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BIOPRINTING PROTOCOL

CELLINK RGD

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 CELLINK RGD using the INKREDIBLE, INKREDIBLE+, BIO X or BIO X6, and covers steps from pre-print mixing with cells, 3D bioprinting and post-print processes such as ionic crosslinking. This protocol was optimized for CELLINK RGD, undiluted as well as using a 10+1 cell suspension dilution. Changing the parameters in the protocol might change the crosslinking time required. This protocol was optimized using the pneumatic printhead on the BIO X.

Materials needed

- CELLINK RGD*
- Cells* + cell culture medium*
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER* (optional)
- Clear cartridges, 3cc*
- Conical bioprinting nozzles, 22-27G 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, where room temperature is between 20-25°C.

1. Preparing the bioink

MATERIAL

CELLINK RGD

3 mL syringe with Luer lock connections

Female/female Luer lock adaptor

Pipette tip or spatula

DESCRIPTION

- Mix the full 3 mL of CELLINK RGD a few times to make sure it is homogenous. Connect the cartridge to a 3 mL syringe using a Luer lock connector and remove the end cap. Push 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. Gently mix the bioink back and forth between the cartridge and syringe to homogenize the bioink, taking care not to introduce air. If not using the entire 3 mL of the bioink in the cartridge, spare the rest of the bioink in the optimal storage conditions.
- Warm up CELLINK RGD to room temperature. Only warm the needed volume.
- *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 CELLINK RGD

Cell suspension

Cartridge, 3cc

CELLMIXER (optional)

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.*
- If preparing for quantities < 2 mL of CELLINK RGD, 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: To avoid introducing air when connecting the syringes, carefully pre-fill the Luer lock adaptor with CELLINK RGD before attaching it to the syringe with the cell suspension.

3. Preparation for printing

MATERIAL

CELLINK RGD mixed with cells (if applicable) in cartridge

Conical bioprinting nozzles, 22-27G

DESCRIPTION

- Cap the cartridge with a printing nozzle of choice and place the room tempered CELLINK RGD in the printhead. Connect the cartridge to the air adapter.

Note: The recommended nozzle size is 22G. Decrease the nozzle diameter to achieve smaller filament diameter, however this also increase the risk of the bioink clogging.

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

- Bioprint structures with parameters according to 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: 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) used for printing continuous filaments at 20-25°C $^{\text{with cells}}/_{\text{without cells}}$. Again, 'with cells' assumes a mixture of one part cell suspension and ten parts bioink. For highly concentrated cell suspensions, 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	6 / 11	8 / 13	9 / 14	11 / 17
25	8 / 12	10 / 15	11 / 17	12 / 17
27	10 / 13	14 / 15	15 / 17	16 / 20

*** 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 bioink temperature of 22°C and with a 10+1 bioink dilution with cell suspension.*

5. Crosslinking

MATERIAL

Crosslinking Agent

Cell culture medium

DESCRIPTION

CELLINK RGD is 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 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 in 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.