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Transfection of Primary Human Hepatocytes

Efficient Transfection and Sustained Long Term Functionality

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Abstract

Primary Human Hepatocytes (PHH) are a standard tool for determining drug metabolism, transport, and toxicity in pre-clinical drug development. However, just as virtually all non-dividing primary cells, PHH are difficult to transfect while maintaining their differentiated and specialized functions, limiting the ability for researchers to study more detailed mechanisms of these critical processes.

In this study, we optimized the thawing, transfection and culture procedure for cryopreserved PHH using the 4D-Nucleofector® System. Transfection efficiency of both pmaxGFP® plasmid DNA and Cleancap® mCherry RNA (TriLink) was assessed by fluorescence microscopy. PHH were plated on collagen-coated cell culture vessels in Matrigel® (Corning) sandwich culture and analyzed for cell viability, bile canaliculi formation, albumin secretion and CYP3A4, CYP1A2 and CYP2B6 metabolite formation.

Two 4D-Nucleofector® Programs, DS-150 and EX-147, were assessed. Using program DS-150 efficiencies of up to 20% for DNA and up to 85% for mRNA were achieved and sustained for the complete culture period of 7 days. Following initial transfection, slight reductions were seen for viability, albumin secretion, and CYP3A4 activity which recovered over the course of the 7 days. Transfected PHH formed complex, branched bile canaliculi network and maintained good morphology throughout the culture period. To achieve higher efficiency, program EX-147 was used resulting in DNA transfection efficiencies of up to 68% at 24 hours post transfection. However, cell viability and characteristic hepatocyte functions were reduced over time compared to non-transfected controls limiting use of this program to shorter term studies.

In summary, we demonstrate efficient DNA and mRNA expression in PHH with preserved functionality for up to 7 days that to enable the improved use of primary human hepatocytes for mechanistic studies.

Introduction

Delivery of nucleic acids into different cell types is often used to create new genetic systems for dissecting molecular mechanisms in cells. Transfecting cells with heterologous DNA and RNA molecules create new cell functions such as overexpression or knockdown of particular pathways and/or molecular reporters to detect certain activities in response to stimulus. While most proliferating, tumor-derived cell lines can be easily transfected by standard transfection methods such as lipofection or polyethylenimine (PEI), transfection of primary cells remains a challenge. Because normal primary cells are thought to represent the *in vivo* cellular functions better than tumor cells, it is highly desirable to use primary cells to work out molecular mechanisms of relevant pathways

The Nucleofector® Technology utilizes a combination of cell-type specific solutions and electrical parameters to successfully deliver DNA and RNA into difficult-to-transfect cell lines and primary cells[1]. High transfection efficiency facilitates implementation of reporter gene assays, cell based assays[2], siRNA[3, 4, 5] and CRISPR applications [6, 7, 8]. The 4D-Nucleofector® X Unit allows for transfection of higher cell numbers (10^5 – 10^7 cells) in the 100 µL Nucleocuvette® Vessel and lower cell numbers (10^4 – 10^6 cells) in the 20 µL Nucleocuvette® Strip in one device[9].

Primary Human Hepatocytes (PHH) are the state-of-the art *in vitro* human liver model system in the field of drug metabolism and toxicology [10, 11]. PHH express functional metabolic enzymes and membrane transporters that reliably reflect the corresponding activities of *in vivo* liver. In contrast to cell lines [12], and just as virtually all non-dividing primary cells, PHH are difficult to transfect with standard methods such as lipofection[13]. Because of this, it has been very difficult to use PHH for probing specific mechanisms of action of the enzymes involved in transporting and metabolizing drugs and toxicants using expression systems and reporter genes. Instead, researchers typically have turned to tumor cell lines such as HEK and MDCK cells where these transporter and metabolic genes are heterologously expressed for demonstrating specific mechanisms and activities. The ability to modify expression and function of drug transporters and metabolic enzymes directly in PHH would allow the genetic manipulation to occur in the appropriate biological background, leading to greater biological relevance of results and potentially better *in-vitro*-to-*in-vivo* extrapolation (IVIVE). In this study, we optimized the thawing, transfection and culture procedure for cryopreserved PHH. Transfection efficiency and hepatocyte functionality were analyzed over 7 days.

Materials and Methods

Sandwich culture: Cryopreserved Primary Human Hepatocytes (PHH; Plateable, Induction Qualified; Catalog # HUCPI, Lonza) were cultured according to the instructions (14) in sandwich culture. Briefly, 50,000 cells per well were plated in collagen coated 96-well plates. 4-6 hours post-seeding, Matrigel® (Corning) overlay was added in Hepatocyte Culture Medium (HCM® Bullet® Kit: CC-3198; Lonza). Media was renewed daily.

Transfection with 4D-Nucleofector® System
Primary human hepatocytes (PHH; Plateable, Induction Qualified; Catalog # HUCPI, Lonza) were thawed in thaw-

ing medium, centrifuged for 8 minutes at 100 xg, carefully resuspended in 3 mL plating medium and counted according to the instructions[14]. Calculated amount of cells was centrifuged for 8 minutes at 80 x g, resuspended in appropriate volume of P3 Nucleofector® Solution and transfected according to the instructions[15] in the X Unit of the 4D-Nucleofector® System. For transfection in 100 µL Nucleocuvette® Vessel, 5×10^5 cells in 100 µL Nucleofector® Solution were transfected with program EX-147 (high efficiency; for short-term experiments) or DS-150 (high functionality; for long-term experiments) using 5 µg pmaxGFP® Vector, 5 µg beta-galactosidase expressing vector pCMVbeta or 5 µg CleanCap® mCherry mRNA (TriLink; Cat. No.: L-7203). After 10 minutes of post-transfection incubation, the complete content of one 100 µL Nucleocuvette® Vessel was transferred in 400 µL plating medium into one well of a 24-well cell culture plate. For transfection in 20 µL Nucleovette® Strips, 1×10^5 cells in 20 µL P3 Nucleofector® Solution were transfected with program EX-147 using 1 µg beta-galactosidase expressing vector. After 10 minutes of post-transfection incubation, the complete content of one 20 µL Nucleovette® reaction was added to 80 µL of plating medium and transferred into one well of a 96-well cell culture plate.

Beta-Galactosidase expression

Cells were transfected with a beta-galactosidase expressing vector in 100 µL or 20 µL volume using program EX-147. Cells were plated in 96-well plates at identical seeding density. Beta-galactosidase expression was quantified by a luminescence assay (Beta-Glo® Assay System, Promega).

Microscopy

Transfection efficiency, cell morphology and bile canaliculi formation was observed and documented by fluorescence and bright field microscopy (Zeiss AxioObserver Z.1 microscope equipped with a Zeiss N-Achroplan 10x objective and an AxioCam MRm camera). For calculation of transfection efficiency, living cells and fluorescent cells were counted manually.

Bile Canaliculi Formation

Cholyl-lysyl-fluorescein (CLF) staining (Corning) was used for visualization of bile canaliculi formation. Cells were incubated for 1 hour at 37°C in medium containing 4 µM CLF followed by three washing steps with 500 µL medium each prior to examination by fluorescence microscopy.

Viability and general metabolic activity

Viability / metabolic capacity of the culture was assessed via CellTiter-Blue® Cell Viability Assay (Promega) according to manufacturer's instructions. Albumin content in the supernatant was quantified with the Human Albumin ELISA Kit (Bethyl) according to manufacturer's instructions.

4D-Nucleofector® System and transfection procedure

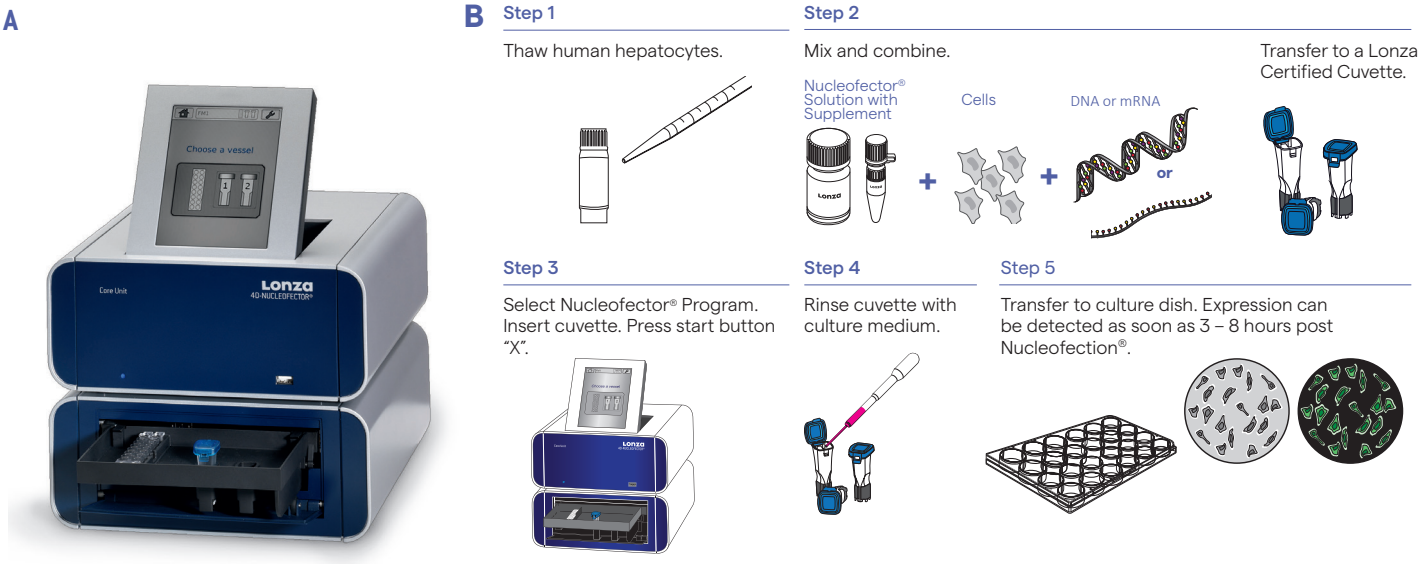


Figure 1.
(A) The 4D–Nucleofector® X Unit supports the transfection in volumes of 20 µL and 100 µL. (B) Transfection procedure: primary human hepatocytes were thawed in thawing medium, centrifuged, resuspended in plating medium, counted, centrifuged again and resuspended in appropriate amount of Nucleofector® Solution. For transfection in 20 µL Nucleovette® Strips, 1×10^5 cells in 20 µL Nucleofector® Solution were transfected with 1 µg DNA or RNA per sample. For transfection in the 100 µL Nucleocuvette® Vessel, 5×10^5 cells in 100 µL Nucleofector® Solution were transfected with 5 µg DNA or RNA per sample.

Cytochrome P450 activity

CYP3A4 activity was assessed via the P450-Glo® CYP3A4 Assay (Promega) using Luciferin-IPA as a substrate according to manufacturer's instructions. For direct measurement of CYP1A2, CYP2B6 and CYP3A4 metabolic activity, cells were incubated with 100 µM phenacetin, 250 µM bupropion or 200 µM testosterone for 15 minutes. Formation of the metabolites acetaminophen, OH-bupropion and 6β-hydroxytestosterone was evaluated, as analyzed by LC/MS/MS (Biotranex).

Results

Cryopreserved primary human hepatocytes are a convenient and widely available tool for a growing number of assays and application. To ensure high viability and typical hepatocyte functionality of the cells, very gentle handling – especially during and after thawing procedure and transfection – is crucial. However, transfection of nucleic acids can be somewhat toxic to cells, at least initially, which for hepatocytes can cause cells to either die off or de-differentiate. Therefore, we sought to establish a program with medium transfection efficiency program that would support longer term healthy culture following transfection. In a second step, we established a high efficiency transfection protocol (> 60% transfected cells), that however had an impact oncell health after transfection.

Sustained transfection and functionality of DS-150 transfected hepatocytes for at least 7 days

To facilitate long term culture of transfected primary human hepatocytes, transfection with Nucleofector® Program DS-150 was optimized. With the described transfection procedure, efficiencies of up to 20% for DNA were observed (Figure 2 and 3A). GFP expression was sustained for the complete culture period of 7 days without any decrease.

Both metabolic capacity, measured by the reduction of resazurin into resorufin, and albumin secretion were slightly reduced on day 1 and 2 after transfection, reaching around 70% of non-transfected control but recovering over time and increasing up to about 90% control on day 7 (Figure 3B, Figure 3C).

Compared to control cultures, initial CYP1A2 and CYP3A4 activity of the transfected hepatocytes was ~ 60%, increasing up to 80% at day 7 (Figure 3D, Figure 3E). Basal CYP2B6 activity was ~75% of control on day 1, persisting at 60% of control for the following days of culture, finally increasing on day 7 (Figure 3F). This high and prolonged cytochrome p450 activity was combined with good overall morphology of the cells (Figure 2).

In comparison to plasmid DNA, sustained and very efficient mRNA expression of up to 85% was observed after transfection with mCherry mRNA (Figure 4). While plasmid DNA must be delivered to the nucleus to initiate transcription and thus protein expression, mRNA translation occurs directly in the cytoplasm. Obviously, mRNA delivery to the cytoplasm was very efficient, even with the very gentle program DS-150, making this program a very good choice for efficient RNA delivery. Similar results could be anticipated when working with siRNA.

Sustained plasmid DNA expression for 7 days in primary human hepatocytes with program DS-150

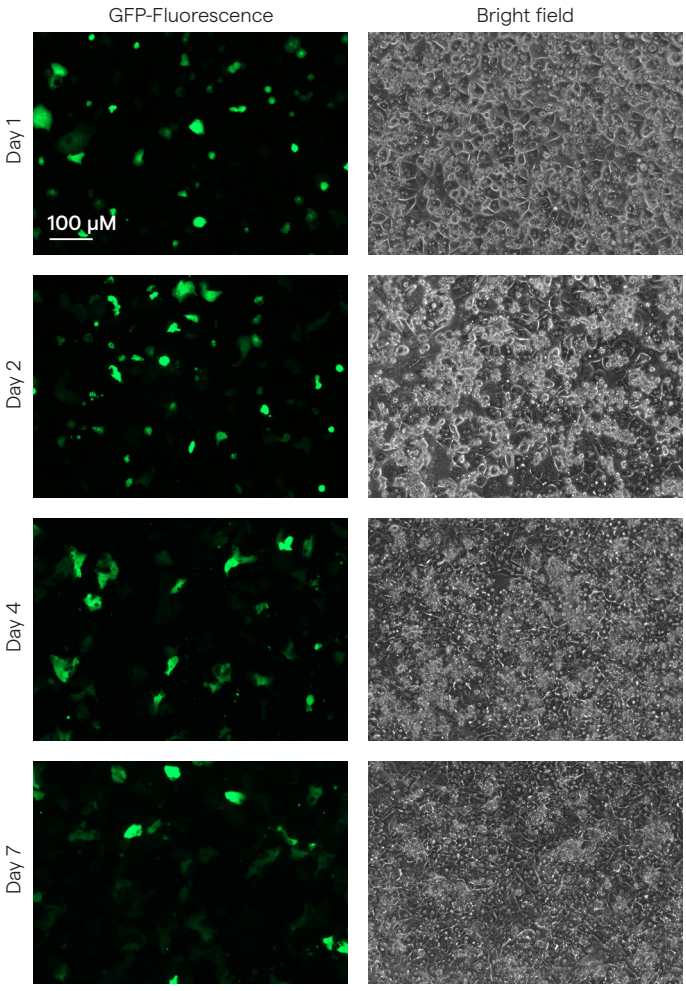


Figure 2.
Primary human hepatocytes (lot HUM4235) were transfected with program DS-150 and pmaxGFP® DNA. On day 1, 2, 4 and 7 after transfection, transfection efficiency and cell morphology was observed and documented by fluorescence and bright field microscopy.

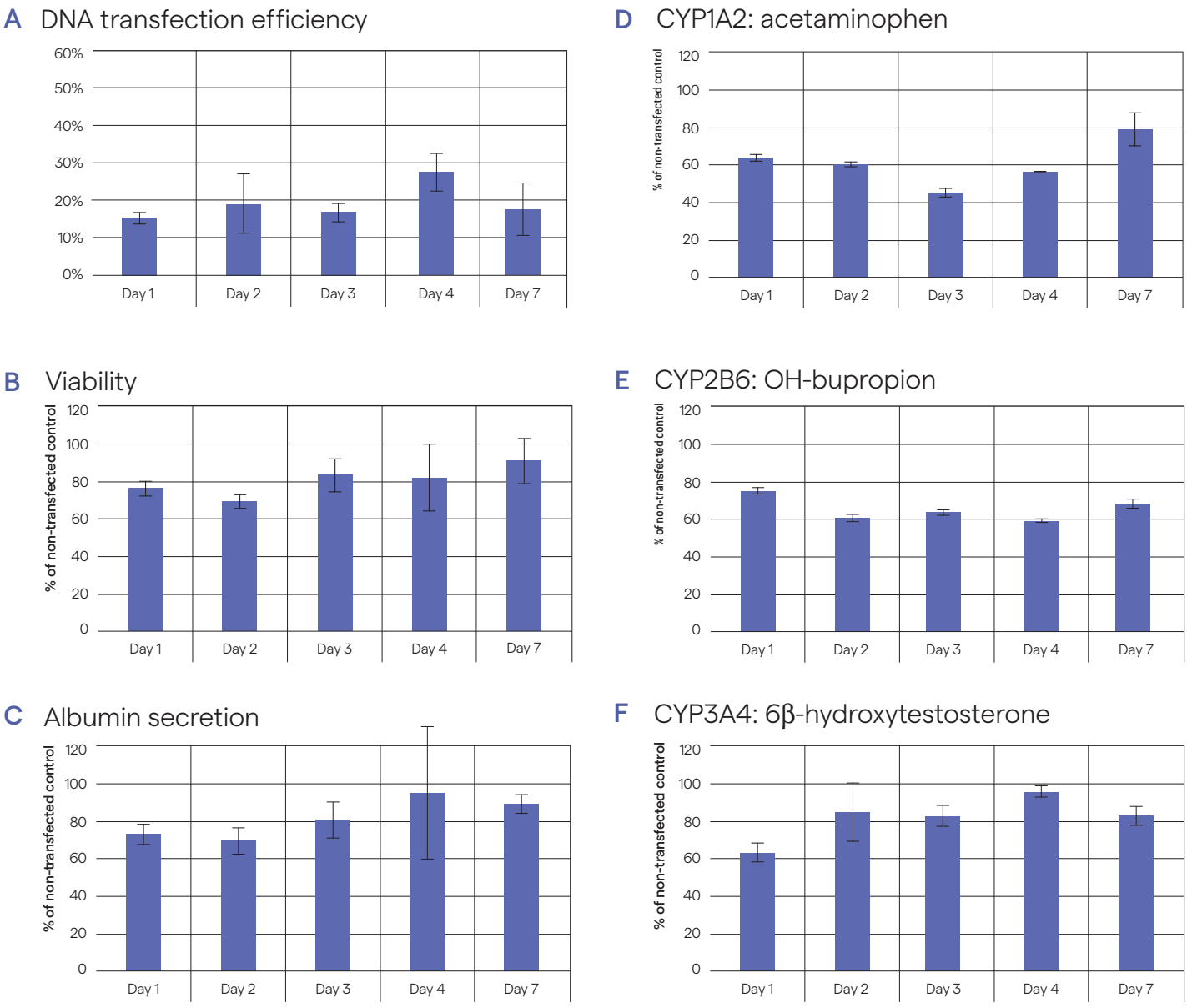
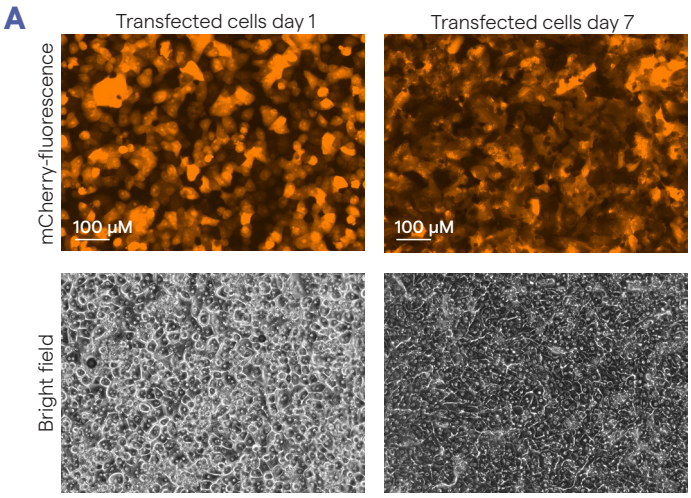


Figure 3.
Primary human hepatocytes were transfected with program DS-150 and pmaxGFP® DNA. (A) For calculation of transfection efficiency, fluorescent cells (lot HUM4235) were counted manually on day 1, 2, 3, 4 and 7 after plating. For both (B) viability and (C) albumin analysis, the respective non-transfected sample was set to 100% for each day for normalization. Results of two donors (lot HUM4235 and HUM4108) are shown, n=6. D – F) For measurement of basal Cytochrome p450 activity, the formation of typical metabolites was evaluated. Non-transfected samples were set to 100% for each day for normalization. Results of one PHH donor (lot HUM4235) are shown. All culture supernatants were analyzed by LC/MS/MS for the indicated metabolite. Error bars indicate standard deviation.

Efficient and sustained mRNA expression and typical healthy hepatocyte morphology after transfection with program DS-150



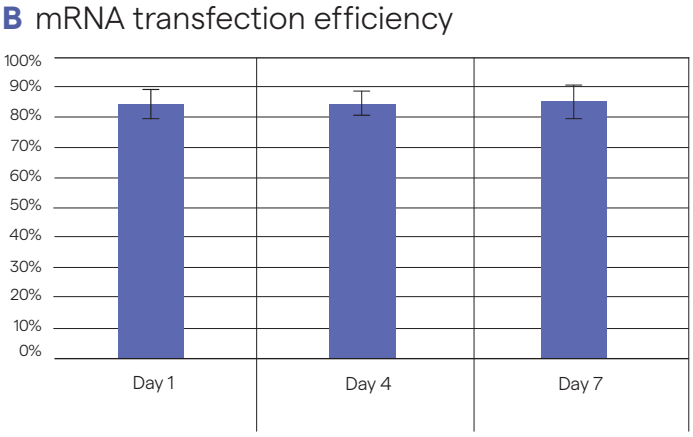
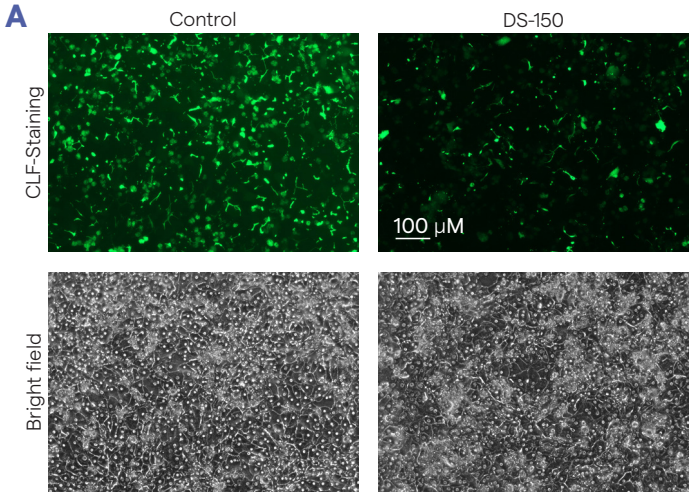


Figure 4.
(A) Primary human hepatocytes (lot HUM4235) were transfected with program DS-150 and Cleancap® mCherry RNA (TriLink). On day 1 and 7 after transfection, transfection efficiency and cell morphology were observed and documented by fluorescence- and bright field microscopy. (B) For calculation of transfection efficiency, fluorescent cells were counted manually on day 1, 4 and 7 after plating.

Transfected and mRNA expressing hepatocytes formed complex, branched bile canaliculi network within 4 days. No difference could be observed between transfected cells and controls (**Figure 5**). To demonstrate that bile canaliculi were formed between successfully transfected cells, images of CLF-staining were combined with corresponding pictures of red mCherry fluorescence (**Figure 5B**).

Branched bile canaliculi network in both transfected and non-transfected hepatocytes on day 4



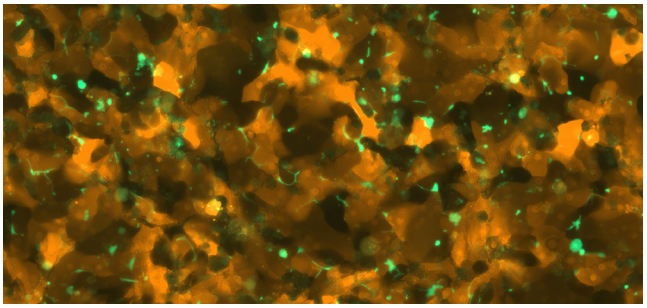


Figure 5.
Primary human hepatocytes (lot HUM4235) were transfected with program DS-150 and Cleancap® mCherry RNA (TriLink). On day 4, Cholyl-lysyl-fluorescein (CLF) staining (Corning) was used for visualization of bile canaliculi formation. (A) Non-transfected and transfected cells in comparison (B) Overlay: mCherry transfection efficiency (visualized by red fluorescence) and CLF bile canaliculi staining (green fluorescence).

Very efficient plasmid DNA transfection in primary human hepatocytes with program EX-147

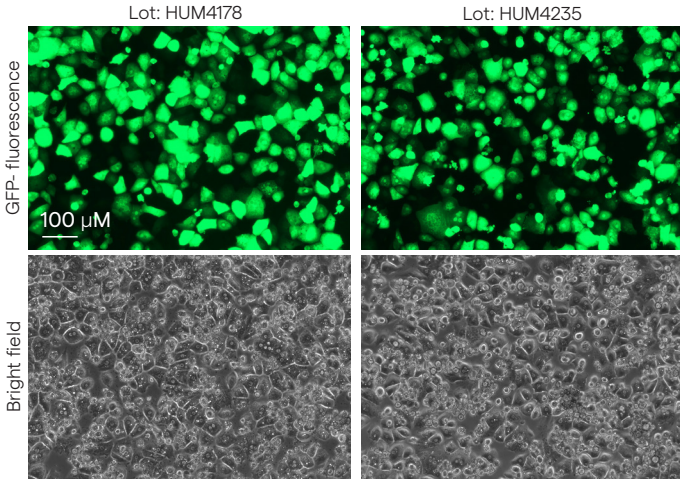
In addition to the high functionality program DS-150, which ensures sustained expression and functionality at intermediate DNA transfection efficiency, we optimized the high efficiency program EX-147 allowing for very efficient transfection of primary human hepatocytes for short term applications.

With program EX-147, DNA transfection efficiency of 68% was observed 24 hours post transfection (Figure 6A, Figure 6B). This program is particularly useful for applications requiring high DNA transfection efficiency. Metabolic capacity and albumin secretion were ≥ 50% 24 hours post transfection (Figure 6C), exhibiting a lot dependent decrease during the following days. Due to this observation, we recommend program EX-147 for short-term culture. Cytochrome p450 activity was reduced to 52-67% of control culture activity 24 hours after transfection, as demonstrated for CYP1A2, CYP2B6 and CYP3A4 (Figure 6D). CYP3A4 activity was recovering over time and reached 80% of non-transfected control at day 4 (data not shown).

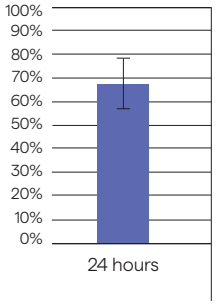
To compare transfection in the 100 µL Nucleocuvette® Vessel and 20 µL Nucleocuvette® Strip, beta-galactosidase expressing vector was used. After transfection with program EX-147in 20 µL Strip, the whole sample was transferred to one well of a 96-well plate. For comparison, after transfection in the 100 µL Vessel, the sample was transferred to 5-wells of a 96-well plate, resulting in a corresponding cell density for both samples. Beta galactosidase expression was identical after transfection in the 100 µL Nucleocuvette® Vessel and 20 µL Nucleocuvette® Strip (Figure 7).

Transfected PHH formed complex bile canaliculi network within few days. First small canaliculi were already visible on day 1, and on day 4 both transfected and non-transfected cultures presented extensive branched canaliculi network which was even more apparent on day 7. Compared with non-transfected cells, bile canaliculi formation was not impaired after transfection (Figure 8). However, for some of the donors, cells were not 100% confluent when plating after transfection. Subconfluence in hepatocyte culture may result in reduced bile canaliculi formation.

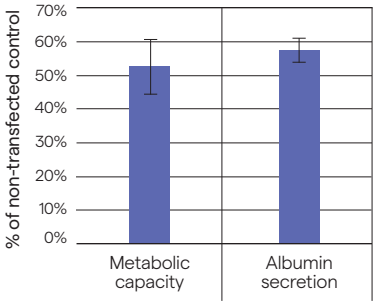
A Efficient GFP expression in primary human hepatocytes 24 hours post transfection.



B DNA transfection efficiency



C Viability and Albumin Secretion



D Cytochrome p450 Activity

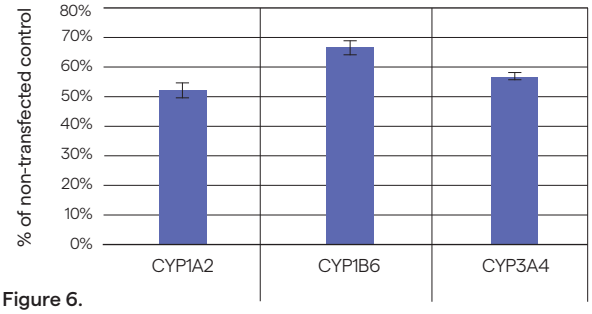


Figure 6.
5×10^5 cells were transfected with program EX-147 and 5 µg pmaxGFP® DNA in 100 µL Nucleocuvette® Vessels in the 4D-Nucleofector® System. Cells were plated in collagen/Matrigel® sandwich culture in 24-well plate. 24 hours after plating, transfection efficiency and cell morphology were observed and documented by fluorescence and bright field microscopy. (A) Typical results are shown. (B) For calculation of transfection efficiency, fluorescent cells were counted manually. Data from 5 independent experiments with 4 different donors are shown, n=17. (C) Viability data of five independent experiments with five different donors is shown, n = 22. Albumin content in the supernatant is shown from one representative donor HUM4235; n=3. The respective non-transfected sample was set to 100% for normalization. (D) For measurement of basal Cytochrome p450 activity, the formation of typical metabolites was evaluated. All culture supernatants were analyzed by LC/MS/MS. The respective non-transfected sample was set to 100% for each day for normalization. Results of one typical donor are shown (lot HUM4235), n = 3. Error bars indicate standard deviation.

Comparable beta-galactosidase expression levels after transfection in 20 µL and 100 µL Nucleocuvette® Vessels

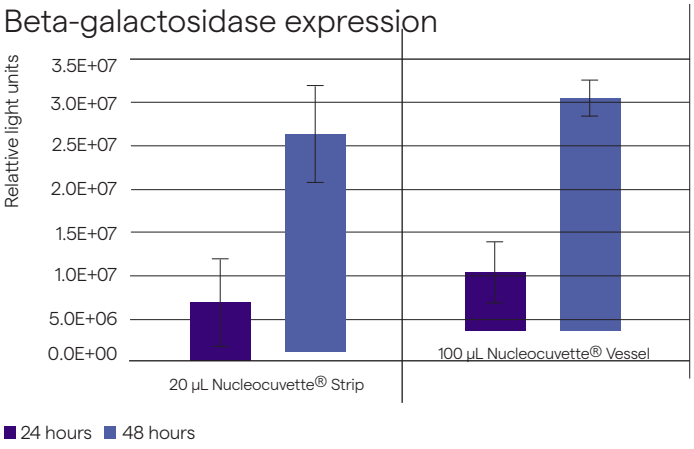


Figure 7.
Primary human hepatocytes were transfected with a beta-galactosidase expressing vector in 20 µL Nucleocuvette® Strip or 100 µL Nucleocuvette® Vessel using program EX-147. Cells were plated in sandwich-culture in 96-well plates at identical density. Beta-galactosidase activity was quantified by a luminescence assay (Beta-Glo® Assay System, Promega). Error bars indicate standard deviation, n ≥3.

Bile canaliculi formation is comparable to non-transfected control

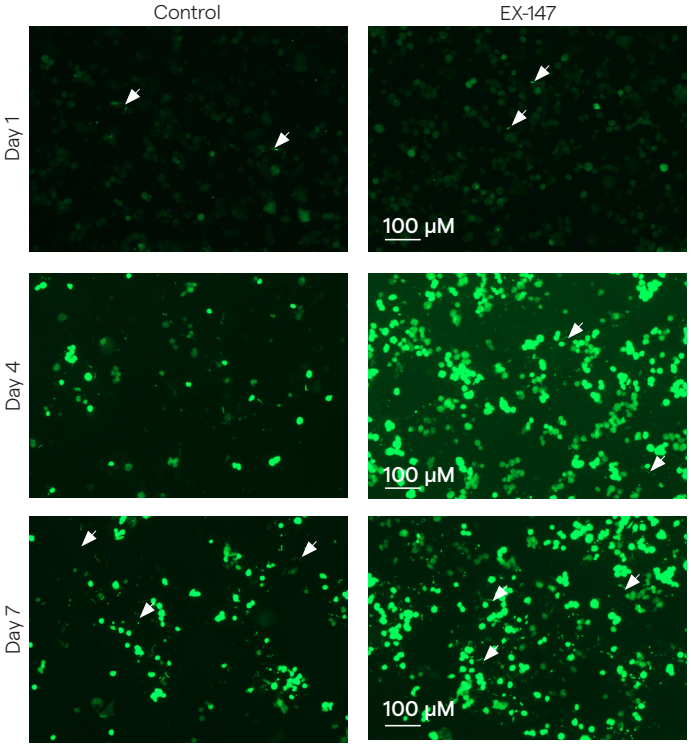


Figure 8.
Primary human hepatocytes (lot HUM4108) were transfected with program EX-147 and Cleancap® mCherry RNA (TriLink). On day 1, 4 and 7, Cholyl-lysyl-fluorescein (CLF) staining (Corning) was used for visualization of bile canaliculi formation. Typical results are shown.

Discussion

Primary human hepatocytes are considered the "gold standard" *in vitro* model for understanding the effects of drugs on hepatic metabolism and function during the preclinical phases of drug development. Freshly isolated and cryopreserved PHH can recapitulate the molecular functions of *in vivo* hepatocytes for up to 7 days when placed into cell culture. However, because of the shortened culture times for PHH and the difficulty to transfect PHH, many researchers turn to tumor cell models when asking specific mechanistic questions. Nevertheless, to determine the biological relevance of these mechanistic studies, they need to then be also observed using primary hepatocytes. Cryopreserved primary hepatocytes, are very sensitive to manipulation and environmental changes, therefore successfully transfecting these delicate cells is very difficult.

Here we describe both a high-functionality and a high efficiency method for transfecting PHH using the 4D-Nucleofector® X Unit. Transfection with program DS-150 resulted in very efficient mRNA transfection efficiency and intermediate DNA transfection efficiency; both sustained expression levels for the culture period of one week. The difference in expression levels for both types of nucleic acid after transfection with program DS-150 may be because plasmid DNA must be delivered into the cell nucleus for transcription, while mRNA translation occurs directly in the cytoplasm. Viability, albumin production and basal cytochrome p450 activity were preserved for 7 days following transfection of either nucleic acid. Primary human hepatocytes also maintained typical healthy hepatocyte morphology, including complex bile canaliculi formation. In summary, program DS-150 is suitable for efficient RNA transfection and intermediate level DNA transfection allowing for prolonged hepatocyte culture.

For short-term experiments, program EX-147 facilitates efficient DNA transfection with donor-dependent reduced cell functionality and viability compared to program DS-150. The inverse relation between hepatocyte viability and transfection efficiency was described previously (16) by Chen et al. Therefore, program EX-147 is particularly useful for applications requiring short-term high DNA transfection efficiency.

In summary, we developed effective and convenient protocols for the transfection of highly functional primary human hepatocytes that can be applied for smaller cell numbers or higher throughput in a 20 µL format and for larger cell numbers in a 100 µL format using the same cell culture and transfection conditions. The results presented demonstrate a reliable method for transfecting PHH, using Nucleofector® Technology, that can facilitate more and better genetic manipulation of PHH for studies of molecular mechanisms of hepatocyte function.

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