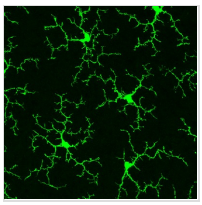

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Feb 06, 2022

Thawing frozen hematopoietic stem cells (HPCs) V.1

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protocol .

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Protocol to thaw frozen hematopoietic stem cells made with the StemDiff Hematopoietic Kit for subsequent differentiation into microglia.

Jessie Buth 2022. Thawing frozen hematopoietic stem cells (HPCs). **protocols.io** https://protocols.io/view/thawing-frozen-hematopoietic-stem-cells-hpcs-b4rgqv3w

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Work quickly, but gently. Expect to revive ~50-95% of frozen cells depending on the vial.

A	B	C
General items:	**Company**	**Catalog #**
Conical tubes (15mL + 50mL)		
Glass pipettes (5mL + 10mL)		
Plastic pipettes (5mL + 10mL)		
Wide bore p1000 tips		
Wide bore p200 tips		
p20 tips		
1.5mL eppendorf tubes		
70% ethanol		
For coating plates:		
GFR Matrigel (or phenol free GFR Matrigel or GFR Cultrex)	Corning	
6w tissue culture treated plates	Corning	
DMEM/F12	Cytiva	
Cell culture media:		
Microglia Base Media (without cytokines)		
Microglia Base Media +3c		
IL-34 (stock 100 ug/mL - used +100ng/mL 1:1000)	Peprotech	
TGFB1 (stock 50 ug/mL - used +50ng/mL 1:1000)	Peprotech	
M-CSF (stock 100 ug/mL - used +100ng/mL 1:1000)	Peprotech	
For thawing cryovials:		
Beaker (500mL or 1L size)		
Hot Water (sink)		
Foam to float cryovial	Fishersci	
For counting cells:		
Trypan Blue	Gibco	
Hemocytometer + cover glass		

Materials Needed

Use the blue cryogloves and tongs when working in the liquid nitrogen tanks.

Make sure you have:

- Enough GFR matrigel + DMEM/F12 to coat the plates
- Microglia base media +/- 3c warmed in the bead bath

Prep Matrigel Plates 40m

1 Coat tissue culture treated plates with 1 mg/mL GFR Matrigel (Growth GFR Matrigel (Growth factor reduced).

Input the matrigel catalog and lot number here to download the quality certificate with its concentration: https://www.corning.com/worldwide/en/products/life-sciences/resource-library.html

1.1 Get a frozen aliquot of GFR Matrigel from the hallway -30. The aliquots are typically labeled "6.5" or "12.5", this is the number of mL DMEM/F12 you will add.

You need:
1mL/well for 6w plates
500uL/well for 12w plates

If you are coating a plate that will be imaged in plate such as the 4w chambers or 24w lumox use the phenol free GFR matrigel. These are labeled "PF GFR Mat" and come in "2.5", "5", or "6.5" sized aliquots.

1.2 Let the aliquot thaw in the fridge. 5m

1.3 

Once thawed, centrifuge the tube with a tabletop centrifuge to get any matrigel off the lid.

1.4 Bring the tube to the tissue culture hood. In a 15mL conical (or 50mL if needed) add the appropriate amount of DMEM/F12.

If using a "6.5" tube, add 6.5mL DMEM/F12.

DMEM/F12 is typically in the bottom two rows of the tissue culture fridge door. If not, more is in the stock fridge down the hall. Second to last room on the left (one before room with cryostat).

1.5 

Get the cold p1000 tips kept at the bottom left of the tissue culture fridge.

Make sure to return them to the fridge for the next person when you are done!!

1.6 Pick up 1mL of DMEM/F12 from the conical tube and mix it into to the GFR matrigel aliquot to start to dissolve it. Add the diluted mixture back to the conical tube.

1.7 Mix the diluted matrigel well with a cold glass pipette or by inverting the tube a few times.

"cold glass pipette" can be a pipette left in the fridge 5-10 minutes or in the -80 freezer 1-2 minutes.

1.8 Use the cold p1000 tips (or cold glass pipette) to aliquot 1mL/well for a 6w plate (or 500uL/well for a 12w plate).

1.9 Shake the plate to distribute the matrigel evenly on the surface.

Shake enough to get it to spread out, but not to bounce around and get on the lid.

1.10

You can prepare coated plates beforehand. Seal the coated plate in parafilm and keep in the 4 degree fridge in the dark for up to 1 week. You will still need to do the next step for 30 mins or 1 hour on the day you use the plate.

1.11 1h 30m

Incubate **00:30:00** at **37 °C** in the incubator (or **01:00:00** **Room temperature** in the tissue culture hood).

If I have multiple plates, I usually lay them all flat (not stacked) to ensure equal heating.

1.12 1h 30m

Once the **00:30:00** (or **01:00:00**) is complete, aspirate the matrigel from the plate. Add 2mL/well microglia base media +3c

+3c = 100ng/mL IL-34, 50ng/mL TGFB1, and 100ng/mL M-CSF

These are all aliquoted in 50uL aliquots in the hallway -80. The boxes are located in the rack on the second shelf from the bottom, farthest on the right. Second column from the front top three boxes. IL-34 has yellow tape, TGFB1 is a brownish/yellow box, and M-CSF has red tape.

TGFB1 aliquots are labeled "TB". Label the date the cytokines were thawed. Thawed cytokines can be kept at 4 degrees for up to 1 week.

Thaw Frozen HPCs 20m

2 Thaw a vial of frozen hematopoietic stem cells (HPCs) previously made using the StemDiff Hematopoietic Kit (StemCell Tech) from the liquid nitrogen.

Bambanker **Contributed by**

These cells are frozen in **users Catalog #CS-02-001** *. This is a modified version of the protocol on their product sheet.*

2.1 Fill a large beaker with hot water from the sink. Feel the water until hot, it can take a minute to warm up.

A clear glass beaker is ideal, not a clouded plastic one.

2.2 ⚠

Float the vial using the foam tube holders near the sink in the beaker of hot water. DO NOT SHAKE.

2.3 Keep watch until the a small iceberg is left. When it floats to the top, retrieve the cryovial from the water and bring to the tissue culture hood.

Make sure to spray it down well with 70% ethanol before putting in the hood.

> *Note: Cryovials will fit in the blue nunc tube holders, but not in the regular ones we use in lab for eppi tubes.*

2.4 ⚠

Pre-wet a 5mL glass pipette i.e. pipette up some media and put it back so the pipette is wet inside.

2.5 Pick up the entire contents of the cryovial and put it into the bottom a 15mL conical tube (not down the side).

2.6 ⚠

Add 10mL microglia base media to the conical tube **drop-wise** to avoid osmotic shock.

2.7 After the 10mL is added, close the lid and invert the tube once slowly to gently mix.

2.8 5m

Centrifuge the cells **300 rcf, Room temperature, 00:05:00**

2.9 During the centrifuge time, prep to count the cells.

Add 10uL trypan blue to an eppi tube and wipe off the hemocytometer with a kim wipe. Place the coverglass on the hemocytometer.

2.10 When centrifugation is complete, retrieve the conical tube. The pellet will be visible, but very small.

2.11 Aspirate the media from the conical tube and tap to loosen the pellet.

2.12 Use a wide bore p1000 tip or 5mL glass pipette to resuspend the cells in 1mL microglia base media +3c.

Try to put the liquid down the sides of the tube to get all the cells that went up the sides during tapping the pellet.

2.13 Pipette up and down just enough to mix. Usually 3-4x if using wide bore tips.

2.14 Add 10uL of cells to the eppi tube containing 10uL trypan blue. Mix up and down 3-5x and then add 10uL to one side of the hemocytometer.

Tip: Use a p20 here to add 10uL to the hemocytometer. It is much easier than using the p10.

2.15 Count the cells. I usually count 2 of the outer squares. For example, red squares "B" and "C" in this image.

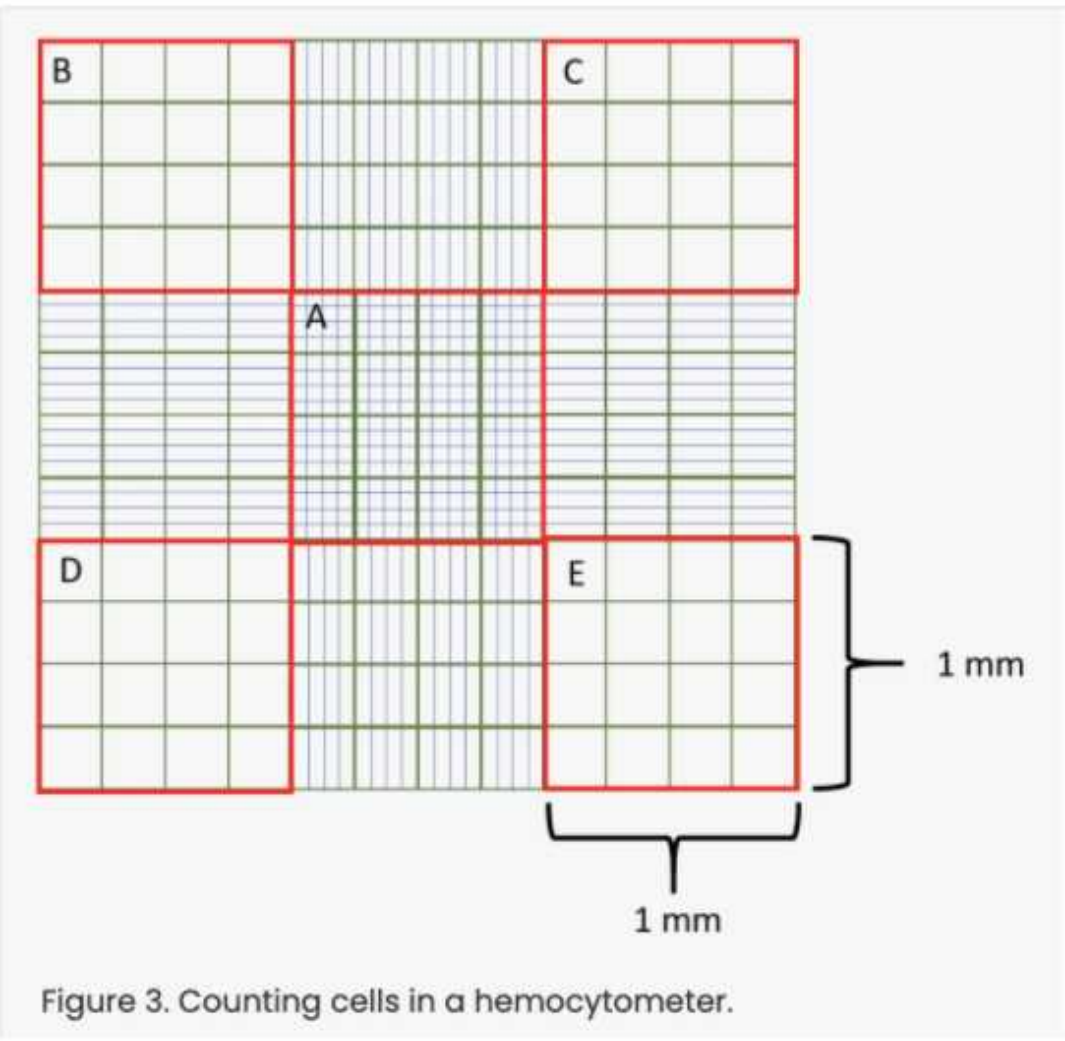

Figure 3. Counting cells in a hemocytometer.

2.16 Calculate the number of cells/mL and how many uL are needed to have 25,000 cells.

For example:

If I count 70 cells in red square "B" and 64 cells in red square "C".

$$\frac{(70+64)\ cells}{2\ squares} * 2 = 134 \text{ cells per red square}$$

Here you divided by the number of squares counted to get the average cells per square. Then multiplied by 2 because of the 1:1 dilution in trypan blue.

We know the size of each red square is equivalent to 1 mm^2 or 1000 um^2

$$134 * 10^4 = 1.34 * 10^6 \text{ cells per 1000uL}$$

Do a ratio to determine number of uL needed for 25K cells.

$$\frac{1{,}340{,}000\ cells}{1000\ uL} = \frac{25{,}000\ cells}{x}$$

Cross multiply to solve for x.

$$x = \frac{(25{,}000\ cells) * (1000\ uL)}{1{,}340{,}000\ cells}$$

This is the number of uL that has 25,000 cells.

$x = 18.66\ uL$ has 25,000 cells

2.17 Add 25,000 cells per well on top of the 2mL/well microglia base media +3c added at the end of matrigel prep.

Use wide bore tips here if possible.

2.18 Distribute the cells evenly by shaking in the incubator. 5x forward-back. 5x side-side. 5x forward-back. Close the magnetic door gently.