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Bioprinting Protocol

CELLINK BONE

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 CELLINK® BONE 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® BONE, 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.

Material needed

- CELLINK® BONE*
- 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)

- 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/*.

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Protocol

This protocol can be performed with printheads and printbed at room temperature, where room temperature is between 20-25°C.

Step	Title	Material	Description
1	Prepare bioink	- CELLINK® BONE	If not printing with cells move directly to step 3. - Warm up CELLINK® BONE a cartridge to room temperature.
2	Mix CELLINK® BONE with cells	- 3 ml syringes with luer lock connections - Prewarmed CELLINK® BONE - 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® BONE before attaching the syringe with the cell suspension. If preparing for quantities < 2 ml of CELLINK® BONE, 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® BONE (and cells) - Sterile Conical Bioprinting nozzles.	- Place the room tempered CELLINK® BONE in the printhead and cap with a printing nozzle of choice. Note: Not recommended to use a nozzle with smaller diameter than 25G (such as 27G) due to risk of clogging.

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4	Printing	- Bioprinter (BIO X or INKREDIBLE series recommended)	- Bioprint structures with parameters according to Table 1 or the Printing parameters in the *Application note*. 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	4 / 13	5 / 15	5 / 17	6 / 19
25	4 / 22	8 / 25	9 / 28	10 / 33

**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 solution - Cell culture medium	CELLINK® BONE can be crosslinked with ions using the $CaCl_2$ crosslinking solution. - Submerge the cell-laden constructs in the crosslinking solution for 30 sec to 5 min depending on construct size. Remove crosslinking solution 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 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 your application.

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