**Time Course Protocol - Percoll Synchronization**

Adapted from: B Witkowski, D Menard, C Amaratunga, RM Fairhurst. Ring‐stage Survival Assays (RSA) to evaluate the in‐vitro and ex‐vivo susceptibility of Plasmodium falciparum to artemisinins. Institute Pasteur du Cambodge – National Institutes of Health Procedure RSAv1.

Material preparation:

* Heparinized RPMI (200 mL, freshly prepared)
  + 5.2 mg porcine heparin in 200 μl 1X PBS
  + Add heparin to 200 mL RPMI
  + Vacuum filter sterilize
* 90% Percoll (160 mL, freshly prepared)
  + 144 mL Percoll
  + 16 mL 10X PBS
* 75% Percoll (180 mL)
  + 150 mL 90% Percoll
  + 30 mL heparinized RPMI
  + Vacuum filter sterilize
* Wash media (500 mL)
  + 100 μl 50 mg/mL gentamicin in 500 mL bottle of RPMI
  + Vacuum filter sterilize
* ACM (1 L)
  + Remove 65 mL from new RPMI 1640 w/ 25 mM HEPES and no L-glutamine
  + Thaw:
    - 5 mL hypoxanthine
    - 5 mL L-glutamine
    - 50 mL Albumax
    - 200 μl gentamicin (50 mg/mL stock)
  + Add the above and 5 mL 45% glucose to RPMI bottle
  + Vacuum filter
* 5% sorbitol

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Pre-warm at 37°C:

* Heparinized RPMI
* Wash media
* ACM
* 5% sorbitol

Set at room temperature:

* 75% Percoll

Prepare

* 4 T75 flasks containing 1.8 mL donor blood in 15 mL ACM at 37°C

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1. **Input parasite cultures: 12 mL of iRBCs at ~2% parasitemia**
   1. **Ensure cultures are well synchronized in lead up to experiment, splitting cultures to <2% parasitemia after each cycle**
2. On day of experiment, make a slide of cultures, ensuring parasites are entering late schizont stage
   1. If the proportion of mature schizonts in culture (10‐12 nuclei) is < 0.5%, wait
   2. If the proportion of mature schizonts in culture (10‐12 nuclei) is > 0.5%, proceed
3. **Transfer the cultures to 50 mL conical tubes** 
   1. **Spin 500g 5 minutes, remove supernatant**
4. Resuspend each pellet in 15 mL heparinized RPMI
   1. Mix gently
5. Incubate at 37°C for 15 minutes
6. During incubation, transfer 20 mL 75% Percoll to 50 mL conical
7. After (4) incubation, carefully layer iRBCs in heparinized RPMI on top of 75% Percoll
   1. Set motorized pipet to “G” and only transfer 5-8 mL at a time from 10 mL serological
8. Spin 1,000g for 15 minutes
9. Transfer schizont layer to 15 mL conical containing 6 mL wash media (pre-warmed to 37°C)
   1. Bring volume to 12 mL with wash media (also at 37°C)
10. Spin 500g for 3 minutes
11. Remove supernatant, wash with 12 mL wash media (37°C) and spin 500g for 3 minutes
    1. **Repeat**
12. **Combine all pellets in 2 mL ACM**
    1. **Check volume to equally distribute maximum volume to flasks (4)**
13. **Split schizont suspension (≥250 μl) between two T25s, each containing 0.9 mL donor RBCs in 8 mL ACM at 37°C**
    1. **Set aside 20 μl of each culture in microfuge tubes to make slides**
    2. **Enclose in hypoxia chamber and fill with gas mixture**
14. Incubate at 37°C for 3 hours on nutator

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**During 3-hour incubation, prepare:**

* + **12 x 12-well plates (3 / patient sample)**
    - **3.5 ml ACM in 32 wells**

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1. **At end of 3-hour incubation, transfer cultures to 50 mL conical tubes (1 per donor sample)**
   1. **Spin 500 g for 5 minutes, remove supernatant**
2. **Add 18 mL 5% sorbitol to each 50 mL tube**
   1. **Vortex, incubate at 37°C for 10 minutes, vortexing again at 5 and 10 minutes**
3. **Spin 500g for 5 minutes, remove supernatant and wash with 20 mL wash media**
   1. **Repeat**
4. **Resuspend each pellet in 15 mL ACM**
   1. **15 mL + 1.8 mL iRBCs = 16.8 mL culture**
5. **Aliquot 500 μl of each sample into 32 wells (3 x 12-well plates per patient sample, already prepared)**
   1. **Wells contain: 3.5 mL ACM at 37°C**
6. **Take 2 samples / patient every three hours**
   1. **Draw off 2.6 mL media with 2 mL pipet**
   2. **Resuspend iRBCs in remaining media**
   3. **Transfer to labeled microfuge tube**
   4. **Put plates back in hypoxia chamber, gas, and set in incubator**
   5. **Transfer 30 μl of each time point into “slide” microfuge tube**
   6. **Centrifuge at 8,000g for 3 minutes**
      1. **Draw off supernatant with p1000**
   7. **Add 1 mL TRIzol to each timepoint sample**
      1. **Close lids tightly**
      2. **Vortex briefly to mix**
   8. **Place in -80°C box on top shelf of freezer in 337**
   9. **Make slides**
      1. **Spread all 30 μl over slide without scraping off much of spot to count and stage parasites**
      2. **Dry, fix in fresh methanol**
      3. **At conclusion of experiment, stain all slides with 10% Giemsa (diluted with PBS) for 20 minutes, wash, and dry**