Protocol 7643

Preparation of Organoid or Spheroid Samples and Lysates

7643 Preparation of Spheroid or Organoid Samples and Lysates
Version 1.0

Contents

1 OVERVIEW

This protocol describes a method for preparation of 3D cultures (e.g. organoids and spheroids) for application on PamChip® kinase profiling assays. The first sections of the protocol details the preparation of pelleted organoid/spheroid samples (Section 4.1) for shipment to PamGene or, when applicable, for sample lysis (Section 4.2). The lysis conditions for 3D cultures can vary by sample type, so it is recommended to follow the lysis optimization protocol described in Section 4.3 when lysing your sample type for the first time. Once the optimal lysis conditions have been determined, the standard lysis protocol in Section 4.2 can be followed. In cases where samples are prepared and directly shipped to PamGene without lysis, we request that an additional 4 to 6 samples are included for lysis optimization.

For kinome analysis we prefer three or more aliquots of snap frozen lysate that contain approx. **1** µg/µl protein concentration (equivalent to 25 µg of total protein).

2 MATERIALS AND REAGENTS

For preparing samples for shipment:

- *1X PBS: Phosphate Buffered Saline without Magnesium and Calcium (ice-cold) (Gibco™ 70011036)*
- *1.5 ml Eppendorf tubes (preferably Safe-Lock)*

For preparing samples and lysis:

- *M-PER® Mammalian Protein Extraction Buffer (M-PER Buffer) (Thermo Fisher Scientific™ 78503) stored at RT*
- *Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific™ 78420) stored @4°C*
- *Halt™ Protease Inhibitor Cocktail, EDTA free (Thermo Fisher Scientific™ 87785) stored @4°C*
- *NaCl (5M stock)*
- *1X PBS: Phosphate Buffered Saline without Magnesium and Calcium (ice-cold) (Gibco™ 70011036)*
- *1.5 ml Eppendorf tubes (preferably Safe-Lock)*
- *Pierce™ Bradford Plus Protein Assay Kit (Thermo Fisher 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 1:50 (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 PROTOCOL

Before beginning:

- Determine if lysis optimization is needed
- Read Notes and FAQ section
- Pre-cool centrifuge to 4°C
- Prepare lysis buffer (if applicable) and place on ice
- Label the required number of Eppendorf tubes

If performing lysis optimization, skip to Section 4.3.

4.1 Preparation of organoids/spheroids

Note: *If preparing organoids/spheroids for direct shipment to PamGene, please include an additional 4 to 6 samples for lysis optimization.*

4.1.1 Label one 1.5 ml Eppendorf tube per sample with adequate information and store on ice.

4.1.2 Transfer the organoid/spheroid samples to its corresponding tube.

4.1.3 Centrifuge for 8 minutes at 500 x g and 4°C. Remove any residual culture medium and place samples on ice.

4.1.4 Gently resuspend samples in 1 ml ice-cold PBS and centrifuge for 5 minutes at 1000 x g and 4°C.

4.1.5 Carefully remove as much PBS as possible by pipetting without disturbing the sample and place on ice.

4.1.6 If shipping samples to PamGene, snap freeze the organoids/spheroids on dry ice or using liquid nitrogen and store at -80°C until shipment. If lysing the organoids/spheroids, continue to Section 4.2.

4.2 Lysis of Organoids/Spheroids

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

4.2.2 Add selected volume (from lysis optimization or previous sample prep experience) of lysis buffer to each tube on ice and start the timer.

4.2.3 Lyse the samples by gently pipetting the volume up and down five times every 5 minutes for 30 minutes. Keep the samples on ice during lysis.

4.2.4 After 30 minutes, centrifuge the lysed samples for 15 minutes at max speed (minimum 10,000 x g) and 4°C.

4.2.5 Collect all lysate (supernatant) at once and transfer to one of its corresponding labelled tubes on ice. From this tube, prepare one aliquot of 5 µl, then divide the leftover lysate equally among the remaining tubes. We recommend aliquots of 10 to 15 µl to avoid freeze-thaw cycles of the lysate. A minimum of one aliquot of 5 µl is required for protein quantification.

Note: *Depending on culture conditions, it is possible to recover a lysate volume larger than the volume of lysis buffer added due to release of culture matrix contents.*

4.2.6 Snap-freeze samples in liquid nitrogen or on dry ice and store at –80°C.

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

4.3 Lysis optimization of organoids/spheroids using 4 samples

4.3.1 Label one 1.5 ml Eppendorf tube per optimization sample with adequate information to use for sample preparation and store on ice.

4.3.2 Label additional 1.5 ml Eppendorf tubes (a minimum of two) per sample with adequate information to use for lysate aliquots and store on ice.

4.3.3 Transfer the organoid/spheroid samples to the tube prepared in Step 4.3.1.

4.3.4 Centrifuge for 8 minutes at 500 x g and 4°C. Remove any residual culture medium and place samples on ice.

4.3.5 Gently resuspend samples in 1 ml ice-cold PBS and centrifuge for 5 minutes at 1000 x g and 4°C.

4.3.6 Carefully remove as much PBS as possible by pipetting without disturbing the sample and place on ice.

4.3.7 Add increasing volumes of lysis buffer (5, 10, 15, and 20 µl) to the 4 sample tubes on ice and start a timer.

4.3.8 Lyse the samples by gently pipetting the volume up and down five times every 5 minutes for 30 minutes. Keep the samples on ice during lysis.

4.3.9 After 30 minutes, centrifuge the lysed samples for 15 minutes at max speed (minimum 10,000 x g) and 4°C.

4.3.10 Collect all lysate (supernatant) at once and transfer to one of the corresponding labelled tubes prepared for lysate aliquots on ice. From this tube, prepare one aliquot of 5 µl, then divide the leftover lysate equally among the remaining tubes. A minimum of one aliquot of 5 µl is required for protein quantification.

Note: *Depending on culture conditions, it is possible to recover a lysate volume larger than the volume of lysis buffer added due to release of culture matrix contents.*

4.3.11 Snap-freeze lysates in liquid nitrogen or on dry ice and store at –80°C until protein determination.

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

4.3.13 After protein determination select the largest lysis buffer volume that yields:

- Total protein concentration of approximately 1 µg/µl (or higher) **and**
- A minimum total protein yield of 25 µg

If both of these criteria are not met, repeat the lysis optimization with a larger organoid/spheroid starting amount.

4.3.14 After selecting optimal lysis buffer volume, prepare and lyse samples according to Sections 4.1 and 4.2

In case of questions or concerns regarding the lysis optimization, please contact the PamGene Support team (support@pamgene.com).

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 If the sample pellet becomes loose when pipetting the supernatant, sample can be centrifuged again.

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

5.5 The preferred protein concentration is at least 1 µg/µl but in cases of extra replicates and/or inhibitor spike-in experiments, more sample may be required. In case of questions or concerns regarding your protein concentration or sample preparation, please contact the PamGene Support team (support@pamgene.com).

5.6 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 may depend on the cell line and the inhibitor.

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

6 FREQUENTLY ASKED QUESTIONS

- *"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

7.1 Disclaimer

FOR RESEARCH PURPOSES ONLY

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