

ProMTag Immunoprecipitation to Mass Spectrometry (ProMTag IP-to-MS) Kit Guide

For unbiased extraction, cleanup, and digestion of target antigens

Contents

<u>Kit component</u>	<u>Quantity</u>	<u>Storage</u>
ProMTag	60 µL	-20°C
Quencher	100 µL	-20°C
MT-Trypsin	100 µL	-20°C
IP Lysis Buffer (IP-LB)*	10 mL	RT
IPP Buffer	12 mL	RT
IP Elution Buffer (IP-EB)	2.5 mL	RT
Elution Buffer (EB)	400 µL	RT
Wash Buffer 1 (IP-WB1)	2 mL	RT
Wash Buffer 2 (IP-WB2)	2 mL	RT
Wash Buffer 3 (IP-WB3)	2 mL	RT
Protein A resin	100 µL	4°C
ProMTag capture resin	100 µL	4°C
0.5 mL ProMTag Resin Capture (RC) tubes	16 tubes	RT
2 mL waste collection (adapter) tubes	16 tubes	RT
1.5 mL sample collection tubes	16 tubes	RT
RC-tube end cap	8 caps	RT

*We recommend adding protease inhibitors (not included) to the IP-LB immediately prior to use

RT- room temperature

Storage

Store the entire kit at room temperature (RT) EXCEPT: Protein A resin and ProMTag capture resin (store at 4°C), and ProMTag, Quencher, and MT-Trypsin (store at -20°C). We recommend using your kit within 6 months of receiving it.

Safety

Always protect yourself appropriately when working with chemicals. This includes, but is not limited to, utilizing an appropriate lab coat, disposable gloves, and protective eye goggles. For more information, please consult the appropriate Safety Data Sheets. These are available online at <https://www.impactproteomics.com/resources>.

The ProMTag, IP-WB1, IP-WB2, and IP-WB3 contain various amounts of acetonitrile. Please dispose of these appropriately and avoid open flames.

EB contains formic acid. Avoid contact with skin and eyes. If skin contact occurs, remove contaminated garments and rinse skin thoroughly with water. If eye contact occurs, remove contact lenses if applicable and rinse with plenty of water.

Equipment and reagents you will need before you start:

- Cell lysate, tissue lysate, or protein source
- Serum, plasma, or other antibody source
- Protease inhibitors (we recommend phenylmethanesulfonyl fluoride (PMSF), pepstatin A, and leupeptin)
- Pipettes and pipette tips
- Benchtop centrifuge (mini or full size)
- Sample rotator (rotisserie or carousel)
- Heating block
- Vortex
- Ultrapure, deionized water

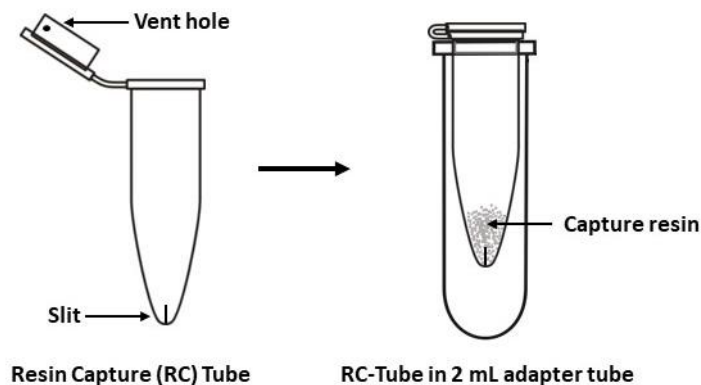
Cell lysis and preparation of the biological sample for ProMTag IP-to-MS processing

The ProMTag IP-to-MS Kit is intended to prepare mass spectrometry-ready target antigens. For the best results, cell lysis must be as thorough as possible. While we recommend utilizing the included lysis buffer, other lysis buffers are compatible with the kit. You can use your own lysis buffer **as long as it does not contain TRIS (or any other buffer with primary amines) and is ~pH 8.0**. If your lysis technique uses TRIS, we recommend switching to 100 mM HEPES pH 8.0. If you need advice on lysis for your particular sample, we are available to help. Simply email us at info@impactproteomics.com.

We **highly** recommend using protease inhibitors in your lysis buffer to prevent protein degradation. Note that certain protease inhibitors contain primary amines and will interfere with ProMTag labeling. We recommend adding phenylmethanesulfonyl fluoride (PMSF), pepstatin A, and leupeptin to the IP-LB immediately prior to cell lysis.

Other notes to consider before you begin

- The RC-tubes have two features that distinguish them from typical spin columns: a fine slit to retain resin instead of a frit, and a hole in the rim of the cap to prevent loss of liquid when closing the tubes.
- Avoid touching the bottom of the RC-tubes. Keep the RC-tube in a waste or collection tube, or a low protein binding tube when incubating or vortexing, except when mixing on a rotisserie.
 - a. We recommend briefly vortexing or tapping the RC-tubes at multiple points throughout the protocol to aid in resuspension of the resin. **Never vortex the RC-tube alone**. Always vortex by placing the RC-tube in a 1.5 mL or 2 mL tube as an adapter to avoid touching the bottom of the RC-tube.
- We do not recommend pipetting to mix at any stage where capture resin is present, as the resin will stick to the tip and result in suboptimal yield.
- All centrifugation steps may be performed on a benchtop centrifuge at room temperature.



- For best results, keep the resin suspended during all incubation steps. This can be done using a 360° rotisserie (recommended) or a carousel. We do not recommend shaking to keep the resin suspended, but if you do ensure you use **gentle** agitation.
- The protocol takes 6-8 hours to perform.

Protocol for antigen extraction, cleanup, and digestion using the ProMTag IP-to-MS kit

Lysate preparation

1. If you are starting with a prepared lysate, skip this step but read the note on Page 3 regarding lysis buffers. If you are starting with un-lysed cells, start here. We recommend using a lysate prepared from K562 cells.
 - a. **Before beginning, supplement IP lysis buffer (IP-LB) with protease inhibitors.**
Only add protease inhibitors to the IP-LB you will be using today. Store the IP-LB on ice after adding protease inhibitors.
 - i. We recommend adding PMSF dissolved in isopropanol to a final concentration of 1 mM and leupeptin and pepstatin A dissolved in DMSO to a final concentration of 10 µg/mL.
 - b. To lyse cells:
 - i. Add IP-LB supplemented with protease inhibitors to your pelleted cells. For 2×10^8 cells we recommend adding 5 mL IP-LB. Rotate the tube on ice with occasional vortexing to fully resuspend the pellet.
 - ii. Sonicate. We recommend 30-40 blasts at 30% power and 30% duty cycle. Keep the tube on ice while sonicating.
 - iii. Aliquot lysate into 1.5 mL tubes and centrifuge at 15k rpm for 20 minutes at 4°C. Transfer the lysate to a fresh tube avoiding any debris and the fatty layer if applicable.
 - c. Assay protein concentration via your method of choice. We recommend a BCA assay.

Lysate Labeling

2. Determine the amount of lysate, IP-LB, ProMTag, and Quencher necessary for **all** samples (n) you will process per day. You will set up a single bulk labeling reaction for all samples that you will process at the same time.

$$n = \# \text{ of reactions}$$

$$\text{lysate } (\mu\text{L}) = n * \frac{100 (\mu\text{g})}{\text{lysate conc. } (\mu\text{g}/\mu\text{L})}$$

$$\text{IP-LB } (\mu\text{L}) = (n * 200) - \text{lysate } (\mu\text{L})$$

$$\text{ProMTag } (\mu\text{L}) = (n * 6)$$

$$\text{Quencher } (\mu\text{L}) = (n * 10)$$

3. Combine lysate and IP-LB.
4. Add ProMTag to the lysate and incubate on ice for 30 minutes.
 - a. **Note: Immediately after beginning this 30-minute incubation proceed to Step 6 below. These steps are done concurrently.**
5. Add Quencher to the lysate and incubate on ice for 30 minutes.

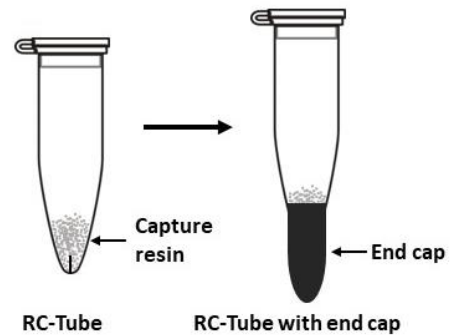
Binding antibodies (Abs) to Protein A resin

6. Place n 0.5 mL RC-tubes, where n is the number of reactions, into 2 mL waste collection tubes. Add 100 μL IPP to each RC-tube.
7. Add 10 μL protein A resin to each tube. Ensure the resin is thoroughly mixed before using.
8. Centrifuge tubes briefly (~2 seconds) in a benchtop centrifuge until all the liquid has passed into the waste collection tube. Discard flowthrough.
9. Wash resin 2 times with 100 μL IPP. All wash steps are carried out as follows:
 - a. Place the RC-tube in a 2 mL waste collection tube.
 - b. Add 100 μL appropriate wash buffer (IPP for Step 9) to RC-tube.
 - c. Centrifuge briefly (~2 seconds) in a benchtop centrifuge until all the liquid has passed into the waste collection tube.
 - d. Discard the flowthrough and, if necessary, tap the waste tube on a paper towel to empty the waste tube and avoid carryover.
10. Add 25 μL IPP to RC-tube.
11. Add 10 μL serum/plasma to RC-tube and tap gently to mix.
 - a. Other antibody sources can be used instead of serum. If the antibody source volume is higher than 25 μL , skip Step 10 and instead add the antibody source directly to the protein A resin.
 - b. Recommended amounts of non-plasma/serum antibody sources:
 - i. Purified antibody: 2-5 μg
 - ii. Bronchoalveolar lavage fluid (BALF): 50 μL
12. Incubate for at least 45 minutes at 4°C with gentle mixing.
 - a. This incubation can be carried out for more than 45 minutes if necessary, but if you begin preparing the protein A resin immediately after Step 4 this 45-minute incubation should be completed at roughly the same time as the Step 5 incubation with Quencher. If Step 5 is completed early, simply keep the lysate on ice until Step 12 is completed.

13. After Steps 5 and 12 are **both** completed, wash the protein A resin 4 times with 100 μ L IPP. Refer to Step 9 for washing instructions.

Capture of ProMTagged antigens on protein A resin

14. Cap the RC-tube with a RC-tube end cap as shown in the diagram to the right.
- The RC-tube cap will not fully push onto the RC-tube. Part of it will extend from the bottom of the RC-tube as shown in the diagram.



15. Add 216 μ L ProMTagged lysate from Step 5 to the washed protein A resin from Step 13 and tap gently to suspend the resin.
16. Incubate for 2 hours at 4°C with gentle mixing.
- We highly recommend rotating the tubes sideways on a rotisserie rather than end-over-end rotation to avoid leakage. Some minor leakage is possible during this incubation period regardless and is not a concern.
17. Wash the protein A resin 2 times with 100 μ L IPP. Refer to Step 9 for washing instructions.

Release of ProMTagged antigens from the protein A resin

18. Add 25 μ L IP-EB to the RC-tube. Ensure the resin is completely covered by the IP-EB.
19. Incubate for 10 minutes at room temperature with gentle mixing.
20. Place the RC-tube into a fresh 1.5 mL tube and centrifuge briefly (~5 seconds) in a benchtop centrifuge until all the liquid has passed into the waste collection tube.
- This is the **IP-Eluate**.

Binding of ProMTagged antigens to ProMTag capture resin

21. To n fresh RC-tubes, where n is the number of reactions, add 10 μ L ProMTag capture resin.
- We recommend pipetting the ProMTag capture resin with a wide-bore pipette tip if possible. If you do not have these, simply pipette slowly and carefully.

22. Wash the ProMTag capture resin 1 time with 100 μ L IP-WB3. Refer to Step 9 for washing instructions.
23. Add the IP-Eluate from Step 20 to the ProMTag capture resin.
24. Incubate for 15 minutes at room temperature with gentle mixing.
25. Wash the ProMTag capture resin as follows.
 - a. Wash 2 times with 100 μ L IP-EB.
 - b. Wash 2 times with 100 μ L IP-WB1.
 - c. Wash 2 times with 100 μ L IP-WB2.
 - d. Wash 1 time with 100 μ L IP-WB3.
 - e. Wash 2 times with 100 μ L ultrapure deionized water.

Release of antigens from the ProMTag capture resin and digestion

26. Place RC-tubes into 1.5 mL sample collection tubes. Add 20 μ L EB (**not** IP-EB) to the ProMTag capture resin.
27. Add 10 μ L MT-Trypsin.
28. Incubate for 1 hour at 37°C.
29. Centrifuge tubes briefly (~5 seconds) in a benchtop centrifuge until all the liquid has passed into the sample collection tube.
30. Add 20 μ L EB (**not** IP-EB) to the ProMTag capture resin.
31. Incubate RC-tube for 15 minutes at room temperature with gentle mixing.
32. Place the RC-tube into **the same 1.5 mL tube you used in Step 29** and centrifuge briefly (~5 seconds) in a benchtop centrifuge until all the liquid has passed into the sample collection tube.
 - a. This tube contains your sample of pure peptides in a volatile, acidic buffer ready for mass spectrometry. We recommend concentrating the sample by drying in a vacuum concentrator.

Quick start guide for ProMTag IP-to-MS

For unbiased extraction, cleanup, and digestion of target antigens

This abbreviated guide is intended for users familiar with the ProMTag IP-to-MS protocol. We highly recommend first time users follow the full-length guide.

Note: Never vortex the RC-tube alone. Always vortex by placing the RC-tube in a 1.5 mL or 2 mL tube as an adapter to avoid touching the bottom of the tube.

1. Before beginning, supplement IP-LB with protease inhibitors. See page 5 of the full-length guide for more details.
2. Label lysate with ProMTag according to the following:

$$n = \# \text{ of reactions}$$

$$\text{lysate } (\mu\text{L}) = n * \frac{100 (\mu\text{g})}{\text{lysate conc. } (\mu\text{g}/\mu\text{L})}$$

$$\text{IP-LB } (\mu\text{l}) = (n * 200) - \text{lysate } (\mu\text{L})$$

$$\text{ProMTag } (\mu\text{L}) = (n * 6)$$

$$\text{Quencher } (\mu\text{L}) = (n * 10)$$

- a. Combine lysate and IP-LB.
 - b. Add ProMTag and incubate for 30 minutes on ice. **Proceed to step 3 below. These steps are done concurrently.**
 - c. Add Quencher and incubate for 30 minutes on ice.
3. After adding ProMTag to lysate, begin preparing protein A resin. To n RC-tubes, add 100 μL IPP, then add 10 μL protein A resin.
 4. Wash protein A resin 2 times with 100 μL IPP.
 5. Add 25 μL IPP to protein A resin, then add 10 μL serum/plasma. See Page 6 of the full-length guide for notes about using non-serum/plasma antibody sources.
 6. Incubate protein A resin with serum/plasma for at least 45 min at 4°C with gentle mixing.
 7. After Steps 2 and 6 are **both** completed, wash the protein A resin 4 times with 100 μL IPP.
 8. Cap the RC-tube with a RC-tube end cap.

9. Add 216 μL ProMTagged lysate from Step 2 to the RC-tube and incubate for 2 hours at 4°C with gentle mixing.
10. Wash Protein A resin 2 times with 100 μL IPP.
11. Add 25 μL IP-EB to the protein A resin and incubate for 10 minutes at RT with gentle mixing. Place the RC-tube into a fresh 1.5 mL tube and centrifuge to collect the **IP-Eluate**.
12. To n fresh RC-tubes, add 10 μL ProMTag capture resin. Wash the ProMTag capture resin 1 time with 100 μL IP-WB3.
13. Add the IP-Eluate from Step 11 to the ProMTag capture resin and incubate for 15 minutes at RT with gentle mixing.
14. Wash the ProMTag capture resin as follows:
 - a. Wash 2 times with 100 μL IP-EB.
 - b. Wash 2 times with 100 μL IP-WB1.
 - c. Wash 2 times with 100 μL IP-WB2.
 - d. Wash 1 time with 100 μL IP-WB3.
 - e. Wash 2 times with 100 μL ultrapure deionized water.
15. Place the RC-tube into a fresh 1.5 mL sample collection tube. Add 20 μL EB (**not IP-EB**) and 10 μL MT-Trypsin to the ProMTag capture resin.
16. Incubate for 1 hour at 37 °C.
17. Centrifuge tubes briefly to collect the eluate.
18. Add 20 μL EB to the ProMTag capture resin and incubate RC-tube for 15 minutes at RT with gentle mixing.
19. Place the RC-tube into the **same 1.5 mL tube used in step 17** and centrifuge briefly to collect the eluate.
20. Concentrate sample with a vacuum concentrator if desired.