

STANDARD OPERATING PROCEDURE

Title: Preparation of samples for intact protein immuno-precipitation

SOP#: M-142

Version #: 1

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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. **MATERIALS**

- 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 μ L
- 5.5. Pipet-Lite LTS Pipette L-1000XLS+ (or equivalent), adjustable 100-1000 μ L
- 5.6. Pipet-Lite LTS Pipette L-20XLS+ (or equivalent), adjustable 0.2-20 μ L
- 5.7. Pipet-Lite LTS Pipette L-2XLS+ (or equivalent), adjustable 0.1-2 μ L
- 5.8. Pipet-Lite Multi Pipette L12-300XLS+, adjustable 20-300 μ L
- 5.9. Rainin LTS 20 tips, 0.1-20 μ L
- 5.10. Rainin LTS 250 tips, 20-250 μ L
- 5.11. Rainin LTS 300 tips, 20-300 μ L
- 5.12. Rainin LTS 1000, 100-1000 μ L
- 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 μ L (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™ Protein G Magnetic Beads, Thermo Scientific 88847 (for Mouse Monoclonal Antibodies)
 - 6.1.1.1. Store in the refrigerator (4°C)
- 6.2. Pierce Protein A Magnetic Beads, Thermo Scientific 88845 (for Rabbit Monoclonal Antibody)
 - 6.2.1.1. Store in the refrigerator (4°C)
- 6.3. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), VWR 0465-50G
- 6.4. PBS 10x, pH 7.4, Gibco/Invitrogen, Cat. Number 7001169
- 6.5. 2X Laemmli Sample Buffer, Bio-Rad 1610737
- 6.6. BME (2-Mercaptoethanol), Bio-Rad 1610710XTU
- 6.7. Urea, Millipore Sigma U5378-500G
- 6.8. Dithiothreitol, Bio-Rad 1610611
- 6.9. 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 SOP XXX (Digest SOP)

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

	<i>Protein solution type</i>	<i>Protein Solution dilution in PBS/CHAPS</i>		<i>Antibody Solution in PBS/CHAPS</i>		<i>Plate Size</i>
	RecProt or Lys	Amount ug	Volume uL	Amount ug	Volume uL	DW or STD
Large	Lys	500-4000*	up to 800	10	100	DW
Small	RecProt	5	100	10	100	STD
	Lys	500	up to 125	10	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. Prepare **antibody component** for the IP mix
- 8.2.4.1. Prepare **antibody component of the IP mix** (large- or small-scale prep) and add to each well as indicated in Table 1
- Prepare antibodies at 0.1 mg/mL in PBS/CHAPS
 - Add 100 uL of antibody solution per well
- 8.2.5. Prepare **beads component of the IP mix** detection (large- or small-scale prep) and add to each well as indicated in Table 1
- Transfer required total volume of beads suspension needed for all wells into 1.5 mL microcentrifuge tube, including a 10% volume excess (for example: for 10 wells, with 20 uL/well, the total volume would be 200 uL; with 10% excess would be a final beads suspension volume of 220 uL).
 - Pellet beads on magnetic rack and remove storage buffer
 - Resuspend beads in equal volume of PBS/CHAPS
 - Repeat last two steps for a total of two washes
 - Resuspend beads in starting volume (20uL) with PBS/CHAPS
 - Add 20 uL of bead suspension to each well
- 8.2.6. Combine antigen, antibody, and beads components into each plate well

- Cover plate with adhesive sealer
- Incubate overnight at 4°C with shaking

8.3. KingFisher Flex Set-up

The prep scale (Table 1) affects the choice of the Tip Comb and the plate type.

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

- Prepare a STD plate with 150 uL of PBS/CHAPS buffer per well. Label this plate as Recovery
- Prepare a DW plate with a DW comb
- Check if the magnetic head installed on the instrument is of the required size (DW) for the beads transfer. If necessary, follow the manual instruction to change the magnetic head manually using the keys and control buttons on the front panel of the KingFisher Flex
- Open the BindIt software and load the Beads Recovery 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. The Recovery plate should be considered as the IP Mix plate for all the following steps (8.4 onward)

8.4. Prepare all necessary material for automated processing.

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 manual instruction to change the magnetic head manually using the keys and control buttons on the front panel of the KingFisher Flex.

8.4.2. Open BindIt software and choose automated method based on preferred application (MS or WB).

8.4.2.1. For IP-MS – use method SC Multiplex Urea

- Prepare 3 wash plates (STD), loading 200 uL of PBS/CHAPS
- Prepare 1 elution plate (STD) with 200 uL TUB (refer to Digestion SOP M-143)
- Prepare 1 PCR TipComb in a STD plate

8.4.2.2. For IP-WB – use method SC_Multiplex

- Prepare 3 wash plates (STD), loading 200 uL of PBS/CHAPS
- Prepare 1 elution plate (PCR) with 30 uL SDS Reducing Buffer (2X Reducing Sample Buffer – Laemmli with 5% BME)
- Prepare 1 PCR TipComb in a STD plate

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

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

8.5. **Sample analysis**

Sample 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 (version #2)

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. SkyLine¹ software is used to generate a transition list with predicted optimum collision energy.

The following is the general strategy to generate an *in-silico* trypsin digestion and create a transition list with predicted optimum collision energies in Skyline². Open Skyline and start a new blank document.

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

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.

- 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.