PROTOCOL NAME: Concentration, clean-up and on-bead digestion using MagReSyn® SAX

PROTOCOL ID: SAX-CLEANUP

DATE LAST MODIFIED: 23 October 2019

INTRODUCTION:

The protocol describes the use of SAX chemistry to bind proteins extracted and solubilized in Urea or GdHCl, followed by efficient on bead digestion using Trypsin. The protocol is particularly suitable for dilute samples such as urine, where precipitation is not feasible for clean-up. **IMPORTANT NOTE**: *This protocol is **NOT** suitable for clean-up of samples extracted in detergents such as **SDS**.* Please contact info@resynbio.com if you have any queries relating to this protocol.

MATERIALS: All reagents and chemicals should be of analytical grade or better, and preferably MS grade.

- MagReSyn® SAX – Catalogue Number MR-SAX002
- Eppendorf LoBind microcentrifuge tubes, 0.5, 1.5 and 2 ml.
- Magnetic Separator or Magnetic bead handling station (e.g. KingFisher™)
- Pipettes
- Ammonium bicarbonate (NH_4Bicarb)
- Trifluoracetic acid (TFA)
- Trizma base (Tris)
- Boric acid
- Sodium hydroxide
- Iodoacetamide (IAA)
- Dithiothreitol (DTT)
- Peptide quantification kit (e.g. Pierce™ Quantitative Colorimetric Peptide Assay)
- MS grade water

REAGENT PREPARATION: NOTE: *Buffers can be stored at 4°C for up to 2 weeks.*

- DTT Stock: 1M DTT (prepare fresh)
- IAA Stock: 1M IAA (prepare fresh, light sensitive)
- 10X SAX Binding Buffer: 1M Tris-Borate pH 10
- Equilibration Buffer: 100mM Tris-Borate pH 10 (prepared by dilution of 10X Binding Buffer)
- Wash Solution: MS grade water
- Trypsin Digestion Buffer: 25 mM NH_4Bicarb

METHOD:

NOTES*:*

- *The method is suitable for concentration and clean-up of proteins from urea and GdHCl, but not suitable for clean-up from detergent solubilized samples containing e.g. SDS.*
- *The current methods are suitable for manual preparation, or may be fully automated on magnetic bead handling systems such as KingFisher™ or similar, protocols are available on request.*
- *The current protocol is sufficient for the clean-up of 20 µg of protein, but can be scaled up or down by keeping a protein to bead ratio of 1:10.*

REDUCTION AND ALKYLATION OF PROTEIN SAMPLE:

1. Quantify your protein sample using a suitable protein quantification technique bearing in mind the potential interfering contaminants in your sample
2. Dilute sample equivalent to 20 µg of protein to ≤ 1µg.µl^{-1} in extraction buffer compatible with clean-up using SAX.
3. Reduce proteins using 10 mM DTT for 30 min at 37°C (diluted into sample from stock solution). **NOTE***: For samples containing urea (such as urine) consider reduction at room temperature to reduce potential carbamylation.*

4. Alkylate proteins using 30 mM IAA for 30 min in the dark (diluted into sample from stock solution)
5. Quench IAA by adding an equivalent volume of 10 mM DTT (diluted into sample from stock solution)
6. Add sufficient ***10X SAX Binding Buffer*** to achieve a final sample concentration of 100 mM Tris-Borate pH 10 (1-part buffer to 9-parts protein solution). This sample is ready to bind to pre-equilibrated MagReSyn® SAX (refer below).

MICROPARTICLE EQUILIBRATION:

7. Re-suspend MagReSyn® SAX thoroughly by vortex mixing or inversion to ensure a homogenous suspension. **NOTE**: *When multiple samples are being prepared, ensure that you maintain a homogeneous suspension by mixing regularly, for example by inversion or pipetting the micro particle mixture up/down before transferring the required volume.*
8. Transfer 10 µl MagReSyn® SAX (200 µg) microparticles to 2 ml Protein Lo-Bind tube.
9. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
10. Remove the shipping solution by aspiration with a pipette and discard.
11. Wash the microparticles by re-suspending in 200 µl of ***Equilibration Buffer*** with agitation (e.g. gentle vortex mixing) for 1 min.
12. Place the tube on the magnetic separator and allow the microparticles to clear.
13. Remove the equilibration solution by aspiration with a pipette and discard.
14. Repeat steps **11 – 13**.

PROTEIN CAPTURE AND ON-BEAD DIGESTION:

15. Add protein sample from **6** to beads from **14** above. **NOTE***: a minimum volume of 25 µl for manual processing or 50 µl for automated processing on a KingFisher® magnetic bead handling station.*
16. Incubate for 30 min at room temperature with continuous mixing (e.g. slow vortexing) to ensure adequate sample and microparticle interaction.
17. Place the tube on the magnetic separator and allow the microparticles to clear. Remove and discard the unbound fraction by aspiration with a pipette.
18. Wash the beads with 200 µl of ***Wash Solution*** (LC MS grade water) and mix for 60 sec with gentle agitation.
19. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear. Remove the supernatant and discard.
20. Repeat steps 13 and 14.
21. Perform on-bead digestion by adding in 20 µl of 25 mM Ammonium Bicarbonate pH 8.0 containing 2 µg sequencing grade Trypsin (1:10 enzyme:protein ratio) for 4 hrs at 37 °C. Ensure sufficient mixing to keep the particles in solution during digestion to ensure good particle liquid interaction. **NOTE**: *For automated sample processing we recommend increasing digestion volume to 200 µl.*
22. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
23. Remove peptide solution and place in a 0.5 ml Eppendorf LoBind tube.
24. Elute peptides with 20 to 100 µl of 1% TFA for 5 minutes. **NOTE**: *Although elution is recommended, this step should be evaluated first to determine possible elution of possible accumulated contaminants under these acidic conditions.*
25. *Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.*
26. *Remove supernatant and combine with eluate from **21**.*
27. Analyse supernatant by LC-MSMS

NOTES:

- *Samples can be vacuum or freeze-dried to reduce the volume of the sample from frozen at low temperature, and resuspended in 40 ul of 0.2% Formic Acid with 2% ACN.*
- *To estimate peptide recovery for LC MS analysis you can use a peptide quantification kit such as Pierce™ Quantitative Colorimetric Peptide Assay*
- *Sample can be desalted with C18 SPE or on-line with a C18 trap cartridge used in a typical pre-concentration LCMS set-up*