

ULTIMATE HIS-UB ASSAY FOR MAMMALIAN CELLS

This assay is performed to detect ubiquitylated proteins in transfected mammalian cells. For this procedure, you will need an expression vector for your protein, together with pMT107 (from Dirk Bohmann: Dirk_Bohmann@urmc.rochester.edu) which expresses polyhistidine-tagged Ub. For each sample, transfect 2 μg of each plasmid into a 50% confluent 10 cm dish of cells. Twenty-four to forty hours after transfection, cells are harvested, total ubiquitylated proteins recovered by nickel-affinity chromatography, and specific proteins probed for by Western blotting.

A. PREPARATION OF NI-NTA-AGAROSE.

1. Each transfection requires 50 μl of 50% Ni-NTA resin. For 16 transfections, transfer 2 x 500 μl of 50% resin into two tubes. Spin in a microfuge 7 sec at top speed at room temperature. Wash each tube of resin 3 times in 1 ml of Buffer A. Resuspend both samples in 250 μl of Buffer A. Pool the two tubes for a total of 1 ml.

B. HARVESTING THE TRANSFECTION.

Prepare:
(i) 3 sets of labeled tubes
(ii) 50 μl of 2 x Laemmli per dish (1 ml for 16 tfns.)
(iii) 50 μl of resin per dish (1 ml for 16 tfns: Prepared in [A])
(iv) 1 ml Buffer A per dish (20 ml for 16 tfns.)
(v) Ice-cold PBS
(vi) Place 50 μl of 2 x Laemmli into one set of tubes.
(vii) Ice bucket
(viii) Rotator on bench.

1. Remove media from cells. Wash in 10 ml PBS. Aspirate media completely.

2. Add 1 ml ice-cold PBS to each dish. Scrape cells; transfer to labeled tube and place on ice.

3. Spin cells 45 sec at top speed. Carefully aspirate supernatant. Resuspend cells in 500 μl of PBS. Vortex to resuspend. Transfer 20 μl of each suspension to a tube containing 50 μl 2 x Laemmli. Mix. Boil 10 minutes, making sure that tubes don't 'pop'. Store these samples frozen. Meanwhile, proceed below.

4. Spin remaining cells 45 sec at top speed. Aspirate supernatant. Resuspend cells in 1 ml of Buffer A. Mix carefully by pipetting. Suspension will be viscous.

Don't forget those samples in the heating block!

5. Sonicate cell suspension using small tip (maximum microtip limit; 8 pulses of 1 second each). Wash probe carefully between samples.

6. Add 50 μl of equilibrated (50%) Ni-NTA-agarose to each tube. Rotate 3 hr at room temperature.

C. ELUTION OF HIS-TAGGED PROTEINS.

Prepare: (i) 2 ml Buffer A per sample (40 ml for a 16 tfn. experiment)
(ii) 2 ml Buffer A/TI per sample (40 ml for a 16 tfn. experiment)
(iii) 1 ml TI per sample (20 ml for a 16 tfn. experiment)
(iv) 100 μl of 2 x Laemmli/Imidazole per sample (2.0 ml for 16 tfns)

1. Spin tubes containing cell lysates for 10 sec, top speed, at room temperature.

2. Carefully discard all but the last ~100 μl of supernatant. Note that the resin can be hard to see.

3. Add 1 ml Buffer A to each tube. Rock back and forth five times. Spin 10 sec. Discard all but the last ~100 μl of supernatant.

4. Repeat Buffer A wash one more time.

5. Wash resin 2 times in Buffer A/TI.

6. Wash resin 1 time in TI.

7. Aspirate supernatant. This time, remove all but the last ~100 µl of supernatant, then spin the samples again. Then, plunging a 26G needle directly into the resin, remove all the liquid. The resin should turn white.

8. Resuspend resin in 100 µl of 2 x Laemmli/Imidazole. Mix by pipeting. Boil 10 minutes. Analyze pre- (20 µl) and post-(50 µl)Ni-NTA-agarose samples by SDS-PAGE/Western blotting.

C. SOLUTIONS.

Buffer A	**For 250 ml**
6 M guanidine-HCl	143.3 g guanidine HCl
0.1 M Na_2HPO_4/NaH_2PO_4	25 ml 1M $Na_2HPO_4/NaH_2PO_4.H_2O$
10 mM imidazole	2.5 ml 1M imidazole
Adjust pH to 8.0 with NaOH.	

TI	**For 200 ml**
25 mM Tris.Cl	5 ml 1M tris.Cl (pH 6.8)
20 mM imidazole	4 ml 1M imidazole
Adjust pH to 6.8.	

Buffer A/TI

1 volume Buffer A

3 volumes TI