Axolotl Biosciences

Bioprinting User Manual

TissuePrint - LV

Aspect Biosystems RX1

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Contents

Welcome!

Axolotl Biosciences is driven by innovation, integrity, and collaboration, and we strive to deliver the best bioprinting experience to our users. This manual will be your guide to printing with Axolotl Biosciences' TissuePrint bioink on using the Aspect Biosystems RX1 bioprinter and Lab-on-a-Chip™ technology. This ink is a xeno-free fibrin-based bioink which supports multiple cell lines such as human induced pluripotent stem cells (HiPSCs), neural progenitor cells (NPCs), and mesenchymal stem cells (MSCs). TissuePrint comes in both high viscosity (HV) and low viscosity (LV) formulations. This user manual is designed specifically for the TissuePrint-LV formulation, which is optimized for microfluidic based bioprinting. TissuePrint-HV, which is optimized for extrusion based bioprinting, is also available, visit www.axolotlbiosciences.com for more information.

1.0 Materials, Reagents, and Equipment

1.1 Product Map

What's inside the box:

1.2 Material and Equipment Required for Printing with the Aspect Biosystems RX1 printer

Purchased from Axolotl Biosciences

- ❑ TissuePrint Component 1
- ❑ TissuePrint Component 2
- ❑ TissuePrint Component 3
- ❑ Crosslinker Component A
- ❑ Crosslinker Component B

Purchased from Aspect Biosystems

- ❑ Buffer solution
- ❑ RX1 Bioprinter + accessories
- ❑ DUO-1 Printhead
- ❑ Material tubing + nylon sheet
- ❑ Vacuum chuck

Material Supplied by User

- ❑ Culture plate
- ❑ Cells + cell culture media
- ❑ 15mL conicals (VWR cat no:
- ❑ CA21008−940)
- ❑ 125 mL bottle (VWR cat no: 16120−741)
- ❑ Autoclave safe rinse bottle (VWR cat no: 16651−904)
- ❑ Anti-adherence rinsing solution (optional) (STEMCELL cat no: 05835)
- ❑ 10mL syringe + 18 gauge blunt needle (optional)
- ❑ Wide bore 1mL pipette tips (or cut off ends of regular 1mL tips)
- ❑ Spatulas (assorted)
- ❑ Tweezers
- ❑ 2 conical racks
- ❑ Small waste beaker (50mLs?)
- ❑ 1 Large beaker (200-250mLs) filled with ice
- ❑ 1 Large waste beaker

2.0 Protocol

The following procedure describes bioink preparation, bioprinting, and tips for culturing and analyzing 3D scaffolds. Troubleshooting information is included at the end of each protocol step where it may apply. A graphical overview of the procedure is included at the end of this section. For additional support and resources please visit www.axolotlbiosciences.com or email info@axolotlbiosciences.com.

2.1 Preparation

Note: You may want to set up the bioprinter (Steps #-# Section 2.2.1) before mixing bioink, especially if you are unfamiliar with this procedure.

2.1.1 Preparing Bioink:

1. Let Component 1-LV and Component 3-LV thaw at 4°C (approximately 4 hours for 5mL solution). Once completely thawed, thaw Component 2-LV at room temperature.
2. Using a p200 pipette, pipette Component 2-LV into Component 1-LV. Component 2-LV may clump upon addition to Component 1-LV. If this occurs, use a p1000 pipette with a wide bore pipette tip and slowly pipette up and down to disperse the clump.
3. Using a p1000 pipette, pipette Component 3-LV into solution. Slowly mix into the bioink by stirring with the pipette or pipetting up and down. Avoid generating air bubbles.

2.1.2 Preparing Bioink to include Cells:

1. Let Component 1-LV thaw at 4°C (approximately 4 hours for 5mL solution).
2. Once Component 1-HV is completely thawed, thaw Component 2-LV at room temperature and Component 3-LV at 37°C.
3. To formulate bioink: Using a p200 pipette, pipette Component 2-LV into Component 1-HV. Component 2-LV may clump upon addition to Component 1-LV. If this occurs, use a p1000 pipette with a wide bore pipette tip and slowly pipette up and down to disperse the clump, avoiding the introduction of bubbles. You can also slowly stir the solution with the pipette tip to disperse any clumps.
4. Prepare desired cells for resuspension in Component 3-LV. Either thaw or harvest from culture dishes as per cell specific harvesting protocols, into a conical tube and use cell specific media to obtain a final volume of 10 mL.
5. Centrifuge at 300g for 5 minutes, or according to culturing protocols.

6. Remove supernatant from conical using an aspirator or 10mL pipette and gently resuspend the cell pellet in Component 3-LV using a p1000 and regular tip (or 5mL pipette etc), avoiding the introduction of air bubbles.
7. Slowly add cells in Component 3-LV solution to the bioink using a p1000 pipette. Slowly mix into the bioink by stirring with the pipette or pipetting up and down. Avoid generating air bubbles.

2.1.3 Preparing Crosslinker

1. Let Component A-LV and Component B-LV thaw at 4°C (approximately 2 hours for a 3mL solution).
2. Pipette Component B-LV to Component A-LV. Slowly mix by stirring with the pipette or pipetting up and down. Avoid generating air bubbles.
3. Crosslinker should be transferred to a 125mL bottle (see Section 1.2) before printing.

2.1.4 Troubleshooting

Avoiding Air Bubbles

Minimizing the formation of air bubbles when preparing bioink will yield the best result. When preparing the bioink and incorporating cells, avoid over dispensing the pipette while pipetting and mixing components, as this can introduce air pockets. For more viscous solutions, using a wide bore pipette when mixing may help decrease air bubble formation. If there are air bubbles in the bioink, centrifuging the bioink conical may help decrease the amount of air bubbles.

2.2 Bioprinting

NOTE: Please consult the Aspect Biosystems RX1 User Manual before printing and for any concerns directly related printer mechanics or software.

The bioprinter should be placed in the proper environment according to your application. If aseptic conditions are required, the bioprinter should be placed inside a biosafety cabinet (BSC), and your culture dish should be placed in a way that maintains clear airflow around it. Everything should be properly sterilized (autoclave, UV, and/or 70% ethanol) before moving it into the BSC. Extreme care is required when manipulating the printhead and tubing, and when transferring scaffolds from the print bed to the culture dish.

2.2.1 Preparing the Printhead

The following items should be properly sterilized and ready to use in the BSC before proceeding:

- Crosslinker and buffer in 125mL bottles
- Bioink in 15mL conical (either before proceeding or at Step 10)
- Pneumatic caps for crosslinker and buffer bottles
- Pneumatic cap(s) for bioink conical(s)
- DUO-1 Printhead
- Tubing (at least three tubes)
- Vacuum chuck
- Nylon sheet
- Waste beaker or petri dish
- Syringe, 18 gauge blunt needle, and anti adherence rinsing solution (optional)
- Rinse bottle with sterile water
- Sterile tweezers
- Sterile spatula
- Culture dish pre-filled with desired media
- 2 Conical rack
- 1 Large beaker (filled with ice)
- 1 Large waste beaker

1. Turn on the printer and Aspect Studios software, and load a design file according to the RX1 User Manual.
2. Place a small waste beaker of petri dish to the right on the print bed.
3. Remove the caps from the crosslinker and buffer and replace them with the pneumatic caps. Connect the crosslinker and buffer to the printer's pneumatic tubes.
4. Open and remove the printhead from its bottle using sterile tweezers, attach it to the printer, and connect the valve tubing.
5. Open a tubing package and connect one end to the buffer inlet on the print head and the other end to the buffer bottle through the brown fitting. Ensure the tube is pushed down far enough into the buffer bottle so it is submerged.
6. Open a tubing package and connect one end to the crosslinker inlet on the print head and the other end to the crosslinker bottle through the brown fitting. Ensure the tube is pushed down far enough into the crosslinker bottle so it is submerged.
7. Move the printhead so it is above the waste beaker and **Set Waste.**
8. Press the **Pressure** source button.
9. Optional steps for rinsing the material channels:
    a. Select **Manual** on the toolbar.
    b. Using an 18 gauge blunt needle, load a syringe with anti-adherence rinsing solution.
    c. Insert the needle into the Material 1 channel, manually open the channel on the software, depress the syringe and flush some solution (~0.5-1.0mL) through the channel. Repeat for Material 2 channel if desired.
    d. Use an ethanol soaked kimwipe and wipe away any excess rinsing solution. This step is crucial as the rinsing solution causes the surface of the printhead to become slippery, and the material tubes may fall out of their channels if excess rinsing solution is present.

10. If not already done, now is the time to prepare bioink and incorporate cells. You should also prepare a culture dish with your desired media to transfer constructs to once they are printed.
11. Move the bioink conical into the BSC, unscrew the cap, and replace it with a material cap.
12. Open a tubing package and connect one end to the Material 1 inlet on the print head and the other end to the bioink bottle through the brown fitting. Ensure the tube is pushed down far enough into the buffer bottle so it is submerged. Connect the bioink conical to the printer's Material 1 pneumatic tube. Repeat this step with Material 2 if you are using more than one material.
13. Set bioink and crosslinker pressures to 60mbar and buffer to 100mbar, then prime the printhead manually or using the priming sequence, according to the RX1 user manual.

2.2.2 Printer Setup and Settings

1. The recommended* settings are:
    a. Material pressure: 20mbar
    b. Crosslinker pressure: 40mbar
    c. Buffer pressure:
    d. Speed: ???

*Printing parameters may vary from printer to printer, sometimes depending on seeding density and environmental conditions. See Troubleshooting for more information. There may be a short delay before the system reaches pressure, wait until desired pressures are reached before printing.

2. Connect the vacuum chuck to the printer, then remove the nylon sheet from its packaging and place it on the vacuum chuck.
3. Press the **Vacuum** button on the software and soak the nylon sheet with sterile water from the rinse bottle.
4. Move the printhead to an appropriate home position (~0.3mm above the surface in the center of the print bed) and press **Set Home**.
5. In the **Distance** box in the software, set the distance to **0.1mm**. If you need to adjust the height of the printhead throughout the process, this is a good increment.

2.2.3 Printing

1. Ensure the desired design file is loaded
2. Press **Print**. If required, you can adjust the height of the printhead during printing. The nozzle should not be too far away that the ink is coming out in droplets, and not too close that it is dragging on the construct. The bioink should appear to be flowing.
3. Once printing has finished, press the **Waste** button.
4. Using a sterile spatula, transfer the construct from the print bed to the culture dish. You may need to use the rinse bottle to remove it from the spatula.

5. Repeat steps 2-4 until the desired amount of constructs have been printed. Periodically check bioink and crosslinker levels (every 1-2 prints) to ensure you have enough to continue.
    a. If you run out of material (bioink, crosslinker, and/or buffer) during printing:
        i. Set the empty material's pressure to 0mbar, then disconnect the material's pneumatic tube.
        ii. Unscrew the empty material's cap from the conical/bottle, remove the empty conical/bottle, then replace with a new conical/bottle with new material. Use extreme caution to ensure the tubing does not come into contact with anything. If the tubing touches anything other than the inside of the material conical/bottle, replace it with new tubing.
        iii. Reset material to pressure described in Section 2.2.2 (or desired printing pressure).
6. Once all desired constructs have been printed, use a p1000 pipette or serological pipette to remove the media from the culture dish and replace with fresh application specific media. There should be enough media to fully submerge the construct. Pipette in the corner furthest away from the construct to avoid disturbing it.
7. For a construct with cells, incubate the construct at 37°C and 5% CO_2 (or according to experiment application). For a construct without cells, store as desired in either 4°C, 37°C, or according to experiment application.

Do we need a section on take down/clean up?

2.3 Culturing 3D Constructs

Culturing protocols will vary based on the cell type and experiment. It is recommended to follow your lab's validated culturing protocols while keeping the following in mind:

- The tissue constructs will be delicate. When changing media on the constructs, it is recommended to use a 1000uL pipette (or be very careful with a serological pipette), and slowly pipette the media in the corner of the well furthest from the construct to avoid breaking it. Do not use an aspirator as this may aspirate pieces of the construct.
- With long term cultures, it is normal to see small pieces of the construct disperse from the construct. Be careful not to suck these pieces into the pipette, as they may still be attached to the main construct.
- Depending on the size of your construct, you may need more media than with a 2D cell culture. The construct should be completely covered by media.

2.4 Analyzing 3D Constructs

The 3D constructs can be analyzed in a variety of ways: live/dead staining, immunocytochemistry, flow cytometry etc. Protocols can be found at www.axolotlbiosciences.com. You might take into consideration the following for analyzing 3D tissues:

- The construct will display autofluorescence. We have found better results using ethidium homodimer-III instead of propidium iodide for staining dead cells.
- It might be necessary to increase washes and incubation times and use a shaker plate when staining the constructs.
- A benchtop instrument for tissue dissociation, such as the gentleMACS™ dissociator, can be used for breaking down the constructs for flow cytometry.

3.0 Terms and Conditions

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1. **DESCRIPTION**. BioInk allows the User to support printing of multiple different cell lines, such as somatic cells, human induced pluripotent stem cells (hiPSCs), neural progenitor cells (NPCs), and mesenchymal stem cells (MSCs). BioInk generates stable and reproducible 3D-structures (constructs) that can remain in culture for over a month, maintain long-term cultures with high viability, and can support tissue differentiation and function. The BioInk products are experimental in nature and must be used with prudence and appropriate caution, since not all of their characteristics are known. The BioInk products have not been approved for use in humans by the U.S. Food and Drug Administration or any other regulatory body and may not be used in humans.
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