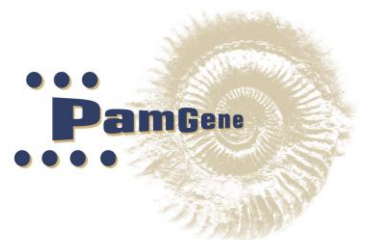
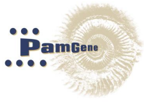
Protocol 1160

For Preparation of Lysates of Cell Lines or Purified Cells





Protocol for Kinase Profiling of Cell Lines or Purified Cells
Version 4.2

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1 INTRODUCTION

1.1 Intended use

The aim of this protocol is to prepare cell lysates from cell lines or purified cells for PamChip® Kinase profiling analysis.

2 PROTOCOL

2.1 Materials & reagents

Material/Equipment	Supplier	Catalog number	Storage
M-PER™ Mammalian Extraction Buffer	Thermo Fischer Scientific	78503 or 78501 or 78505	RT
Halt™ Phosphatase Inhibitor Cocktail (100x)	Thermo Fischer Scientific	78420 or 78426 or 78427 or 78428	+4°C
Halt™ Protease Inhibitor Cocktail, EDTA free (100x)	Thermo Fischer Scientific	78437 or 78425 or 78439	+4°C
PBS: Phosphate Buffered Saline (ice-cold)			
Pierce™ Coomassie Plus (Bradford) Assay Kit	Thermo Fischer Scientific	23236	RT

Cells for lysis

Cell culture

- Adherent (recommended 80-90% confluency)
- Suspension cells

Purified cells, e.g.:

- Peripheral Blood Mononuclear Cells (PBMC's)
- Monocytes (*For cells purified from blood, be sure no (or as few as possible) erythrocytes are present, for the large amount of protein in red blood cells disturbs the kinase assay).*

2.2 Equipment

Centrifuge (pre-cooled to 4°C).

2.3 Safety & Precautions

Standard laboratory safety regulations apply.

2.4 Cleaning Tools and Equipment

No special cleaning required.

2.5 Procedure

Handle all samples in the same way. Keep all samples as cold as possible by using ice-cold solutions, keeping the samples on ice and pre-cooling all tubes. Handle lysates gently, avoid foaming (protein denaturation), never vortex lysates. Read 3 Notes AND FAQs before proceeding.

1. Pre-cool centrifuge to 4ºC. Pre-cool all solutions and tubes on ice and work fast (do not process more than 4 samples in parallel). Use ice cold lysis buffer and keep lysates on ice.
2. Prepare lysis buffer by diluting Halt Phosphatase Inhibitor Cocktail and Halt Protease Inhibitor Cocktail EDTA free 1:100 in M-PER Mammalian Extraction Buffer and store on ice. For more detailed information we refer to the manufacturer's protocol, see 2.1.
3. Prepare for every sample at least 3 tubes with adequate labelling and store on ice.
4. Process 4 to 6 samples simultaneously.
5. For **suspension cells**, proceed with step 6,
   For **loosely attached adherent cell**s, proceed with step 11,
   For **adherent cells**, proceed with step 20.
6. For **suspension cells**, transfer cell suspension(s) to centrifuge tube(s). Centrifuge tubes for 8 min 500 x g at 4°C (In case of unequal volumes, tare tubes with culture medium).
7. Pour off medium in one movement. When pellet detaches, stop and centrifuge again. Place tube on ice.
8. Carefully resuspend pellet in 1 ml ice-cold PBS and transfer this to an ice-cold 1.5ml Eppendorf vial and centrifuge for 5 min 1000xg at 4°C.
9. Carefully remove as much supernatant as possible by pipetting without disturbing the pellet and place on ice.
10. Continue with step 14.
11. For **loosely adhering cells**, pour off the culture medium. Place flask/ plate on ice. Pipette ice-cold PBS in flask/ plate, pipette gently over the cells to detach them. When cells are detached, transfer them to a cold centrifuge tube/ 1.5ml vial. Suggestions for volumes to be used are given in Note 5.
12. Centrifuge for 8 min 500 x g at 4°C (In case of unequal volumes, tare tubes with PBS).
13. Carefully remove as much supernatant as possible by pipetting without disturbing the pellet, and place tube/ vial on ice.

14. Lyse cell pellet in the 1.5 ml Eppendorf vial with lysis buffer (M-PER containing phosphatase and protease inhibitors, See Step 2; Use 100 µl lysis buffer for $1x10^6$ cells). Start lysis by gently pipetting up and down 8 times. To reduce shear stress, the pipette tip can be cut.
15. Lyse samples on ice for 15 min in total. Pipette each lysate up and down once every 5 min to homogenize the lysate. Check visually whether solution is homogenous, or check lysate under a microscope.
16. Centrifuge the vials for 15 min in a pre-cooled centrifuge at maximum speed (>10,000 x g) at 4°C.

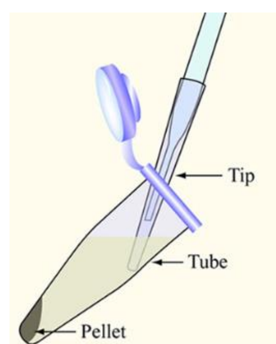


Figure 1. Collect supernatant carefully

17. Collect the supernatant lysate (*Figure 1*) and transfer to a pre-cooled clean labelled vial. Divide this lysate over at least 3 vials. To avoid freeze-thaw cycles of the lysate we recommend aliquots of 15 µl. Collect 5 µl sample for protein quantification purposes.
18. Store samples at –80°C (preferably snap-freeze samples on dry ice or liquid nitrogen before storage; Use the same procedure consistently for all samples).
19. Perform protein quantification.
20. For **adherent cells**, pour off the culture medium. Place flask/ plate on ice. For additional information on adherent cells, see Notes 6, 7.
21. Pipette ice-cold PBS in flask/ plate. Swirl PBS gently over cells, and pour off.
22. Repeat this washing step.
23. Keep flask/plate on ice. Lyse cells with lysis buffer (M-PER containing phosphatase and protease inhibitors, See Step 2). For volumes per flask/ well see Note 5.
24. Use a cell scraper to loosen the cells. Pipette the lysate up and down every 5 min to homogenize the lysate. The frequency of pipetting depends on how easy the cell pellet dissolves in lysis buffer. If a more concentrated cell lysate is required, use the first lysate to lyse a second flask/ well. Shorten the incubation time per cycle, but be sure to transfer all cell material/ debris.
25. Check visually whether solution is homogenous, or check lysate under a microscope.
26. Collect the lysate in pre-cooled 1.5 ml vials.

27. Centrifuge the vials for 15 min in a centrifuge at maximum speed (>10,000 x g) at 4°C.
28. Collect the supernatant lysate (*Figure 1*) and transfer to a pre-cooled clean vial. Divide this lysate over at least 3 vials. To avoid freeze-thaw cycles of the lysate we recommend aliquots of 15 µl. Collect 5 µl sample for protein quantification purposes.
29. Store samples at –80°C (preferably snap-freeze samples on dry ice or liquid nitrogen before storage; Use the same procedure consistently for all samples).
30. Perform protein quantification.

2.6 Waste disposal

Use the accepted internal procedures for disposal of tissue residues and for laboratory waste.

2.7 Quality control

Lysis can be checked visually under a microscope to ensure cells are disrupted completely.

3 NOTES AND FAQS

3.1 Notes

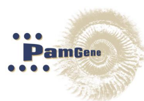
1. Biological replicates should be cultured under the same experimental conditions. Samples that will be compared should be cultured and processed under identical conditions.
2. Cell pellets can be harvested, snap frozen and stored at -80 ºC to be lysed at a later stage. However, immediate lysis gives better results. When using frozen cell pellets, leave them on ice for 2 min, before adding ice-cold M-PER lysis buffer. Don’t allow the cell pellet to thaw completely.
3. Step 14: When less than 10E6 cells are available, reduce the volume of lysis buffer. The minimal volume that can be handled easily is 25 µl.
4. Step 14: When cells are small, the number of cells per 100 µl lysis buffer can be increased or the volume of lysis buffer can be reduced.
5. Step 11 and 23: Add an appropriate volume of lysis buffer to cover (most of) the culture surface area. As a general rule, use 100 µl lysis buffer for $1x10^6$ cells
6. Step 20: For adherent cells, trypsin treatment to detach cells must be avoided because this influences kinase activity. Scrape cells in lysis buffer.
7. Scraping cells in PBS must be avoided, because scraping may damage cells and lead to leakage of cell contents (Ref 1). Scraping in lysis buffer is advised, contents of broken cells are collected in the lysis buffer.

8. Steps 17 and 28: when accidentally a bit of the pellet is transferred, samples can be centrifuged again to remove this debris.
9. In general, the numbers of cells needed per array for the STK assay is about 40,000 cells, and for PTK assay 100,000 – 200,000.
10. Freeze-thawing may affect kinase activity. Always use a never-thawed aliquot for a PamChip® assay.
11. When cells have been cultured in the presence of a kinase inhibitor, they should be washed carefully to remove any external inhibitor molecules. Inhibitor taken up by the cells might end up in the lysate and interfere with inhibitor studies. This depends on the cell line and the inhibitor.
12. The preferred protein concentration is at least 1 µg/µl; however, 0.5 µg/µl is also acceptable, but lower concentrations give poor results.
13. Step 19 and 30: Recommended Coomassie Plus (Bradford) Assay Kit see 2.1. Follow instructions of the supplier.
14. Step 19 and 30: Perform protein quantification of all samples simultaneously.
15. Step 19 and 30: Prevent variation in protein quantification. Use 3 technical replicates or a dilution series of minimal 3 with readout within the linear part of the calibration curve
16. Step 19 and 30: Use a sample with known protein concentration as internal control for protein quantification.
17. Step 19 and 30: When reading the extinction, make sure that no air bubbles are present in the light path, they disturb the reading and protein quantification

Ref.1. Batista U., Garvas M., Nemec M., Schara M., Veranic P., Koklic T.; Effects of different detachment procedures on viability, nitroside reduction and plasma membrane heterogeneity of V-79 cells Cell Biol Int 2010 34(6) 663-8

3.2 Frequently asked questions on materials & reagents

- "*Which protease inhibitors are present in the recommended Protease inhibitor cocktail #78437?*"
  Product #78437 contains the protease inhibitors aprotinin, bestatin, E-64, Leupeptin, AEBSF and pepstatinA.



- *"Which phosphatase inhibitors are present in the Phosphatase inhibitor cocktail #23236?"*
  Product #23236 contains the phosphatase inhibitors NaF, NaOrthovanadate, Na PPi and beta-glycerol-phosphate.
- *"Can I use the Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (catalog number 78441) instead of the two separate inhibitor cocktails?"*
  No, product 78441 is not identical to the combination of the protease and phosphatase inhibitors mentioned above. Product number 78441 contains the same four phosphatase inhibitors as product #23236, but 4 instead of 6 protease inhibitors (aprotinin, bestatin, E-64 and Leupeptin).
- *"Can I use Protease and Phosphatase inhibitor cocktails from other suppliers?"*
  Compatibility of PamChip Cell Lysate Kinase Profiling assays with other lysis protocols, buffers and inhibitor cocktails has been demonstrated, but could require further optimization and might result in different profiles.
- *"How can I calculate the correct rotor speed (RPM) to use for pelleting my cells by centrifugation?"*
  g Force or Relative Centrifugal Force (RCF) is the amount of acceleration to be applied to the sample. It depends on the revolutions per minute (RPM) and radius of the rotor. It is relative to the force of Earth's gravity. A good and precise protocol for centrifugation instructs you to use the g force rather than RPMs because the rotor size might differ, and g force will be different while the revolutions per minute stay the same. Centrifuges may have an automatic converter. To convert the g force to rpm for a particular centrifuge you can check whether the supplier of your centrifuge has a conversion tool on its website. Alternatively, you can measure the maximum radius of your rotor and enter the information into the formula:

  g Force (RCF) = (rpm)2 × 1.118 × 10-5 × r
  with
  g = Relative Centrifuge Force
  r = rotational radius (cm)
  N = Revolutions Per Minute (RPM)

4 TRANSPORT OF SAMPLES

For studies conducted by PamGene, please contact PamGene before preparing samples about exact amounts needed for specific studies.
In case of shipment to PamGene, the samples need to be clearly labelled and packed in a sufficient amount of dry ice accompanied by a list of samples, with preferable an available tracking number.

Shipping address:

D.A. Pijnenburg BSc
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5 SUPPORT

For questions contact our support team.

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6 RIGHTS AND RESTRICTIONS

6.1 Disclaimer

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