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RECONSTITUTION AND BIOPRINTING PROTOCOL

PhotoGel® 95% DS

This is a suggested procedure, please adjust it 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 on how to reconstitute PhotoGel® 95% DS (lyophilized methacrylated gelatin with 95% degree of methacrylation) to your desired concentration using Reconstitution Agent P. It also provides instructions for bioprinting of PhotoGel 95% DS using the BIO X/BIO X6. The protocol covers pre-print mixing with cells, 3D bioprinting and post-printing processes such as crosslinking through photocuring. The bioprinting instructions are optimized for concentrations of PhotoGel at 10% w/v and LAP at 0.25% w/v. Changing bioink and LAP concentrations will directly affect the final stiffness of the bioink. Time and distance from the light source will also reflect on the material stiffness and cell viability. Refer to the *Photocrosslinking Optimization Protocol* to adjust accordingly.

Materials needed

- PhotoGel® 95% DS (500 mg)*
- Reconstitution Agent P* or an alternative buffer of choice
- Photoinitiator (PI) *
- Syringes in different volumes
- 0.22 µm sterile syringe filter
- 50 mL Falcon tubes
- pH paper
- NaOH and/or HCl solutions (optional)
- UV-shielding cartridges, 3 mL*
- Cell suspension and cell culture medium
- Female/female Luer lock adaptor*
- Conical bioprinting nozzles, 22-27G recommended*
- Temperature-controlled Printhead*
- BIO X or BIO X6 3D bioprinter*
- Petri dish* or well plate

*The product can be purchased in the CELLINK shop at www.cellink.com/shop/.

Protocol for reconstitution

This protocol describes the reconstitution of 500 mg of PhotoGel® 95% DS to obtain bioinks of different concentrations. We recommend concentrations from 5 to 20% w/v. A photoinitiator, e.g., LAP, should be added to the reconstitution solution to ensure photocrosslinking of the PhotoGel® 95% DS at 365 or 405 nm and provide various gel stiffness. Different LAP concentrations and light exposure times will directly affect the final stiffness of the bioink. Protect the bioinks from light when mixed with LAP. Employ aseptic practices to maintain the product's sterility throughout the preparation and handling of PhotoGel and other solutions.

1. Preparing PhotoGel® 95% DS

MATERIAL

PhotoGel® 95% DS

DESCRIPTION

- Take a vial with 500 mg PhotoGel® 95% DS from storage (-20°C) and let it reach room temperature, this takes at least 1 hour.

2. Preparing PI solution

MATERIAL

Reconstitution Agent P

Photoinitiator (PI), e.g., LAP

20 mL syringe

0.22 µm sterile syringe filter

Two 50 mL Falcon tubes

DESCRIPTION

- Weigh the desired amount of PI in a 50 mL Falcon tube. See Table 1 for suggested LAP concentrations.
- Add 20 mL of a Reconstitution Agent P to the tube and dissolve the PI by slightly shaking the tube.

Note: Reconstitution Agent P is a specially designed buffer that maintains a physiologic pH in the final PhotoGel bioink.

Note: Remember to protect all PI-containing solutions from light.

- Transfer the PI solution (Reconstitution agent + PI) to a 20 mL syringe and sterile filter it into another sterile 50 mL Falcon tube using a 0.22 µm sterile syringe filter.
- Warm the PI solution to ≈50°C.

Table 1. Suggestions of LAP concentration and the corresponding mass.

LAP concentration in bioink	LAP mass for 20 mL of Reconstitution Agent P
0.10% (1 mg/mL)	20 mg
0.25% (2.5 mg/mL)	50 mg

3. Reconstituting PhotoGel® 95% DS

MATERIAL

PhotoGel® 95% DS

Warmed PI solution

pH paper

NaOH and/or HCl solutions (optional)

Syringes and/or UV-shielding cartridges

DESCRIPTION

- Add the desired volume of prepared PI solution to the PhotoGel® 95% DS vial; see Table 2 for suggested bioink concentrations.
- Mix on a shaker table or rotator plate until fully solubilized. Keep warm (>37°C) if possible (e.g., place your rotator in an incubator) to speed up the solubilization process.
- Double check that the pH is in the 7.0-7.4 range since pH is important for the proper viscosity of the bioink. If needed, balance it with small volumes and low concentrations of NaOH or HCl solutions.

Note: Be careful with adding NaOH since high pH degrades the bioink.

- Transfer the PhotoGel bioink solution to syringes/UV-shielding cartridges for further use.
- Store it protected from the light at 4-8°C.

Note: PhotoGel® 95% DS reconstituted in Reconstitution Agent P is stable for at least 6 months without a photoinitiator, and 4 months with a photoinitiator, if stored at 4-8°C protected from light.

Note: If the material gels, warm it to >30°C for it to become liquid again.

Table 2. Suggestions of final bioink concentrations for reconstitution of 500 mg PhotoGel® 95% DS.

Concentration of PhotoGel bioink (w/v)	Volume of PI solution needed
5%	10 mL
10%	5 mL
20%	2 mL

Protocol for bioprinting

PhotoGel 95% DS bioink has been optimized for the use of BIO X and BIO X6 systems and the Temperature-controlled Printhead with thermal nozzle cover and the use of a cooled print bed. 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 and bioprinter for printing

MATERIAL

Syringe containing reconstituted PhotoGel 95% DS with LAP

BIO X or BIO X6 3D bioprinter

Temperature-controlled Printhead

DESCRIPTION

- Set the Temperature-controlled Printhead to 27°C.
- Set the print bed to 15°C.
- Warm the reconstituted PhotoGel 95% DS with LAP at >30°C until it is liquid.
- If not printing with cells, move directly to Step 3.

2. Mixing the bioink with cells

MATERIAL

Pre-warmed syringe containing reconstituted PhotoGel 95% DS with LAP

3 mL syringe

Cell suspension and cell culture medium

Female/female Luer lock adaptor

UV-shielding cartridge, 3 mL

Conical bioprinting nozzles, 22-27G

DESCRIPTION

- Prepare a cell suspension with the desired number of cells dispersed in cell culture medium. The volume of the cell suspension should be 10% of bioink volume. Transfer the cell suspension to a 3 mL syringe.
- Connect the pre-warmed PhotoGel 95% DS syringe to the syringe with cell suspension using a female/female Luer lock adaptor.
- Mix ten parts of PhotoGel 95% DS solution with one part of the cell suspension without introducing air bubbles to the mixture. Other ratios are possible, change accordingly. For detailed instructions see the *Mixing cells and bioink Protocol.*

Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock adaptor with PhotoGel 95% DS solution before attaching the syringe to the cell suspension.

- Mix the bioink with the cell suspension by gently pushing the solutions back and forth between the syringes.
- Transfer the mixture to a UV-shielding cartridge by connecting the syringe to the cartridge using the Luer lock adaptor.
- Cap the cartridge with the desired bioprinting nozzle.

Note: The mixing with cells needs to be performed while the bioink is warmed. If viscosity increases due to the temperature decrease, warm again at >30°C until it is liquid.
Note: Always protect the bioink from light.

3. Preparation for printing

MATERIAL

UV-shielding cartridge containing PhotoGel 95% DS mixed with cells (if applicable)

Temperature-controlled Printhead

BIO X or BIO X6 3D bioprinter

DESCRIPTION

- Connect the cartridge to the air pressure adapter tube.
- Place the cartridge in the pre-heated Temperature-controlled Printhead set to 27°C.
- Wait at least 10 minutes for the temperature in the bioink to equilibrate with the temperature of the printhead.

4. Printing

MATERIAL

BIO X or BIO X6 3D bioprinter

Well plate or Petri dish

DESCRIPTION

- Calibrate the nozzle to the well plate or Petri dish surface.
- In the pressure settings, test the flow of the bioink. Start with low pressure and gradually increase until the bioink extrudes from the nozzle.
- Bioprint structures onto the well plate or Petri dish.
  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 100 ms pre-flow delay, the suggested pressure range is between 25-30 kPa without cell suspension dilution and 15-20 kPa if diluted with cell suspension in 10/1 volume ratio.

Note: If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material at different speeds.

- If proper viscosity and printability are not achieved by extending temperature equilibration time or tuning pressure, follow the tips below:
  - If bioink has too low viscosity (wide filaments despite using low pressure) – decrease the printhead temperature by 0.5-1°C to increase the viscosity and equilibrate an additional couple of minutes.
  - If bioink has too high viscosity (chunky filaments and high pressure required) – increase the printhead temperature by 0.5-1°C to decrease the viscosity and equilibrate an additional couple of minutes.

Note: If waiting is too long between extrusions the bioink can dry in the nozzle causing it to clog. If this occurs, take a sterile tweezer and remove the dried bioink from the edge of the nozzle or replace it with a new nozzle.

5. Crosslinking

MATERIAL

405 or 365 nm LED modules for photocuring

DESCRIPTION

- PhotoGel 95% DS with LAP can be photocrosslinked using either the 405 or 365 nm photocuring module.

Note: It is recommended to use the 405 nm LED module instead of 365 nm if possible, with a maximum of 30 seconds of light exposure. Overexposure might damage the cells.

- Ensure that the bioprinted constructs are thermally gelled by cooling them on the print bed (15°C) on the BIO X or BIO X6 for a couple of minutes prior to starting the photocrosslinking.
- Photocrosslink the constructs for 10 seconds if the construct is 1 mm thick, or 30 seconds if the construct is 3 mm thick.
- Let the structure sit for 3-5 minutes to allow crosslinking after the light source is turned off.

Note: To verify that the photocrosslinking is sufficient, add 37°C cell culture medium to one printed well and observe that it doesn't dissolve.

6. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- After photocrosslinking, add the desired medium to the constructs and place them in the incubator.
- Incubate the constructs in a cell culture medium in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to application. Replace medium regularly.