Culturing malaria guide

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1 Washing blood

Blood, once received, should be washed before use. Which wash method to use depends on whether the blood was drawn by a volunteer phlebotomist or received from the blood bank. Regardless, it should be stored at 4 °C, and used within a week.

The washed blood will be at **50% hæmatocrit**.

Washing blood drawn by phlebotomist:

This procedure is more involved, as it removes the white cells (leukocytes) and platelets (thrombocytes) found in freshly drawn blood.

- If not already sedimented, centrifuge blood at 3,000RPM for 8 minutes
- Aspirate supernatant, and resuspend the blood in incomplete medium at a 1:1 ratio
- Into a 15 mL sterile centrifuge tube, transfer 4 mL of LymphoPrep
- Carefully layer ≈ 8 mL of resuspended blood on top of the LymphoPrep. Pour against side of tube, from a height
- Centrifuge at 3,000RPM for 15 minutes
- Aspirate everything except the bottom-most layer of red blood cells. Make sure to get everything, even if you take some red blood cells as well
- Resuspend the cells in 10 mL wash medium
- Centrifuge at 3,000RPM for 8 minutes
- Aspirate and resuspend in wash medium at a 1:1 ratio

Washing blood from the blood bank:

This procedure is simpler, as the blood has already been filtered of non-red blood cells.

- If not already sedimented, centrifuge blood at 3,000RPM for 8 minutes
- Aspirate supernatant, and resuspend the blood in incomplete medium at a 1:1 ratio
- Centrifuge at 3,000RPM for 10 minutes
- Aspirate everything except the bottom-most layer of red blood cells. Make sure to get everything, even if you take some red blood cells as well
- Resuspend in wash medium at a 1:1 ratio

2 Freezing cultures

Cultures can be frozen in liquid nitrogen for long-term storage, and thawed later.

Only ring-stage parasites can be frozen and thawed; later stages will not survive the process. Therefore, freezing should be done with a high percentage of rings, 5% or so. This is easier to achieve if the culture has recently been synchronized.

This process is also very time sensitive, as the cells do not enjoy being in glycerolyte for any longer than they have to be.

Freezing procedure

- Transfer culture to a sterile centrifuge tube
- Centrifuge at 1,800 RPM for 5 minutes
- Aspirate supernatant and resuspend in small volume of remaining supernatant
- Estimate the volume of the pellet. Add 0.5 times this volume of glycerolyte drop-wise, while mixing by swirling. This should be done slowly
- Let sit at room temperature for 5 minutes
- Add 1.5 times the original volume of the pellet drop-wise, while mixing by swirling
- Aliquot into a sterile freezing vial and place at $-80\,^{\circ}\mathrm{C}$ for 24 hours
- Transfer vials to liquid nitrogen for long-term storage. Make sure this transfer is done quickly and so that the vial does not warm.

3 Thawing frozen cultures

Malaria parasites are frozen in liquid nitrogen for long-term storage, and must be thawed carefully. Before starting, make sure you have read the instructions carefully, as it is a time-sensitive process.

It is important that the process not take longer than necessary, as the cells are unhappy in their glycerolyte solution. It is also important to not rush the drop-wise process; it should feel tedious.

Thawing procedure

- Remove the frozen vial of infected cells from the liquid nitrogen container, and warm it in your hand until it has melted.
- Transfer the infected cells to a 50 mL sterile centrifuge tube, ideally one with volume markings all the way down
- Estimate the volume of infected cells. Add 0.2 times this volume of 12% NaCl drop-wise, while mixing by swirling. This should be done slowly.
- Allow to stand at room temperature for 5 minutes
- Add 10 mL of 1.8% NaCl drop-wise, while mixing by swirling
- Pellet infected cells by centrifugation at 1,800RPM for 5 minutes
- Remove the supernatant, and resuspend the pelleted infected cells by adding 10 mL of 0.9% drop-wise, while mixing by swirling
- Pellet infected cells by centrifugation at 1,800RPM for 5 minutes
- Remove the supernatant
- Resuspend in enough growth medium and fresh blood to achieve the desired hæmatocrit and volume (usually 3% Hct in 10 mL)
- Transfer to a sterile tissue culture flask, add gas (1% O_2, 3% CO_2) for a minute, seal, and place in the 37 °C incubator

Immediately afterwards, smear to check whether the thawing process succeeded and to check the parasitæmia. There should only be rings. The culture should be allowed a few days to stabilize before being used for an experiment.

4 Caring for your culture

A malaria culture can be kept indefinitely by properly caring for it. Each strain has its own favored conditions. Here, I will describe the best conditions for the 3D7 strain.

To properly care for a culture, the following is necessary:

Maintain a healthy parasitæmia: If the percentage of infected cells becomes too high (more than 6% or so), the culture can "crash," or die out. Therefore, we want to keep the culture below this percentage. While for freezing or synchronization parasitæmia of 5% or so is necessary, it is otherwise safer to stay around 1%. Once a week it is suggested that the parasitæmia be brought down to 0.5% or so.

Keeping fresh medium: The parasites use up the medium, and so it must be periodically replaced. When diluting heavily, the majority of the medium will be fresh, so this is sufficient. Likewise, if the cells are below 1%, there is no need to change the medium. Otherwise, the medium can be easily changed. Sediment the cells either by centrifuging at 1,800RPM for 5 minutes, or leaving the flask standing for some time. Remove the supernatant, and add fresh complete medium.

The volume fraction of red blood cells is the **hæmatocrit**. For 3D7, anything between 3-5% should work, but I find 3% works best, and I use this exclusively.

Adding fresh red blood cells: The parasites need fresh red blood cells to invade. As the parasite population increases, it will have to be split, which will involve the addition of fresh blood cells. The fresher the blood, the better.

Predicting parasitæmia: The simple rule for predicting what your culture will look like in the future is:

- To find the population of rings tomorrow, take today's trophozoite and schizont population, and multiply by 5.
- To find the population of trophozoites tomorrow, take today's ring population

Therefore, a population of 1.2% rings and 0.5% trophozoites on Monday will likely be around 2.5% rings and 1.2% trophozoites on Tuesday.

Splitting: To keep the parasitæmia for getting too high, the culture must be split. If we have a culture with 2% trophozoites today, there will be 10% rings tomorrow, which is far too high. If we wish to have, for example, 2% rings instead, we must split the culture by five.

To split a culture by, say, five, we do as follows:

- Dispose of all but $10\,\text{mL} \div 5 = 2\,\text{mL}$
- Add 8 mL of 3% hæmatocrit blood:
  - Since $0.03 \times 8\,\text{mL} = 240\,\mu\text{L}$, this is how much 100% blood we need
  - However, our blood is stored at 50%, so we need $2 \times 240\,\mu\text{L} = 480\,\mu\text{L}$ of 50% Hct blood
  - Since $8\,\text{mL} - 480\,\mu\text{L} \approx 7.5\,\text{mL}$, this is how much complete medium we need
- Gas, seal, and put in incubator

Preparing for the weekend: Unless you plan on coming in during the weekend, you should decrease the parasitæmia of your culture to 0.5% on Friday. Then, make sure to tend to your culture on Monday morning, as the parasitæmia is likely high.

5 Smearing and staining

To determine the parasitæmia, and the stage of the culture, we perform a blood smear, fixing a thin layer of blood cells onto a glass slide. We then stain the parasites, and image it with a 100X oil-immersion objective.

- Transfer roughly 100 µL sample of cells from the culture into an Eppendorf Tube
- Spin the sample for a few seconds in the microcentrifuge
- Remove roughly 5 µL of the pellet with a pipette, mixing just a bit to get some supernatant as well
- Place this drop of blood on a clean slide, towards the opaque end
- Smear the blood by dragging the blood towards the label side, then quickly smearing away
- Wait a few seconds until the blood dries
- Rinse with methanol, and air dry immediately with a hair dryer
- Flood slide with fresh, filtered Giemsa in phosphate buffer
- Wait 10 minutes
- Flood slide with RO water
- Air dry immediately with a hair dryer

The blood smear can now be imaged under the microscope. The immersion oil can be placed directly on the surface, without a coverslip.

We can now quantify the parasitæmia and stage by counting cells. Move the image to a "randomly selected" place, and stop. Count the number of healthy red blood cells, the number of cells with ring-stage parasites, with trophozoites, and with schizont parasites. If a cell has multiple parasites, count as if it only had one. Count until you have 500 or more cells.

It is worth noting the limitation with counting. The posterior distribution of the parasiæmia p after counting a total of N cells has a standard deviation $= \sqrt{\frac{p(1-p)}{N}}$. Therefore, if one counts a parasitæmia of $p = 1\%$, the true value is likely to be anywhere between $p = 0.5\%$ and $p = 1.5\%$.

6 Synchronizing

To keep parasites within a narrow age range, we must synchronize the culture. A synchronized culture which is allowed to "free-wheel" will drift apart, and within two days, will be unsynchronized.

6.1 Sorbitol lysis synchronization

Parasitized red blood cells become metabolically active roughly 20 hours after invasion. At this point, parasite proteins are inserted into the red cell membrane, allowing the active transport of extracellular substances into the cell cytoplasm. Therefore, after this stage—and only after this stage—the parasitized red blood cells are permeable to Sorbitol, and, therefore, can be selectively lysed. The final washed pellet will contain only the younger, ring-forms.

If the goal is to see schizonts, then aim to do the experiment 26 hours after synchronization.

- Warm the Sorbitol solution, wash medium, and growth medium to 37 °C in the incubator
- Transfer the culture to a clean, sterile, centrifuge tube of the appropriate volume
- Pellet the culture cells by centrifugation at 1,800RPM for 5 minutes.
- Remove supernatant
- Resuspend pellet in ten times the volume of 5% Sorbitol solution
- Leave in the 37 °C incubator for 5 minutes

- Pellet cells by centrifugation at 1,800RPM for 5 minutes.
- Remove supernatant and resuspend cells in 20 times the volume of wash medium
- Pellet cells by centrifugation at 1,800RPM for 5 minutes.
- Check the volume of pellet (i.e., the haematocrit). Subtract this volume from 2.5 mL, and multiply the result by two. Add this quantity of washed blood
- Add enough growth medium to reach 50 mL. Resuspend the mixture of pelleted infected cells, washed blood, and growth medium
- Transfer to a sterile tissue culture flask, add gas (1% O_2, 3% CO_2) for a minute, seal, and place in the 37 °C incubator

6.2 Magnetic separation

To separate out the schizont-stage infected erythrocytes, we use magnetic separation, since the hemazoin present in these cells are paramagnetic.

- Set up the magnet on its metal stand in the hood
- Place the autoclaved column in the purple magnet
- Place an empty tube beneath column. Wash the column with 3 mL of wash medium. Let the medium flow through, drop-wise, with the plunger on top. Press on plunger if necessary.
- Dispose of flow-through
- Place an empty tube beneath column. Flow blood through column. Add ≈ 2.5 mL at a time, and let it flow through.
- Wash the column with 3 mL of wash medium.
- The collected flow-through blood can be disposed off, as above, or kept, as above.
- Remove column from magnet, and put it on top of a clean centrifuge tube
- Wash the column with 3 mL of wash medium
- Centrifuge collected schizont cells at 1,500RPM for 5 minutes
- There should be only a tiny red dot of a pellet. Remove supernatant (leave a little bit to not suck up pellet!)
- Add 400 µL of growth medium. Resuspend.
- Divide into two containers
- Add 1 µL of washed blood into each, to achieve approximately 0.05% Hct
- Put into incubator

To clean the column

- Flow ≈ 3 mL distilled water down the column
- Flow ≈ 3 mL distilled water down the column, a second time
- Flow ≈ 3 mL ethanol down the column
- Autoclave column

The cells will begin to erupt roughly 30 minutes hence.

7 Recipes and ingredients

7.1 Media

Most of the work is done using "complete medium," occasionally called "growth medium." In addition, an "incomplete medium" (alternatively "wash medium") is occasionally used to wash.

These media last for a month or more at 4 °C. Since the medium is often needed warm, a small amount of medium can be kept in the incubator for ready use.

Incomplete medium a.k.a. wash medium (≈ 500 mL):

- To a 500 mL bottle of RPMI-1640, add:
  - 18.75 mL of 1M HEPES
  - 5 mL 20% glucose solution
  - 3 mL 1M NaOH solution
  - 1.25 mL gentamicin sulphate solution (10 mg/mL). Final concentration will be 25 mg/mL.
  - 5 mL of 200 mM glutamine solution
  - 0.5 mL of 100 mM hypoxanthine solution (in 1M NaOH). Final concentration will be ≈ 0.1 mM.

Complete medium a.k.a. Growth medium (≈ 200 mL, final concentration = 0.5%):

- Weigh out 1 g of Albumax II, and dissolve it in 30 mL in wash medium
- Filter-sterilize the Albumax II solution by passing it through a syringe filter (pore size 0.2 µm) into a sterile flask
- Add wash medium to the sterile flask to a final volume of 200 mL.

7.2 Freezing and thawing

Glycerolyte solution:

- 57 g glycerol
- 1.6 g sodium lactate (lactic acid, sodium salt)
- 30 mg KCl
- 1.38 g Sodium dihydrogen phosphate (Sodium phosphate monobasic)
- pH to 6.8 with NaOH (1M or higher)
- Make up to 100 mL with water
- Sterilize through 0.2 µm filter

Thawing solutions:
Thawing involves three different solutions of salt. They will last forever, essentially.

Thaw 1 - 12% (w/v) NaCl solution in double distilled water (12 g NaCl in 100 mL DDW)

Thaw 2 - 1.8% (w/v) NaCl solution in double distilled water (1.8 g NaCl in 100 mL DDW)

Thaw 3 - 0.9% (w/v) NaCl solution in double distilled water, plus 0.2% glucose (0.9 g NaCl and 0.2 g glucose in 100 mL DDW)

Filter all three solutions through a 0.2 µm filter.

7.3 Smearing

10% filtered Giemsa:
This should be made fresh, daily

- Mix roughly 0.5 mL of pure Giemsa and 9.5 mL of PBS into a syringe
- When using, pass solution through a 0.2 µm filter directly onto glass slide