EpiQuant™ Sample Preparation Kit

Cat. # MPEQ-SP

MILLIPLEX® MAP EpiQuant™ Sample Preparation Kit

#MPEQ-SP

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

MILLIPLEX® MAP EpiQuant™ technology uses computational algorithms to identify unique, continuous linear sequences (EpiQuant Sequences) in proteins. EpiQuant antibodies generated against EpiQuant sequences have pre-defined target specificity. Protein abundance and/or protein phosphorylation measurements are made at the peptide level, using EpiQuant antibodies, following proteolytic fragmentation of samples and liberation of EpiQuant-bearing peptides.

A unique feature of the EpiQuant sample processing protocol is fragmentation or digestion of the protein sample. This process has a number of benefits:

- Protein:protein interactions are disrupted
- A synthetic peptide can be used as a quantitative standard
- Specificity of the antibodies can be narrowed to a small linear peptide sequence
- Cross-reactivity with similar peptide sequences in other proteins can be predicted and avoided
- Residual enzymatic activity of the sample (e.g. phosphatases and proteases) is quenched

EpiQuant technology represents a major advance in the design and implementation of quantitative immunoassays.

The MILLIPLEX MAP EpiQuant Sample Preparation kit is an integral component to the successful application of EpiQuant technology. Cell lysate samples MUST be prepared using this kit. The Sample Preparation kit provides reagents for the reduction of cystine thiols (MPEQ-RA), alkylation of these reduced thiols (MPEQ-AA), proteolytic fragmentation of lysate proteins (MPEQ-DE) and finally inhibition of proteolytic activity (MPEQ_PI) so that digested samples may be analyzed utilizing MILLIPLEX EpiQuant immunoassays (sold seperately).

This kit contains sufficient reagents for the lysis and preparation of 5 mL (10 mg total protein, total protein concentration of 2 mg/mL) of sample lysate. This volume (5 mL) is sufficient for the preparation of five 100 mm plates/ T75 flasks, 16 wells from 6 well plates, 48 wells from 24 well plates, or one 96 well plate.

This kit is for research purposes only.

Please read entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
EpiQuant Lysis Buffer	MPEQ-LB	30 mL	1 bottle
EpiQuant Nuclease (250X)	MPEQ-NCLS	40 µL	1 tube
EpiQuant Reducing Agent (100X)	MPEQ-RA	100 µL	1 tube
EpiQuant Alkylating Agent (50X)	MPEQ-AA	lyophilized	1 vial
EpiQuant Digestion Enzyme (50X)	MPEQ-DE	lyophilized	1 vial
EpiQuant Protease Inhibitor (100X)	MPEQ-PI	100 µL	1 tube

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. BCA, or other detergent compatible assay, based total protein assay
2. TBS (or PBS, for lysate preparation)
3. Though not necessary for successful sample preparation, SDS-PAGE analysis and Coomassie staining may be utilized to verify protein digestion.

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 1 µL to 1000 µL
2. Low Volume (1-10 µL) Multichannel Pipette (if performing sample preparation from a 96 well plate)
3. Cell Scraper (as needed)
4. Polypropylene Microfuge Tubes
5. Laboratory Vortex Mixer
6. Orbital or plate shaker
7. Laboratory oven or heat block for incubating samples at 85°C.
8. Laboratory oven or incubator for incubating samples at 37°C.

SAFETY PRECAUTIONS

- All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, sodium azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- After hydration, the alkylating agent and digestion enzyme should be used immediately or aliquoted and stored at -20°C.

SAMPLE COLLECTION AND STORAGE

A. Considerations for Cell Stimulation.

- Treating cells with growth factors (ex. EGF), cytokines (ex. TNFα), or other compounds (ex. Arsenite) induce a multitude of signaling cascades. The duration of stimulation in addition to the concentration of the respective factor/compound should be considered since they influence the degree of phosphorylation of any given analyte.
- Cellular responses to growth factors are typically improved when cells have been serum starved prior to treatment.
- Cell lines will differ in the robustness of their signaling response for any given stimulation.

NOTE: This kit contains sufficient reagents for the digestion of up to 5.0 mL of sample lysates. Experiments should be planned and digestion conducted to allow for complete sample coverage.

B. Preparation of Cell Lysates (for 96-well tissue culture plates, see supplemental protocols)

EpiQuant lysis buffer preparation

1. Warm lysis buffer to room temperature prior to use. Lysis buffer may contain precipitate at 4°C, however this precipitate should go completely into solution when the buffer is warmed to room temperature.
2. Determine the amount of lysis buffer needed (1.0 mL per 100 mm dish or T-75 flask, 0.3 mL per well of a 6-well plate or 0.1 mL per well of 24-well plate) and combine the following reagents immediately prior to use:

	Total Volume Required		
Reagent:	**1mL**	**2.5mL**	**5mL**
EpiQuant Lysis Buffer	1mL	2.5mL	5mL
EpiQuant Nuclease (250X)	4µL	10µL	20µL

NOTE: Do not place the lysis buffer on ice. If a precipitate forms in the lysis buffer, warm it to **room temperature** and vortex until precipitate returns to solution.

Cell lysis protocol for adherent cells

1. After cell treatment, wash cells with ice-cold Tris Buffered Saline (TBS, or PBS) and remove wash buffer.
2. Add prepared Lysis Buffer to cells (1.0 mL per 100 mm dish or T-75 flask, 0.3 mL per well of a 6-well plate or 0.12 mL per well of 24-well plate).
3. Scrape adherent cells from the dish with a cell scraper. Transfer the cell suspension into a centrifuge tube and gently rock for 10-15 minutes at 4°C. When using multi-well plates scraping and transfer may not be practical. If desired, lyse samples in the plate by placing on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4°C after addition of lysis buffer. **NOTE: If the lysate is viscous, additional nuclease may be added.**
4. Place samples on ice.
5. Remove an aliquot of lysate (at least 20 µL recommended) for protein concentration determination by BCA or other detergent compatible assay. Dilute the lysate at least 1:4 for BCA assay. Based on determined total protein concentrations of samples, adjust each sample to a final concentration of 2 +/- 0.1 mg/mL by diluting with lysis buffer (nuclease need not be added at this point). **NOTE:** If necessary, due to protein concentrations less than 1.9 mg/mL, the lysates may be adjusted to the protein concentration of the most dilute sample without adjusting this protocol. Dilution to concentrations less than 1 mg/mL is not recommended.
6. Transfer **at least** 100 µL of lysate of each prepared sample to a labeled 1.5 mL microcentrifuge tube and place on ice, proceed to sample preparation procedure. If desired, any remaining sample may be snap frozen for analysis by other techniques.

NOTE: The EpiQuant lysis buffer does not contain protease inhibitors because the EpiQuant protocol requires protease digestion. However, we recommend adding protease inhibitors (Millipore #20-201 or equivalent) prior to snap freezing samples reserved for other techniques (e.g. Western blots) to preserve protein integrity upon storage.

Cell lysis protocol for non-adherent cells

1. Pellet cells by centrifugation (500–1000 x g) in a tabletop centrifuge for 5 minutes.
2. Wash cells in ice cold TBS (or PBS), centrifuge and remove wash buffer.
3. Add prepared Lysis Buffer to cells (1 mL per 1 x 10^7 cells).
4. Gently rock the lysate for 10-15 minutes at 4°C.
5. Place samples on ice.

6. Remove an aliquot of lysate (at least 20 µL recommended) for protein concentration determination by BCA or other detergent compatible assay. Dilute the lysate at least 1:4 for BCA assay. Based on determined total protein concentrations of samples, adjust each sample to a final concentration of 2 +/- 0.1 mg/mL by diluting with lysis buffer (nuclease need not be added at this point). **NOTE:** If necessary, due to protein concentrations less than 1.9 mg/mL, the lysates may be adjusted to the protein concentration of the most dilute sample without adjusting this protocol. Dilution to concentrations less than 1 mg/mL is not recommended.
7. Transfer **at least** 100 µL of lysate of each prepared sample to a labeled 1.5 mL microcentrifuge tube and place on ice, proceed to sample preparation procedure If desired, any remaining sample may be snap frozen for analysis by other techniques.

NOTE: The EpiQuant lysis buffer does not contain protease inhibitors because the EpiQuant protocol requires protease digestion. However, we recommend adding protease inhibitors (Millipore #20-201 or equivalent) prior to snap freezing samples reserved for other techniques (e.g. Western blots) to preserve protein integrity upon storage.

REAGENT PREPARATION

50X Alkylating Agent and Digestion Enzyme Preparation

- Prepare 50X Alkylating Agent by adding 200 µL ultrapure water to the EpiQuant Alkylating Agent vial.
- Prepare 50X Digestion Enzyme by adding 120 µL ultrapure water to the EpiQuant Digestion Enzyme vial.

Place the reconstituted vials on ice and use immediately or aliquot to polypropylene tubes and store at -70°C for future use.

SAMPLE PREPARATION PROCEDURE

1. For each collected sample, add 100X Reducing Agent to a final concentration of 1X by adding 1 µL of the 100X Reducing Agent per 100 µL of sample. Vortex mix.
2. Heat the samples at 85°C for 10 min to reduce and denature each sample.
3. Remove from heat and allow the samples to cool to room temperature, approximately 5-10 minutes.
4. Add Alkylating Agent to a final concentration of 1X by adding 2 µL of 50X Alkylating Agent per 100 µL of sample and incubate in the dark at room temperature for at least 30 minutes, but not to exceed 3 hours.
5. If desired, set aside ~10 µL of the 2 mg/mL sample for SDS-PAGE analysis and Coomassie staining as a control to verify digestion. Label and freeze this sample at -20°C or below.
6. Add 2 µL of the 50X Digestion Enzyme for each 100 µL of sample and incubate samples at 37°C with end-over-end mixing or orbital mixing at ~300 rpm for 2 hours. **NOTE**: Incubate digesting samples **at least** 2 hours at 37°C. Samples may be digested overnight if desired.
7. After sample incubation, for each 100 µL of sample, add 1 µL 100X Protease Inhibitor to the digested sample. If desired, retain ~10 µL for SDS-PAGE analysis and Coomassie staining.
8. Snap freeze all samples in either liquid nitrogen or a dry ice ethanol bath and store at -70ºC or proceed immediately to Immunoassay Protocol. If needed, aliquot samples to minimize freeze-thaw cycles.

- Treat, wash and lyse cells
- Remove a sample aliquot for total protein determination
- Transfer at least 100 µL lysate to a microfuge tube
- Add MPEQ-RA to a final concentration of 1X

Heat samples for 10 minutes at 85°C Cool to room temperature

Add MPEQ-AA to a final concentration of 1X

Incubate samples in the dark at RT for at least 30 minutes.

- If desired, remove a pre-digestion aliquot of lysate for digestion verification by SDS-PAGE
- Add MPEQ-DE to a final concentration of 1X

Incubate samples at 37°C for at least 2 hours

- Add MPEQ-PI to a final concentration of 1X
- If desired, remove a post-digestion aliquot of lysate for digestion verification by SDS-PAGE
- Proceed immediately to sample analysis or snap freeze samples and store at -70°C for later analysis.

Cell Lysis Protocol for 48 or 96-well Tissue Culture Plates

EpiQuant lysis buffer preparation

1. Warm lysis buffer to room temperature prior to use. Lysis buffer may contain precipitate at 4°C, however this precipitate should go completely into solution when the buffer is warmed to room temperature.
2. Combine 3.5 mL lysis buffer and 15 µL nuclease

NOTE: Do not place the lysis buffer on ice. If a precipitate forms in the lysis buffer, warm it to room temperature and vortex until precipitate returns to solution.

Cell lysis protocol for cells in sterile 48 or 96-well tissue culture plates

Adherent or non-adherent cells grown in sterile 48 or 96-well tissue culture grade plates can be treated, washed, lysed and digested in the same plate. Cells should be seeded and incubated to achieve a final cell density of approximately 300-400,000 cells/ well (150-200,000 cells/ well for 96-well plate) at the time of lysis.

1. Remove supernatant via aspiration, or centrifuge (non-adherent cells) and remove supernatant via aspiration wash 1X with 100 µL ice cold TBS (or PBS), if desired.
2. Add 65 µl/well (48-well plate) or 35 µL/well (96-well plate) of prepared Lysis Buffer.
3. Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4°C.
4. Remove the plate from the orbital shaker and place on ice. If desired, remove a 5µL aliquot of lysate for protein concentration determination by BCA or other detergent compatible assay. Dilute the lysate at least 1:4 for BCA assay. Based on determined total protein concentrations of samples, adjust each sample to a final concentration of 2 +/- 0.1 mg/mL by diluting with lysis buffer (nuclease need not be added at this point). **NOTE:** If necessary, due to protein concentrations less than 1.9 mg/mL, the lysates may be adjusted to the protein concentration of the most dilute sample without adjusting this protocol. Dilution to concentrations less than 1 mg/mL is not recommended.
5. Proceed to Sample Digestion.

Reagent Preparation

25X Alkylating Agent and Digestion Enzyme Preparation

- Prepare 25X Alkylating Agent by adding 400 µL ultrapure water to the EpiQuant Alkylating Agent vial.
- Prepare 25X Digestion Enzyme by adding 240 µL ultrapure water to the EpiQuant Digestion Enzyme vial.

Place the reconstituted vials on ice and use immediately or aliquot to polypropylene tubes and store at -70°C for future use.

25X Reducing Agent Preparation

- Prepare 25X Reducing Agent by combining 100 µL 100X and 300 µL EpiQuant Lysis Buffer. 25X Reducing Agent may be stored at room temp prior to use.

SAMPLE PREPARATION PROCEDURE

1. Add 2 µL / well (4 µL/well, 48-well) 25X Reducing Agent, mix on an orbital shaker for 30 seconds.
2. Seal the plate with sealing tape and heat the samples at 85°C for 10 min to reduce and denature samples**.
3. Remove from heat and allow the samples to cool to room temperature, approximately 5-10 minutes.
4. Add 2 µL/ well (4 µL/well, 48-well) 25X Alkylating Agent, mix on an orbital shaker for 30 seconds, then incubate in the dark at room temperature for at least 30 minutes, but not to exceed 3 hours.
5. Add 2 µL/ well 25X Digestion Enzyme (4 µL/well, 48-well), incubate the samples at 37°C on an orbital shaker at ~300 rpm for 2 hours. **NOTE**: Incubate digesting samples **at least** 2 hours at 37°C. Samples may be digested overnight if desired.
6. Prepare 25X Protease Inhibitor immediately prior to use by combining 100µL Protease Inhibitor and 300 µL lysis buffer. Add 2 µL/ well (4 µL/well, 48-well) 25X Protease Inhibitor.
7. Store at -70ºC or proceed immediately to Immunoassay.

****NOTE: Step 7 may be performed utilizing either a lab oven set for 85°C, or the 96-well plate may be covered with sealing tape and floated in a glass baking dish or large flask containing 85°C water, a fully skirted solid 96-well plate is needed to float the plate.**

- Treat, wash and lyse cells
- Remove a sample aliquot for total protein determination, if desired
- Add MPEQ-RA to a final concentration of 1X

↓ Heat samples for 10 minutes at 85°C Cool to room temperature

Add MPEQ-AA to a final concentration of 1X

↓ Incubate samples in the dark at RT for at least 30 minutes.

- Add MPEQ-DE to a final concentration of 1X

↓ Incubate samples at 37°C for at least 2 hours

- Add MPEQ-PI to a final concentration of 1X

Samples are now prepared for analysis using MILLIPLEX MAP EpiQuant Cell Signaling Panels, proceed immediately to sample analysis or store at -70°C for later analysis.

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Samples are overly viscous after lysis	Highly concentrated lysates	Add additional nuclease to lysate and/or dilute samples with lysis buffer.
Low molecular weight band observed on SDS-PAGE	Nuclease band	No action is needed.
Samples are less than 2 mg/mL		Adjust samples to lowest sample concentration, sample protein concentration should be greater than 1 mg/ mL

REPLACEMENT REAGENTS	Catalog #
EpiQuant Lysis Buffer	MPEQ-LB
EpiQuant Nuclease (250X)	MPEQ-NCLS
EpiQuant Reducing Agent (100X)	MPEQ-RA
EpiQuant Alkylating Agent (50X)	MPEQ-AA
EpiQuant Digestion Enzyme (50X)	MPEQ-DE
EpiQuant Protease Inhibitor (100X)	MPEQ-PI

ORDERING INFORMATION

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To assure the clarity of your custom panel order, please FAX the following information to our customer service department:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® EpiQuant Analytes

FAX: (636) 441-8050
Toll-Free US: (800) MILLIPORE
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St. Charles, Missouri 63304 U.S.A.

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