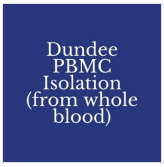

Mar 01, 2021

Dundee peripheral blood mononuclear cell (PBMC) isolation protocol (from whole blood)

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bnhxmb7n

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SUBMIT TO PLOS ONE

ABSTRACT

We describe a fast and efficient peripheral blood mononuclear cell (PBMC) isolation for LRRK2 kinase pathway analysis

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 PBMCs by facilitated density gradient centrifugation, 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. PBMC lysates can then be used to quantify LRRK2 kinase pathway activity by measuring LRRK2-mediated phosphorylation of its endogenous RabGTPase substrates in human peripheral blood neutrophils either by quantitative immunoblotting or targeted mass-spectrometry. The benefits of using PBMCs are that they are routinely used as a biomatrix for Parkinson's research and their isolation procedure is significantly faster and easier than that for example of neutrophils or monocytes from peripheral blood. However, their heterogeneity in terms of cellular composition and highly variable expression levels of LRRK2 and its substrates including Rab10 make PBMC a suboptimal biomatrix for reliably measuring LRRK2 kinase pathway activity. A potential use could be target engagement studies in clinical trials targeting LRRK2.

ATTACHMENTS

Dundee_peripheral_blood_mononuclear_cell_(PBMC)_isolation_protocol_(from_whole_blood).pdf

DOI

dx.doi.org/10.17504/protocols.io.bnhxmb7n

PROTOCOL CITATION

Adil R. Sarhan, Andrew J.M. Howden, Dario R. Alessi, Esther M. Sammler 2021. Dundee peripheral blood mononuclear cell (PBMC) isolation protocol (from whole blood). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bnhxmb7n

KEYWORDS

peripheral blood mononuclear cell, PBMC, PBMC isolation, whole blood

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CREATED

Oct 16, 2020

LAST MODIFIED

Mar 01, 2021

OWNERSHIP HISTORY

Oct 16, 2020	Emily Hasser	University of Washington
Nov 16, 2020	Dario Alessi	

PROTOCOL INTEGER ID

43287

MATERIALS TEXT

MATERIALS

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

DMSO **Sigma Catalog #D8418**

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

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

Ficoll-Paque PREMIUM **Ge Healthcare Catalog #17-5442-02**

2% Fetal bovine serum (FBS) **Contributed by users Catalog #S-001-BR**

Consumables:

- SepMate™ tube (STEMCELL, Cat# 85450), 50 mL capacity.
- BD Vacutainer sodium heparin 6ml tubes, green BD Hemogard Closure 13x100mm (BD, cat# 367876). (3 tubes are needed for each PBMC preparation).
- 20 ml syringes (BD, cat# 300613)
- 21-G needle (for example BD, # 304432)
- 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:

- Dulbecco's phosphate-buffered saline (PBS) (ThermoFisher Cat# 14190094) with 2% Fetal bovine serum (FBS), (Life Sciences Production, Cat # S-001- BR). Prepare fresh on day of use. 50ml of PBS with 2% FBS are needed per PBMC preparation: add 1ml of FBS to 49ml of PBS. Use at room temperature.
- MLi-2 (LRRK2 inhibitor diluted in DMSO at a stock concentration of 200 µM (1000X concentration), available from MRC-

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PPU Reagents to order please email: MRCPPUreagents@dundee.ac.uk.

- 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, 1 µg/ml Microcystin-LR, 0.27 M sucrose, 0.1% (v/v) βmercaptoethanol, 1x cOmplete(EDTA-free) protease inhibitor cocktail (Roche) + 1 mM diisopropylfluorophosphate (DIFP).

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 °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.
- 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.
- Sharps waste container.
- 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.

DISCLAIMER:

BEFORE STARTING

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

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1

Collect 3 x **6 mL blood** into BD Vacutainer sodium heparin 6ml tubes, green BD Hemogard Closure 13x100mm (BD, cat# 367876). Mix gently by inverting tubes 8 - 10 times.

2 Add density gradient medium Ficoll-Paque PREMIUM to the SepMate™ tube (STEMCELL, Cat# 85450, 50 mL capacity) using a 20ml syringe with a large bore needle (at least 21G) by carefully adding the **15 mL** through the central hole of the SepMate™ insert. Dispose of needle appropriately. The top of the density gradient medium will be above the insert. NOTE: Small bubbles may be present in the density gradient medium after pipetting. These bubbles will not affect performance.

3

Using a 5 ml pipette, transfer **15 mL blood** from the BD Sodium heparin tubes into a 50 ml Falcon tube and dilute at a 1:1 ratio by adding **15 mL PBS with 2% FBS** . Mix gently by inversion.

4 Keeping the SepMate™ tube vertical, add the diluted blood sample by pipetting it down the side of the tube. Take care not to pour the diluted blood sample directly through the central hole that lies in the centre of the insert disk.

5

Centrifuge at **1200 x g, Room temperature , 00:10:00** , with the brake ON.

6

After centrifugation, carefully pipette off the top plasma transparent layer and discard appropriately.

7

Using a new pipette, collect the remainder of the sample above the insert disk (which contains the PBMCs) into a 15 mL falcon tube.

8

Top up the tube to **15 mL PBS with 2% FBS** . Mix gently by inversion.

9

Centrifuge at **1000 x g, 00:02:00** , brake ON, to pellet the PBMCs.

10 Carefully discard the supernatant without disturbing the PBMC pellet.

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11 Carefully resuspend the cell pellet in **10 mL PBS with 2% FBS** .

12 

Centrifuge at **1000 x g, 00:02:00** , brake ON to pellet the PBMCs.

13 During the centrifugation Step 12, take MLi-2 inhibitor stock solution (200 µM / 1000x) out of the **-80 °C** freezer, and leave at **Room temperature** to thaw for subsequent use (Steps 19 and 21)

14 Immediately after the centrifugation, carefully discard the supernatant without disturbing the PBMC pellet.

15 

Resuspend the PBMC cell pellet in **10 mL PBS with 2% FBS** by gently pipetting cells up and down 4 times.

16 Divide resuspended PBMCs equally into 2 tubes (**5 mL** in each).

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

18 

To “DMSO” labelled tube, add **5 µl DMSO** and mix gently by pipetting up and down 2 times with a 10 ml pipette.

19 

To “MLi-2” labelled tube, add **5 µl of 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.

20 

40m

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

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21 During the incubation period:

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

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

21.3 Defrost an aliquot (**0.5 mL**) of the lysis buffer by taking it out of the freezer allow to defrost at **Room temperature** and then place it **On ice** for subsequent use (Step 27)

21.4 Prepare **1 mL PBS with 2% FBS** containing **1 µl DMSO** and call this “DMSO Resuspension Buffer”

21.5 Prepare **1 mL PBS with 2% FBS** containing **1 µl 200 µM MLi-2 stock solution** and call this “MLi-2 Resuspension Buffer”

22 30m

After the **00:30:00** 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 PBMCs.

23 Carefully discard the supernatant in each tube without disturbing the PBMC pellets.

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

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

26

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

27 15m

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During this time proceed with preparing the lysis buffer: In the fume hood carefully to the **0.5 mL lysis buffer** add **0.5 µl 0.5 M DIFP solution** as well as **0.5 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 (Steps 31 and 32) as DIFP relatively unstable in aqueous solution.

28 Return 0.5M DIFP to **-80 °C** freezer.

29 

Immediately after the centrifugation in Step 26, carefully discard supernatant by using a pipette while taking care not to disturb the PBMC pellets.

30 Place the tubes with the PBMC cell pellets **On ice** .

31 

Immediately add **200 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).

32 

Next add **200 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).

33 

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** .

34 Transfer "DMSO" and "MLi-2" supernatants (containing PBMC lysates) into new Eppendorf tubes. Discard debris pellet.

35 Immediately snap freeze samples in liquid nitrogen.

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

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