

PROTOCOL: Positive Pressure 96-Well Desalt (PP96D)

Purpose

Remove salts introduced during 8M urea lysis and subsequent digestion prior to phosphopeptide enrichment by IMAC. IMAC is sensitive to salt because it relies on the electrostatic interaction between Fe³⁺ and phosphate groups for enrichment.

Preparation

1. Keep samples from PROTOCOL: Automated Protein Digestion (DIG) on ice prior to desalting

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {PP96D-M01}
- Acetonitrile (ACN), EMD Millipore, Cat. No. AX0156-1 {PP96D-M02}
- Trifluoroacetic acid (TFA), Sigma-Aldrich, Cat. No. T6508-25ML {PP96D-M03}
- 25 mg capacity C18 Sep-pak, Waters, Cat. No. 186002319 {PP96D-M04}
- Breath-EASIER seal, Diversified Biotech, Cat. No. BERM-2000 {PP96D-M05}
- Axygen -80 C rated foil seal, Axygen, Cat. No. PCRAS200 {PP96D-M06}
- 96-Well 2.2 mL Deep Well "Waste" plate, VWR, Cat. No. 82006-448 {PP96D-M07}
- 96-Well 1.2 mL Deep Well "Elution" plate, VWR, Cat. No. 82007-292 {PP96D-M08}

Assets

- Positive pressure manifold {Waters Positive Pressure-96}
- Thermo Scientific Savant SC210A Concentrator {A02}

Reagent Mixes

ID	Name	Step	Composition	Stock Volume	Use
MIX01	100% ACN/ 0.1% TFA	PP96D	100% {PP96D-M02}/ 0.1% {PP96D-M03} in {PP96D-M01}	1000 mL	wash prior to sample load
MIX02	50% ACN/ 0.1% TFA	PP96D	50% {PP96D-M02}/ 0.1% {PP96D-M03} in {PP96D-M01}	1000 mL	peptide elution
MIX03	0.1% TFA	PP96D	0.1% {PP96D-M03} in {PP96D-M01}	1000 mL	equilibrate and wash after sample load

Buffer Prep:

MIX01:

1. Mix 999 mL ACN {PP96D-M02} with 1 mL TFA {PP96D-M03} in graduated cylinder
2. Pour buffer into conditioned 1 L P100 bottle and check pH

- a. Expected pH~ 1

MIX02:

1. Mix 499.5 mL ACN{PP96D-M02} with 499.5 mL HPLC grade water {PP96D-M01} 1 mL TFA {PP96D-M03} in graduated cylinder
2. Pour buffer into conditioned 1 L P100 bottle and check pH
 - a. Expected pH~ 1-2

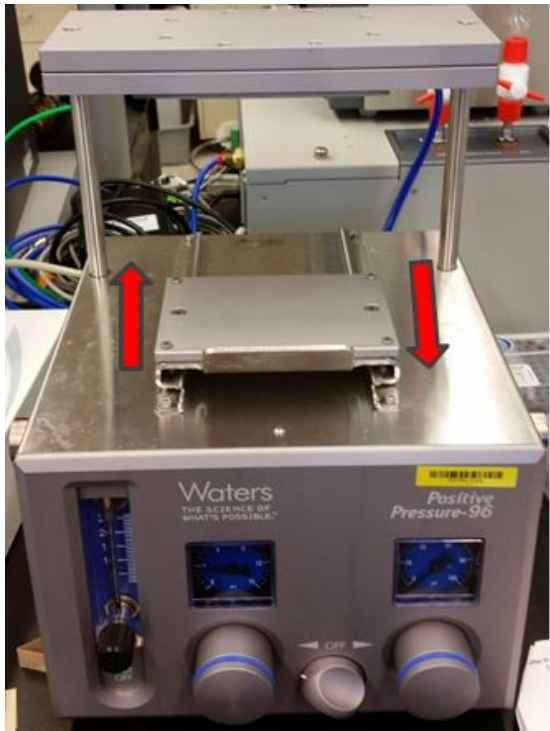
MIX03:

1. Mix 999 mL HPLC grade water {PP96D-M01} with 1 mL TFA {PP96D-M03} in graduated cylinder
2. Pour buffer into conditioned 1 L P100 bottle and check pH
 - a. Expected pH~ 5

Procedure

Manifold Setup

1. Fit C18 Sep-pak on top of "Waste" plate and place on sleigh
2. Move sliding sleigh backwards to the end of the tracks
3. Set middle dial facing left and the left dial at "0"
4. Press and hold blue buttons on the right and left sides of the manifold for several seconds to apply pressure
5. Slowly turn left dial clockwise to increase pressure
6. Only use pressure dial on the right side if the Sep-pak clogs
7. Press and hold blue buttons on the right and left sides of the manifold to release pressure



NB: Samples should flow at a rate 1 drop/second. If some wells are flowing slower, allow normally flowing wells to complete and cover the plate with parafilm. Poke a hole in the wells that are flowing slowly and increase the pressure until the desired flow rate of 1 drop/second is achieved.

Peptide Desalting

1. Flow 1 mL 100% ACN /0.1% TFA {PP96D-MIX01} without pressure through C18 Sep-pak
2. Repeat step 1
3. Equilibrate with 1 mL of 0.1% TFA {PP96D-MIX03} (~9psi)
4. Repeat Step 3
5. Repeat Step 3
6. Add 0.2 mL of 0.1% TFA {PP96D-MIX03} immediately before adding sample
7. Pipette Sample up and down 2x to mix and load sample onto C18 Sep-pak (~3psi)
8. Wash digestion plate wells with 0.5 mL of 0.1% TFA {PP96D-MIX03} and load onto C18 Sep-pak (~3psi)
9. Wash/desalt C18 Sep-pak with 1mL of 0.1% TFA {PP96D-MIX03} (~6psi)
10. Repeat Step 9
11. Repeat Step 9
12. Remove "Waste" plate and replace with 1.2 mL "Elution" plate
 - 12.1.1. Label elution plate with "Date_Plate#_Experiment Name_LINCS"
13. Elute with 300uL of 50 % ACN / 0.1 % TFA {PP96D-MIX02} (~3psi)
14. Repeat Step 13
15. Cover plate with Breath-EASIER seal and then with Beckman Coulter foil seal
16. Make balance plate containing 600 uL of 50 % ACN / 0.1 % TFA {PP96D-MIX02} in each well
17. Weigh balance plate to confirm its weight is within 1 gram of the sample plate
18. Freeze sample and balance plates (-80 C)
19. Remove foil seal and leave Breath-EASIER seal on sample plate
20. Speed vac sample and balance plates to dryness overnight