# Imunoprecipitation.v1

This is a protocol for immunoprecipitation in muscle homogenate based on a protocol at:
http://www.abcam.com/ps/pdf/protocols/Immunoprecipitation%20protocol%20(IP).pdf

**Procedures:**

**Muscle homogenization**

1. Harvest gastrocnemius muscle (GA) from a mouse under anesthesia and mince it with a razor blade on a parafilm, and homogenize a quarter of the minced GA muscle (~25 mg) in 3 ml lysis buffer with protease and phosphatase inhibitors (for other muscles, the sample buffer to muscle weight ratio could be used) using by glass-on-glass homogenizer on ice. Transfer the homogenate to a XXX tube.
2. Shake the samples on a gyratory shaker at XXX setting for 2 hrs at 4°C (what does this mean? Why we need to do this?).
3. Centrifuge for 20 min at 12,000 rpm (XXXX g) at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge, place them on ice and transfer the supernatant to a fresh tube on ice.

**Immunoprecipitation**

1. Transfer 300 µl muscle homogenate to a fresh tube on ice. Add 3 µg antibody (rabbit anti-mouse p38 gamma pAb, R&D, AF1347). Transfer another 300 µl muscle homogenate to a fresh tube and add 3 µg rabbit IgG (Sigma, I-8140) as control. Note: Polyclonal antibody is usually better than monoclonal antibody.
2. Add 50 µl protein A/G PULS-agarose beads (Santa Cruz, sc-2003). Note: please transfer well mixed beads into the samples before the beads set.
3. Shake the samples overnight at 4°C on a gyratory shaker (Make sure that the beads are constantly mixed during this time).
4. Spin the samples at 1000 x g for 5 min at 4°C, remove the supernatant carefully and wash the beads in 1 ml Lysis buffer with protease and phosphatase inhibitors three times. Note: Tap the tubes after adding Lysis buffer until the beads are resuspended. DO NOT vortex.
5. Add 25µl of 2X Sample buffer complete to the beads and heat at 95°C for 5 min (DO NOT forget the sample. Overheating will ruin your samples). Load the sample on the SDS-PAGE gel and store the remaining samples in -20°C.

**Reagents**

Mistu, please see the protocols below how we make our solutions. It should contain all the information in precise way so people can follow well.

Tris-HCl: 1.5 mM Tris-HCl (pH 8.8)

- NaCl: Fisher, S671-3
- glycerol: Fisher, G33-1
- TritonX-100: MP biomedical, 807426
- EDTA: ACROS, 118430010
- Protease inhibitor: Roche, complete mini, 11836153001
- Phosphatase inhibitor cocktail-I: Sigma, P2850
- Phosphatase inhibitor cocktail –II: Sigma, P5726

**Non-denaturing lysis buffer**
(Tris-HCl, 20 mM, pH 8, NaCl 137 mM, Glycerol 10%, TritonX-100 1%, EDTA 2 mM)
Prepared reagent and saved at 4ºC. Add protease (4 tablets), phosphatase-I and -II (400 ml/each) inhibitors in 20ml non-denaturing lysis buffer immediately before experiment.
* Non-denaturing lysis buffer (without inhibitors) can store up to 6 month at 4°C.
(1 tablet for 10 ml) and (100 µl for 10 ml).

**Sample buffer stock solution (4.5X) 44 ml**
(Tris-HCl 0.23 M, pH 6.8, SDS 4.5%, Glycerol 45%, Bromophenol blue 0.04%)
1. Pipette 20 ml of Tris-HCl (1 M, pH 6.8) into a 100-ml beaker containing a stir bar.
2. Transfer 20 ml of glycerol (ICN 800688, 100%) to the stirring solution with a 25-ml pipette. Rinse pipette repeatedly with liquid from the beaker to completely transfer the glycerol.
3. Add SDS (Fisher Biotech BP166-500 MW 289) and Bromophenol blue (Fisher Biotech BP115-25 MW 670) to the solution.
4. Transfer solution to a graduated cylinder. Use purified water to rinse beaker and adjust volume to 44 ml in the graduated cylinder.
5. Mix by gentle inversion to avoid formation of foam.
6. Divide into 5-ml aliquots and store at 4°C.

**Sample buffer (4X) 5 ml**
(Sample buffer stock 4X, DTT 80 mM, 2-Mercaptoethanol 0.57 M)
1. Allow 4.5X sample buffer stock solution (stored at 4 °C, crystals form) and DTT (stored at -20 °C) to warm to room temperature and dissolve completely.
2. Pipette 4.4 ml of 4.5X Sample buffer stock into a 15-ml conical tube.
3. Add 0.4 ml of DTT (1 M).
4. Add 0.2 ml of 2-Mercptoethanol (EM Science 6010, 14.2 M).
5. Cap the tube and mix by gentle inversion to avoid formation of foam.
6. Use it fresh to prepare Sample buffer complete. Store the remaining buffer at -20 °C.

**Sample buffer complete (2X) 5 ml**
(Protease inhibitor cocktail 2X, Sample buffer 2X, Phosphatase inhibitor cocktails 2X)
1. Allow phosphatase inhibitor cocktails 1&2 (Sigma-Aldrich P2850, 100X, and Sigma-Aldrich P5726, 100X, stored at 4°C) to thaw and dissolve completely. This step will vary in time, depending on volume of aliquot.
2. Add a tablet of protease inhibitor (Roche 1836153) to 2.3 ml purified water in a 15-ml conical tube, and vortex to dissolve completely.
3. Add 2.5 ml of freshly made 4X sample buffer (containing reducing agents), 100 µl phosphatase inhibitor cocktail 1, and 100 µl phosphatase inhibitor cocktail 2 to the solution.
4. Cap tube and mix slowly by inversion (to avoid formation of foam).
5. Divide into 0.5-ml aliquots and store at -20°C.