Efficient Transfection and Sustained Long Term Functionality of Primary Human Hepatocytes

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Purpose

Primary Human Hepatocytes (PHH) are the state-of-the art **in vitro** human liver model system in the field of toxicology. PHH are known to be difficult to transfect with classical transfection methods. Furthermore, PHH tend to lose their typical liver functions rapidly in culture. In this study, we optimized the thawing, transfection and culture procedure for cryopreserved PHH. Transfection efficiency and hepatocyte functionality were analyzed over 7 days.

Methods

Lonza's cryopreserved plateable human hepatocytes were transfected using the 4D-Nucleofector® System. Prior transfection, cryopreserved PHH were gently thawed and resuspended in P3 Nucleofector® Solution. Following transfection using program EX-147 or DS-150, PHH were plated on collagen-coated cell culture vessels in Matrigel™ (Corning) sandwich culture. We characterized specific hepatocyte functions of the resulting transfected sandwich cultures for up to 7 days. Transfection efficiency of both pmaxGFP™ Plasmid DNA and Cleancap® mCherry RNA (TriLink) was assessed by fluorescence microscopy. PHH were analyzed for cell viability, bile canaliculi formation, albumin secretion and CYP3A4, CYP1A2 and CYP2B6 metabolite formation. Experimental details can be found in the figure legends below.

A) 4D-Nucleofector® Device

B) Transfection Process

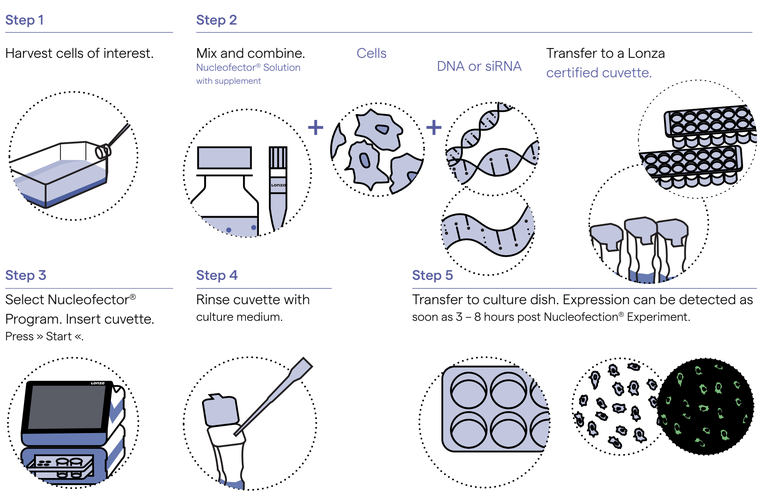

Figure 1: 4D-Nucleofector® Device for efficient transfection of difficult to transfect primary cells and cell lines

Conclusion

We present reliable protocols for efficient DNA and mRNA expression in cryopreserved PHH. We demonstrate highly preserved functionality of transfected hepatocytes for 7 days when using program DS-150. Our protocols enable transfection of human hepatocytes for generation of more sophisticated long-term *in vitro* liver models.

Results

A) GFP-Fluorescence

B) Bright-Field

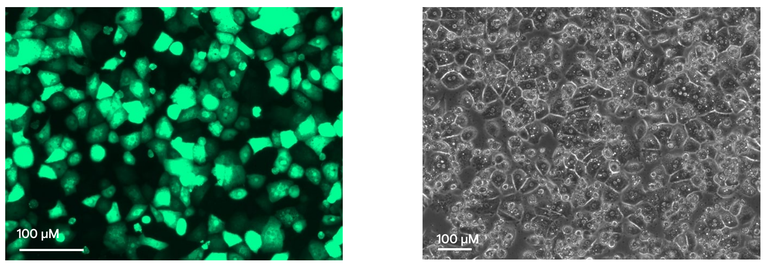

Figure 2: High GFP expression 24 hours after transfection with the high efficiency program EX-147
24 hours after plating, GFP fluorescence and cell morphology was observed and documented by (A) fluorescence and (B) bright field microscopy. (Zeiss AxioObserver Z.1 microscope).

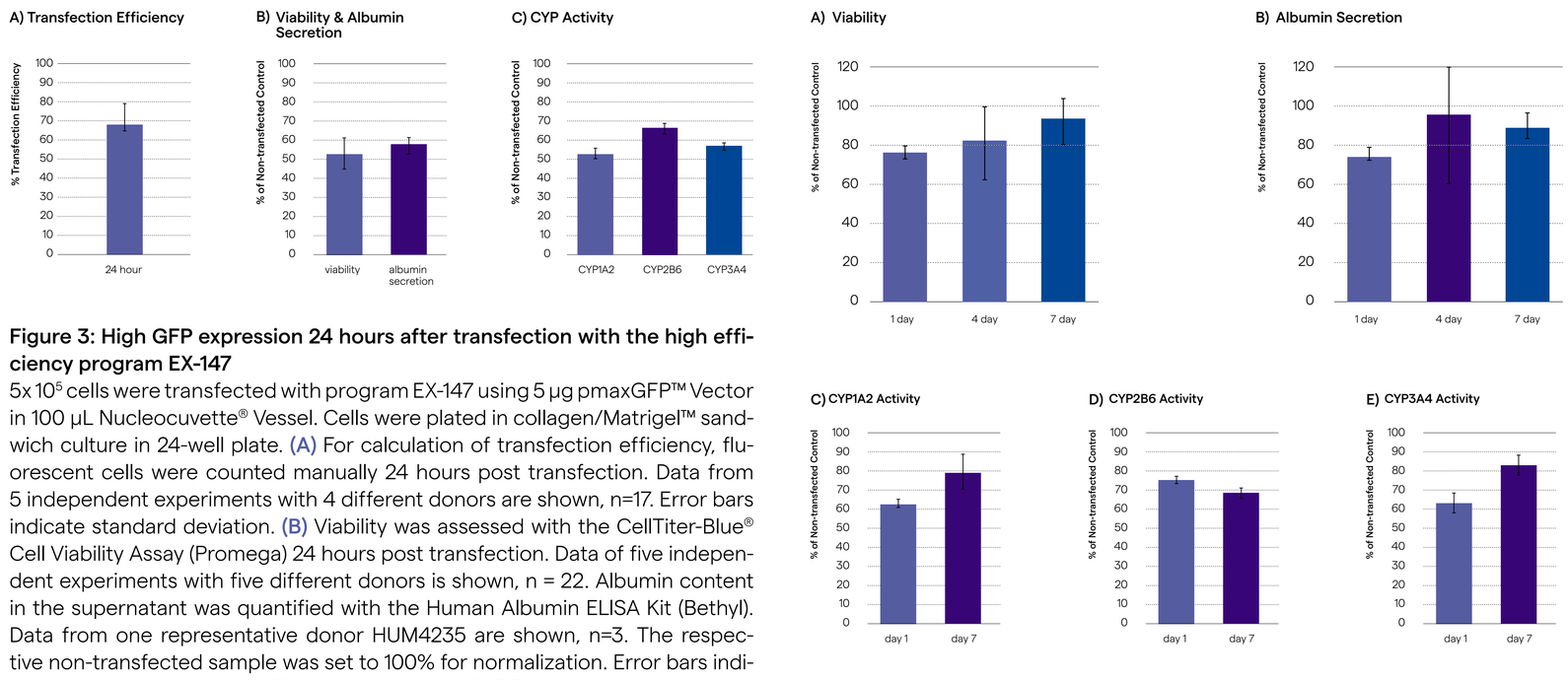

Figure 3: High GFP expression 24 hours after transfection with the high efficiency program EX-147
5x 10^5 cells were transfected with program EX-147 using 5 µg pmaxGFP™ Vector in 100 µL Nucleocuvette® Vessel. Cells were plated in collagen/Matrigel™ sandwich culture in 24-well plate. (A) For calculation of transfection efficiency, fluorescent cells were counted manually 24 hours post transfection. Data from 5 independent experiments with 4 different donors are shown, n=17. Error bars indicate standard deviation. (B) Viability was assessed with the CellTiter-Blue® Cell Viability Assay (Promega) 24 hours post transfection. Data of five independent experiments with five different donors is shown, n = 22. Albumin content in the supernatant was quantified with the Human Albumin ELISA Kit (Bethyl). Data from one representative donor HUM4235 are shown, n=3. The respective non-transfected sample was set to 100% for normalization. Error bars indicate standard deviation. (C) For measurement of CYP3A4 activity 24 hours post transfection cells were incubated with 200 µM testosterone for 15 minutes and the formation of 6ß-hydroxytestosterone was evaluated. For measurement of CYP2B6 activity PHH were incubated with 250 µM bupropion for 15 minutes and the formation of OH-bupropion was evaluated. For measurement of CYP1A2 activity PHH were incubated with 100 µM phenacetin for 15 minutes and the formation of acetaminophen was evaluated. All culture supernatants were analyzed by LC/MS/MS. The respective non-transfected sample was set to 100% for normalization. Results of one typical lot (HUM4253) are shown, n = 3. Error bars indicate standard deviation.

With program EX-147, high DNA transfection efficiencies of 68% were observed. Albumin secretion and Cytochrome p450 activity were clearly detectable 24 hours post transfection, but partially not sustained for a longer period of time (data not shown). Therefore, we recommend program EX-147 for highly efficient short term DNA transfection.

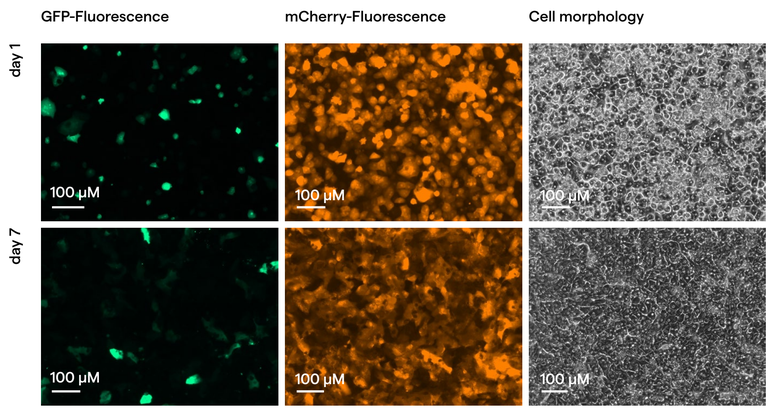

Figure 4: Sustained plasmid DNA and efficient mRNA expression for 7 days in primary human hepatocytes maintaining typical healthy hepatocyte morphology after transfection with program DS-150
Primary human hepatocytes (lot HUM4235) were transfected with program DS-150 using 5 µg pmaxGFP™ Vector or 5 µg Cleancap® mCherry RNA (TriLink) in 100 µL Nucleocuvette® Vessel 4D-Nucleofector® Device. Cells were plated in collagen/Matrigel™ Sandwich culture in 24-well plate. On day 1 and 7 after transfection, transfection efficiency and cell morphology was observed and documented by fluorescence- and bright field microscopy.

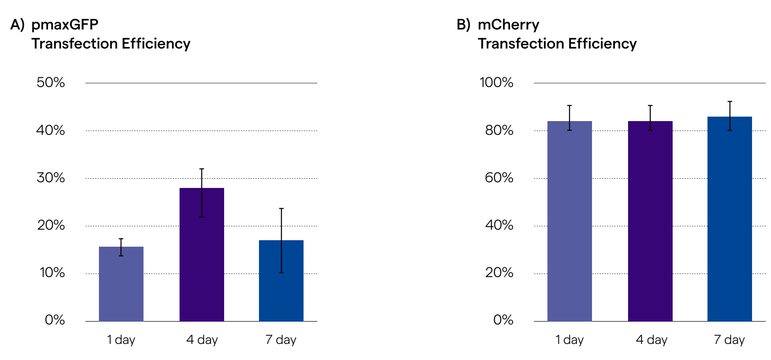

Figure 5: Sustained GFP and mCherry expression for at least 7 days after transfection with program DS-150
Primary human hepatocytes (lot HUM4235) were transfected with program DS-150 using (A) 5 µg pmaxGFP™ Vector or (B) 5 µg Cleancap® mCherry RNA (TriLink) in 100 µL Nucleocuvette® Vessel 4D-Nucleofector® Device. Cells were plated in collagen/Matrigel™ Sandwich culture in 24-well plates. For calculation of transfection efficiency, fluorescent cells were counted manually on day 1, 4 and 7 after plating.

Figure 6: Preserved viability and typical hepatocyte functionality of the cells for at least 7 days after transfection with program DS-150
Primary human hepatocytes were transfected with program DS-150 using 5 µg pmaxGFP™ Vector in 100 µL Nucleocuvette® Vessel 4D-Nucleofector® Device. Cells were plated in collagen/Matrigel™ Sandwich culture in 24-well plates. (A) Viability was assessed with the CellTiter-Blue® Cell Viability Assay (Promega). (B) Albumin content in the supernatant was quantified with the Human Albumin ELISA Kit (Bethyl). (C) For measurement of CYP3A4 activity 24 h post transfection cells were incubated with 200 µM testosterone for 15 minutes and the formation of 6ß-hydroxytestosterone was evaluated. (D) For measurement of CYP2B6 activity PHH were incubated with 250 µM bupropion for 15 minutes and the formation of OH-bupropion was evaluated. (E) For measurement of CYP1A2 activity PHH were incubated with 100 µM phenacetin for 15 minutes and the formation of acetaminophen was evaluated. All culture supernatants were analyzed by LC/MS/MS. The respective non-transfected sample was set to 100% for normalization. For viability and albumin content, data of two donors (lot HUM4235 and HUM4108) are shown, n=6. For Cyp Acticity, data of one typical donor (lot HUM4235) is shown, n=3.

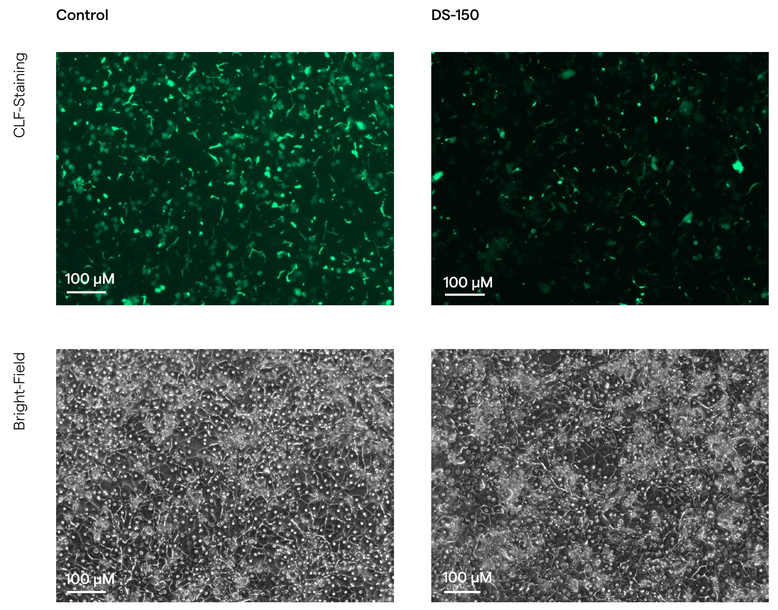

Figure 7: Branched bile canaliculi network in both transfected and non-transfected hepatocytes on day 4
Primary human hepatocytes (lot HUM4235) were transfected with program DS-150 using 5 µg Cleancap® mCherry RNA (TriLink) in 100 µL Nucleocuvette® Vessel 4D-Nucleofector® Device. Cells were plated in collagen/Matrigel™ Sandwich culture in 24-well plate. Culture medium was changed daily. On day 4, Cholyl-lysyl-fluorescein (CLF) staining (Corning) was used for visualization of bile canaliculi formation. Cells were incubated for 1 hour in medium containing 4 µM CLF followed by three washing steps with culture medium prior to examination by fluorescence microscopy. Typical results are shown.

To address the needs of long-term culture of transfected hepatocytes, conditions preserving cell functionality over time were identified. Following transfection with program DS-150, efficiencies of up to 20% for DNA and up to 85% for mRNA were achieved and sustained for 7 days. Viability and albumin secretion at 24h after transfection were slightly reduced, but recovering over time. In comparison to control cultures, CYP1A2, CYP2B6 and CYP3A4 activity was between 60% and 80%. Transfected PHH formed complex, branched bile canaliculi network. We recommend program DS-150 for efficient transfection of siRNA and mRNA as well as intermediate levels of DNA transfection for long-term culture after transfection.