Pi $^{3\text{TM}}$ Methionine Reagent
(*Pi*^{3}-Met)

Instruction Manual
Version 3.0

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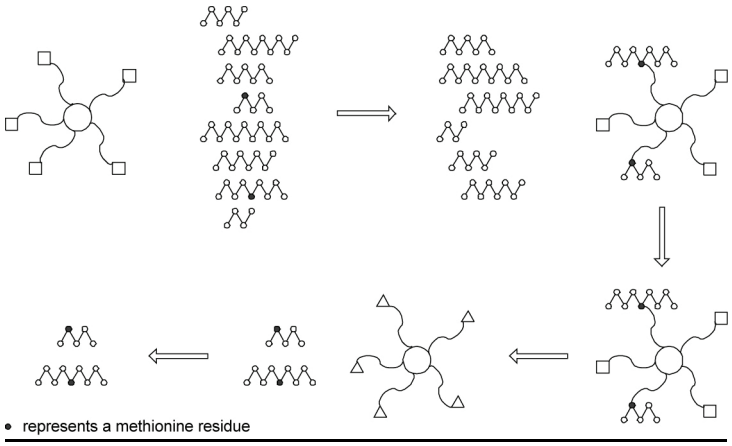
General Suggestions for Maximizing Sample Recovery at Low Levels

- Rinse any 1.5 ml microcentrifuge tubes to be used, first with 1 ml 2% TFA in water and then with 1 ml of 70% CH_3CN/0.1% TFA in water.
- Use the same pipette tip for multiple transfers of the same sample.
- Use a 250 µl pipette tip for sample transfer, instead of a 1 ml pipette tip.

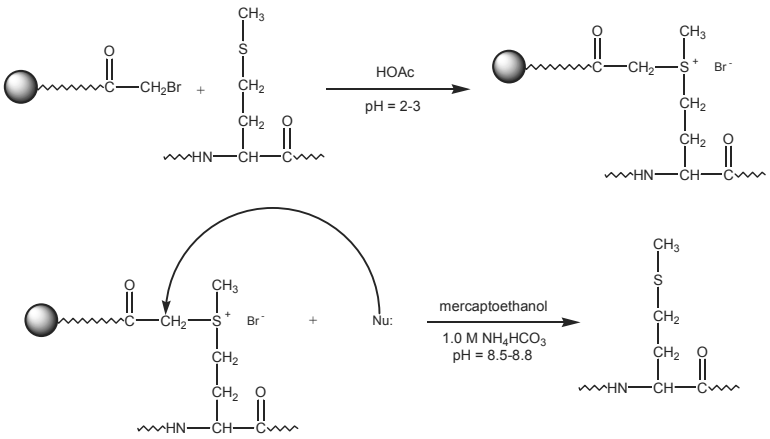
Introduction

Pi[3] Methionine Reagent (*Pi*[3]-Met) is a solid-phase reagent designed to isolate methionine-containing peptides from peptide mixtures. Methionine peptides are covalently bound to the *Pi*[3]-Met, allowing for the removal of non-methionine peptides, and the subsequent release of the methionine peptides.

Schematic



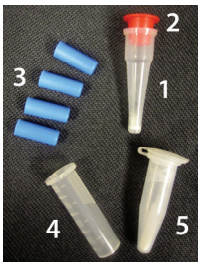
Chemistry



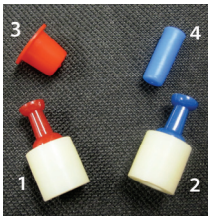
The Kit

Each *Pi*^{3}-Met kit contains material enough for two isolation reactions. Included are:

A. Two isolation packs, each comprising:
   1. Spin tube containing *Pi*^{3}-Met (qty. 1)
   2. Red cap for spin tube top (qty. 1)
   3. Blue caps for spin tube bottom (qty. 4)
   4. Collection tube (qty. 1)
   5. Centrifuge tube containing reverse phase silica (50mg) (qty. 1).



B. One accessory pack, comprising:
   1. Top cap puncher (red handle) for red cap
   2. Bottom cap puncher (blue handle) for blue cap
   3. Extra red cap
   4. Extra blue cap.



Each isolation pack is designed to be used on a digestion of up to 5 nmol of protein.

Required Equipment

A. Vortex mixer with micro tube insert:
   1. The holes in the insert must be, or must be modified to be, 3/8" (9mm) in diameter.
   2. If the holes in the insert are not through holes, the depth of the holes must be 1 3/4" (45mm) deep.
   3. We recommend the Vortex Genie II fitted with the 6" platform head and the 60 micro tube insert. This equipment is widely available from most laboratory suppliers.

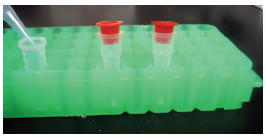
B. Bench top centrifuge:
   1. Capable of 10000 RPM
   2. Capable of holding 1.5-2 ml microcentrifuge tubes.

Required Material for One Isolation

A. Chemicals and solutions:
   1. DI water (H_2O) (~10 ml)
   2. Methanol (MeOH) (~3.5 ml)
   3. β-Mercaptoethanol (BME) (~20 µl)
   4. 1.0 M Ammonium Bicarbonate (NH4HCO3), pH 9.0-9.1 (~150 µl)
   5. 15% HOAc in H_2O (~30 µl)
   6. 2% Trifluoroacetic Acid (TFA) in water (~3 ml)
   7. 0.2 M BME in 25% HOAc (~1.5 ml)
   8. 20% Acetonitrile (CH_3CN)/0.1% TFA in water (~75 µl)
   9. 70% CH_3CN/0.1% TFA in water (~5 ml).

- **All chemicals used above are reagent grade or better.**
- **All solutions should be freshly made.**

B. Other supplies:
   1. Microcentrifuge tube rack (qty. 1)
   2. 1.5 ml microcentrifuge tube (qty. 2)



Procedure for Use

Notes:

- **A microcentrifuge tube rack is used as a holder for the spin tube and the collection tube throughout this procedure.**
- **All liquid additions to the spin tube are done with the spin tube sitting in the collection tube.**
- **All vortexing steps are performed using the micro tube insert, except where noted.**
- **All centrifuge spins are done at 10000 RPM for 1 min.**
- **The following is a general procedure. Wash steps may occasionally need to be adjusted, depending on sample nature, to decrease non-specific binding.**
- **Longer reaction times may be required for more complex samples.**

A. Sample Preparation

1. Bring the sample volume to 75 µl with H_2O. Mix well. Add 25 µl 15% HOAc. Mix well.

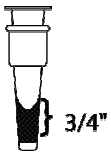
B. *Pi*^{3}-Met Pretreatment

1. Take the spin tube containing the *Pi*^{3}-Met and spin in the centrifuge to ensure that all material is settled at the bottom of the spin tube.
2. Remove the red cap from the spin tube. Using the top cap puncher, punch a hole in the cap to prevent pressure build-up in the tube.

Caution!

Never vortex the spin tube without having the red cap firmly in place.

3. Place a blue cap on the spin tube. Add 200 µl of MeOH to the spin tube. Replace the red cap.
4. Place the spin tube in the vortex. Adjust the vortex speed to give a liquid level as shown in the drawing. Vortex for 15 min.
5. Remove the spin tube from the vortex. Using the bottom cap puncher, punch a hole in the blue cap. Remove the blue cap. Place the spin tube in the collection tube. Remove the red cap. Place the spin tube with the collection tube into a centrifuge. Spin, Discard the liquid from the collection tube into a waste container. Place the spin tube in the col1ection tube.



6. Add 400 µl of H_20 to the spin tube. Replace the red cap. Hold the collection tube together with the spin tube by hand, and vortex for 15 sec., adjusting the vortex speed until the liquid just reaches the red cap. Remove the red cap. Place the spin tube with the collection tube into a centrifuge and spin.
7. Repeat Section B6 two (2) more times.

C. Capture of Methionine Peptides

1. P1ace a new blue cap on the spin tube. Add the sample to the spin tube and replace the red cap. Insert the cap firmly.
2. Place the spin tube in the vortex. Vortex for 1.5 hr in a manner similar to Section B4 above.

D. Wash after Capture

1. Remove the spin tube from the vortex.
2. Remove the red cap and add 100 µl of 0.2 M BME to the spin tube. Replace the red cap firmly and vortex for 30 min.
3. Remove the spin tube from the vortex.
4. Using the bottom cap puncher, punch a hole in the blue cap.
5. Remove the blue cap. Place the spin tube in the collection tube. Remove the red cap. Place the spin tube with the collection tube into a centrifuge. Spin. Remove the solution from the collection tube and save the solution if desired.
6. Repeat the procedure described in Section B6 above, but with the following solutions and volumes:
   a) 3 times with 400 µl of 0.2 M BME
   b) 3 times with 400 µl of 70% CH_3CN/0.1% TFA
   c) 3 times with 400 µl of H_2O.

E. Methionine Peptide Release

1. Place a new blue cap on the spin tube. Add 86 µl of 1.0 M NH_4HCO, and 14 µl of BME to the spin tube. Place the red cap firmly.
2. Vortex the spin tube for 2 hr. in a manner similar to Section B4 above.

F. Sample Collection

1. Rinse the collection tube with 1 m1 of 70% CH_3CN/0.l% TFA followed by 1ml of H_2O.
2. Holding the spin tube upright, tap it gently three times on the bench. Using the bottom -cap puncher, punch a hole in the blue cap and remove the blue cap carefully from the spin tube.
3. Place the spin tube into the rinsed collection tube. Remove the red cap carefully. Add any liquid remaining on the red cap to the spin tube. Spin.
4. Transfer the solution from the collection tube to a clean 1.5 ml microcentrifuge tube.
5. Add 50 µl of 20% CH_3CN/0.l% TFA to the spin tube. Place the red cap and a new blue cap on the spin tube.
6. Vortex the spin tube for 15 min. in a manner similar to Section B4 above.
7. Holding the spin tube upright, tap it gently three times on the bench. Using the bottom -cap puncher. Punch a hole in the blue cap .and remove the blue cap careful1y from the spin tube.
8. Place the spin tube into the collection tube. Remove the red cap carefully. Add any liquid remaining on the red cap to the spin tube. Spin.
9. Remove the solution from the collection tube and add to the solution already in the 1.5 m1 mlcrocentrifuge tube.
10. Add 400 µl of 2% TFA to the spin tube and replace the red cap. Hold the collection tube with the spin tube by hand, and vortex for 15 sec., adjusting the vortex speed until the liquid just reaches the red cap.

11. Holding the spin tube/collection tube upright, tap them gently three times on the bench. Remove the red cap carefully. Add any liquid remaining on the red cap to the spin tube. Spin.
12. Remove the solution from the collection tube and combine with the solution already in the 1.5 ml microcentrifuge tube. Add 400 µl of 2% TFA to the solution already in the 1.5 ml microcentrifuge tube.

G. Sample Clean-Up

1. Discard the used glass beads in the spin tube in an appropriate manner.
2. Rinse the spin tube with H_2O until no visible glass beads remain.
3. Repeat the procedure described in Section B6 above, but with following solutions and volumes:
   a) 2 times with 400 µl of H_2O
   b) 2 times with 400 µl of MeOH.
4. Weigh and add an amount of silica, per the following table, to the spin tube.

Protein Sample Amount	Suggested Amount of Silica	Suggested Amount of Eluent
< 1 nmol	10 mg	30 µl
1-2 nmol	20 mg	40 µl
2-5 nmol	50 mg	50 µl

Add 400 µl of MeOH to the spin tube. Repeat the procedure described in Section B6 above.

5. Add 400 µl of 2% TFA to the spin tube. **DO NOT VORTEX!** Spin and discard the solution from the collection tube into a waste container.
6. Add half of the sample solution (475 µl) in the 1.5 ml microcentrifuge tube from Section F12 to the spin tube. **DO NOT VORTEX!** Spin and discard the solution from the collection tube into a waste container.
7. Repeat Section G6 but using the remaining second half of the sample.
8. Repeat Section G5 two (2) times.
9. Repeat Section F1.
10. Add a volume of the eluent, 70% CH_3CN/0.1% TFA per the table in Section G4. **DO NOT VORTEX!** Spin. Transfer the solution in the collection tube to a new 1.5 ml microcentrifuge tube.
11. Repeat Section G10 two (2) times.
12. Retain the sample in the 1.5 ml microcentrifuge tube for analysis.
13. Dispose the waste material in an appropriate manner.
14. **Analyze sample as soon as possible to avoid loss or breakdown.**

Appendix – Mass Spectrometry Search for Methionine-Containing Peptides

A. Search Strategy for Captured Methionine-Containing Peptides When Using MALDI and MS-Fit

1. Creating Methionine/Methionine Oxidation Doublets:
   a) For each peak in the mass table, create an artificial oxidation doublet by adding 15.99 Da. to each peak.
   b) Search each doublet individually using the methionine oxidation option.
   c) Require that there be two matches for each doublet.
   d) Require that any correctly identified protein have at least one methionine moiety in every searched peptide.
   e) Pool data from b)–d) above to determine protein(s).