Protocol 7644

For Preparation of Lysates from Adherent Cells

PamGene

7644 Preparation of Lysates From Adherent Cells
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Contents

1 OVERVIEW

This protocol describes a method for lysing strongly (Section 4.1) and loosely (Section 4.2) adherent cells for application on PamChip® kinase profiling assays.

For kinomic analysis we prefer three or more aliquots of snap frozen cell lysates that contain approx.1 µg/µl total protein concentration (equivalent to 25 µg of total protein). Please culture the cells between 70-80% confluency as culturing too densely may affect cell viability and kinase activity. We suggest to use 6 well plates (or larger). Trypsin treatment to detach cells must be avoided when collecting cells for lysis because this influences kinase activity. Instead, perform direct lysis by scraping cells in lysis buffer according to the protocol described below.

2 MATERIALS AND REAGENTS

- *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)*
- *PBS: Phosphate Buffered Saline without Magnesium and Calcium (ice-cold) (Gibco™ 70011036)*
- *1.5 ml Eppendorf vials (preferably Safe-Lock)*
- *Cell scraper*

3 LYSIS BUFFER PREPARATION

- 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 1:50 (2x) 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	102.3 µl	-
100x HALT Protease Inhibitors EDTA-free	2.2 µl	2x
100x HALT Phosphatase inhibitor cocktail	2.2 µl	2x
5M NaCl	3.3 µl	150 mM
Total volume lysis buffer	110 µl	-

4 LYSIS PROTOCOL

Before beginning:

- Precool centrifuge to 4°C
- Prepare lysis buffer (Section 3)
- Label the required number of Eppendorf tubes and conical tubes, where applicable

Note: Process a maximum of four 100 mm plates or one 6 well plate at a time.

4.1 Procedure for STRONGLY adherent cells

4.1.1 Label four or five 1.5 ml Eppendorf tubes for each sample (plate/well) with adequate information and store on ice.

4.1.2 Remove culture medium from cells and put the plates on ice.

4.1.3 Wash cells by pipetting ice-cold PBS onto plate (use same volume as cell culture medium). Swirl gently over cells and remove.

4.1.4 Repeat wash step.

4.1.5 After the second wash step, it is important to **remove all PBS** and keep plate on ice**!**

4.1.6 To each plate/well add lysis buffer with inhibitors and NaCl: 40 µl lysis buffer for 6 well plate; 50 µl for 100 mm plate. Start a timer.

4.1.7 Scrape the cells in the lysis buffer off the bottom of the plate using a cell scraper.

4.1.8 Collect the cells of each plate/well in a labelled pre-cooled 1.5 ml Eppendorf tube.

4.1.9 Lyse the cells by pipetting the volume up and down five times carefully (to prevent foam) every 5 min to homogenize the lysates.

4.1.10 Lysis time from applying the lysis buffer on the plates to the centrifuge step is approx. 30 min and should be consistent all samples.

4.1.11 Centrifuge lysates for 15 min at max. speed (minimum 10,000 x g) at 4°C.

4.1.12 Collect all the lysate (supernatant) at once and transfer to its respective labelled tube on ice. From this tube, prepare three to four aliquots. Recommended are two or more aliquots of 10 µl, and one aliquot of 6 µl for protein quantification.

4.1.13 Snap-freeze samples in liquid nitrogen or on dry-ice.

4.1.14 Store samples at –80°C until shipment.

4.2 Procedure for LOOSELY Adherent Cells

4.2.1 Label four or five 1.5 ml Eppendorf tubes AND one 10-15 ml conical (V-bottom) tube for each sample (plate/well) with adequate information and store on ice.

4.2.2 Gently remove culture medium from cells and put the plates on ice.

4.2.3 Wash cells by pipetting ice-cold PBS onto plate (use same volume as cell culture medium). Swirl PBS gently over cells and collect the PBS in the labelled 10-15 ml conical tube.

4.2.4 Centrifuge the conical tubes for 5-10 min at 125 x g at 4°C.

4.2.5 Remove the PBS with a pipet.

4.2.6 Wash the plate as described in step 4.2.3 and use this to resuspend the pelleted cells in the 10-15 ml conical tube. It is important to remove **all PBS** and keep plate on ice**!**

4.2.7 Centrifuge the conical tubes once more for 5-10 min at 125 x g at 4°C.

4.2.8 Remove all PBS from the conical. It is important to remove **all PBS** and keep the conical on ice!

4.2.9 To each plate/well add lysis buffer with inhibitors and NaCl: 40 µl lysis buffer for 6 well plate; 50 µl for 100 mm plate. Start a timer.

4.2.10 Scrape the cells in the lysis buffer off the bottom of the plate using a cell scraper.

4.2.11 Collect the cells in lysis buffer of each plate/well into their respective 10-15 ml conical tubes on ice and use to resuspend the cell pellet.

4.2.12 Transfer the resuspended cells in lysis buffer into labelled pre-cooled 1.5 ml Eppendorf tubes.

4.2.13 Lyse the cells by pipetting the volume up and down five times carefully (to prevent foam) every 5 min to homogenize the lysates.

4.2.14 Lysis time from applying the lysis buffer on the plates to the centrifuge step is approx. 30 min and should be consistent all samples.

4.2.15 Centrifuge lysates for 15 min at max. speed (minimum 10,000 x g) at 4°C.

4.2.16 Collect all the lysate (supernatant) at once and transfer to its respective labelled tube on ice. From this tube, prepare three to four aliquots. Recommended are two or more aliquots of 10 µl, and one aliquot of 6 µl for protein quantification.

4.2.17 Snap-freeze samples in liquid nitrogen or on dry-ice.

4.2.18 Store samples at –80°C until shipment.

5 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 °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.
3. Trypsin treatment to detach cells must be avoided when collecting cells for lysis because this influences kinase activity. Instead, perform direct lysis by scraping cells in lysis buffer according to the protocol described below.
4. 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 so that the contents of broken cells are collected in the lysis buffer.
5. If a bit of the pellet is transferred when pipetting the supernatant, samples can be centrifuged again to remove this debris.
6. Repeated freeze-thawing may affect kinase activity. Always use a freshly thawed aliquot for a PamChip® assay.
7. 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 be present in the lysate and interfere with inhibitor studies. This occurrence depends on the cell line and the inhibitor.
8. 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).
9. 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.

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.

6 FREQUENTLY ASKED QUESTIONS

- *"How many cells are needed to yield at least 1 µg/µl protein concentration?"*
  Protein yields can vary by cell type, shape, and viability. We recommend culturing cells to 70-80% confluency in a 6 well plate or 100 mm dish to achieve a high enough cell density to yield the target protein concentration. Keep in mind that culturing cells too densely may affect viability and kinase activity. In case of uncertainty for the expected protein yield for your cell line, a pilot optimization for lysis is recommended.

- *"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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