Protocol 7642

For Preparation of Cell Pellets and Lysates

PamGene

7642 Preparation of Cell Pellets and Lysates
Version 1.0

Contents

1 OVERVIEW

This protocol describes a method for preparing cell pellets (Section 4.1) for shipment to PamGene or, when applicable, lysed (Section 4.2) for application on PamChip® kinase profiling assays.

We recommended starting with 1 to 2 million cells per treatment/replicate to achieve the preferred total protein concentration yield of 1 µg/µl.

The cells being prepared may be suspension cell lines or purified cells, such as peripheral blood mononuclear cells (PBMCs) or sorted immune cells (e.g. monocytes, T cells, B cells). For cells purified from blood, make sure that as few erythrocytes as possible are present. The large amount of protein in erythrocytes can negatively impact the kinase assay.

2 MATERIALS AND REAGENTS

2.1 For preparing cell pellets for shipment only:

- *1X Phosphate Buffered Saline (PBS) without Magnesium and Calcium (ice-cold)*
- *Conical tubes (if pelleting large volumes of cell suspensions)*
- *1.5 ml Eppendorf tubes (preferably SafeLock®)*

2.2 For preparing cell pellets and lysis:

- *M-PER® Mammalian Protein Extraction Buffer (M-PER Buffer)*
  (Thermo Fischer Scientific™ 78503) stored at RT
- *Halt™ Phosphatase Inhibitor Cocktail*
  (Thermo Fischer Scientific™ 78420) stored @4°C
- *Halt™ Protease Inhibitor Cocktail, EDTA free*
  (Thermo Fischer Scientific™ 87785) stored @4°C
- *NaCl (5M stock)*
- *1X Phosphate Buffered Saline (PBS) without Magnesium and Calcium (ice-cold)*
- *Conical tubes (if pelleting large volumes of cell suspensions)*
- *1.5 ml Eppendorf vials (preferably Safe-Lock)*
- *Pierce™ Bradford Plus Protein Assay Kit*
  (Thermo Fischer Scientific™ 23236) stored @4°C

3 LYSIS BUFFER PREPARATION (IF APPLICABLE)

- Lysis buffer should be made just prior to use and stored on ice.
- Prepare lysis buffer by diluting both Halt Phosphatase Inhibitor Cocktail and Halt Protease Inhibitor Cocktail EDTA-free 1:100 (1x) and NaCl to 150 mM final concentration in M-PER Buffer. Always prepare an excess of 10% of the total volume of MPER buffer required for all samples.

  For example, if 100 µl Lysis buffer is required:

	Volume	Final conc.
M-PER Protein Extraction Reagent	104.5 µl	-
100x HALT Protease Inhibitors EDTA-free	1.1 µl	1x
100x HALT Phosphatase inhibitor cocktail	1.1 µl	1x
5M NaCl	3.3 µl	150 mM
Total volume lysis buffer	110 µl	-

4 PROTOCOL

Before beginning:

- Read Notes and FAQ section
- Pre-cool centrifuge to 4°C
- Prepare lysis buffer (if applicable) and place on ice
- Place 1X PBS on ice
- Label the required number of Eppendorf tubes and conical tubes (where applicable)

Note: Do not process more than 8 samples at a time.

4.1 Preparation of cell pellets

4.1.1 Label a 1.5 ml Eppendorf tube for each sample with adequate information and store on ice. For large volumes of cell suspensions, label an additional conical tube for each sample to use for collecting cells and store on ice.

4.1.2 Transfer at least 1-2 million cells of cell suspension to a labelled Eppendorf tube or conical tube if starting with large volume. Centrifuge the tubes for 8 minutes at 500 x g and 4°C.

Note: Large volumes of cell suspensions may require a longer centrifugation time to form a pellet.

4.1.3 Slowly pipette or pour off medium in one movement into a waste beaker. If the pellet detaches, stop and centrifuge again. Place pellet on ice.

4.1.4 Gently resuspend the pellet in 1 ml ice-cold PBS. If a conical tube was used, transfer the resuspended pellet to its corresponding 1.5 ml Eppendorf tube on ice. Centrifuge for 5 minutes at 1000 x g and 4°C.

4.1.5 Remove all PBS from the tube without disturbing the pellet. It is important to **remove all PBS** and keep the pellets on ice!

4.1.6 If shipping cell pellets to PamGene, snap freeze the pellets on dry ice or using liquid nitrogen and store at –80°C until shipment. If lysing the cell pellets, continue to Section 4.2 Lysis of Cell Pellets) or store pellets at –80°C until ready to lyse (see Notes Sectio 5.3).

4.2 Lysis of Cell Pellets

4.2.1 Label four or five 1.5 ml Eppendorf tubes for each sample with adequate information and store on ice.

4.2.2 Add cold lysis buffer with inhibitors and NaCl (See Section 3 Lysis Buffer Preparation) to each sample pellet. Do not process more than 8 samples at a time.

For samples of $1x10^6$ to $2.5x10^6$ cells we recommend using 25 µl lysis buffer. For samples with higher cell counts, use 100 µl lysis buffer per $1x10^7$ cells. Start a timer.

4.2.3 Gently pipette the volume up and down five times every 5 minutes for 30 minutes in total to lyse the samples. Keep the samples on ice during lysis.

4.2.4 Centrifuge the lysates for 15 minutes in a pre-cooled centrifuge at maximum speed (> 10,000 x g) and 4°C.

4.2.5 Collect all of the lysate supernatant (see Figure 1) and transfer to its respective labelled Eppendorf tube on ice.

4.2.6 Aliquot 5 µl lysate in a pre-cooled labelled tube to use for protein quantification. Divide the remaining lysate equally between the remaining three or four tubes. We recommend aliquots of 10 to 15 µl to avoid freeze-thaw cycles of the lysate.

4.2.7 Snap-freeze the lysate aliquots on dry ice or using liquid nitrogen and store at –80°C until use.

4.2.8 Perform protein quantification using Pierce™ Bradford Plus Protein Assay Kit according to the instructions of the supplier.

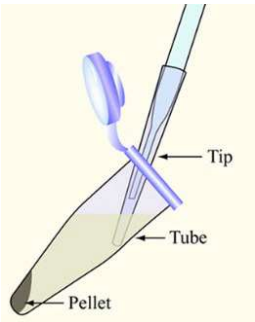

Figure 1. Collect supernatant carefully

5 NOTES

5.1 Biological replicates should be cultured under the same experimental conditions. Samples that will be compared should be cultured and processed under identical conditions.

5.2 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.

5.3 Cell pellets can be harvested, snap frozen and stored at -80 °C to be lysed at a later stage. However, immediate lysis yields better results. If lysing frozen cell pellets, leave the pellets on ice for 2 min before adding ice-cold M-PER lysis buffer. Do not allow the cell pellet to thaw completely.

5.4 If a bit of the pellet is transferred when pipetting the supernatant, samples can be centrifuged again to remove this debris.

5.5 Repeated freeze-thawing may affect kinase activity. Always use a freshly thawed aliquot for a PamChip® assay.

5.6 The preferred protein concentration is at least 1 µg/µl. In case of questions or concerns regarding your protein concentration, please contact the PamGene Support team (support@pamgene.com).

5.7 Recommended for protein quantification is the Coomassie Plus (Bradford) Assay. Follow instructions of the supplier.

a. Perform protein quantification of all samples simultaneously.
b. Use 3 technical replicates or a dilution series of minimal 3 with readout within the linear range of the calibration curve.
c. Use a sample with known protein concentration as an internal positive control for protein quantification.
d. Before reading the absorbance, make sure that no air bubbles are present in the light path, they interfere with the absorbance reading and can thus affect the protein concentration calculation.

5.8 Typically, PamGene requires 1 to $2x10^6$ cells in a cell pellet for testing conditions. In case of extra replicates and/or inhibitor spike-in experiments, more cells may be required. Please contact the PamGene Support team (support@pamgene.com) before preparing samples if you have questions or concerns regarding the number of cells required.

5.9 When cells have been cultured in the presence of a kinase inhibitor, wash carefully to remove any external inhibitor molecules.

6 FREQUENTLY ASKED QUESTIONS

- *"Is it really necessary to provide a minimum of 1 million cells?"*

  A minimum 1 million cells per sample is recommended to ensure sufficient protein yield after lysis. However, protein yields can vary by cell type, shape, and viability. In case of lack of clarity on starting cell number, refer your question to PamGene Support team (support@pamgene.com) or appointed project leader.

- *"Which protease inhibitors are present in the recommended Halt™ Protease Inhibitor Cocktail?"*

  Halt™ Protease Inhibitor Cocktail, EDTA-free (Thermo Fischer Scientific™, Cat# 78437) includes the protease inhibitors AEBSF, aprotinin, bestatin, E-64, Leupeptin, and pepstatin A.

- *"Which phosphatase inhibitors are present in the recommended Halt™ Phosphatase Inhibitor Cocktail?"*

  Halt™ Phosphatase Inhibitor Cocktail (Thermo Fischer Scientific™, Cat# 78420) includes the phosphatase inhibitors sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and β-glycerophosphate.

- *"Can I use the Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (Thermo Fischer Scientific™, Cat# 78441) instead of the two separate inhibitor cocktails?"*

  No, this product (Thermo Fischer Scientific™, Cat# 78441) is not identical to the combination of the protease and phosphatase inhibitors mentioned above – it contains fewer protease inhibitors.

- *"Can I use Protease and Phosphatase inhibitor cocktails from other suppliers?"*

  Compatibility of PamChip® kinase profiling assays with other lysis protocols, buffers and inhibitor cocktails has been demonstrated, but could require further optimization and may result in different profiles.

- *"How can I calculate the rotor speed in RPM to use for pelleting my cells by centrifugation?"*

  G-force or Relative Centrifugal Force (RCF) is the amount of gravitational force to be applied to the sample and takes into account the revolutions per minute (RPM) and radius of the rotor. It is preferable to use RCF rather than RPM because the rotor size might differ, and RCF will be different, while the revolutions per minute stay the same. Most modern centrifuges have the functionality that measures both. To convert RCF to RPM for a centrifuge that only has an RPM setting, 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:

  $$RPM = \sqrt{\frac{RCF}{(r \times 1.118)}} \times 1000$$

  r = rotational radius (mm)

7 RIGHTS AND RESTRICTIONS

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Notes:

Customer Support
PamGene International B.V.
Wolvenhoek 10
5211 HH ’s-Hertogenbosch
The Netherlands
☏ +31 (0)73 615 80 80 General
☏ +31 (0)73 615 89 00 customer support
🖷 +31 (0)73 615 80 81
✉ support@pamgene.com