethylene oxid sterilization

NOTE: Nucleofector™ Kits and Devices are for research use only and are not intended for use in humans!

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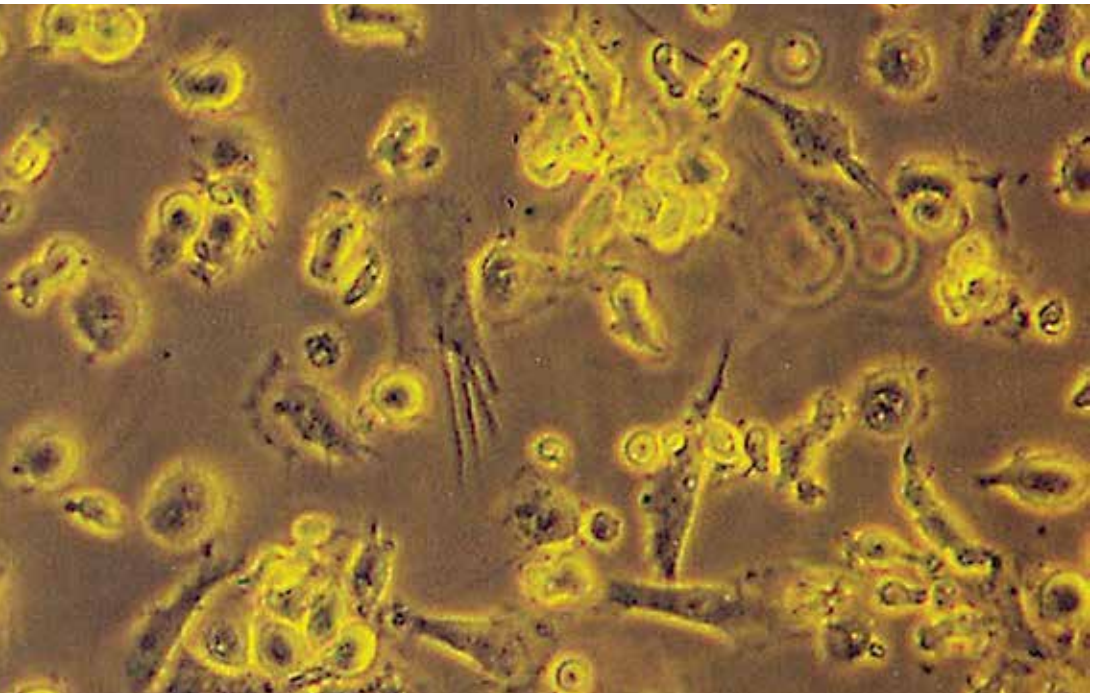

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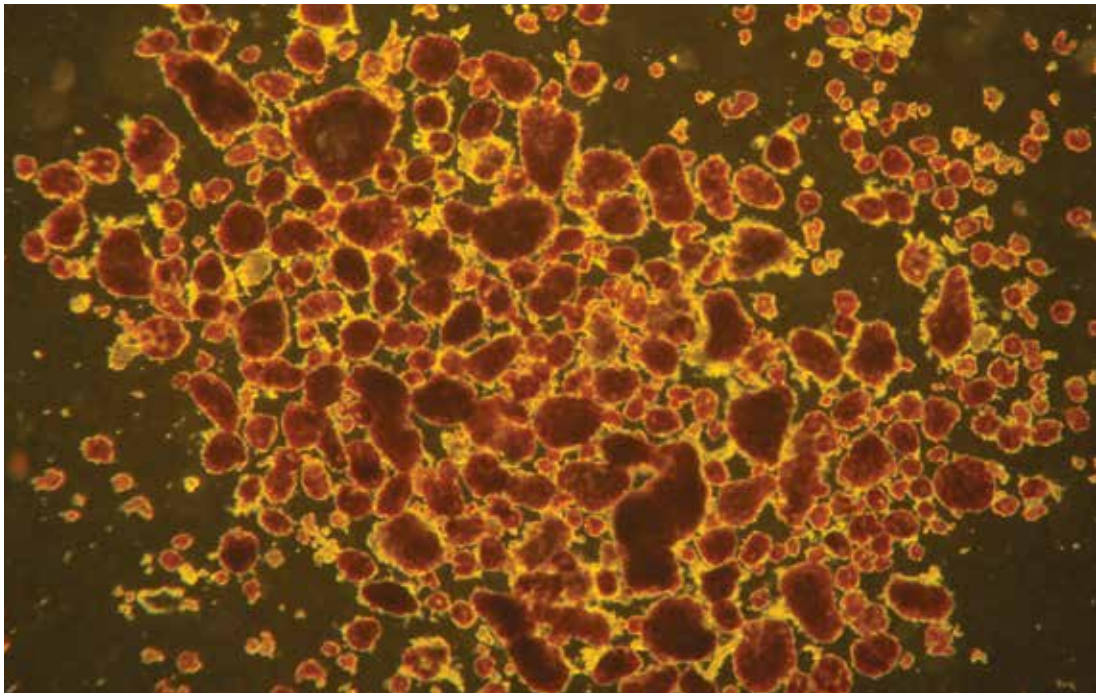
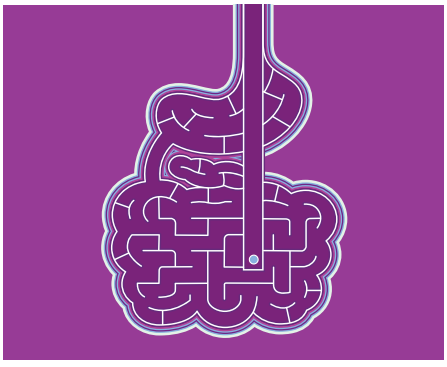


Avoid Roadblocks in Diabetes Research

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

Let's Talk About Malaria

In this issue of our **TechTalk**, we will provide you with guidelines for genetic modification of Plasmodium parasites using the 4D-Nucleofector™ X Unit. We will also respond to frequently asked questions on this topic.

Q. What impact has the Nucleofector™ Technology made in the field of malaria research?

A. Malaria is caused by a parasite called Plasmodium, which is transmitted via the bites of infected mosquitos. In the human or animal body, the parasites multiply in the liver, and then infect red blood cells. Malaria remains a significant cause of human mortality with the vast majority of cases resulting from infection with *Plasmodium falciparum*[1]. A deeper understanding of the molecular biology of this parasite is necessary to develop new therapeutic drugs or vaccines. As a model system in this research field, the rodent malarial parasite *Plasmodium berghei* is often used due to easier cell culture handling. In 2006, Janse *et al.* published their great success in *Nature Protocols*[2, 3] which describes their findings, indicating they were able to achieve 100–1,000 times higher efficiency using Nucleofection™ versus common electroporation protocols. Such high transfection efficiency greatly reduces the time, number of laboratory animals, and amount of materials required to generate transfected parasites.

Q. Does Lonza offer a protocol for transfecting *Plasmodium berghei* using the Nucleofector™ I/ II or 2b Device?

A. Yes, a detailed online protocol is available from Janse *et al.* in *Nature Protocols*[2, 3]. This protocol starts with a collection of schizonts and guides users, step-by-step, through the complete process, including critical steps and troubleshooting suggestions. The best combination for successful Nucleofection™ is the program U-033 (U-33 on Nucleofector™ I Device) in combination with the Basic Parasite Nucleofector™ Kit 2 (Lonza, cat. no. VMI-1021).

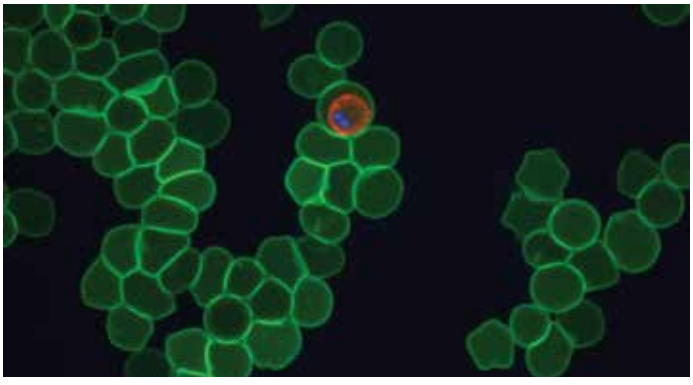


Figure 1
Mouse erythrocytes with infected *Plasmodium berghei*. Red: mCherry expressing parasite in erythrocyte. **Green**: anti-TER staining. **Blue**: Hoechst/Dapi staining for nuclei.

Q. Can I use the same protocol[2, 3] for other rodent malaria species?

A. The optimized conditions do work for other rodent malarial parasites such as *Plasmodium yoelii* and *Plasmodium chabaudi*[4, 5] as well. In some of the published references, the 88A6 Test Nucleofector™ Solution is described in the Materials and Methods section. Please note that 88A6 Test Nucleofector™ Solution is now utilized as the Nucleofector™ Solution in the Basic Parasite Kit 2.

Q. Do you have conditions for genetic modification of *Plasmodium berghei* compatible with the 4D-Nucleofector™ X Unit?

A. Please note that it is not possible to simply translate the Nucleofector™ II/2b Programs to 4D-Nucleofector™ Programs. The difference between the metal electrodes used in the original system and the conductive polymer used in the new system require unique solutions and electrical parameters which must be determined empirically. However, customers using the 96-well Shuttle™ System have achieved results comparable to the published protocol[8] and for some strains with even better viability. They used program FF-167 in combination with P5 Primary Cell Solution. As the 96-well Shuttle™ System also uses the conductive polymer electrodes, these conditions are transferable to the 4D-Nucleofector™ X Unit.

Q. How does Nucleofection™ compare to standard electroporation?

A. The use of transfection to study the biology of malarial parasites has been limited due to poor transfection efficiencies (integration frequency of 10^{-6}–10^{-9}). By using non-viral Nucleofector™ Technology for the rodent parasite *Plasmodium berghei*, the transfection efficiency obtained (episomal and targeted integration into the genome) is in the range of 10^{-2}–10^{-3}. That is 100–1,000 times more clones per experiment[3]. The lower number of required parasites and lower amount of DNA, combined with this success rate, significantly reduces the number of laboratory animals used. Combined with the short period of only 5–6 days to select the transfected parasites, this greatly simplifies the entire transfection technology and will therefore provide opportunities for the use of genetic modification in larger scale and high throughput approaches to investigate the biology of Plasmodium.

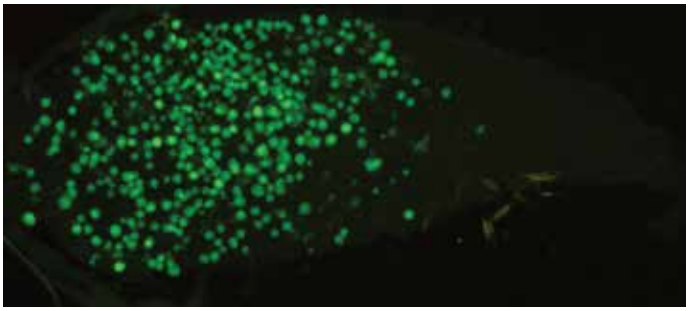


Figure 2
Nucleofection™ of *Plasmodium berghei*. GFP expressing parasite oocytes on mosquito midgut.

Q. Can I use Lonza's pmaxGFP™ Control Vector with this protocol for parasite transfection?

A. Using the pmaxGFP™ Vector is not recommended for parasite transfection because this control vector has a mammalian CMV promoter. This construct is not well expressed in parasites. We suggest using parasite-specific reporters and vectors. Once the optimal Nucleofection™ Conditions are determined with a positive control vector, they can be used with your specific DNA or a variety of substrates including dsDNA, shRNA or siRNA.

Q. Where can I find Lonza's protocols for Nucleofection™ of Plasmodium?

A. To determine if an optimized protocol is already available, search for 'Optimized Protocols' at the Lonza website (www.lonza.com). For parasites where no optimized conditions are currently available, we offer our customers published results located in our Cell Transfection database (http://lonza.com/celldatabase). Here we list the combination of programs and suitable Nucleofector™ Solutions showing the best results. You can also use our online citation database to identify Nucleofector™ Publications with parasites. If you don't find any results, please contact us at scientific.support@lonza.com for assistance.

Q. What about the human malarial parasite *Plasmodium falciparum*? Can I use the conditions established for *Plasmodium berghei*?

A. The transfection of *Plasmodium falciparum* is much more difficult and progress in this area is still limited due to poor transfection efficiency. Over the last decades, most electroporation protocols have been based on two publications; either the spontaneous uptake of DNA from transfected erythrocytes[6] or the transfection of parasitized red blood cells[7]. In 2012, Joseph deRisi, from the University of California, published a transfection protocol using the Nucleofector™ 96-well Shuttle™ System for use with *Plasmodium falciparum*, which included a variety of improvements over the established protocols. One major development is the adaptation of electroporation, culture maintenance, and monitoring to a 96-well plate format for both transient and stable transfection experiments. Plate-based transfection requires 20 fold less plasmid DNA yet results in an approximate 7 fold increase in transient transfection efficiency and stable transfection success rates of >90% using the Nucleofector™ 96-well Shuttle™ System[8]. This protocol shows Nucleofection™ of red blood cells (RBC) using P3 Primary Cell Solution with selected programs (CM-150, CM-138, CM-113) followed by superinfection with *Plasmodium falciparum* schizonts, culture maintenance, and monitoring in the 96-well plate format for both transient and stable transfection experiments.

Q. Can I use the published protocol[8] for transfection of RBCs, described above, with the 4D-Nucleofector™ X Unit?

A. Yes, the 20 µl Nucleocuvette™ Strips used in the Nucleofector™ 96-well Shuttle™ System and 20 µl on the 4D-Nucleofector™ X Unit are essentially identical and share the same programs and solutions. With the conductive polymer cuvettes and the specific Nucleofector™ Solutions, a great improvement in transfection is possible with this technology.

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Figures 1 and 2 provided by Dr. Chris J. Janse, Leiden Malaria Research Group, Parasitology, Center of Infectious Diseases, Leiden University Medical Center (LUMC), Netherlands.

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