Protocol 1150

For Preparation of Lysates of Tissue Sections

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Version 4.1

Contents

1 INTRODUCTION

1.1 Intended use

The aim of this protocol is to prepare cell lysates of tissue sections for use in 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

- *Tissue Sections*
  One 60 µm tissue sections of fresh frozen tissue (see PamGene Protocol 1140 Preparation of Tissue Sections). Optionally: 2 x 30 µm or 6 x 10 µm sections of ~5 x 5 mm can be used. For core biopsies: cut a number of tissue sections that gives about 1.0 mm^3 of tissue.
- *Pieces of fresh frozen tissue*.
  The size of pieces must be reduced (at -80 °C) to preferably < $1mm^3$ by e.g. a Mixer Mill, dismembrator or using other mechanical means, e.g. a hammer. See the section Sample Preparation on https://www.pamgene.com/en/KinaseAssays.htm for more details.

Tissue-Tek® OCT™ embedded material is not compatible with PamChip® analysis. If Tissue-Tek® OCT™ Compound[1] is used, cut away as much as possible. Final sections must contain less than 10% Tissue-Tek®.

[1] Tissue-Tek® OCT™ Compound: Optimal Cutting Temperature embedding medium, brand name Tissue-Tek, https://www.sakura.eu/Our-products/item/11/Cryotomy/48/Tissue-Tek-OCT-Compound-and-Cryomolds .

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 to 6 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. Use a 1:50 dilution for tissues with a high protease activity, e.g. lung tissue. 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. Take the vials with the frozen slices out of –80°C and keep on ice. Leave them on ice for 2 min, before Step 6 of adding the cold lysis buffer but don't allow the tissue to thaw.
6. Add 100 µl lysis buffer (M-PER containing phosphatase and protease inhibitors to 60 µm frozen tissue section and keep on ice. When less tissue is used, reduce the volume (use at least 25 µl).
7. Promote lysis by gently pipetting the mixture up and down (approx. ten times). When larger pieces are present, the tip of the pipette can be cut. Avoid expelling the fluid too forcefully in order to prevent foaming and denaturation of the proteins. Leave the tip in the solution during incubation for pipetting 3 times up and down every 10 minutes.

8. Incubate lysate for 30 minutes on ice (check lysis visually or use a microscope, see 2.7). Check whether the solution is homogenous (no large particles). To exert more pressure on the tissue pieces, a disposable pestle can be used to grind the tissue.
9. Centrifuge the vials for 15 min in a pre-cooled table-top centrifuge at maximum speed (>10,000 x g) at 4°C.
10. Collect the supernatant lysate 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 5µl sample for protein quantification purposes.
11. 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).
12. 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 tissues are lysed completely.

3 NOTES AND FAQS

3.1 Notes

1. Samples that will be compared should be prepared and processed under identical conditions.
2. Determine the % of tumor in a HE stained 5 µm section. Check also for the presence of necrosis. Exclude samples that show a lot of necrosis. Consider excluding samples with less than 30 % tumor.
3. Optional: The % of Tumor Infiltrating Lymphocytes can be counted in HE stained sections as described in Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Penault-Llorca F, Perez EA, Thompson EA, Symmans WF, *et al*. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015; 26: 259-71.
4. Step 10: When a fatty layer is visible on the lysate after centrifugation, transfer the lysate underneath the layer to a clean tube on ice while leaving the fatty layer (and pellet) behind. Centrifuge this tube and transfer lysate to a second clean tube (leaving the fatty layer, if present, behind and transfer to a clean pre-cooled tube.
5. Steps 10: when accidentally a bit of the pellet is transferred, samples can be centrifuged again to remove this debris
6. Freeze-thawing may affect kinase activity. Always use a never-thawed aliquot for a PamChip® assay.
7. Step 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.
8. Step 12: Recommended Coomassie Plus (Bradford) Assay Kit see 2.1.

9. Step 12: Perform protein quantification of all samples simultaneously.
10. Step 12: 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.
11. Step 12: Use a sample with known protein concentration as internal control for protein quantification.
12. Step 12: When reading the extinction, make sure that no air bubbles are present in the light path, they disturb the reading and protein quantification.

3.2 Frequently asked questions

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.

4 SUPPORT

For questions contact our support group.

Please contact support on:
☏ +31 (0)73 615 89 00
✉ support@pamgene.com

5 TRANSPORT OF SAMPLES

For studies conducted by PamGene, at their premises, please contact PamGene before preparing samples and aliquots about exact amounts needed for specific studies.
In case of shipment to PamGene the samples must be labelled clearly and packed in a bag or box in a sufficient amount of dry ice, accompanied by a list of samples

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