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BIOPRINTING PROTOCOL

Alginate 5%

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 bioprinting of Alginate 5% using the INKREDIBLE, INKREDIBLE+, BIO X or BIO X6. The protocol covers the steps of pre-print mixing with cells, 3D bioprinting and post-print processes such as ionic crosslinking. It was optimized for Alginate 5% diluted with a cell suspension at a 10+1 volume ratio. Changing the parameters in the protocol might change the crosslinking time required. This protocol was optimized for the pneumatic printhead on the BIO X and BIO X6. For printing complex multi-layered structures, it is recommended to mix your alginate hydrogel with a thickener such as Nanofibrillated Cellulose, Xanthan Gum or Glucomannan also available from CELLINK.

Materials needed

- Alginate 5%
- Cells* + cell culture medium*
- Female/female Luer lock adaptors*
- 3 mL syringes with Luer lock connections
- Positive displacement pipette + pipette tips (optional)
- CELLMIXER* (optional)
- Cartridges, 3cc*
- Bioprinting nozzles or needles, 22G-27G recommended*
- BIO X*, BIO X6* or INKREDIBLE series* 3D bioprinter
- Well plate or Petri dish*
- 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 printheads and print bed at room temperature, i.e. 20-25°C.

1. Preparing the bioink

MATERIAL

Alginate 5%

DESCRIPTION

- If applicable, dilute Alginate 5% to the desired concentration, see *Dilution Protocol Alginate 5%.*
- Warm up the Alginate solution to 37°C to make it softer and thus easier to mix with cells (refer to Figure 1).
- *If not printing with cells, transfer the alginate to a cartridge and move directly to step 3.*

2. Mixing the bioink with cells

MATERIAL

3 mL syringes with Luer lock connections

Female/female Luer lock adaptor

Pre-warmed Alginate solution

Cell suspension

Cartridge, 3cc

CELLMIXER (optional)

Positive displacement pipette + pipette tips (optional)

DESCRIPTION

- At this point, mix ten parts of bioink with one part of cell suspension, taking care not to introduce air bubbles to the mixture. For detailed instructions see the *Mixing cells with bioink Protocol.*
- If preparing for quantities < 2 mL of Alginate, it is recommended to connect two 3 mL Luer lock syringes, one with the bioink and the other with the cell suspension and gently mix back and forth between the syringes until homogeneous. 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.
- If using larger quantities, the CELLMIXER can be used:
    - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor.
    - Transfer the bioink to the 12 mL syringe (PART 2) using a female/female Luer lock adaptor.
    - Clip both syringes to the Dispensing unit (PART 3).
    - Connect the two syringes to the Mixing unit (PART 4), then connect the Empty cartridge (PART 5) to the Mixing unit's other side.
    - Apply gentle pressure onto the Dispensing unit to mix the content of both syringes into the empty cartridge. Cap the cartridge with a tip cap.

Note: Transferring viscous Alginate solutions may be facilitated by using a positive displacement pipette.

Note: To avoid introducing air when connecting the syringes, carefully pre-fill the Luer lock adaptor with Alginate before attaching it to the syringe with the cell suspension.

3. Preparation for printing

MATERIAL

Alginate mixed with cells (if applicable) in cartridge

Bioprinting nozzles or needles, 22-27G

DESCRIPTION

- If applicable, set the desired temperature on the Temperature-controlled printhead or pneumatic printhead.
- Cap the cartridge with a printing nozzle or needle of choice and place in printhead. Connect the cartridge to the air adapter.

Note: Test the flow of the bioink after the calibration is performed and start with a low pressure and increase stepwise.

4. Printing

MATERIAL

BIO X, BIO X6 or INKREDIBLE series bioprinter

Well plate or Petri dish

DESCRIPTION

- Bioprint structures using suggested parameters (Table 1) on to a well plate or Petri dish. If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material.

Note: Using the Syringe Pump printhead in combination with the BIO X allows for better control over extrusion rates.

Table 1. Recommended minimal extrusion pressure** (±2 kPa) used for printing continuous filaments at 20-25°C $^{diluted}/_{undiluted}$ bioink. 'Diluted' assumes a mixture of one part of cell suspension and ten parts of bioink. For smaller dilutions, the pressure needs to be increased towards the pressure used for undiluted bioink.

Printing speed (mm/s) → Nozzle size (G) ↓	**5**	**10**	**15**	**20**
22	9 / 16	11 / 20	13 / 23	16 / 29
25	9 / 16	13 / 21	16 / 25	19 / 31
27	10 / 16	13 / 22	16 / 30	19 / 36

***Note this is only a recommended reference of starting pressures. The actual pressure needed will vary depending on the preparation procedures (amount of bioink and actual temperature of the bioink) as well as the fitting of the piston in the cartridge and the leveling of the print surface. This table was generated with printhead temperature of 24°C and with a 10+1 bioink dilution with PBS.*

5. Crosslinking

MATERIAL

Crosslinking Agent

Cell culture medium

DESCRIPTION

Alginate hydrogels can be crosslinked with ions using the $CaCl_2$ containing Crosslinking Agent.

- Submerge the cell-laden constructs in the Crosslinking Agent for 30 seconds to 5 minutes depending on construct size, infill density and desired construct stiffness. Remove the Crosslinking Agent and rinse constructs with basal culture media once.

Note: 30 seconds is recommended for 10 µL droplets while 10 minutes might be required for dense 1 cm^3 blocks. In addition, optimize the crosslinking depending on the cell type.

6. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- After crosslinking and washing, 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 your application.

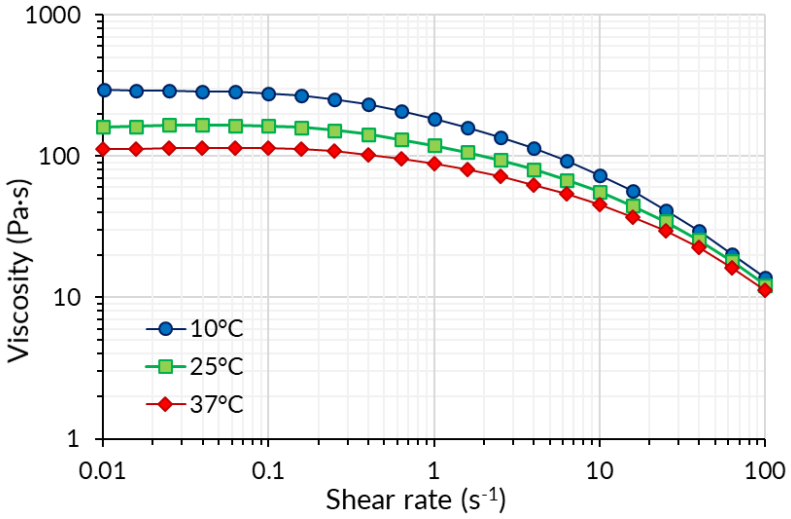

***Figure 1**. Viscosity behavior of Alginate 5% at different temperatures, over a shear rate range of 0.01 to 100 s^{-1}.*