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Bioprinting Protocol

CELLINK SKIN

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

Material needed

- CELLINK SKIN*
- Clear cartridges, 3cc*
- Sterile Conical Bioprinting nozzles*
- BIO X* or INKREDIBLE-series* 3D Bioprinter
- Well plate or Petri dish
- Crosslinking agent (included with the bioink purchase)
- Vial with 100 U thrombin (included with the bioink purchase)
- Cells + cell culture medium
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptor*
  or
- 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 at room temperature, where room temperature is between 20-25°C.

Step	Title	Material	Description
1	Prepare bioink	- CELLINK SKIN	If not printing with cells move directly to step 3.

			- Warm up the CELLINK SKIN cartridge to room temperature.
2	Mix CELLINK SKIN with cells	- 3 mL syringes with Luer lock connections - Prewarmed CELLINK SKIN - Female/female Luer lock adaptor - Cell suspension in syringe	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 Protocol CELLINK Series*. - 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 units other 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 adaptor with CELLINK SKIN before attaching the syringe with the cell suspension. If preparing for quantities < 2 mL of CELLINK SKIN, it is recommended to connect two 3 mL Luer lock syringes and mix back and forth between the syringes until homogeneous.
3	Cool and load the cartridge	- Clear cartridges, 3cc loaded with CELLINK SKIN (and cells) - Sterile Conical Bioprinting nozzles.	- Place the CELLINK SKIN on the counter to reach room temperature. If the bioink has been pre-heated, it can instead be placed in a fridge for 3-5 min. - Attach a nozzle to the cartridge and mount it into the printhead. The recommended nozzle size is 22G. Decrees the nozzle diameter to achieve smaller filament diameter, however this also increase the risk of the bioink clogging.
4	Printing	- Bioprinter (BIO X or INKREDIBLE series recommended) - 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) used for printing continuous filaments at 21-25°C $^{\text{with cells}}/_{\text{without cells}}$. Again, 'with cells' assumes a mixture of one part cell suspension to ten parts bioink. For information about filament diameter, see Printing parameters in the *Application note*. For highly concentrated cell suspensions, the pressure needs to be increased towards the pressure used for undiluted bioink.

Printing speed (mm/sec) → Nozzle size (G) ↓	5	10	15	20
22	9 / 11	11 / 12	12 / 14	13 / 15
25	10 / 12	11 / 14	13 / 15	15 / 17
27	11 / 15	13 / 16	14 / 17	15 / 20

**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 at 23°C and with a bioink dilution with a low concentration of cells.*

Step	Title	Material	Description
5	Crosslinking	- Crosslinking Agent - Thrombin - Cell culture medium	CELLINK SKIN is crosslinked with ions using the $CaCl_2$ Crosslinking Agent. Thrombin is reconstituted in cell culture medium and supplied with the constructs overnight. - Ionic crosslinking: Submerge the cell-laden constructs in the Crosslinking Agent for 30 s to 5 min depending on construct size. Remove the Crosslinking Agent and rinse the constructs with basal culture media once. - Thrombin: Reconstitute the thrombin in 10 mL of cell culture medium by adding 1 mL of the medium to the thrombin vial. Then transfer the 1 mL of thrombin solution back to the 9 mL of medium to receive a 10 U/mL dilution. Mix gently by pipetting up and down 2-3 times. Submerge the samples in the thrombin containing medium and incubate overnight in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.
6	Incubation	- Cell culture medium	- The next day, switch the thrombin containing medium to regular cell culture medium and incubate in standard culture conditions or according to your application.