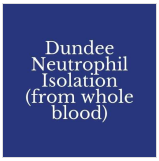

Mar 01, 2021

Dundee neutrophil isolation protocol (from whole blood)

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1 Works for me dx.doi.org/10.17504/protocols.io.bnhtmb6n

asap

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SUBMIT TO PLOS ONE

ABSTRACT

We describe a fast and efficient immunomagnetic negative isolation method to purify peripheral blood neutrophils for LRRK2 kinase pathway analysis in humans

Gain of kinase function mutations in the Leucine rich repeat kinase 2 (LRRK2) are associated with causing Parkinson's disease. LRRK2 phosphorylates a subgroup of Rab GTPases and their phosphorylation levels mirror LRRK2 kinase activation status. Here, we describe a facile and robust method for isolating peripheral blood neutrophils by immunomagnetic negative selection, subsequent treatment with and without the specific LRRK2 kinase inhibitor MLi-2 to ensure that any effect seen is LRRK2 kinase dependent and cell lysis. Neutrophil lysates can then be used to quantify LRRK2 kinase pathway activity by measuring LRRK2-mediated phosphorylation of its endogenous Rab GTPase substrates either by quantitative immunoblotting or targeted mass-spectrometry. The benefit of using peripheral blood neutrophils is that they are an abundant and homogenous white blood cell population that express relatively high levels of LRRK2, Rab10 and other Rab GTPases. The downside is their high intrinsic elastase serine protease activity that necessitates using the neurotoxic, but efficient serine protease inhibitor Diisopropyl fluorophosphate (DIFP) when lysing the cells. Using this assay, we have demonstrated significantly elevated LRRK2 kinase pathway activity in human peripheral blood neutrophils of patients carrying specific mutations such as LRRK2 R1441G (doi:https://doi.org/10.1101/2021.01.28.21249614) and VPS35 D620N (PMID:29743203). This assay can also be used for pharmacodynamic and target engagement studies of small molecule LRRK2 kinase inhibitors.

ATTACHMENTS

Dundee_neutrophil_isolation_protocol_(from_whole_blood).pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

neutrophil, isolation , neutrophil isolation, whole blood

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MATERIALS TEXT

MATERIALS

☒ 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #14190094**

☒ DMSO **Sigma Catalog #D8418**

☒ EasySep™ Direct Human Neutrophil Isolation Kit For processing 100 mL whole blood **Stemcell Technologies Catalog #19666**

☒ RPMI 1640 Medium **Thermo Fisher Catalog #21875034**

☒ 2-Propanol (99.5 %) **Millipore Sigma Catalog #278475**

☒ DIFP (Diisopropylfluorophosphate) **Sigma Aldrich Catalog #D0879**

☒ Ethylenediaminetetraacetic acid (EDTA) **Sigma Aldrich Catalog #E6758**

Consumables:

- BD Vacutainer (BD Vacutainer™ Hemogard Closure Plastic K2-EDTA Tube, Cat# BD 367525)
- 50 mL falcon tube (CELLSTAR, Cat #227 261).
- 15 mL falcon tube (CELLSTAR, Cat #188 271).
- 1.5 mL Eppendorf tubes.
- Marker pen.
- Ice.
- Liquid nitrogen.
- Dry Ice and large polystyrene box for shipping
- Ethanol, in spray bottle.
- Pipette tips (serological 10 mL, 25 mL & 1 mL, 200 µL, 10 µL).
- Personal protection equipment:
  -Disposable gloves.
  -Lab coat.
  -Safety glasses.

Additional Reagents:

- MLi-2 (LRRK2 inhibitor diluted in DMSO at a stock concentration of 200 µM (1000X concentration), available from MRC-PPU Reagents; to order please email: MRCPPUreagents@dundee.ac.uk and address request to both Hilary McLauchlan and James Hastie.
- Lysis buffer (50 mM Tris-HCl pH 7.5, 1%(v/v) Triton X-100, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) β-mercaptoethanol, 1x cOmplete(EDTA-free) protease inhibitor cocktail (Roche), 1 µg/ml Microcystin-LR, 0.5 mM diisopropylfluorophosphate (DIFP).

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Note: Diisopropylfluorophosphate (DIFP) is prepared as a 0.5 M stock solution in isopropanol (it is unstable in aqueous solution) and stored at - 80 °C. Please note that DIFP is toxic and should be handled with care in the fume hood. DIFP can be added to the lysis buffer and used immediately. Alternatively, complete lysis buffer containing DIFP and all other components can be aliquoted and stored at -80 °C for subsequent use for at least 4 weeks.

Note we purchase Microcystin-LR from Enzo Life Sciences, Cat# number ALX-350-012-M001) and make 1 mg/ml stock in DMSO and store at -80 o C.

We can provide frozen lysis buffer in aliquots without Microcystin-LR and DIFP; to order please email MRCPPUreagents@dundee.ac.uk and address request to both Hilary McLauchlan and James Hastie.

Equipment:

- Category 2 biological safety cabinet.
- Easy 50 EasySep™ Magnet (Stemcell technologies, Catalog #18002)
- Centrifuges (Beckman Coulter Allegra X-15R & Eppendorf centrifuge 5417R) or equivalent. Swinging Bucket Rotor for 15 mL and 50 mL falcon tubes at speed of 1000-1200 xg.
- Pipette tips and pipettes.
- Freezer, -80 °C.
- Liquid nitrogen carrier

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

According to local UK regulation we undertake all manipulations and pipetting of human blood in a category 2 biological safety cabinet.

Please note that DIFP is toxic and should be handled with care in the fume hood.

DISCLAIMER:

This method was also described in PMID:29743203 and was also published in the JoVE Journal (doi:10.3791/58956).

BEFORE STARTING

For DIFP, prepare 0.5M stock solution in isopropanol using special precautions.

Prepare two stocks of EDTA solution:

Prepare **0.1 mL EDTA Stock Solution 1** containing 100 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma, Cat #E6758) in phosphate-buffered saline (PBS) (ThermoFisher Cat# 14190094). This is called (EDTA stock solution 1). **100 µl EDTA stock solution 1** is added to each **10 mL human blood** (Step 3). This solution is made by dissolving EDTA powder in PBS and then adjusting pH to 8.0 to allow EDTA to dissolve. This solution is available to order please email: MRCPPUreagents@dundee.ac.uk and address request to both Hilary McLauchlan and James Hastie.

Prepare **60 mL EDTA stock solution 2** containing 1 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma, Cat #E6758) in Dulbecco's phosphate-buffered saline (PBS) (ThermoFisher Cat# 14190094). This is called (EDTA stock solution 2). About **40 mL EDTA stock solution 2** needed for Step 8 in the protocol and approximately

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6 mL - **11 mL** is required for Step 19.

1

Collect **10 mL blood** into BD Vacutainer. Ensure **10 mL blood** is collected by not prematurely discontinuing blood collection. Mix gently by inverting tubes 7-8 times.

2 Transfer **10 mL blood** into a 50 mL falcon tube.

3

Add to blood **100 µl "EDTA Stock Solution 1" (0.1M EDTA-PBS solution)** . Mix gently.

4

Add **0.5 mL "isolation cocktail solution"** from the neutrophil isolation kit to the whole blood sample.

5 Vortex the "RapidSpheres magnetic beads" from the neutrophil isolation kit for **00:00:30** before use in order to resuspend very fine magnetic beads. 30s

6

Add **0.5 mL RapidSpheres magnetic beads** to the blood sample.

7 5m

Mix blood sample gently by inverting the tube and incubate at **Room temperature** for **00:05:00** .

8 Top tube up to 50 ml with **"EDTA Stock Solution 2"** (1 mM EDTA-PBS solution).

9

Mix by very gently pipetting up and down 2-3 times.

10 10m

Place the tube into the magnet and remove the lid to avoid subsequent agitation of tube. Incubate for **00:10:00** at **Room temperature** .

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11 

Carefully pipette the enriched cell suspension that contains neutrophils into a new 50 ml falcon tube. Take care not to touch the side of the tube that is in contact with the magnet and avoid collection and perturbation of the red blood cells at the bottom of the tube (leave approximately **10 mL red blood cell suspension** behind at the bottom of the tube).

12 

Add **0.5 mL RapidSpheres magnetic beads** to the tube from Step 11.

13  5m

Mix blood sample gently by inverting the tube and incubate at **Room temperature** for **00:05:00** .

14 Place the tube into the magnet, remove the lid, and leave for **00:05:00** at **Room temperature** . 5m

15 Carefully pipette the enriched cell suspension that contains neutrophils into a new 50 ml falcon tube. Take care not to touch the side of the tube that is in contact with the magnet. Leave approximately **5 mL suspension** at the bottom of the tube.

16 To ensure complete removal of magnetic beads from cell mixture, place the new tube from Step 15 into the magnet, remove the lid, and leave for **00:10:00** at **Room temperature** . 10m

17 Carefully pipette the enriched cell suspension that now contains pure neutrophils into a new 50 ml falcon tube. Take care not to touch the side of the tube that is in contact with the magnet. Leave approximately **5 mL suspension** at the bottom of the tube.

18 The neutrophils will be in a volume of 30-35 ml at this stage.

19 

Top up isolated cells with **1 mM EDTA stock solution 2** to a final volume of approximately **41 mL** . Pipette up and down to mix. Divide solution equally into 2 tubes with approximately **20 mL** in each tube.

20 

Centrifuge both tubes at **335 x g, 00:05:00** . The setting for acceleration and deceleration is both 5 using a

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Beckman Coulter Allegra X-15R Centrifuge (maximum is 10).

21 During this centrifugation take MLi-2 inhibitor stock (200 µM / 1000X concentration) out of the **-80 °C** freezer, leave at **Room temperature** for subsequent use (Steps 26 and 28).

22 Immediately after the centrifugation, carefully discard the supernatant without disturbing the neutrophil pellet.

23 

Resuspend each cell pellet in **10 mL RPMI medium** at **Room temperature** by gently pipetting cells up and down 4 times.

24 Label one tube “DMSO” and other tube “MLi-2”.

25 

To “DMSO” labelled tube, add **10 µl DMSO** and mix gently by pipetting up and down 2 times with a 10 ml pipette.

26 

To “MLi-2” labelled tube, add **10 µl 200 µM MLi-2 stock solution (final concentration 200 nM)** and mix gently by pipetting up and down 2 times with a 10 ml pipette.

27 

40m

Incubate samples for **00:30:00** at **Room temperature** . Mix gently by inversion every **00:10:00** during the incubation.

28 During the incubation period,

28.1 Remove 0.5 M DIFP stock from **-80 °C** freezer and place in fume hood **On ice** .

28.2 Remove 1 mg/ml Microcystin-LR stock from **-80 °C** freezer and leave at **Room temperature** to thaw.

28.3 Defrost an aliquot (**0.25 mL**) of the lysis buffer by taking it out of the freezer, allowing it to defrost

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at **Room temperature** and then placing it **On ice** for subsequent use (Step 34).

28.4 Prepare **1 mL RPMI medium** containing **1 µl DMSO** and call this "DMSO Resuspension Buffer".

28.5 Prepare **1 mL RPMI medium** containing **1 µl 200 µM MLi-2** and call this "MLi-2 Resuspension Buffer".

29 

After the 30 min incubation, centrifuge both tubes at **335 x g, 00:05:00** . The setting for acceleration and deceleration is both 5 using a Beckman Coulter Allegra X-15R Centrifuge (maximum is 10) to pellet the neutrophils.

30 Carefully discard the supernatant in each tube without disturbing the neutrophil pellet.

31 For the DMSO labelled sample gently resuspend pellet in **1 mL "DMSO resuspension buffer"** and transfer to Eppendorf tube labelled "DMSO".

32 For the MLi-2 labelled sample gently resuspend pellet in **1 mL "MLi-2 resuspension buffer"** and transfer to Eppendorf tube labelled "MLi-2".

33 

Centrifuge both tubes at **300 x g, 00:03:00** .

34 During this time proceed with preparing the lysis buffer: In the fume hood carefully to the **0.25 mL lysis buffer** 15m add **0.25 µl 0.5 M DIFP solution** as well as **0.25 µl 1 mg/ml microcystin-LR** . Mix and leave **On ice** until use. DIFP should be added to lysis buffer within **00:15:00** of cell lysis (Step 38 and 39) as DIFP relatively unstable in aqueous solution.

35 Return 0.5M DIFP stock to **-80 °C** freezer at this stage.

36 After the centrifugation in Step 33, immediately proceed to carefully remove all supernatant with a pipette without disturbing the neutrophil pellets.

37 Place the tubes with the neutrophil cell pellets **On ice**

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38

Immediately add **100 µl lysis buffer containing DIFP** to the “DMSO” labelled tube. Using a 100-200 µL pipette, re-suspend the cell pellet by pipetting up and down until all cells are lysed (5-10 times).

39

Next add **100 µl lysis buffer containing DIFP** to the “MLi-2” labelled tube. Using a 100-200 µL pipette and using a 100-200 µL pipette, re-suspend the cell pellet by pipetting up and down until all cells are lysed (5-10 times).

40 10m

Incubate cell lysates **On ice** for **00:10:00** . Place “DMSO” and “MLi-2” labelled tubes into a centrifuge and remove cell debris by centrifugation at **14000 rpm, 4°C, 00:15:00** .

41 Transfer ”DMSO” and “MLi-2” supernatants (containing neutrophil lysates) into new Eppendorf tubes. Discard debris pellet.

42 Immediately snap freeze samples in liquid nitrogen.

43 Store samples at **-80 °C** until further use or shipment on dry ice keeping samples frozen at all times.

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