Ref No: BPR-5270-1KIT
Date: 28-MAR-2023
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RECONSTITUTION, NEUTRALIZATION AND BIOPRINTING PROTOCOL

PhotoCol®

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 for the reconstitution of lyophilized PhotoCol (methacrylated type I collagen) into a stock solution, pH neutralization and supplementation with LAP, as well as mixing with cells and bioprinting. This protocol has been optimized for use with the BIO X and BIO X6 systems and the Temperature-controlled Printhead with a thermal nozzle cover.

Materials needed

- PhotoCol® LAP kit *
- 1x PBS
- 0.22 µm sterile syringe filter
- Eppendorf tubes
- Positive displacement pipette
- pH paper
- NaOH and/or HCl solutions (optional)
- Ice bath
- 3 mL syringes
- Cell suspension and cell culture medium
- Female/female Luer lock adaptor
- 3cc amber cartridge* including tip cap
- Bioprinter (BIO X or BIO X6)*
- Temperature-controlled Printhead*
- 22G conical bioprinting nozzle*
- Well plate

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

Protocol

This protocol provides instructions for the reconstitution of PhotoCol into a stock solution concentration of 10 mg/mL. The steps of neutralization and LAP addition lead to a dilution of a stock solution, allowing the possibility of bioink preparation with different concentrations. The bioprinting section has been optimized to bioprinting different concentrations of collagen mixed with cells. It is recommended that the collagen and other working solutions are kept on ice during the preparation. Employ aseptic practices to maintain the product's sterility throughout the preparation and handling of the collagen and other solutions. Protect the bioinks from light when mixed with LAP.

1. Reconstitution of PhotoCol® 10 mg/mL stock solution

MATERIAL

PhotoCol® lyophilizate (100 mg vial)

20 mM acetic acid solution

5 mL Eppendorf tube

DESCRIPTION

- In a 5 mL Eppendorf tube, weigh the desired mass of PhotoCol for the stock solution preparation. Store the remaining PhotoCol material at 2-10°C.
- Add the corresponding volume of 20 mM acetic acid to the Eppendorf tube to achieve the target concentration of 10 mg/mL.

Note: For example, to prepare 3 mL of 10 mg/mL stock solution, add 3 mL of 20 mM acetic acid to 30 mg of lyophilized PhotoCol.

- Mix on a shaker table or rotator plate at 2-10°C overnight or until fully solubilized. Alternatively, place the container in the fridge and gently rotate it a couple of times every other hour.

Note: The reconstituted stock solution with acetic acid is stable for 2 months when stored at 2 to 10°C.

2. Neutralization and LAP addition

MATERIAL

PhotoCol 10 mg/mL stock solution

Neutralization Solution (NS)

Photoinitiator (PI) LAP

1x PBS

0.22 µm sterile syringe filter

Eppendorf tube

Positive displacement pipette

pH paper

NaOH and/or HCL solutions (optional)

Ice bath

DESCRIPTION

- Define the desired final concentration for the PhotoCol bioink (See Table 1 for suggested concentrations).

Note: We suggest 8 mg/mL, 6 mg/mL and 4 mg/mL. For other concentrations, recalculations need to be made, but the same protocol can be followed.

- Weigh the necessary amount of LAP to achieve the desired concentration of PI in the final bioink.

Note: In our bioinks, we commonly use PI at 0.25% (w/v) concentration (see Table 1).

- Dissolve the LAP in the required volume of 1x PBS to achieve the desired final concentration (see Table 1).

Note: Always prepare some extra solution to compensate for losses during filtration and transfer from one container to another.

- Sterile filter the LAP solution using a 0.22 µm syringe filter.
- Place the PhotoCol 10 mg/mL stock solution, NS solution and LAP solution on ice.
- For the preparation of, for example, 1 mL of PhotoCol 6 mg/mL, transfer 600 µL of PhotoCol 10 mg/mL stock solution into a sterile Eppendorf tube using a positive displacement pipette.
- Add 75 µL of NS solution. Use the positive displacement pipette to pipette it up and down and homogenize the resulting solution.

Note: Vortexing or vigorous shaking is not recommended at any step to avoid bubble formation.

Note: If the neutralization solution is chilled too long, the salts can precipitate. Keep the solution at room temperature until it becomes homogeneous again.

- Check if pH is in the range of 7.0-7.4. If needed, balance it with small volumes and low concentrations of NaOH or HCl solutions.
- Add 325 µL of LAP solution and pipette it up and down using a positive displacement pipette until complete homogenization. Protect he bioink from light.
- Keep the neutralized PhotoCol 6 mg/mL solution on ice while preparing for the next steps.

Table 1. Preparation of PhotoCol with different concentrations.

V_{bioink} (mL)	$C_{final\ bioink}$ (mg/mL)	$C_{stock\ solution}$ (mg/mL)	$V_{stock\ solution}$ (µL)	V_{NS} (µL)	M_{PI} (mg)	$V_{LAP\ solution}$ (µL)
1	8	10	800	100	2.5	100
	6		600	75		325
	4		400	50		550

3. Mixing neutralized PhotoCol solution with cells

MATERIAL

Neutralized PhotoCol solution

Two 3 mL syringes

Cell suspension and cell culture medium

Female/female Luer lock adaptor

3cc amber cartridge including tip cap

DESCRIPTION

- Transfer the neutralized PhotoCol solution into a 3 mL syringe using the following procedure: remove a syringe plunger → cap the syringe → transfer the bioink solution to the syringe using a positive displacement pipette → insert the plunger → flip the syringe → release the tip cap and evacuate the air by raising the plunger.
- Prepare a cell suspension with the desired number of cells dispersed in a cell culture medium of choice. The volume of the cell suspension should be 10% of bioink volume. Transfer the cell suspension to a 3 mL syringe.
- Connect the neutralized PhotoCol solution syringe to the syringe with cell suspension using a female/female Luer lock adaptor.

- Mix ten parts of neutralized PhotoCol solution with one part of the cell suspension without introducing air bubbles to the mixture. If using other ratios, adjust printing parameters suggested in Table 2. 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 neutralized PhotoCol solution before attaching the syringe with the cell suspension.

- Mix the bioink with the cell suspension by gently pushing them back and forth between the syringes.
- Transfer the mixture to a 3cc amber cartridge by connecting the syringe to the cartridge using the Luer lock adaptor.
- Add a tip cap to the cartridge containing the mixture and keep it on ice while preparing to the next step.

4. Bioprinting PhotoCol

MATERIAL

Bioprinter (BIO X or BIO X6)

Temperature-controlled Printhead

3cc amber cartridge with PhotoCol mixed with cells

22G conical bioprinting nozzle

Well plate

DESCRIPTION

- Set the Temperature-controlled Printhead to 10°C.
- Attach the 22G conical nozzle to the 3cc cartridge with the PhotoCol mixed with cells.
- Place the cartridge in the Temperature-controlled Printhead.

Note: Make sure the printhead reaches the correct temperature before placing the cartridge with bioink. If the temperature is too high, the collagen may suffer premature self-assembly leading to an increase in viscosity and altered parameters for printing.

- Calibrate the nozzle to the well plate.
- In the pressure settings, test the flow of the bioink. Start with low pressure and gradually increase until liquid comes out of the nozzle.
- Bioprint droplets with the desired size. Use the suggested parameters (see Table 2) as a guide for your printing to achieve droplets of approximately 10-15 µL.

Note: If printability is not as desired, adjust the pressure up/down to extrude more/less material at different extrusion times.

Note: Different nozzle sizes and concentrations of bioink can be used if desired. Adjust the parameters for printing accordingly.

Table 2. Recommended starting parameters for bioprinting PhotoCol at different concentrations*.

PhotoCol concentration (mg/mL)	Pressure (kPa)	Extrusion time (s)
8	16	1.5
6	12	1.5
4	10	1.5

*Bioprinting parameters were obtained with PhotoCol prepared at a specific concentration and further mixed with cells (10:1). The cell density and different proportionality may affect the final viscosity of the bioink. If changes are made, printing parameters need to be adjusted accordingly.

5. Crosslinking and incubation

MATERIAL

Well plate with printed droplets

Bioprinter (BIO X or BIO X6) with LED modules of choice for photocuring

Cell culture medium

DESCRIPTION

- Incubate the droplets at 37°C (5% CO_2 and 95% relative humidity) for thermal self-assembly of PhotoCol. 15-30 min of incubation is recommended but depends on the droplet size.

Note: Make sure the droplet is thermally crosslinked before moving to the next step. When gelled, the translucent collagen turns into an opaque white hydrogel.

- If photocrosslinking is desired, after thermal self-assembly, subject the droplets to light with a wavelength necessary to activate the PI of choice. See Figure 1 for the comparative stiffness of collagen constructs at different concentrations with and without photocrosslinking.

Note: It is recommended to use the 405 nm photocuring module for 30 seconds instead of 365 nm, if possible, when photocuring PhotoCol with LAP photoinitiator. Overexposure at the 405 nm and 365 nm wavelength might damage the cells.

Note: Time and distance will directly affect the stiffness of the material and the viability of cells. Use a distance between 3 and 5 cm from the plate to the LED module, and a maximum of 30 seconds of light exposure.

- Submerge the constructs in the cell culture medium and place them back in the incubator (37°C, 5% CO_2 and 95% relative humidity) for 3D cell culturing according to your application.

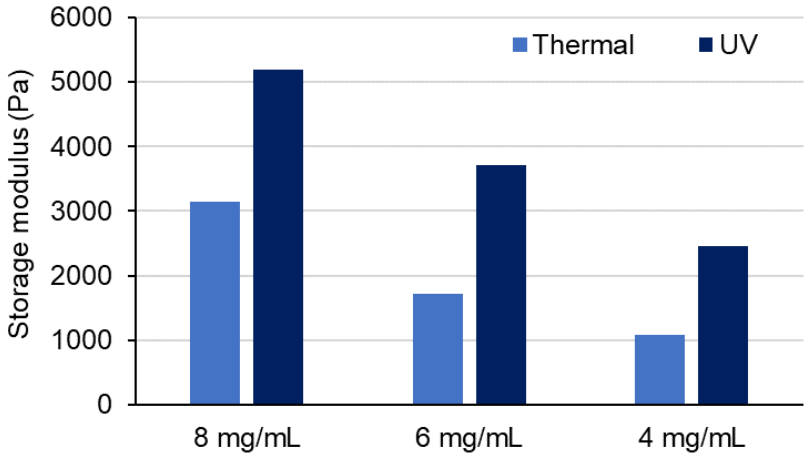

Figure 1. *PhotoCol stiffness at different concentrations with thermal only and with additional photocrosslinking. Rheology tests were performed on a Discovery Hybrid Rheometer, HR 20, TA instruments. Thermal crosslinking was done at 37°C, followed by an in situ photocrosslinking with exposure at 405 nm (LAP as a PI), for 30 seconds and at 5 cm distance from the light source.*