# Preparation of RBC Ghosts for Proteomic Analysis

**RBC Isolation:**

1. Draw blood:

    - fill syringe with 5cc Anti-CoAg
    - draw 35 cc blood

2. Divide equally into two 50 mL flacon tubes (~20 mL each).

3. Add PBS to ~50 mL and mix gently by inversion.

4. Spin @ 2500 RPM, 18ºC for 15 min.

    SUP = platelets + plasma proteins
    Interface = WBCs
    Pellet = RBCs

5. Remove supernatant by aspiration.

6. Resuspend in PBS, repeat spin, and remove supernatant.

7. Repeat 4X.

**RBC Lysis:**

8. Pool RBCs into one tube.

9. Add 50 mL lysis buffer-pefabloc and vortex.

    (Need ~ 10 volumes for efficient lysis)

10. Divide into two 50 mL corex tubes and spin @ 8K, 4ºC for 20' on SS34.

    (10 min prerun to cool centrifuge to 4ºC)
    ? spin at 11,000 rpm for 15 minutes at 4ºC?

11. Remove supernatant without disturbing loose pellet – **SAVE SUP**

    (tilt tube, remove sup, remove white clump)

12. Resuspend in 10 mL lysis buffer-pefabloc and repeat spin.

13. Resuspend pellet in lysis buffer (without pefabloc), aliquot in microfuge tubes, and spin 11,000 rpm for 15 minutes.
14. Store pellet at –80°C until processing for mass spectrometry.

## MATERIALS

acid-citrate-dextrose anticoagulant

syringe with 5cc Anti-CoAg

50 mL flacon tubes

PBS (10 mM NaCl, 155 mM KCl, 10 mM glucose, 1 mM MgCl2, 2.5 mM KHPO4, pH 7.4)

PBS + 2.5 mM Pefabloc

Aspirator

Lysis buffer (5 mM $NaHPO_4$, 1 mM EDTA, pH 7.4)

Lysis buffer + 2.5 mM Pefabloc

two 50 mL corex tubes

SS34 rotor, sorvall centrifuge

microfuge tubes

The murine RBC ghost preparation was further solubilized in 0.1% SDS and 6M urea and digested with trypsin, chymotrypsin, or AspN plus 1/500 trypsin. Following digestion, the samples were boiled for 10 minutes to inactivate the enzymes and inhibitors leupeptin 1M , chymostatin 20 M , PMSF 0.1mM were added and the digests were combined. The resulting mixture was acidified with 10% phosphoric acid and separated on an Integral 100Q separation system (Applied Biosystems, Foster City, CA, USA) using cation-exchange chromatography with a 100mm x 4.6mm column containing 5mm particle Polysulfoethyl-A resin with 300 Å pore (PolyLC, Columbia, MD, USA). The buffers used were: buffer A, 20mM $KH_2PO_4$, 25% acetonitrile, pH 3.0; buffer B 20 mM $KH_2PO_4$, 350 mM KCl, 25% acetonitrile, pH 3.0. A flow rate of 700ml/min was run with a gradient of 0-30% buffer B over thirty minutes then 20-100% over 20 minutes. Thirty fractions were collected, each 90 seconds long.

**Preparation of Human Triton Insoluble Ghosts**

RBC ghosts were prepared by hypotonic lysis and extensive buffer wash.
Five hundred microliters of 2% Triton X-100 in PBS were added to 500 microliters of RBC ghosts for a final concentration of 1% Triton X-100.
They were vortexed and incubated on ice for 30 minutes,
then centrifuged at 100,000 x g for one hour in a Beckman ultracentrifuge at 4oC.
The supernatant was removed and the pellet was resuspended in 1 ml of 1M NaCl, 10 mM $NaHPO_4$, 5 mM EDTA, pH 7.4.
The sample was vortexed and spun again at 100,000 x g for one hour.
The salt-extracted, Triton insoluble protein pellet was solubilized with 0.1% SDS and stored at -80oC until analysis.
It was then solubilized further with 6 M urea, digested with trypsin, chymotrypsin, or AspN plus 1/500 trypsin and subsequently combined for strong cation exchange chromatography as above collecting 15 fractions.