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

For unbiased extraction, cleanup, and digestion of target antigens

Contents

Kit component	Quantity	Storage
ProMTag	60 µL	-20°C
Quencher	100 µL	-20°C
MT-Trypsin	100 µL	-20°C
IP Lysis Buffer (IP-LB)*	9.89 mL*	RT
IPP Buffer (IPP)	12 mL	RT
IP Elution Buffer (IP-EB)	2.5 mL	RT
Elution Buffer (EB)	400 µL	RT
Wash Buffer 1 (IP-WB1)	3 mL	RT
Wash Buffer 2 (IP-WB2)	3 mL	RT
Wash Buffer 3 (IP-WB3)	3 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. See notes on pages 3 and 5.
RT- room temperature

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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:

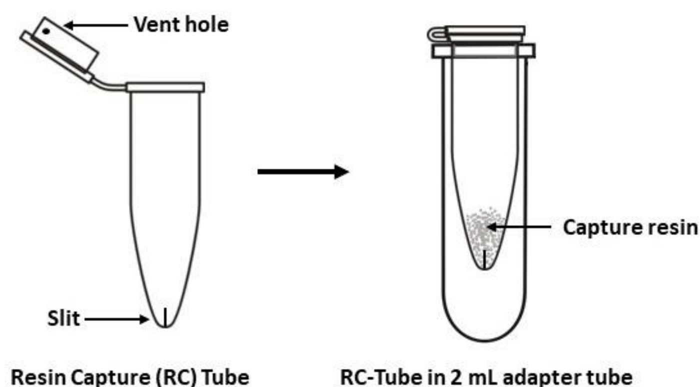
- Cell lysate, tissue lysate, or protein source
- Serum, plasma, or other antibody source
- Protease inhibitors (we recommend phenylmethanesulfonyl fluoride (PMSF), pepstatin A, and leupeptin)
- Pipettes and pipette tips
- Benchtop centrifuge (mini or full size)
- Sample rotator (rotisserie or carousel)
- 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 antigen peptides. 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. The total volume of provided IP-LB is 9.89 mL, leaving 110 µL of volume for protease inhibitors. If you do not add protease inhibitors, add 110 µL distilled water prior to using IP-LB.

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 or tissue, 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. The volume of added protease inhibitors should not exceed 1.1% of IP-LB volume. 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.
         1. For example, to bring the provided 9.89 mL IP-LB to volume, add 100 µL of 17.4 mg/mL PMSF and 10 µL of 10 mg/mL leupeptin+pepstatin A.
   b. <u>Suggested cell lysate preparation protocol:</u>
      i. Thoroughly wash cells. We recommend washing in 100 mM HEPES pH 8.0, but cell culture grade PBS is also suitable.
      ii. Add 5 mL of IP-LB per $2x10^8$ pelleted cells.
      iii. Rotate tube on ice with occasional vortexing to completely resuspend the pellet.
      iv. Sonicate for 30-40 blasts while keeping tubes on ice and avoiding foaming (recommended settings: 30% power, 30% duty cycle). Check that lysate is no longer viscous and continue sonicating if necessary.
      v. Pellet cell debris by centrifugation at 15,000 xg for 20 minutes at 4°C. Transfer supernatant to a fresh tube, avoiding fatty fractions on the surface and pelleted debris.
      vi. Measure protein concentration with protein assay of your choice. We recommend a BCA assay. The expected protein concentration is 5-6 mg/mL if the suggested procedure is followed.
   c. <u>Suggested tissue lysate preparation protocol:</u>
      i. Add 25 volumes (in microliters) of IP-LB per milligram of tissue. For example, add 250 µL IP-LB to 10 mg tissue.
      ii. Homogenize the tissue in 1.5 mL tubes for 3-5 minutes with a pellet pestle fitted to 1.5 mL microtubes while keeping tube with tissue on ice.

iii. (Optional) For viscous homogenates, sonicate for 30-40 blasts while keeping tubes on ice and avoiding foaming (recommended settings: 30% power, 30% duty cycle).

iv. Pellet tissue debris by centrifugation at 15,000 xg for 20 minutes at 4°C. Transfer supernatant to a fresh tube, avoiding fatty fractions on the surface and pelleted debris.

v. Measure protein concentration with protein assay of your choice. We recommend a BCA assay. The expected protein concentration is 1.5-2.5 mg/mL if the suggested procedure is followed.

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 lysate labeling reaction for all samples.

$$n = \#\ of\ reactions$$

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

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

$$ProMTag\ (\mu L) = (n * 6)$$

$$Quencher\ (\mu L) = (n * 10)$$

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.

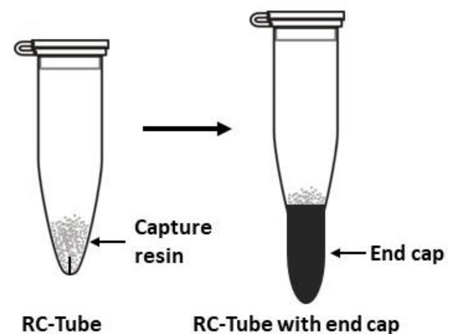
Binding antibodies to Protein A resin

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 µL 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.
    a. 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 µL 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.



    a. 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 4 times with 100 µL IPP. Refer to Step 9 for washing instructions.

Release of ProMTagged antigens from the protein A resin

18. Add 25 µL 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 1.5 mL tube.
    a. 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 µL ProMTag capture resin.
    a. 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 µL 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 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.

Release of antigens from the ProMTag capture resin and digestion

26. Place RC-tubes into **fresh** 1.5 mL sample collection tubes. Add 20 µL EB (**not** IP-EB) to the ProMTag capture resin.
27. Add 10 µL 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 µL EB (**not** IP-EB) to the ProMTag capture resin.
31. Incubate RC-tube for 10 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:

$$n = \#\ of\ reactions$$

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

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

$$ProMTag\ (\mu L) = (n * 6)$$

$$Quencher\ (\mu L) = (n * 10)$$

   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 an RC-tube end cap.

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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 4 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 10 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.