Ref No: BPR- IKA325000503
Date: 18-DEC-2020
Author: PT, JB, AB, VK. Version: 2

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 installed at 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%*
- Crosslinking Agent*, included with the product
- Cartridges, 3cc*
- Sterile bioprinting nozzles or needles, 22G-27G recommended*
- BIO X*, BIO X6* or INKREDIBLE series* 3D Bioprinter
- Well plate or Petri dish*
- Cells + cell culture medium*
- Female/female Luer lock adaptors*
- Syringes with Luer lock connections
- Positive displacement pipette + pipette tips (optional)
- CELLMIXER*

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

Protocol

This protocol can be performed with printheads and print bed set to room temperature, *i.e.* 20-25°C.

Step	Title	Material	Description
1	Preparation of the bioink	- Alginate 5%	If not printing with cells move directly to step 3. - Warm up Alginate 5% to room temperature to make it softer and thus easier to mix with cells (refer to Figure 1).
2	Mixing the bioink with cells	- Cell suspension - CELLMIXER - Luer lock adaptor - Syringes with Luer lock connections - Prewarmed Alginate 5%. - Positive displacement pipette + pipette tips (optional)	- Mix ten parts of the bioink with one part of cell suspension without introducing air bubbles to the mixture. Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock adaptor with Alginate 5% before attaching the syringe with the cell suspension. Note: Transferring viscous Alginate 5% may be facilitated by using a positive displacement pipette. Note: If preparing bioink quantities >2 mL, use CELLMIXER. If preparing for bioink quantities <2 mL, it is recommended to connect two 3 mL Luer lock syringes and mix the bioink and the cell suspension back and forth between the syringes until a homogeneous mixture is achieved.
3	Preparation for printing	- Cartridge, 3cc - Alginate 5% (mixed with cells if applicable) - Luer lock adaptor - Sterile bioprinting nozzles or needles, 22-27G	- Load a cartridge with the bioink using a Luer lock adaptor. - Place the bioink in the printhead and cap with a printing nozzle or needle of choice. - Choose the desired printing temperature for the bioink (refer to Figure 2).
4	Printing	- Bioprinter (BIO X or INKREDIBLE series recommended) - Well plate or Petri dish	- Bioprint structures using suggested parameters (Table 1). 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.

5	Crosslinking	- Crosslinking Agent - Cell culture medium	Alginate hydrogels can be crosslinked with multivalent ions such as Ca^{2+}, which is present in the Crosslinking Agent. - Submerge the cell-laden constructs in the Crosslinking Agent for 30 seconds to 5 minutes depending on construct size and desired construct stiffness. Remove the Crosslinking Agent and rinse constructs with basal culture media once.
6	Incubation	- Cell culture medium	- 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.

Table 1. Recommended minimal extrusion pressure** (±2 kPa) used for printing continuous filaments at 20-25°C using $^{\text{diluted}}/_{\text{undiluted}}$ bioink. 'Diluted' assumes a mixture of one part of PBS to ten parts of bioink, which is the simulation of bioink and cell suspension mixing conditions. 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.*

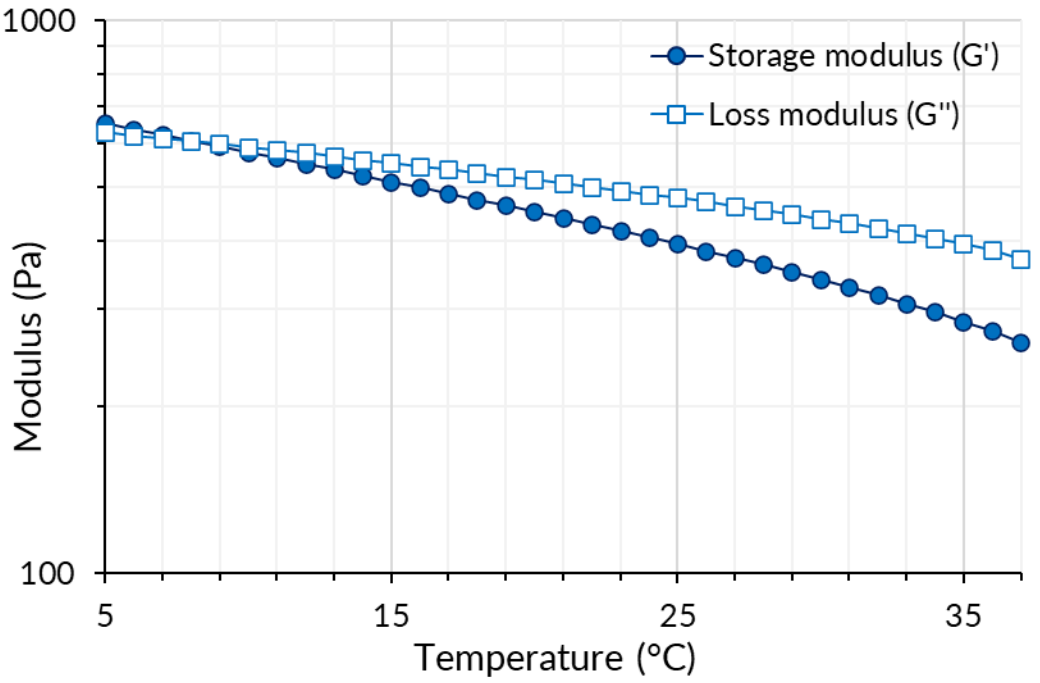

Figure 1. Solidification of Alginate 5% at temperatures below ≈8°C expressed as crossover of storage and loss moduli curves.

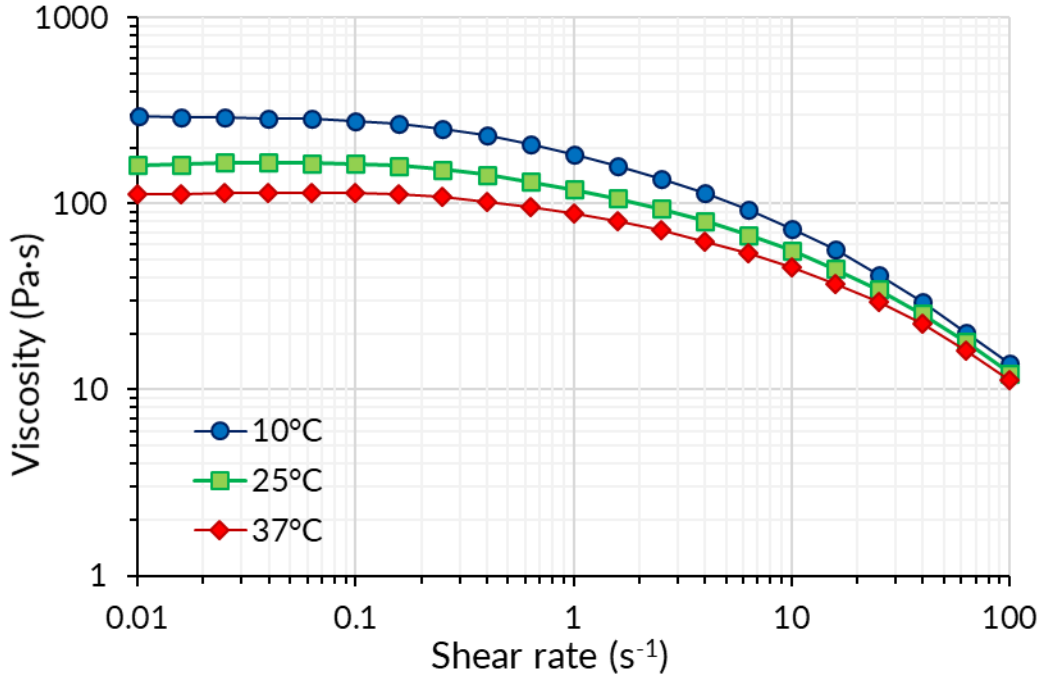

Figure 2. Viscosity behavior of Alginate 5% at different temperatures, over a shear rate range of 0.01 to 100 s^{-1}.