Ref No: BPR-IK-3X0102
Date: 25-NOV-2019
Author: EP, JB, VK. Version: 3

Bioprinting Protocol

GelXG

This is a suggested procedure, please adjust according to your experimental needs.

Protocol aim

The aim of this protocol is to provide instructions for bioprinting with the GelXG bioink using the INKREDIBLE, INKREDIBLE+, or BIO X, with and without cells. This document covers pre-print mixing with cells, 3D bioprinting and post-print processes of crosslinking ionically or through photocuring. This protocol was optimized for GelXG with LAP 0.25% undiluted as well as a 10+1 cell suspension dilution. Changing the concentration of LAP or bioink to cell suspension ratio will change the photocrosslinking time. Reference the *Photocrosslinking Optimization Protocol* to adjust and determine these numbers. This protocol was optimized using the Temperature-controlled printhead using the BIO X.

Materials needed

- GelXG bioink*
- UV shielding cartridges, 3cc*
- Sterile conical bioprinting nozzles, 22-27G*
- BIO X or INKREDIBLE series 3D Bioprinter*
- Well plate or Petri dish*
- 405/365 nm light modules for photocuring
- Cells + cell culture medium
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
  or
- CELLMIXER*

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

KEEP THE BIOINK PROTECTED FROM LIGHT IF TRANSFERRED FROM THE ORANGE UV PROTECTED CARTRIDGES TO AVOID CROSSLINKING BEFORE PRINTING. WORK WITH 3D PRINTERS IN DARK MODE. THE PHOTOINITIATOR IS SENSITIVE TO REPEATED OR PROLONGED EXPOSURE TO HEAT.

Boston, USA
75 Kneeland Street
Boston, MA 02111

Gothenburg, Sweden
Arvid Wallgrens Backe 20,
Gothenburg, 41346

Blacksburg, USA
2000 Kraft Dr, Suite 2125
Blacksburg, VA 24060

Kyoto, Japan
46-29 Yoshida-Shimo Adachi-cho, Sakyo-ku, Kyoto

www.cellink.com | +1 (833) CELLINK

Protocol

This protocol works best using the BIO X with the cooled print bed at 15°C and the Temperature-controlled printhead at 24°C. The GelXG can also be extruded using the BIO X pneumatic printheads or the INKREDIBLE series, but with decreased shape fidelity if ambient temperature exceeds 25°C and the printhead heats up. If using the INKREDIBLE series, the printing substrates such as Petri dishes or well plates should be placed on ice or another cooled surface to thermally gel the construct after printing prior to photocrosslinking.

Step	Title	Material	Description
1	Prepare bioink	- GelXG	If not printing with cells move directly to step 3. - Heat up GelXG in a cartridge/syringe at 37°C for 10 min. The heating of the GelXG can be performed in a pneumatic printhead, water bath or incubator. Prolonged and repeated heating could negatively affect the photoinitiator stability and the homogeneity of the bioink, requiring additional mixing before adding cells. If not using the entire 3 mL of the bioink in the cartridge, transfer the needed amount to a syringe using a female/female Luer lock adaptor and spare the rest of the bioink in the optimal storage conditions. Note: If there are bubbles in the bioink, make a quick centrifugation for 1.5 min at 1600 rpm.
2	Mix GelXG with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelXG	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 Protocol GelX Series*. - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor. - Transfer GelXG 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 units from another side. - Apply gentle pressure onto the dispensing unit to mix the content of both syringes into the empty cartridge. Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock

Boston, USA
75 Kneeland Street
Boston, MA 02111

Gothenburg, Sweden
Arvid Wallgrens Backe 20,
Gothenburg, 41346

Blacksburg, USA
2000 Kraft Dr, Suite 2125
Blacksburg, VA 24060

Kyoto, Japan
46-29 Yoshida-Shimo Adachi-cho, Sakyo-ku, Kyoto

www.cellink.com | +1 (833) CELLINK

			adaptor with GelXG before attaching the syringe with the cell suspension. If preparing for quantities <2 ml of GelXG, it is recommended to connect two 3 mL Luer lock syringes and mix the bioink back and forth between the syringes until it becomes homogeneous.
3	Cool and load the cartridge	- UV shielding cartridges, 3cc loaded with GelXG (and cells) - Sterile conical bioprinting nozzles, 22-27G	- Place cartridge on counter for 20 min to reach room temperature. If the bioink has been pre-heated, it can instead be placed in a fridge for 3-5 min, or in the Temperature-controlled printhead at 24°C for 5 min for faster cooling. Note: Room temperature is within 20-25°C. - Place the room tempered GelXG in the printhead and cap with the printing nozzle. If using the BIO X, pre-cool the print bed to 15°C. Note: When printing with GelXG, the recommended printhead temperature for the highest printing fidelity is 20-25°C, though the bioink can be dispensed up to 32°C.
4	Printing	- Bioprinter (BIO X or INKREDIBLE series) - Well plate or Petri dish	- Bioprint structures with parameters according to Table 1. 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.

Table 1. Recommended minimal extrusion pressure** (±2 kPa) for printing continuous filaments at 20-25°C using $^{diluted}/_{undiluted}$ 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	27 / 29	29 / 34	30 / 39	32 / 40
25	24 / 32	29 / 35	30 / 38	33 / 40
27	30 / 35	34 / 40	36 / 39	41 / 41

***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.*

Step	Title	Material	Description
5	Crosslinking	- 405/365 nm light modules for photocuring	GelXG can be photocrosslinked using the 405 or 365 nm light modules. See Table 2 below for recommended crosslinking times. Ensure that the bioprinted GelXG construct is thermally gelled after printing by cooling the print bed (if using the BIO X) or placing the printing substrates with the construct on ice for 10 s (if using the INKREDIBLE series). If photocrosslinking during bioprinting, set the crosslinking parameters appropriately in the G-code for the INKREDIBLE series or the printhead setup page for the BIO X. Note: It is recommended to use the 405 nm light module instead of 365 nm one if possible. Overexposure might damage the cells. Note: To verify the crosslinking is sufficient, add 37°C media to one printed well and observe that it doesn't dissolve.

Table 2. Recommended time of the construct photocrosslinking***. Distance from each light module to construct was set to 5 cm using the BIO X photocuring modules. If using the INKREDIBLE series photocuring modules, the time required can possibly be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelXG with mesenchymal stem cells. Don't exceed the exposure time to more than 120 s when printing with cells. To achieve the best structural integrity when printing thicker constructs, it is recommended to apply 3/5 s photocrosslinking with 365/405 nm LED every fourth layer. If bioink is used above 25°C, the best results can be achieved when applying 3/5 s photocrosslinking with 365/405nm LED every second layer.

	365 nm, LAP 0.25%	405 nm, LAP 0.25%
Construct depth (mm) /time (s)	1/5	1/10
	3/20	3/45

****Note: This is only a recommended reference of crosslinking times to start with. The actual time needed for crosslinking will vary depending on the size and temperature of the constructs as well as the intensity of the photocuring module and the distance to the construct.*

Step	Title	Material	Description
6	Incubation	- Cell culture medium	- After photocrosslinking, 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.