Leucocyte depletion of 2.0mL of *Plasmodium*-infected whole blood using MN2100ff cellulose columns

Protocol

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About this protocol

This protocol was developed by MalariaGEN Resource Centre members based at the Wellcome Trust Sanger Institute, UK. The efficacy of leucodepletion, and ease of blood sampling and filtration was tested in the laboratory and during a pilot field study carried out in 2015.

To watch a video demonstration of this protocol, visit the MalariaGEN YouTube page: https://youtu.be/JGqVDYC-4_U

Version history

Version #	Revision(s)	Release date
1.0	Created protocol	11 May 2016

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1. Purpose and Scope

This protocol describes the preparation and use of a cellulose-based column for the depletion of human leucocytes from a 2.0 mL sample of *Plasmodium*-infected human blood. A blood sample drawn from the arm of a patient suffering from malaria contains a high proportion of contaminating human host DNA (>99%) in comparison to *Plasmodium* DNA. The cellulose column traps human leucocytes thereby enriching the flow-through fraction for parasitised red blood cells (RBCs).

CF11 cellulose powder, first marketed by Whatman® and then by Sigma-Aldrich (as C6288 medium fibre powder) has historically been the preferred material for cellulose columns to remove leucocyte contamination. However, this product is no longer manufactured, prompting us to look for alternatives.

We now present a modified protocol that utilises a different powder called '**MN2100ff**' which is manufactured by Macherey-Nagel GmBH. Having evaluated powder performance through a series of laboratory simulations and field studies, this new protocol is ready to be used on *in-vitro* cultured and field isolates of *Plasmodium*-infected human blood.

Note: This protocol is being shared pre-publication to allow for further field-testing and therefore may be subject to change. Any modifications to the protocol will be included in subsequent updates and listed in the version history (see page 2).

2. Abbreviations

MN	Macherey-Nagel & Co. (GmBH)
mL	millilitre(s)
g	gram(s)
PBS	Phosphate Buffered Saline
rpm	Revolutions per Minute
Rcf (or g)	Relative Centrifugal Force
WBC	White Blood Cell
RT	Room Temperature

3. Materials and Equipment

3.1 Materials

Listed below are the products that we found suitable to use while performing this protocol. There are also other equivalent brands on the market available for use.

During blood sample collection:

- Plastic Whole Blood vacutainer with spray-coated K_2EDTA (6.0ml - lavender caps) [*Becton Dickinson - 367863*]
- Ice packs/cold box or refrigeration (+4°C) **– see Appendix 3a for details**

During cellulose column preparation:

- BD Plastipak 10ml Hypodermic Syringe Luer Lock tip [*Becton Dickinson - 300912*]
- Cellulose MN 2100ff (defatted) [*Macherey-Nagel GmbH & Co. - 815070.1*]
- Whatman® lens cleaning tissue, Grade 105 (W × L: 100 mm × 150 mm, 25 wallets of 25 sheets) [*Sigma-Aldrich - WHA2105841*]
- Spatula
- Scissors
- Box of tissues – **see Appendix 1a for details**
- Rubber-tipped pencil
- Desiccant sachets (Silica gel) **– see Appendix 1b for details**

During blood sample leucodepletion:

- Liquid disinfectant (DuPont™ Rely+On™ Virkon® tablets or an equivalent that is suitable to use on blood-related contents) [*Anachem – 95015661*]
- 70% ethanol or 60% isopropanol (to sterilise/clean work surfaces) – optional
- Pipetboy (or pipettor/pipette controller for 5mL serological pipettes)

- Corning® Costar® Stripette® 5.0ml serological pipettes, individually paper/plastic wrapped [*Sigma-Aldrich CLS4487-200EA*]
- Phosphate buffered saline (PBS), pH 7.4 [*Gibco - 10010-056*]. Alternatively, use PBS tablets [*Gibco - 18912-014*] dissolved in deionised/distilled water.
- 50mL centrifuge tube (sterile) – (*BD Falcon™ Conical Tubes, high-clarity, flat-top screw cap*) [*Becton Dickinson – 352098*]
- Adhesive tape

3.2 Equipment

During cellulose column preparation:

- Laboratory weighing balance

During blood sample leucodepletion:

- Table-top centrifuge for 50mL tubes reaching 1000xg
- Ice packs/cold box/refrigeration (+4°C) or freezer (-20°C) – **see Section 4.4 and Appendix 3a for details**

4. Procedure

4.1 Blood sample collection

This procedure is suitable for the collection of 2-6mL of parasitised whole blood.

A minimum parasite density of 7,000/µl is recommended. For lower parasitemia samples collect larger volumes of blood and leucodeplete in several aliquots of 2.0 ml using more than one cellulose column.

1. Collect whole blood in EDTA-coated vacutainers tubes.

Warning: Do **not** use heparin-coated tubes as it can interfere with the downstream stages of sample processing.

2. Label the vacutainers tubes with the study ID, unique sample identifier, and date of blood collection.
3. Mix the blood sample by gently inverting the tube 10 times.

4. Immediately store the sample at +4°C and commence leucodepletion within 3 hours of blood collection (**see Important reminder below**). If a fridge is not available, use ice packs to cool the sample until it can be leucodepleted.

Warning:

- Storing samples at room temperature for longer than 2 hours after collection can lead to haemolysis and significant loss of DNA.
- It is possible to store blood samples at +4°C for up to 24 hours prior to extraction but significant DNA losses do occur.
- Try to avoid placing your tubes directly into ice as this can lead to cell death and haemolysis around the vacutainer tube wall. Either wrap the tubes in several layers of tissue before placing into the ice, or alternatively use ice packs and tube racks. Where ice packs are not available fill plastic bags with crushed ice. Place the tube rack on top of the ice-bags/packs and place extra ice-bags/packs around the outside of the rack. The aim is to avoid direct contact of the ice, ice-bags/packs with the blood tubes.

Watch a video demonstration

Sections 4.2- 4.4 are demonstrated in a step-by-step video on how to prepare MN2100ff cellulose columns and leucodeplete a 2.0 ml blood sample.

Visit: https://youtu.be/JGqVDYC-4_U

4.2 Cellulose column preparation

Note:

- Cellulose columns can be prepared up to 2 weeks in advance provided they are stored in dry conditions, for example, in sealable plastic bags with desiccant sachets.
- When opening a new jar of cellulose powder, drop a desiccant sachet into it to help prevent future clumping.

4.2.1 Preparing the syringe barrel

Watch video demo: https://youtu.be/JGqVDYC-4_U?t=1m35s

1. Remove the plunger from the 10.0 mL syringe.
2. Using the end of the plunger as a template, cut out two discs of lens cleaning tissue that measure the diameter of the syringe barrel.
3. Place the two discs into the syringe. Use the rubber tip of a pencil to lay the two discs flat on top of each other at the bottom of the syringe barrel. This serves as a barrier to any powder leaking through the nozzle. Make sure the discs cover the entire base of the syringe.

4.2.2 Adding cellulose powder to the syringe

Watch video demo: https://youtu.be/JGqVDYC-4_U?t=2m21s

4. Fill the syringe with 1.0g of MN2100ff cellulose powder **using one of the following two ways**:

Method 1 – Pick up the syringe barrel and hold it over the jar of cellulose. Using a spatula, add small quantities of powder to the syringe. Tap the sides of the syringe a few times and place it on the weighing balance. Make a note of the weight. In case there is any visible cellulose residue on the weighing balance surface, please dust this off, as it will interfere with the weighing. If more cellulose needs to be added, repeat this process until you have added 1.0g of cellulose powder. Tap the sides of the syringe a few times again till the powder settles down and there are no visible air gaps.

OR

Method 2 – Weigh out 1.0g of MN2100ff powder into a small weighing boat or onto a piece of clean paper. Carefully pour this into the syringe barrel, making sure not to spill any powder. Tap the sides of the syringe a few times using a spatula until the powder settles down and there are no visible air gaps.

5. The final top level of the cellulose powder should be between the 6.5-7.0 mL mark on the syringe. If it is too high, then gently tap

the syringe sides once more to settle the powder, or you can use the plunger to gently push the powder down to the correct level.

6. Using a tissue, wipe the exterior of the syringe to remove any residual powder.
7. The cellulose column is now ready for use - **proceed to section 4.3**.
8. If you plan to store the column, then gently insert the plunger halfway down the top of the loose powder without compressing it (if you have not already done so). Leave the plunger in the column until ready to use. Label the syringe column with the date of preparation. Place the column in a sealable plastic bag with a desiccant sachet and use within 2 weeks.

4.3 Blood Sample leucodepletion

This procedure is suitable for the leucodepletion of 2.0 mL of blood. For larger volumes, the procedure should be repeated using a new cellulose column for each extra 2.0 mL of blood

4.3.1 Wetting the cellulose column

Watch video demo: https://youtu.be/JGqVDYC-4_U?t=3m37s

9. Label a sterile, 50.0 mL centrifuge tube with the study ID, unique sample identifier, and the date of leucodepletion.
10. Suspend the cellulose column prepared in section 4.2 securely over the uncapped 50.0 mL centrifuge tube. Make sure the lower tip of the syringe sits above the 20.0 mL mark on the tube. This can be done using adhesive tape. Alternatively, a standard laboratory clamp stand can be used to fasten the column with a collection tube placed directly below it - **see Appendix 3b for details**
11. Slowly and carefully remove the plunger using a gentle upward twisting motion so the powder remains undisturbed. If the cellulose becomes disturbed/displaced when removing the plunger, tap the column on the bench and try re-inserting the plunger to move the cellulose back into place.
12. Gently pipette 4.0 mL of PBS onto the top of the powder without disturbing the surface.

13. Allow the PBS to flow into the column by gravity until no liquid is visible above the powder surface.

4.3.2 Loading the sample into the cellulose column

Watch video demo: https://youtu.be/JGqVDYC-4_U?t=5m8s

14. Make sure you have followed all of the instructions in 4.3.1 before performing the next series of steps.
15. Pipette 2.0 mL of the *Plasmodium*-infected whole blood sample onto the top of the powder, again without disturbing the surface.
16. Gently insert the plunger into the syringe barrel and slowly push down to the top of the powder. This will help the blood pass through the column.
17. Slowly remove the plunger as in step 11.

4.3.3 Eluting the leucodepleted RBCs

Watch video demo: https://youtu.be/JGqVDYC-4_U?t=5m38s

18. Gently pipette 4.0 mL of PBS onto the top of the powder without disturbing the surface.
19. Gently insert the plunger into the syringe barrel and slowly push down to the top of the powder. This will help elute all the parasitised RBCs out of the column. Remove the plunger as in step 11.
20. Repeat steps 18 and 19 once again.
21. The cellulose should now be clean and should no longer be red in colour. If any red colouration persists, remove the plunger as described in step 11 and repeat steps 18 and 19 one more time.
22. Detach the cellulose column from the collection tube and immerse it in liquid disinfectant prior to appropriate disposal.

4.4 Preparation for genomic DNA extraction

Watch video demo: https://youtu.be/JGqVDYC-4_U?t=6m58s

23. Centrifuge the collection tube at 1000xg (**see Appendix 2 for details**) at RT for 5 minutes to pellet the parasitized erythrocytes.

If a centrifuge or cold storage isn't readily available, **see Appendix 3c**.

24. Carefully remove and discard the supernatant using a Pasteur pipette.
25. The sample can now be frozen until gDNA extraction. Typically samples can be stored for up to 3 months at -20°C, or for longer periods if stored at -80°C.

Appendix 1

If unsure of what materials you require, here are some of the suggested products to use while performing the protocol:

a. Any available brand of non-alcoholic, dry wipes are suitable for use. For example, KIMTECH Science Precision Wipes [*Kimberley-Clark Professional – 05511*]
b. Any available brand of granulated or powdered silica gel sachets (1g each) will be suitable for use. For example, 1g Silica Gel pouches (Pack of 50) can be purchased through various sellers on the internet-based retailer Amazon Inc. [*example URL:* http://www.amazon.co.uk/Silica-Gel-Pouches-Sachets-Weight/dp/B004XX2W5G]

Appendix 2

Relative centrifugal force (rcf) is measured in times gravity (x g) and depends on both the speed (rotations per minute; rpm) and the radius of the centrifuge rotor. Hence,

Rcf = (1.118 x 10-5)*(radius of rotor in cm)* (rpm)2

A converter between rcf and rpm conversion calculator can be found at: http://insilico.ehu.es/mini_tools/rcf_rpm.php

Appendix 3

This appendix contains additional details on aspects of the procedure, and how it can be adapted to certain field conditions.

Appendix 3a

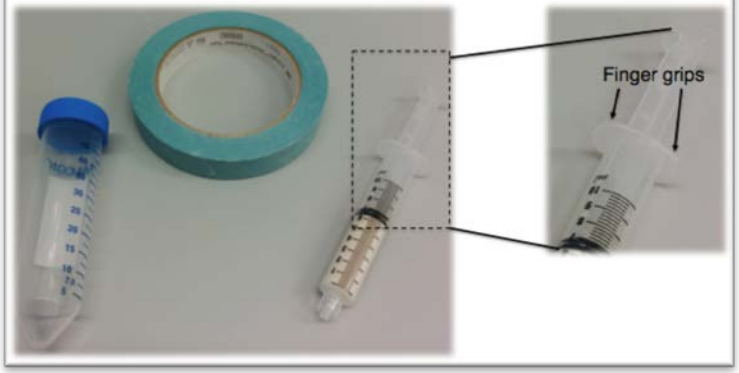
Use ice packs to keep the collected blood or leucodepleted eluate cool and in order to prevent direct contact with bare ice crystals. Alternatively, you can also use a tube box (or tube racks) as a barrier (as shown). Where ice-packs are not available, then crushed ice can be placed into plastic bags and tube boxes/racks used.

Appendix 3b

A step-by-step guide to fastening the cellulose column to the inside of the collection tube

1.

You will need: Adhesive tape, 50ml Falcon tube, cellulose powder column (already prepared)



2.

Remove the lid off the 50ml tube and cut a length of tape approximately 20cm in length.

3.

Wrap the middle of the tape 1/2 way around the syringe below the finger grips at a 45 degree angle and, with the syringe above the 20ml mark inside the tube, attach one half of the sticking tape to the outside of the tube.

4.

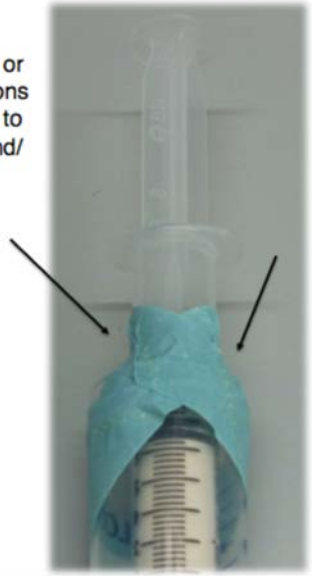
Wrap the other half of the tape forward around the syringe barrel and stick to the outside of the front of the tube.

5.

Stick both of the remaining unstuck sections of tape to the tube by continuing to wrap them around towards the back of the tube.

6.

Press any loosely stuck or unstuck sections of tape firmly to the syringe and/ or tube.



7.

A rear view of the arrangement shows the overlapping tape around the tube.

Appendix 3c

Below are some options to adjust the protocol to meet sample storage and transport limitations. Although alternatives are provided for certain scenarios, these methods may impact on DNA yield.

High yield — **Predicted DNA yield/quality** — Low yield

Scenario 1

Centrifuge, freezer storage, and refrigeration readily available

Sample leucodepletion

Centrifuge at RT for 5 minutes

Store the pellet at -20°C for up to 3 months or at -80°C indefinitely

Extract gDNA

Scenario 2

Freezer storage **NOT** available

(Use one of the following methods)

Method 1:

Sample leucodepletion

Centrifuge at RT for 5 minutes

Refrigerate the pellet at +4°C or store in a cold box/icepack for only up to 3 hours

Extract gDNA immediately

OR

Method 2:

Sample leucodepletion

Centrifuge at RT for 5 minutes

Add lysis buffer and Protease enzyme to the pellet

Refrigerate at +4°C for only up to 48 hrs

Extract gDNA immediately

Scenario 3

Freezer storage and refrigeration **NOT** available

Sample leucodepletion

Centrifuge at RT for 5 minutes

Add lysis buffer and Protease enzyme to the pellet

Store in a cold box/icepack for only up to 12 hours

Extract gDNA immediately

Scenario 4

Centrifuge **NOT readily** available

Sample leucodepletion

Refrigerate the eluate at +4°C or store in a cold box/icepack for only up to 3 hours

Get access to a centrifuge within 3 hours

Centrifuge at RT for 5 minutes

Extract gDNA immediately

Scenario 5

Centrifuge **NOT** available

Sample leucodepletion

Allow the erythrocyte fraction to settle to the bottom for only up to 1.5 hours if at RT, only up to 3 hours if at +4°C

Remove the supernatant with a sterile Pasteur pipette

Store the pellet at -20°C for up to 3 months or extract gDNA immediately