CLINICAL PROTEOMIC TECHNOLOGIES FOR CANCER

STANDARD OPERATING PROCEDURE
Title: Preparation of samples for intact protein immuno-precipitation
SOP#: M-142

Version #: 2	Authors: Simona Colantonio, Joseph Knotts
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1. PURPOSE

This procedure will describe how to prepare an immuno-precipitation (IP) reaction using monoclonal antibodies against full length proteins contained in lysates or as purified recombinant proteins.

2. SCOPE

This procedure applies to cell lysates and purified proteins and provides details for the preparation of samples that will be analyzed by mass spectrometry (MS) or western blot (WB)

3. RESPONSIBILITIES

It is the responsibility of the person(s) executing this procedure to be familiar with laboratory safety procedures and to have basic laboratory skills. It is the responsibility of the analyst to follow the procedure and document any deviations and all observations in the laboratory notebook.

4. EQUIPMENT

4.1. Thermo LP Vortex Mixer (Thermo Scientific, 88880017) or equivalent

4.2. Spectrafuge Mini Centrifuge, Labnet International Inc. (Millipore-Sigma, S6941) or equivalent

4.3. Mettler AJ100 Analytical Balance (Mettler Toledo, discontinued) or equivalent

4.4. Eppendorf ThermoMixer C (Eppendorf, 022627040) or equivalent

4.5. Magnetic Separation Rack for 1.5 mL tubes

4.6. KingFisher Flex Purification System (Thermo Scientific, 5400620)

5. <u>MATERIALS</u>

5.1. Weighing Paper, 3" x 3"

5.2. Falcon BLUE MAX Jr. 15 ml Polypropylene Conical Tube

5.3. Falcon BLUE MAX Jr. 50 ml Polypropylene Conical Tube

5.4. Pipet-Lite LTS Pipette L-200XLS+ (or equivalent), adjustable 20-200 µL

5.5. Pipet-Lite LTS Pipette L-1000XLS+ (or equivalent), adjustable 100-1000 µL

5.6. Pipet-Lite LTS Pipette L-20XLS+ (or equivalent), adjustable 0.2-20 µL

5.7. Pipet-Lite LTS Pipette L-2XLS+ (or equivalent), adjustable 0.1-2 uL

5.8. Pipet-Lite Multi Pipette L12-300XLS+, adjustable 20-300 µL

5.9. Rainin LTS 20 tips, 0.1-20 uL

5.10. Rainin LTS 250 tips, 20-250 µL

5.11. Rainin LTS 300 tips, 20-300 uL

5.12. Rainin LTS 1000, 100-1000 µL

5.13. Hard-Shell PCR Plates 96-well, thin-wall, Bio-Rad 480000742

5.14. KingFisher 96 tip comb for PCR Magnets, Thermo Scientific 97002514

5.15. KingFisher 96 tip comb for DW Magnets, Thermo Scientific 97002534

5.16. KingFisher 96 plate 200 uL (STD, standard), Thermo Scientific 97002540

5.17. KingFisher deep-well 96 plate (DW, deep well), Thermo Scientific 95040450

5.18. Eppendorf Centrifuge Tubes, 1.5 ml

5.19. Corning 50 ml Reagent Reservoir, Polystyrene

5.20. Adhesive Plate Sealers

6. **REAGENTS**

6.1. Pierce™ NHS-Activated Magnetic Beads, Thermo Scientific 88826

- Store in the refrigerator (4°C)

6.2. Hydrocholoric acid, Sigma-Aldrich H1758-500ML

6.3. Deionized water

6.4. Glycine, pH 2.5, Alfa Aesar J61855

6.5. Ethanolamine pH 8, Forte Bio 18-1071

6.6. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), VWR 0465-50G

6.7. PBS 10x, pH 7.4, Gibco/Invitrogen, Cat. Number 7001169

6.8. 2X Laemmli Sample Buffer, Bio-Rad 1610737

6.9. BME (2-Mercaptoethanol), Bio-Rad 1610710XTU

6.10. Urea, Millipore Sigma U5378-500G

6.11. Dithiothreitol, Bio-Rad 1610611

6.12. Trizma hydrochloride solution pH 8.0, 1M, Millipore Sigma T2694 – 100ML

7. **DEFINITIONS**

IP – Immuno-Precipitation

MS – Mass Spectrometry

WB – Western Blot

PCR – 96-well 50 uL PCR plate or magnetic head and tip comb that fits this size plate

STD – 96-well 250 uL plate or magnetic head and tip comb that fits this size plate

DW – Deep well, 96-well 1000 uL deep well plate or magnetic head and tip comb that fits this size plate

TUB – Tris-Urea buffer, see Digest SOP

DI H2O – Deionized water

8. PROCEDURE

8.1. Preparation of Dilution Buffer

8.1.1. Prepare 1X PBS/CHAPS solution from 20X Stock fresh for each experiment

8.1.1.1. Prepare a 20X stock of PBS/CHAPS (1X PBS/1% CHAPS) as needed

- Weigh out 0.1 g CHAPS and add to 15 mL conical tube
- Add 9 mL DI H2O and 1 mL 10X PBS
- Mix until CHAPS is dissolved
- Store at room temperature for up to 4 weeks

8.1.1.2. Prepare 1X PBS/CHAPS

- Label a 50 mL conical tube "1X PBS/CHAPS"
- Add 47.5 mL 1X PBS
- Add 2.5 mL 20X stock

8.2. Prepare IP mixture

Choose appropriate **IP mix prep scale**. The prep scale depends on the protein concentration in the sample and amount of input material required for each capture. Generally large-scale prep is required when using lysate, while small-scale prep is appropriate for purified protein capture.

8.2.1. Consult Table 1 to prepare the **components** of the IP mixture with purified recombinant proteins or cell lysates in large- and small-scale preps.

Table 1

	Protein solution type	Protein Solution dilution in PBS/CHAPS		Antibody+ Beads Solution in PBS/CHAPS		Plate Size
	RecProt or Lys	Amount ug	Volume uL	Amount ug	Volume uL	DW or STD
Large	Lys	500-4000*	up to 800	5	100	DW
Small	RecProt	5	100	5	100	STD
	Lys	500	up to 125	5	100	STD

*: 500 ug input material is sufficient for WB detection, while for MS detection a larger input is needed.

8.2.2. On the chosen **IP mix plate** indicate the number and location of wells to be used with a marker

8.2.3. Prepare **protein component** for the IP mix based on the chosen scale prep as determined in Table 1.

8.2.3.1. To prepare protein component of the IP mix for **large-scale**, add to each well as indicated in Table 1

- Lysate diluted up to 800 uL, to achieve 500 ug as a minimum, and up to 4 mg of total protein

8.2.3.2. To prepare protein component of the IP mix for **small-scale,** add to each well as indicated in Table 1

- Purified proteins at 0.05 mg/mL in PBS/CHAPS, add 100 uL of solution in the IP mix plate well

- Lysate diluted up to 125 uL, to achieve 500 ug of total protein

8.2.4. **Antibody conjugation to NHS activated beads**

8.2.4.1. Calculate volume of bead suspension required to capture the amount of each antibody needed for the experiment

$$Volume\ of\ beads\ (mL) = \frac{x\ ug\ IgG}{50\ ug\ IgG\ mg^{-1}\ beads\ \cdot\ 10\ mg\ beads\ mL^{-1}}$$

8.2.4.2. Briefly vortex and invert stock container of NHS activated beads to resuspend

8.2.4.3. Transfer required volume of NHS beads to labeled tubes corresponding to each antibody

8.2.4.4. Prepare antibody solutions in 1X PBS with a final volume of 150 uL

- Concentration may vary but maintain constant volume of 150 uL; if antibody is too dilute, load directly from the antibody stock

8.2.4.5. Pellet beads by placing tube on magnetic rack and remove storage buffer

8.2.4.6. Wash beads once with 300 uL ice old 1 mM HCl by gently pipetting up and down about 15 times

8.2.4.7. Pellet beads and remove wash buffer as before (step 8.2.4.5)

8.2.4.8. Add prepared antibody solution (from step 8.2.4.4) to beads and resuspend as before (step 8.2.4.6)

8.2.4.9. Incubate antibody + beads at room temperature with ~1000-1200 rpm shaking for 1-2 hours

- Alternatively, antibody + beads can be incubated overnight at 4°C at 1000-1200 rpm

8.2.4.10. Pellet beads and remove flow through from capture (step 8.2.4.5)

8.2.4.11. Wash beads three times by resuspending in 300 uL 0.1 M Glycine pH 2.5; pellet beads and remove wash buffer between washes (step 8.2.4.5 – 8.2.4.6).

8.2.4.12. Wash beads once by resuspending in 300 uL DI water by gently pipetting up and down about 15 times

8.2.4.13. Pellet beads and remove wash buffer as before (step 8.4.2.5)

8.2.4.14. Add 300 uL 1 M ethanolamine pH 8 blocking buffer and resuspend beads; incubate at room temperature for 2 hours with 1000-1200 rpm shaking

8.2.4.15. Pellet beads and remove blocking buffer as before (step 8.2.4.5)

8.2.4.16. Wash beads once by resuspending in DI water by gently pipetting up and down about 15 times

8.2.4.17. Pellet beads and remove wash buffer as before (step 8.4.2.5)

8.2.4.18. Wash beads three times by resuspending in 1x PBS/CHAPS (step 8.1.1.2); pellet beads and remove wash buffer between washes as before (step 8.2.4.5)

8.2.4.19. Pellet beads and remove third wash

8.2.4.20. Dilute beads to a final antibody concentration of 0.05 mg/mL in 1x PBS/CHAPS

- Store unused beads at 4°C

8.2.4.21. Add 100 uL of antibody + beads solution per well

8.2.5. Combine antigen, antibody, and beads components into each plate well

- Cover plate with adhesive sealer

- Incubate overnight at 4°C with 1000-1200 rpm shaking

8.3. **Intermediate KingFisher Processing for Large-scale prep**

The prep scale (Table 1) affects the choice of the Tip Comb and the plate type. *Large-scale prep* processing begins here. For *small-scale prep* processing, skip to section 8.4.

8.3.1. After overnight incubation, the *large-scale prep* in the DW plate requires an extra step before further processing to transfer the beads from the IP mix DW plate to a STD plate

- Load a STD size plate with 150 uL of 1x PBS/CHAPS buffer per well. Label this plate Recovery.

- Insert a DW size tip comb into a DW size plate. Label this plate Tip Comb. Check if the magnetic head installed on the instrument is of the required size (DW) for the beads transfer. If necessary, follow the user manual instructions to change the magnetic head using the keys and control buttons on the front panel of the KingFisher Flex

- Open the BindIt software and load the Volume Reduction DW to PCR Method

8.3.2. Run the method and follow KingFisher instructions to load plates in proper order

8.3.3. After the run is completed, follow the KingFisher instruction to remove plates

8.3.4. For *small-scale prep*, the Recovery plate will be substituted for the IP Mix plate for all the following steps (8.4 onward)

8.4. **KingFisher Flex Processing**

Small-scale prep processing begins here; *large-scale prep* requires additional processing before starting this step. Refer to section 8.3.

8.4.1. Check if the magnetic head installed on the instrument is of the required size for the assay (PCR). If necessary, follow the user manual instructions to change the magnetic head using the keys and control buttons on the front panel of the KingFisher Flex.

8.4.2. Open BindIt software and load the IP Sample Processing PCR Head method.

8.4.3. Load 3 STD size plates with 200 uL PBS/CHAPS buffer per well. Label plates Wash 1 – Wash 3.

8.4.4. Insert a PCR sized tip comb into a STD size plate. Label this plate Tip Comb).

8.4.5.   Elution plate size, elution buffer and elution buffer volume differ based on the intended downstream application. Prepare elution plates as follows:

8.4.5.1.   For IP-MS

- Load 1 STD size plate with 200 uL TUB (refer to Digestion SOP M-143) per well. Label this plate Elution.

8.4.5.2.   In BindIt software, update plate size and buffer volume to match experimental parameters as needed.For IP-WB

- Load 1 PCR size plate with 30 uL SDS Reducing Buffer (2X Reducing Sample Buffer – Laemmli with 5% BME) per well. Label this plate Elution.

8.4.6.   In BindIt software, update plate size and buffer volume to match experimental parameters as needed. Run the method and follow KingFisher instructions to load plates in proper order

8.4.7.   Remove all plates and properly store or dispose of them after method has finished running

8.5.   **Sample analysis**

Samples can be analyzed according to the following SOPs

8.5.1.   For IP-MS, follow indications in *Addendum*

8.5.2.   For IP-WB, follow SOP # M-103

9.   REFERENCED DOCUMENTS

- KingFisher Flex - User Manual, Thermo Fisher Scientific, Inc.

1.   ADDENDUM

General Description of how to generate a transition list with software Skyline for a Multi Reaction Monitoring (MRM) Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) system with triple quadrupole for the detection of synthetic or endogenous peptides.

The transition list contains predicted fragment MWs for each peptide based on charge state and is used by the MS to select precursors and detect fragments. SkyLine[1] software is used to generate a transition list with predicted optimum collision energy.

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[1] https://doi.org/10.1093/bioinformatics/btq054,
https://skyline.ms/project/home/software/Skyline/begin.view

The following is the general strategy to generate an *in-silico* trypsin digestion and create a transition list with predicted optimum collision energies in Skyline[2]. Open Skyline and start a new blank document.

1.1.1. Import protein sequence in FASTA format by copy and pasting into the target's column in a blank document or by using File->Import->FASTA.

1.1.2. Under **Settings->Peptide settings->Digestion**, set enzyme to Trypsin [KR | P] and indicate the max allowed missed cleavages.

1.1.3. Use Skyline to generate a list of peptides and transitions with predicted optimum collision energy for the specific triple quadrupole instrument available for analysis

1.1.4. Under **Settings->Peptide settings->Filter**, set the maximum and minimum peptide length for the experiment.

1.1.5. Under **Settings->Peptide settings->Modifications**, indicate any expected modifications to the peptide (e.g.: Cam C, Heavy labeling)

1.1.6. Under **Settings->Transition Settings->Prediction**, set precursor and ion mass to Monoisotopic, set Collision energy to specific triple quadrupole instrument available for analysis

1.1.7. Under **Settings->Transition Settings->Filter**, set Precursor charges to 2,3,4, set Ion charges to 1 and set Ion types to y. Set the Product ion selection window from ion2 to last ion. Check the box to auto select all matching transitions.

1.1.8. Under **Settings->Transition Settings->Instrument**, set Min m/z to 50 and Max m/z to 1200.

1.1.9. Export the filtered transition list for import to triple quadrupole LC-MS/MS instrument

1.1.10. Under **File->Export->Transition List**, set Instrument type to specific triple quadrupole LC-MS/MS instrument, select single method and set the Run duration (min).

1.1.11. Press OK, then name the file and save it in a location accessible from the LC-MS/MS instrument computer.

1.1.12. From the Shimadzu LCMS 8050 instrument computer, import the file to add a transition list to a method file.

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[2] Other proteolytic enzymes are available in place of Trypsin. If the target peptide cannot be predicted with in silico enzymatic digestion, sequences can be entered manually. In silico trypsin digestion is the most used method.