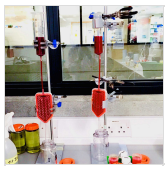

Jan 28, 2021

isolating human malaria parasites for RNA-sequencing at ultra-low densities

Kathryn Milne[1], Florian Bach[1], Wiebke Nahrendorf[1], J. Alexandra Rowe[2], Philip J Spence[2]

[1]Institute of Immunology & Infection Research, University of Edinburgh, Edinburgh, UK;

[2]Institute of Immunology & Infection Research and Centre for Immunity, Infection & Evolution, University of Edinburgh, Edinburgh, UK

1 *Works for me* dx.doi.org/10.17504/protocols.io.brgjm3un

Wiebke Nahrendorf

ABSTRACT

The large subtelomeric multi-gene families encoded in the *Plasmodium* genome play critical roles in parasite virulence, chronicity and transmission. However, quantifying parasite gene expression *in vivo* has been hampered by a lack of sensitive and unbiased RNA-sequencing methodologies that can perform effectively on human blood samples containing small numbers of parasites. The biggest stumbling block is not parasite number per se but host contamination (e.g. white cell transcripts and globin mRNA). These host-derived transcripts outnumber parasite mRNA by many orders of magnitude, preventing their measurement regardless of the depth of sequencing. The solution to this problem is to isolate intact, viable malaria parasites from whole blood and to process these parasites for RNA-sequencing in the absence of host material. Here we present a step-by-step guide to isolating *P. falciparum* and *P. vivax* blood-stage parasites (at any life-cycle stage) from healthy volunteers participating in human challenge studies. We have optimised the effective removal of white cells, the gentle permeabilisation of red cell membranes - leaving the parasitophorous vacuole intact - and maximised the recovery of viable parasites. Crucially, this protocol is highly effective at ultra-low densities (just 5,000 parasites per ml) and has been used to track *var* gene switching *in vivo* and perform PacBio sequencing of new clonal field isolates. Other downstream applications, such as *ex vivo* single cell RNA-sequencing, are possible and this protocol could also be adapted for patient samples from endemic areas and returning travellers. The ability to isolate malaria parasites directly from human blood at ultra-low densities will empower the community to address the role of parasite variant surface antigens in naive and semi-immune hosts.

EXTERNAL LINK

https://doi.org/10.1101/2020.09.04.20188144

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Diverse outcomes of controlled human malaria infection originate from host-intrinsic immune variation and not *var* gene switching. Kathryn Milne, Alasdair Ivens, Adam J. Reid, Magda E. Lotkowska, Áine O'Toole, Geetha Sankaranarayanan, Diana Muñoz Sandoval, Wiebke Nahrendorf, Clement Regnault, Nick J. Edwards, Sarah E. Silk, Ruth O. Payne, Angela M. Minassian, Navin Venkatraman, Mandy Sanders, Adrian V.S. Hill, Michael P. Barrett, Matthew Berriman, Simon J. Draper, J. Alexandra Rowe, Philip J Spence medRxiv 2020.09.04.20188144; doi: https://doi.org/10.1101/2020.09.04.20188144

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Diverse outcomes of controlled human malaria infection originate from host-intrinsic immune variation and not *var* gene switching. Kathryn Milne, Alasdair Ivens, Adam J. Reid, Magda E. Lotkowska, Áine O'Toole, Geetha Sankaranarayanan, Diana Muñoz Sandoval, Wiebke Nahrendorf, Clement Regnault, Nick J. Edwards, Sarah E. Silk, Ruth O. Payne, Angela M. Minassian, Navin Venkatraman, Mandy Sanders, Adrian V.S. Hill, Michael P. Barrett, Matthew Berriman, Simon J. Draper, J. Alexandra Rowe, Philip J Spence medRxiv 2020.09.04.20188144; doi: https://doi.org/10.1101/2020.09.04.20188144

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KEYWORDS

Plasmodium, human malaria, low input RNA-sequencing, parasite transcriptome, var genes

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GUIDELINES

We work with blood from volunteers infected with human malaria parasites - a class 3 pathogen. Since all volunteers tested negative for other common blood-borne infections we can work under derogated CAT3 conditions allowing access to specialised equipment (inc. centrifuges). Before you start carefully assess infection risk of your blood samples, wear appropriate PPE and have decontamination and waste disposal procedures in place. Adhere to your local safety guidelines at all times!

MATERIALS TEXT

materials

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A	B	C	D
	supplier	**catalogue number**	**additional information**
BD Vacutainer® - **Plasma tube, spray coated with 158 USP units of lithium heparin**, 10 ml	Becton Dickinson	367880	
Leucoflex LXT filter	Macopharma		
50 ml Luer Slip Tip Syringe	Becton Dickinson	309654	
250 ml PC Centrifuge Bottle with Silicone O-ring Cap	Corning	431842	before each use: carefully wash with hot soapy water, rinse extensively with water to remove any soap residues and autoclave
Nalgene™ **Oak Ridge High-Speed PPCO Centrifuge Tubes with Sealing Cap, 50 ml**	ThermoFisher	3139-0050	
Gibco™ **1x PBS, pH 7.2**, sterile	ThermoFisher	20012019	
Saponin from quillaja bark	Sigma-Aldrich	S7900	test each batch (see buffers and solutions), WARNING see safety information
TRIzol™ Reagent	ThermoFisher	15596026	DANGER see safety information
50 ml centrifuge tubes, sterile	Corning	430829	
15 ml centrifuge tubes, sterile	Corning	430791	
1.5 ml Eppendorf® tubes, PCR clean	Eppendorf	0030123328	
Corning® Costar® Stripette® **serological pipettes, 50 ml**	Sigma-Aldrich	CLS4490	
Corning® Costar® Stripette® **serological pipettes, 10 ml**	Sigma-Aldrich	CLS4488	
Chemgene™ High Level Surface Disinfectant	Fisher-Scientific	15176686	

buffers and solutions

Saponin: Sapogenin content varies considerably between each batch and we therefore strongly recommend that you determine the optimal Saponin concentration to permeabilise the red cell membrane whilst leaving the parasitophorous vacuole around the malaria parasite intact. We test every batch of Saponin that we receive by spiking cultured P. falciparum (clone 3D7) into 20 ml whole human blood. Mock samples are then processed through this parasite isolation protocol using a range of Saponin concentrations (e.g. 0.005-0.02%) and the efficiency of red cell lysis is assessed on thick smears prepared with the parasite pellets obtained in steps c and d.

as a guide these are the concentrations optimal for our latest batch of Saponin:

stock Saponin solution 0.15% (w/v)
500 ml PBS + 0.75 g saponin
store at 4°C for no longer than 1 week

working Saponin solution
57 ml per volunteer: 53.4 ml PBS + 3.6 ml stock saponin
prepare fresh and keep on ice until use

equipment

- tall metal stand with clamps to hold a 50 ml syringe and Leucoflex LXT filter in place during leukocyte depletion (see photo)
- this protocol requires specialised centrifuges:

1. a centrifuge able to spin 250 ml centrifuge bottles at 1000 xg (room temperature, break OFF) - we use Beckman Coulter's *Avanti* with JA-14 rotor (fixed angle, biosafety lid, 6 x 250 ml, up to 30,000 xg)
2. a refrigerated centrifuge that can spin 50 ml Oak Ridge High-Speed Centrifuge Tubes at 18,000 xg (4°C, break OFF) - we use Beckman Coulter's *Allegra 25R* with TA-14-50 rotor (fixed angle, biosafety lid, 8 x 50 ml, up to 21,000 xg)
3. a refrigerated tabletop microfuge to spin 1.5 ml eppendorf tubes at 18,000 xg (4°C)

- precision scales (accurate to within 0.01 g)

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- electric pipette controller with adjustable aspiration speed

SAFETY WARNINGS

Saponin
WARNING H319 – causes serious eye irritation, H335 - may cause respiratory irritation.
Wear PPE (labcoat, chemical-resistant gloves and goggles) and work in a well ventilated area.

TRIzol Reagent
DANGER H301 - toxic if swallowed, H311 - toxic in contact with skin, H331 - toxic if inhaled, H314 - causes severe skin burns and eye damage, H335 - may cause respiratory irritation, H341 - suspected of causing genetic defects, H373 - may cause damage to organs through prolonged or repeated exposure, H412 - harmful to aquatic life with long lasting effects.
Wear PPE (labcoat, chemical-resistant gloves and goggles) and work in a well ventilated area. All materials that come into contact with TRIzol (including pipette tips) need to be disposed of appropriately - follow your local guidelines.

prepare in advance

1 securely insert the correct rotor into the centrifuges you will use

2 carefully clean centrifuges as well as your bench and equipment (including pipettes) with 1% chemgene and 70% ethanol

3 set water-bath to 37°C, collect ice & dry ice

4 prepare fresh working saponin solution (57 ml per volunteer) using stock saponin. keep on ice until use (for details see buffers and solutions in the materials tab)

5 measure out 230 ml room temperature PBS for each volunteer

6 set up the stand for leukocyte depletion using a 50 ml syringe (plunger removed), Leucoflex LXT filter and 250 ml centrifuge bottle (rinse bottles with sterile PBS, check for any cracks or warps - do not use damaged bottles)

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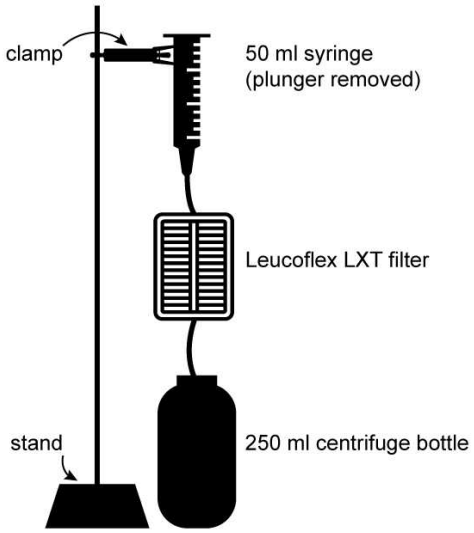

leukocyte depletion

7

*Removing virtually all white blood cells is essential for successful down-stream analysis of the parasite transcriptome. Each white cell contains at least 200 times the amount of RNA compared to a ring-stage parasite and at a density of 5000 parasites*ml^{-1} white cells outnumber parasites by 1000 to 1. As such, if leukocyte depletion is inefficient it will not be possible to detect parasite transcripts (and increasing the depth of sequencing will not overcome the white cell noise).*

8 draw 20 ml of venous blood from a malaria infected volunteer into two 10 ml lithium heparin Vacutainers - keep at room temperature

*In our hands a total of 100,000 ring-stage parasites in 20 ml whole blood (or 5,000 parasites*ml^{-1}) will routinely generate a high quality parasite transcriptome with sufficient genome coverage to analyse expression of the subtelomeric multi-gene families (e.g.* var *and* rifin *genes in* P. falciparum*). See publication: https://www.medrxiv.org/content/10.1101/2020.09.04.20188144v1*

9 mix the blood carefully by inverting each Vacutainer ten times

10 transfer the blood from both Vacutainers to one 50 ml centrifuge tube

11 rinse both Vacutainers with a total of 30 ml room temperature PBS to maximise recovery of red blood cells and add to

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the same 50 ml centrifuge tube

12 pour the 50 ml of diluted whole blood into the syringe body on your leukocyte depletion stand
! take care to not create bubbles

13 allow the liquid to pass through the Leucoflex LXT filter by gravity alone
! do not use the plunger

14 when the diluted whole blood reaches the 10 ml syringe mark (this happens quickly!) start washing the filter by adding 30 ml room temperature PBS to the syringe body
! never let the filter run dry

15 keep topping up the syringe until a total volume of 200 ml PBS has been used to wash the filter

16 allow all liquid to pass through the filter into the 250 ml centrifuge bottle

Extensive washing maximises the recovery of red cells after leukocyte depletion. We have confirmed that infected erythrocytes and reticulocytes - even those containing mature trophozoites and schizonts - can pass through the filter.

spin 1 : pellet red blood cells

17 use precision scales to accurately balance all 250 ml centrifuge bottles to within 0.1 g by adding PBS drop-wise with a pasteur pipette

18 make sure all Silicone O rings are secure and seal the bottles tightly

19 

spin at 1,000 xg for 10 min. at room temperature with the break OFF

20 *note that the Avanti centrifuge takes approx. 1 hour to slow down*
use this time to:
- cool the centrifuge you will use for spin 2 to 4°C
- inspect the Oak Ridge High-Speed Centrifuge Tubes for any cracks or warps - do not use defective tubes

21 once the centrifuge has stopped spinning gently remove the 250 ml centrifuge bottles taking care not to disturb the delicate red cell pellet

22 carefully aspirate and discard the supernatant with a 50 ml serological pipette using an electric pipette controller set to the lowest aspiration setting

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23

the residual volume left in the centrifuge bottle should now be approx. 15 ml (8 ml packed red cells + 7 ml supernatant)

! aspirating too much supernatant will disturb the pellet and lead to red cell loss but leaving too much supernatant will reduce the concentration of saponin in the next step and impair the permeabilisation of red cell membranes

permeabilise red blood cell membranes

24 resuspend the red cell pellet by gently swirling the centrifuge bottle

25 add 57 ml ice-cold **working saponin solution** to achieve a final concentration of 0.0075% saponin (total volume should be approx. 72 ml)

In our hands, this final working concentration of saponin will permeabilise red cell membranes but leave the parasitophorous vacuole around the malaria parasite intact. Carefully determine the optimal concentration of each batch of saponin before use (for details see buffers and solutions in the materials tab).

26 seal and invert the centrifuge bottle to mix thoroughly

27 split the saponin-red cell solution equally between two 50 ml Oak Ridge High-Speed Centrifuge Tubes and place immediately on ice
! Oak Ridge tubes must not be filled above 75% capacity

28 incubate on ice for 10 min. inverting the Oak Ridge tubes every 2 min. to mix

Red cell lysis is complete when the solution becomes transparent - check by holding each tube up to the light. If necessary, extend the incubation time to 15 min.

spin 2 : pellet malaria parasites

29 use precision scales to accurately balance all Oak Ridge tubes to within 0.05 g by adding PBS drop-wise with a pasteur pipette

30 make sure all Silicone O rings are secure and seal the tubes tightly

31 use a permanent marker pen to indicate on each tube the expected location of the parasite pellet and place the tubes into the pre-cooled centrifuge with this mark facing outwards

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32 **If you anticipate the presence of mature parasites (such as in mixed-stage vivax infections) proceed with the next step. If you only expect to find ring-stage parasites (such as in synchronous falciparum infections) then go directly to step 37.**

33 

slow spin to pellet **mature parasites** : 3,000 xg for 30 min. at 4°C with the break OFF

note that the Allegra 25R centrifuge takes approx. 20 min. to slow down

34 gently remove the Oak Ridge tubes from the centrifuge and place onto ice

you should be able to see a small dark parasite pellet and around the pellet a smear of red cell membranes (so-called ghosts)

35 carefully transfer the supernatant to a new Oak Ridge High-Speed Centrifuge Tube using first a 10 ml serological pipette with an electric pipette controller set to the lowest aspiration setting and then a P1000 manual pipette
! do not touch or dislodge the parasite pellet

36 keep the mature parasite pellet on ice

37 

collect ring-stage parasites from the supernatant with a fast spin: 18,000 xg for 20 min. at 4°C with the break OFF

note that the Allegra 25R centrifuge takes approx. 25 min. to slow down

38 gently remove the Oak Ridge tubes from the centrifuge and place onto ice

you should be able to see a small dark parasite pellet and around the pellet a smear of red cell membranes (so-called ghosts)

39 carefully aspirate and discard the supernatant using first a 10 ml serological pipette with an electric pipette controller set to the lowest aspiration setting and then a P1000 manual pipette
! do not touch or dislodge the parasite pellet

40 keep the ring-stage parasite pellet on ice

wash isolated parasites to remove globin

41

Washing the parasite pellet is important to remove as much globin mRNA as possible. Globin mRNA is highly

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abundant and very stable, and in excess will impair analysis of the parasite transcriptome.

42 **If you have mature and ring-stage parasites wash these pellets separately. Remember that for each volunteer there will be two Oak Ridge tubes for each parasite fraction. Take care to choose the correct centrifugation speed.**

43 add 1 ml of ice-cold PBS to the parasite pellet in the first Oak Ridge tube. rinse the walls of the tube well and mix by gently pipetting up and down at least 20 times

44 then transfer to the second Oak Ridge tube containing the same parasite fraction (mature or ring-stage) and repeat the rinse and mix step to pool together samples that were split across two tubes in step 27

45 transfer the pooled PBS-parasite solution to a 1.5 ml eppendorf tube and keep on ice

46 

now choose the **correct centrifugation speed** for each tube:
Step 46 includes a Step case.
mature parasite
ring-stage parasites

step case

mature parasite

to pellet mature parasites spin at 3,000 xg for 8 min at 4°C in a tabletop microfuge

47 after spinning, gently remove the eppendorf tubes from the microfuge and place onto ice

you should be able to see a small dark parasite pellet and few red cell ghosts

48 carefully transfer the supernatant to a new 1.5 ml eppendorf tube and put the first parasite pellet back on ice

49 

spin each supernatant at the appropriate speed and time for mature or ring-stage parasites (see step 46)

Centrifuging the supernatant maximises parasite recovery.

50 gently remove the eppendorf tubes from the microfuge and place onto ice

you may be able to see a very small parasite pellet but very few or no red cell ghosts

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51 carefully aspirate and discard the supernatant and put this second parasite pellet onto ice

52 For each volunteer and parasite fraction (mature or ring-stage) you should now have two 1.5 ml eppendorf tubes on ice, each containing a parasite pellet.

preserve parasites for RNA-sequencing

53

If you have mature and ring-stage parasites you can preserve them separately or pool to maximise the material available for RNA-sequencing. If choosing the second option, make sure that the volume of TRIzol used to pool all four parasite pellets does not exceed 1 ml.

54 for each volunteer add 1 ml TRIzol to the first parasite pellet from step 48. incubate for 5 min. in a 37°C waterbath
! do not exceed this time

55 remove from the waterbath, wipe the tube dry and pipette up and down gently (using a P1000) until the parasite pellet has completely dissolved

56 transfer the TRIzol reagent to the tube containing the second parasite pellet from the same sample (step 51) and pipette gently to dissolve
! do not repeat the 37°C incubation

57 snap-freeze each 1.5 ml eppendorf tube (containing parasites preserved in 1 ml of TRIzol) on dry ice and store at -80°C

58

We have used this protocol to obtain high quality/coverage *P. falciparum* transcriptome data (including subtelomeric *var* and *rifin* genes) from volunteers enrolled in human challenge studies. These data are publicly available from the European Nucleotide Archive (ENA) https://www.ebi.ac.uk/ena/browser/view/PRJEB33557. For details of the downstream steps required for RNA isolation, cleanup, quality control and sequencing please refer to our publication (https://www.medrxiv.org/content/10.1101/2020.09.04.20188144v1). In brief, RNA was purified using a modified phenol-chloroform extraction protocol, DNase-treated and depleted of globin and ribosomal RNA using the Globin-Zero® Gold kit (Illumina, GZG1224, now discontinued. see https://emea.illumina.com/products/selection-tools/rrna-depletion-selection-guide.html for replacements). cDNA was synthesised using a modified low-input *P. falciparum* Smart-Seq2 protocol (Picelli et al. 2013, PMID: 24056875, doi.org/10.1038/nmeth.2639 and Reid et al. 2018, PMID: 29580379, doi.org/10.7554/eLife.33105) and indexed libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (NEB E7370, an updated version of this kit is now available: NEB 7645). Libraries were sequenced on an Illumina Hiseq v4

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flowcell (75 bp paired end reads).

alternative downstream applications

59

Step 59 includes a Step case.

PacBio

scRNA-seq

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