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PROTOCOL

LIVER TISSUE MODEL KIT

This is a suggested procedure, please adjust according to your experimental needs. To maintain the sterility of the product, work under sterile conditions.

Protocol aim

The aim of this protocol is to provide instructions for how to use the Liver Tissue Model Kit A and B. It includes instructions of bioprinting using CELLINK LAMININK 111 (Kit A) or GelXA LAMININK 111 (Kit B) as well as directions of use for the three antibodies included in the kit.

Materials needed

- Liver Tissue Model Kit A or B*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- Liver cells such as hepatocytes and stellate cells in suspension
- Cell culture medium*
- Temperature-controlled printhead (optional)
- BIO X*
- 96-well plate
- Cartridges, 3cc*
- Conical bioprinting nozzles, 22G*
- Crosslinking Agent* (included with the bioink purchase)

*The product can be purchased in the CELLINK shop at www.cellink.com/shop.

Protocol

This protocol can be performed with standard pneumatic printheads at room temperature and print bed cooled to 15°C, where room temperature is between 20-25°C. If using Liver Tissue Model Kit B with GelXA LAMININK 111, it is recommended to use the Temperature-controlled printheads at 24°C for best shape fidelity. Make sure to protect GelXA LAMININK 111 from light.

1. Preparation of bioink

MATERIAL

CELLINK LAMININK 111/GelXA LAMININK 111

3 mL syringe with Luer lock connections

Female/female Luer lock adaptor

Pipette tip or spatula

DESCRIPTION

- Kit A: Warm the CELLINK LAMININK 111 to room temperature.
- Kit B: Heat up GelXA LAMININK 111 at 37°C until it becomes liquid. The heating of the GelXA LAMININK 111 can be performed in a printhead or incubator and usually requires 30-60 min. When GelXA LAMININK 111 is warm, keep the cartridge horizontal when tip and end cap are removed. This is to prevent air from entering the cartridge or bioink from dripping out.
- For both: Connect the cartridge to a 3 mL syringe using a Luer lock connector and remove the end cap. Push approximately half of the bioink from the cartridge into the syringe by gently pushing the cartridge piston using a pipette tip or small spatula while simultaneously pulling the syringe plunger. To remove any air bubble derived from the dead volume in the syringe, separate the syringe and cartridge maintaining the Luer lock on the syringe. Hold the syringe with the tip facing upwards and gently tap the syringe to move air bubbles towards the tip. Carefully extrude air and pre-fill the Luer lock adaptor with the bioink before re-attaching the cartridge. Gently mix the bioink back and forth between the cartridge and syringe to homogenize the bioink. Leave the desired amount of bioink in one 3 mL syringe.

Note: If there are bubbles in the bioink, make a quick centrifugation for 30 s at 600 x *g*.

2. Mixing the bioink with liver cells

MATERIAL

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Pre-warmed CELLINK LAMININK 111 or GelXA LAMININK 111

Liver cell suspension with desired concentration of cells

Cartridge, 3cc

DESCRIPTION

- At this point, mix ten parts bioink with one part cell suspension, taking care not to introduce air bubbles to the mixture. For detailed instructions see the *Mixing cells with bioink Protocol*.
    - Take the syringe with pre-warmed bioink and attach it to an empty 3 mL syringe using a female/female Luer lock adaptor. Push half of the bioink over to the syringe attached.
    - Detach the syringes and pipette the desired cell suspension into one of the bioink-containing syringes. Attach the syringes again gently mix the bioink back and forth between the syringes until the cell suspension is homogeneously incorporated.

Note: To avoid introducing air into the bioink when mixing, carefully pre-fill the Luer lock adaptor with bioink before attaching the empty syringe.

- Transfer the mixture to an empty 3cc cartridge by connecting the syringe to the cartridge using the Luer lock adaptor. Cap the cartridge with a tip cap.

3. Preparation for printing

MATERIAL

Conical bioprinting nozzles, 22G

BIO X

Temperature-controlled printheads (optional)

DESCRIPTION

- Make sure that sterile 22G nozzles, the BIO X and other equipment needed for printing are in place before proceeding to next step.
- Set up the BIO X for printing droplets or small discs.

If using the Liver Tissue Model Kit A:

- Nozzle type and size: Conical tip, 22G
- Printing pressure: 12 kPa**
- Printing speed = 10 mm/s**
- Printhead temperature: -
- Print bed temperature: -

If using the Liver Tissue Model Kit B:

- Nozzle type and size: Conical tip, 22G
- Printing pressure: 10 kPa**
- Printing speed = 10 mm/s**
- Printhead temperature: 24°C
- Print bed temperature: 15°C

** This is recommended starting parameters for the print. The pressure needed and optimal speed depends on the temperature of the surroundings and preparation procedures (amount of bioink and actual temperature of the bioink). ALWAYS test the pressure before starting the print and be prepared to adjust the speed and pressure during the print, especially for long printing sessions.

- *If using the Liver Tissue Model Kit A, go to 4a. If using the Liver Tissue Model Kit B, go to 4b.*

4a. Preparation for printing CELLINK LAMININK 111

MATERIAL

Cartridge with CELLINK LAMININK 111 with liver cells

Conical bioprinting nozzles, 22G

DESCRIPTION

- Attach a 22G nozzle to the cartridge and mount the cartridge in the printhead. Attach the air adaptor to the cartridge.

Note: Be careful not to touch the printhead with the nozzle tip when inserting the cartridge. Alternatively, the cartridge can be placed in the printhead with the tip cap on and when in place switched to a nozzle.

- *Move to Step 5.*

4b. Preparation for printing GelXA LAMININK 111

MATERIAL

Cartridge with GelXA LAMININK 111 with liver cells

Temperature-controlled printhead (optional)

Conical bioprinting nozzles, 22G

DESCRIPTION

- If the cartridge is just taken from the heat or if the cartridge still feels warm after mixing in the cells, place it in the pre-heated Temperature-controlled printhead at 24°C for 5 minutes. If not using the Temperature-controlled printhead, place the cartridge on counter for 5-10 minutes to reach approximately 24°C.
- If the cartridge has cooled down below 22°C, re-heat the cartridge at 37°C for 5 min to reset.
- Attach a 22G nozzle to the cartridge and mount the cartridge in the printhead. Attach the air adaptor to the cartridge.

Note: When printing with GelXA LAMININK 111, the recommended printhead temperature for the highest printing fidelity is 24°C, though the bioink can be dispensed up to 32°C. Below 22°C the bioink can become too viscous resulting in chunky filaments and too high extrusion pressures needed.

Note: Be careful not to touch the printhead with the nozzle tip and if using very liquid materials, make sure that the bioink does not drip through the nozzle especially when attaching the air adapter. Alternatively, the cartridge can be placed in the printhead with the tip cap on and when in place switched to a nozzle.

5. Printing

MATERIAL

BIO X

96-well plate

DESCRIPTION

- Calibrate the nozzle to the 96-well plate surface. Test the flow of the bioink first after calibration and start with a low pressure and increase stepwise. Bioprint droplets or small discs onto the well plate. If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material.

Note: If waiting too long between extrusions the bioink can dry in the nozzle causing it to clog. If this occurs, replace with new nozzle.

- If printing with GelXA LAMININK 111 and proper viscosity and printability is not achieved by extending temperature equilibration time or tuning pressure:
    - Too low viscosity (wide filaments despite using low pressure): decrease the printhead temperature 0.5-1°C to increase the viscosity and equilibrate an additional couple of minutes.
    - Too high viscosity (chunky filaments and high pressure required): increase the printhead temperature 0.5-1°C to decrease the viscosity and equilibrate an additional couple of minutes. If unsuccessful, the bioink might have over-gelled. In this case, re-heat the cartridge at 37°C for 5 min to reset and choose a 1°C higher printhead equilibration temperature.
- *If using the Liver Tissue Model Kit A, go to 6a. If using the Liver Tissue Model Kit B, go to 6b.*

6a. Crosslinking

MATERIAL

Crosslinking Agent

Cell culture medium

DESCRIPTION

- CELLINK LAMININK 111 is crosslinked with ions using the $CaCl_2$- containing Crosslinking Agent.
- Submerge the cell-laden constructs in the Crosslinking Agent for 30 s to 2 min depending on construct size, infill density and desired construct stiffness. Remove the Crosslinking Agent and rinse the constructs with basal culture media once.

Note: 30 seconds is recommended for 10 µL droplets while 2 minutes might be required for thicker discs. In addition, optimize the crosslinking depending on the cell type.

- *Move to Step 7.*

6b. Crosslinking

MATERIAL

Crosslinking Agent

AND/OR

405 or 365 nm LED modules for photocuring

Cell culture medium

DESCRIPTION

- GelXA LAMININK 111 can be photocrosslinked using the 405 or 365 nm LED modules or ionically crosslinked using the $CaCl_2$-containing Crosslinking Agent. If using both, begin with photocrosslinking. It is recommended to use either photocrosslinking alone for softer constructs or both crosslinking methods which will generate more robust constructs. Optimize the crosslinking according to application.

Note: It is recommended to use the 405 nm LED module instead of 365 nm if possible. Overexposure might damage the cells.

- **Photocrosslinking:** Recommended photocrosslinking times for 1 mm thick construct is 10 seconds and 30 seconds for 3 mm thick construct when using the 405 nm module. Ensure that the bioprinted GelXA LAMININK 111 construct is thermally gelled after printing by cooling the print bed.

Note: To verify that the photocrosslinking is sufficient, add 37°C to one printed well and observe that it doesn't dissolve.

- **Ionic crosslinking:** Submerge the cell-laden constructs in the Crosslinking Agent for 30 seconds to 2 minutes depending on construct size, infill density and desired construct stiffness. Remove Crosslinking Agent and rinse constructs with basal culture media once.

Note: 30 seconds is recommended for 10 µL droplets while 2 minutes might be required for thicker discs. In addition, optimize the crosslinking depending on the cell type.

7. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- After crosslinking, add the desired medium to the constructs and place them in an incubator.
- Incubate the constructs in cell culture medium in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to application. Replace medium regularly.
- Time recommendations: Incubate for at least 7 days to analyse the cell viability and morphology of the liver models.

8. Histological analysis

MATERIAL

Collagen Type 1 antibody

ABCC 2 antibody

CYP3A43 antibody

DESCRIPTION

The antibodies provided with the Liver Tissue Model Kit A and B represents antibodies selective of key factors in liver tissue. The antibodies included in the Liver Tissue Model Kit are for the single or co-culture of hepatocytes and stellate cells in bioprinted constructs. The collagen type I antibody is to evaluate the production of collagen type I by the stellate cells during fibrotic inductions. ABCC2 antibody is to evaluate the transporter multidrug resistance protein 2 (MRP2) in hepatocytes, while the CYP3A43 antibody is for analyzing one the of cytochrome P450 enzymes responsible for metabolism of drug compounds.

- After culturing of the liver model for at least 7 days, fixate the constructs and prepare for the histological analysis. Follow the *Fixation Protocol*, *Embedding Protocol*, *Sectioning Protocol,* and *Immunofluorescence staining Protocol* found on CELLINKs webpage to prepare and to stain the tissue model with the antibodies.
- The dilutions for collagen type 1, ABCC 2 and CYP3A43 antibodies are shown in Table 1. Host: Rabbit, Verified Species Reactivity: Human.

Table 1. Recommended dilutions for the antibodies for use on sectioned slides.

Antibody	Dilution range	Recommended dilution
Collagen type 1	1:50 – 1:200	1:50
ABCC 2	1:20 – 1:50	1:20
CYP3A43	1:1000 – 1:2500	1:1000

- In-house data of immunofluorescence analysis with the included primary antibodies; collagen type I (Figure 1, A and B), ABCC2* (Figure 1, C and D) and CYP3A43 (Figure 1, E and F), demonstrate optimized usage for different liver cell types and sources. **The ABCC2 antibody used in Figure 1 is from a previous version of the ABCC2 antibody product using a 1:200 dilution. New product is validated in IHC by vendor.*

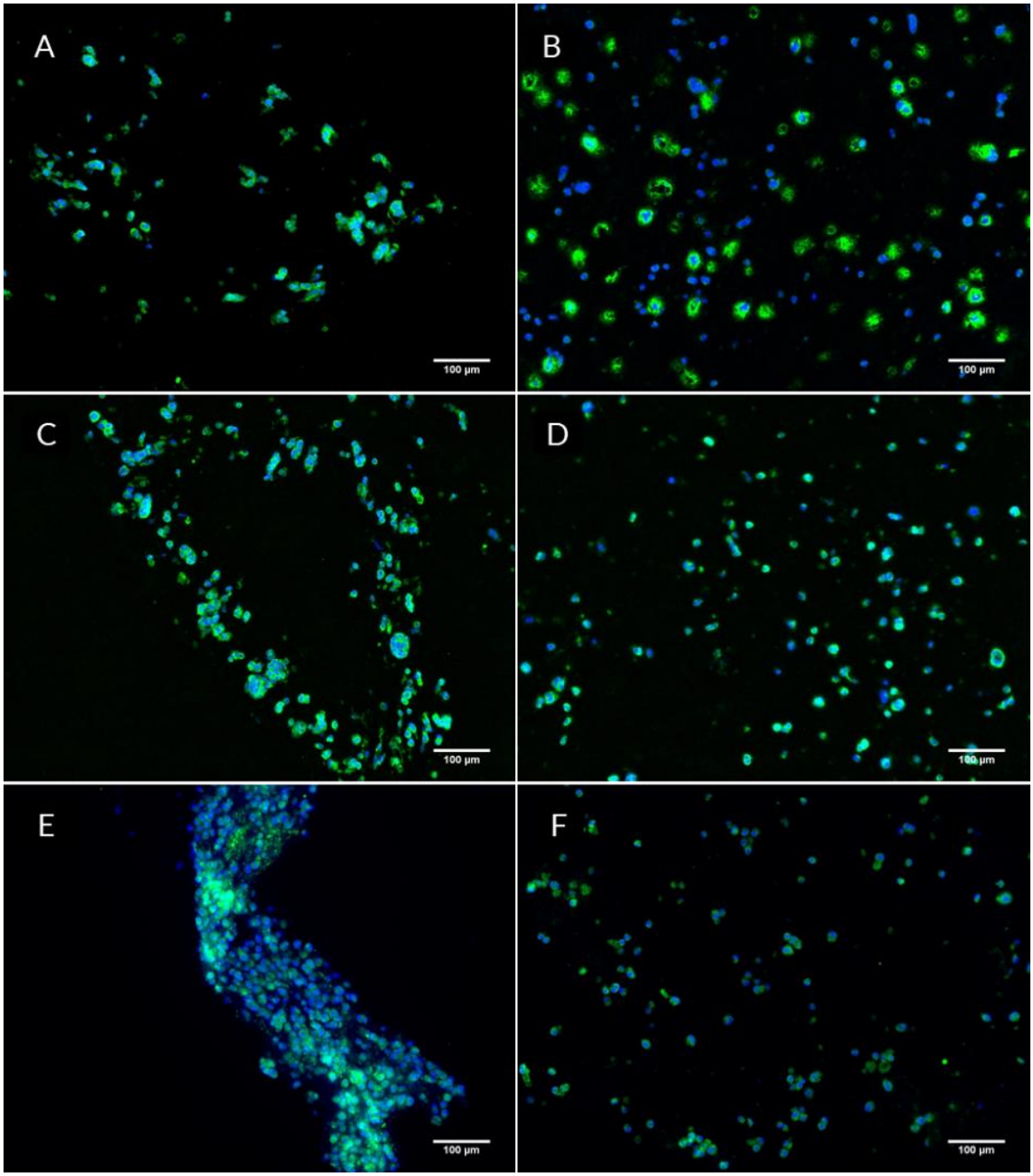

Figure 1. *A-B) Collagen type 1 production in co-cultured HepG2 and LX2 in (A) GelXA LAMININK 111 and (B) CELLINK LAMININK 111 at day 7 of culture. Collagen type 1 expression appears in green (1:50 dilution), and nuclei stained with DAPI appear in blue (1:50 dilution). Secondary antibody: Alexa Fluor 488 conjugated. Scale bar: 100 µm. C-D) The ABCC2 (MRP2) antibody* staining of hepatocyte HepG2 in co-cultured constructs bioprinted with (C) GelXA LAMININK 111 and (D) CELLINK LAMININK 111. ABCC2 expression appears in green, and nuclei stained with DAPI appear in blue (1:50 dilution). E-F) The CYP3A43 antibody staining of primary hepatocytes in E) clusters and F) single cells. CYP3A43 expression appears in green (1:1000 dilution), and nuclei stained with DAPI appear in blue (1:50 dilution). Secondary antibody: Alexa Fluor 488 conjugated. Scale bar: 100 µm.*