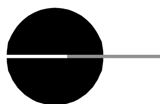
Pi^{3}™ Tryptophan Reagent (*Pi*^{3}-Trp)

Instruction Manual

Revision 8.01.01



BioMolecular Technologies, Inc.
525F Del Rey Ave. Sunnyvale, CA 94085
Phone: 408-617-0609 Fax: 408-617-0669
E-mail: bmt@bmtusa.com

8/1/01

Table of Contents

Introduction 1
Schematic 1
Chemistry 1
The Kit 2
Required Equipment 3
Required Material for One Isolation 3
Procedure for Use 4
A. Sample Preparation 4
B. *Pi*[3]-Trp Pretreatment 4
C. Capture of Tryptophan Peptides 5
D. Wash after Capture 5
E. Tryptophan Peptide Release 5
F. Sample Collection 5
G. Sample Clean-Up 6
Appendix – Mass Spectrometry Search for Tryptophan-Containing Peptides 6
A. Search Strategy for Captured Peptides Containing 1 or 2 Tryptophans When Using MALDI and MS-Fit 6

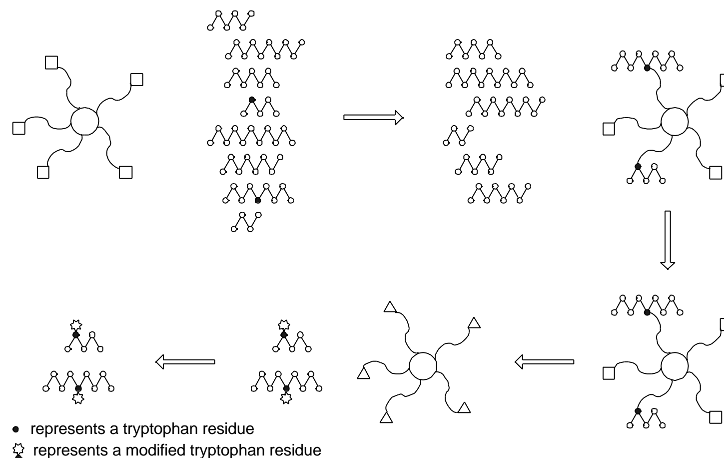
General Suggestions for Maximizing Sample Recovery at Low Levels

- Rinse any 1.5 ml microcentrifuge tubes to be used, first with 1 ml 2% TFA and then with 1 ml of 70% CH_3CH/0.1% TFA.
- 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

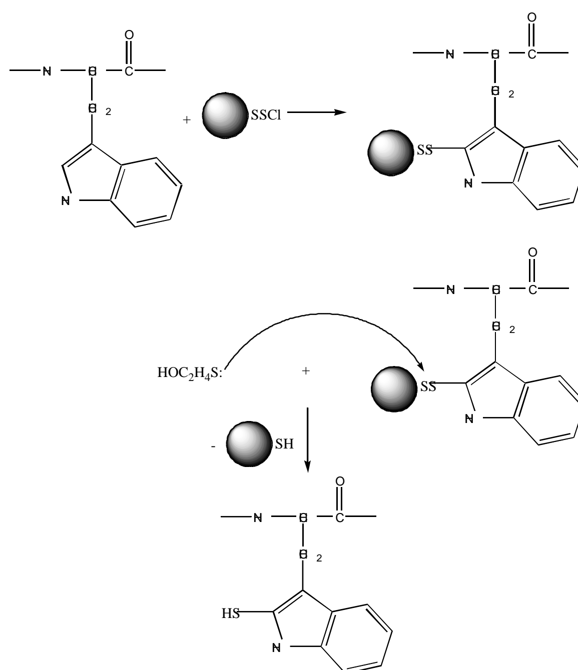
Pi^3 Tryptophan Reagent (*Pi*^{3}-Trp) is a solid-phase reagent designed to isolate tryptophan-containing peptides from peptide mixtures. Tryptophan peptides are covalently bound to the *Pi*^{3}-Trp, allowing for the removal of non-tryptophan peptides, and the subsequent release of the tryptophan peptides.

Schematic



Chemistry

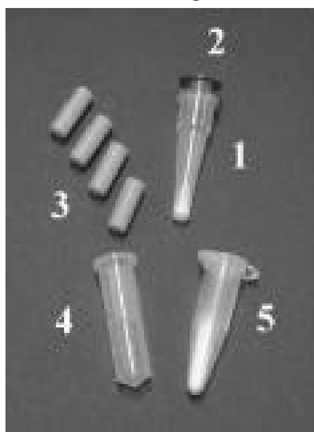
The *Pi*^{3}-Trp solid-phase reagent is composed of porous glass beads designed to perform the following chemistry:



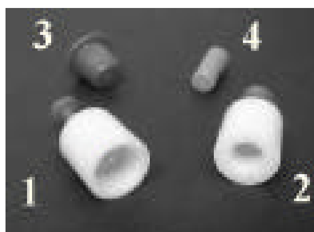
The Kit

Each *Pi*^{3}-Trp kit contains material to perform two tryptophan peptide isolation reactions. Included are:

A. Two isolation packs, each comprising:
   1. Spin tube containing *Pi*^{3}-Trp (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 (50 mg) (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 2 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.

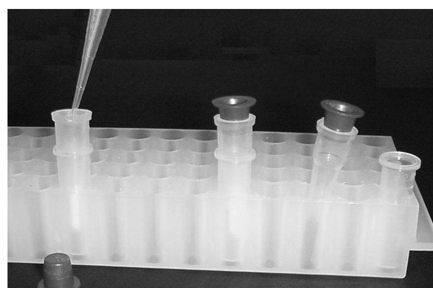
B. Bench top centrifuge:
  1. Capable of 10000 RPM
  2. Capable of holding 1.5-2 ml microcentrifuge tubes.

Required Material for One Isolation

A. Chemicals and solutions:
  1. DI water (H_2O) (~3.5 ml)
  2. Methanol (MeOH) (~2 ml)
  3. Glacial Acetic Acid (HOAc) (~75 µl)
  4. Mercaptoethanol (BME) (~25 µl)
  5. 1.0M Ammonium Bicarbonate (NH_4HCO_3), pH 9 (~500 µl)
  6. 8.0M Guanidine Hydrochloride (GnHCl) in water (~100 µl)
  7. 2% Trifluoroacetic Acid (TFA) in water (~2.2 ml)
  8. 3% Urea in water (~500 µl)
  9. 3.0M GnHCl/1.0M NH_4HCO_3, pH 9 (~100 µl)
  10. 20% Acetonitrile (CH_3CN)/2% TFA in water (~225 µl)
  11. 70% CH_3CN/0.1% TFA in water (~3.5 ml).

  All chemicals used above are reagent grade or better.

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.**

A. Sample Preparation

1. Bring the sample volume to 30 µl with H_2O. Mix well. Add 84 µl of 8.0M GnHCl. Mix well. Add 50 µl of HOAc. Mix well.

B. *Pi*[3]-Trp Pretreatment

1. Take the spin tube containing the *Pi*[3]-Trp 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. Add 400 µl of 70%CH_3CN/0.1%TFA 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. Discard the liquid from the collection tube into a waste container.
4. Repeat Section B3 one (1) more time.
5. Place a blue cap on the spin tube. Add 100 µl of 2% TFA to the spin tube. Replace the red cap. Place the spin tube in the vortex. Adjust the vortex speed to give a liquid level in the tube approximately 1/3 to 1/2 the height of the tube. Vortex for 1 min. Using the bottom cap puncher, punch a hole in the blue cap. Remove the blue cap. Save this punched blue cap for later wash steps. Remove the red cap.
6. Place the spin tube into the collection tube and spin. Discard the liquid in the collection tube into a waste container.

C. Capture of Tryptophan Peptides

1. Place a new blue cap on the spin tube.
2. Add the sample to the spin tube and replace the red cap firmly.
3. Place the spin tube in the vortex. Adjust the vortex speed per Section B5. Vortex for 3.5 hr.

D. Wash after Capture

1. Remove the spin tube from the vortex.
2. Using the bottom cap puncher, punch a hole in the blue cap.
3. 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.
4. Place a new blue cap on the spin tube. Add 200µl of 20% CH_3CN/2%TFA to the spin tube. Replace the red cap.
5. Repeat the procedure of C3 above, but vortex for 15 min.
6. Repeat Sections D1-D3 above.
7. Repeat Sections B5-B6 above, but using the following solutions and volumes:
   a) 2 times with 200 µl of 70%CH_3CN/0.1%TFA
   b) 2 times with 200 µl of 3% urea
   c) 2 times with 200 µl of 1.0M NH_4HCO_3, pH9
   d) 2 times with 200 µl of H_2O.

E. Tryptophan Peptide Release

1. Place a new blue cap on the spin tube. Add 79 µl of 3.0M GnHCl/1.0M NH_4HCO_3, pH 9 and 21 µl of BME to the spin tube. Replace the red cap.
2. Vortex the spin tube in a manner similar to Section C3 above for 1.5 hr.

F. Sample Collection

1. Rinse the collection tube with 1 ml of 70% CH_3CN/0.1% TFA followed by 1 ml 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 collection tube contents to a clean 1.5 ml microcentrifuge tube.
5. Place a new blue cap on the spin tube. Add 400 µl of 2% TFA to the spin tube. Replace the red cap.
6. Vortex the spin tube in a manner similar to Section C3 above for 1 min.
7. Repeat the procedure of F2 above.
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 spin tube from the collection tube. Transfer the contents of the collection tube to the 1.5 ml microcentrifuge tube of Section F4 above. Add 450 µl of 2% TFA to the solution already in the 1.5 ml microcentrifuge tube.

G. Sample Clean-Up

1. Discard the used beads in the spin tube in an appropriate manner.
2. Rinse the spin tube with water until no visible glass beads remain.
3. Repeat the procedure described in Section B3 above, but with 400 µl MeOH.
4. Repeat Section G3 one (1) more time.
5. Weigh and add an amount of silica, per the following table, to the spin tube. Repeat the procedure as described in Section G3 above.

Protein Sample Amount	Suggested Amount of Silica	Suggested Amount of Eluent
1 nmol	20 mg	35 µl
<1-2 nmol	40 mg	40 µl

6. 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.
7. Add half of the sample solution (475 µl) in the 1.5 ml microcentrifuge tube from Section F9 to the spin tube. **DO NOT VORTEX**! Spin and discard the solution from the collection tube into a waste container.
8. Repeat Section G7 but using the remaining second half of the sample.
9. Repeat Section G6 two (2) times.
10. Repeat Section F1.
11. Add a volume of the eluent, 70% CH_3CN/0.1% TFA per the table in Section G5. **DO NOT VORTEX!** Spin. Retain the collection tube containing the solution for use in Section G12.
12. Repeat Section G11 two (2) times.
12. Transfer the solution in the collection tube to a new 1.5 ml microcentrifuge tube and retain the sample for analysis.
13. Dispose the waste material in an appropriate manner.

<u>Appendix</u> – Mass Spectrometry Search for Tryptophan-Containing Peptides

A. Search Strategy for Captured Peptides Containing 1 or 2 Tryptophans When Using MALDI and MS-Fit

1. For Mass Spectra Exhibiting Mass Doublets:
   a) Identify any and all mass doublets differing by 32 Da.
   b) Subtract 64 Da. from the larger mass of each doublet and use the resulting mass for a search.
   c) Search for the largest number of matches possible.
   d) Require that any correctly identified protein have two tryptophan moieties in every searched peptide.
2. For Mass Spectra Exhibiting No Mass Doublets:
   a) Subtract 32 Da. from every mass and use the resulting mass for a search.
   b) Search for the largest number of matches possible.
   c) Require that any correctly identified protein have at least one tryptophan moiety in every searched peptide.