

BIOPRINTING PROTOCOL

GeIXA BONE

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 GeIXA BONE bioink using the INKREDIBLE, INKREDIBLE+, BIO X or BIO X6, with and without cells. This document covers pre-print mixing with cells, 3D bioprinting and post-print processes such as ionic crosslinking and photocuring. This protocol was optimized for GeIXA BONE with LAP 0.25% undiluted as well as a 10+1 cell suspension dilution. Changing the bioink to cell suspension ratio and thus also the concentration of photoinitiator, will change the photocrosslinking time. Refer to 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

- GeIXA BONE bioink*
- Cells* + cell culture medium*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER* (optional)
- UV shielding cartridges, 3cc*
- Conical bioprinting nozzles, 22-27G*
- Temperature-controlled printhead (optional)
- 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.

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.

Protocol

This protocol works best using the BIO X or BIO X6 with the cooled print bed at 15°C and the Temperature-controlled printhead at 27°C. The GelXA BONE can also be extruded using the BIO X/BIO X6 pneumatic printheads or the INKREDIBLE series, but with decreased shape fidelity if ambient temperature exceeds 27°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. First time users of GelMA based bioinks are recommended to optimize the printing conditions without cells before proceeding to bioprint with cells. Perform the desired dilution using medium or PBS.

1. Preparing the bioink

MATERIAL

GelXA BONE

Female/female Luer lock adaptor

3 mL syringes with Luer lock connections

Pipette tip or spatula

DESCRIPTION

- Heat up the GelXA BONE cartridge at 37°C until it becomes liquid. The heating of the GelXA BONE can be performed in a printhead or incubator and usually requires 30-60 min if the bioink is taken directly from the fridge.

Note: 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.

- To make sure that the bioink is homogeneous after heating: 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 GelXA BONE before re-attaching the cartridge. Gently mix the bioink back and forth between the cartridge and syringe to homogenize the bioink. If not using the entire 3 mL of the bioink in the cartridge, keep the rest of the bioink in the optimal storage conditions. Prolonged and repeated heating could negatively affect the photoinitiator stability.

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

- *If not printing with cells 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 GelXA BONE

Cell suspension

UV shielding cartridge, 3cc

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

3. Preparation for printing

MATERIAL

GelXA BONE mixed with cells (if applicable) in UV shielding cartridge

Temperature-controlled printhead (optional)

Conical bioprinting nozzles, 22-27G

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 27°C for 10 minutes. If not using the Temperature-controlled printhead, place the cartridge on counter for 5-10 minutes to reach approximately 27°C.
- If the cartridge has cooled down below 26°C, re-heat the cartridge at 37°C for 5 min to reset, then restart equilibration in printhead.
- Cap the cartridge with a bioprinting nozzle and place the GelXA BONE in the printhead. Connect the cartridge to the air pressure adapter. If using the BIO X or BIO X6, pre-cool the print bed to 15°C.

Note: When printing with GelXA BONE, the recommended printhead temperature for the highest printing fidelity is 27°C, though the bioink can be dispensed up to 32°C. Below 26°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.

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

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

Example: If printing continuous filaments with a Temperature-controlled printhead set to 27°C, a 25G nozzle, a printing speed of 5 mm/s and with 300 ms pre-flow delay, the suggested pressure range is between 25-35 kPa without cell suspension dilution and 20-30 kPa if diluted with cell suspension.

- If 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 temperature than before. Let the bioink reach the new temperature before starting to print again.
- During print sessions longer than 20 min at 27°C the bioink can become too viscous due to continued gelling resulting in chunky filaments and too high extrusion pressures needed. To avoid this, a 0.5°C increase in printhead temperature after 20 min printing can extend the time the bioink remains at good printability.
- If the regular pneumatic printheads are used for a long period, they might heat up above the desired printing temperature. Then the bioink also heats up which decreases its viscosity, observed as extrusion of very thick filaments even at low pressures. Remove the cartridge from the printhead and allow to cool down to 27°C. In addition, remove the printheads from the BIO X/BIO X6 to let them cool down before continuing to print.

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.

5. Crosslinking

MATERIAL

Crosslinking Agent

AND/OR

405/365 nm LED modules for photocuring

Cell culture medium

DESCRIPTION

- GelXA BONE can be photocrosslinked using the 405 or 365 nm LED modules or ionically crosslinked using the CaCl₂-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.

- See Table 1 below for recommended photocrosslinking times.
- **Photocrosslinking:** Ensure that the bioprinted GelXA BONE construct is thermally gelled after printing by cooling the print bed (if using the BIO X or BIO X6) or placing the printing substrates with the construct on ice for 10 seconds (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 or BIO X6.

Note: To verify that the photocrosslinking is sufficient, add 37°C medium 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 5 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 μ L droplets while 10 minutes might be required for dense 1 cm³ blocks. In addition, optimize the crosslinking depending on the cell type.

Table 1. Recommended time of the construct photocrosslinking**. Distance from each light module to construct was set to 5 cm using the BIO X/BIO X6 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 GelXA BONE with mesenchymal stem cells. Don't exceed 120 seconds of exposure time when printing with cells. To achieve the best structural integrity when printing thicker constructs, it is recommended to apply 3 or 5 seconds photocrosslinking with 365 or 405 nm light respectively, every fourth layer.

	365 nm, LAP 0.25%	405 nm, LAP 0.25%
1 mm construct thickness	5 seconds	10 seconds
3 mm construct thickness	15 seconds	30 seconds

**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.

6. 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% CO₂ and 95% relative humidity) or according to your application. Replace medium regularly.