

ULTIMATE HIS-UB ASSAY FOR YEAST

This assay is performed to detect ubiquitylated proteins in yeast. Yeast are first transformed with the vector pUB221 (from Dan Finley: Daniel_Finley@hms.harvard.edu), which expresses polyhistidine-tagged Ub under the control of the copper-inducible CUP1 promoter. Yeast are grown, induced with copper, and harvested. Total ubiquitylated proteins are then recovered by nickel-affinity chromatography, and specific proteins probed for by Western blotting.

A. GROWTH AND INDUCTION.

1. Grow 200 ml of yeast culture overnight. When OD_{600} is 0.4–1.0, induce culture with 400 µl 250 mM $CuSO_4$. Grow 4 hr at 30ºC.

2. Measure OD_{600}. Collect 5 ml for total lysate and 195 ml for purification of His-tagged proteins. Spin down yeast, remove all liquid, and freeze pellets in liquid nitrogen. Store pellets at –80ºC until ready.

B. PURIFICATION.

His-tagged proteins.

1. To each large (195 ml pellet) frozen sample, add Buffer A to give a final OD of approximately 150.

2. Vortex. Add glass beads so there is approximately 1-2 mm of free liquid above the beads.

3. Vortex each sample: 20s vortex/30 s ice. Repeat 6 times.

4. Add 2 ml Buffer A. Vortex.

5. Spin 3000 rpm bench top centrifuge for 15 minutes at 4ºC.

6. Transfer supernatant to fresh tube.

7. Measure the [protein] in 1 µl of remaining sample, using the Bradford Assay.

8. For each sample, transfer an equal amount of protein to a fresh tube. Add 250 µl of 50 % Ni-NTA beads (Quiagen; equillibrated in Buffer A).

9. Rotate on bench for 1-2 hr.

10. Pellet beads by spinning 2 minutes at 3000 rpm in microfuge.

11. Aspirate supernatant. Wash beads in 1 ml Buffer A.

12. Wash beads for a total of three times in Buffer A, three times in Buffer A/TI, and one time in buffer TI.

13. Pellet beads after final wash. Remove all liquid from beads.

14. Resuspend in 200 µl of 2 x Laemmli with 0.2 M imidazole. Boil for five minutes. Run 100 µl on appropriate SDS-PAGE gel.

Total Proteins.

1. Bring tubes up from –80 degree freezer on ice.

2. To each tube, add appropriate amount of EZ Buffer™. Mix by pipeting up and down.

3. Place in 95 degree block for 10 minutes.

4. Transfer 1 µl of each sample to a fresh tube. Measure protein concentration using Bradford assay. Include 1 µl of EZ Buffer™ in each of the standards. Freeze the remaining samples until ready to run gel.

5. Transfer equal amounts of protein for each sample (~75 µg) to a fresh tube. Add 2 x Laemmli buffer to make the final volume approximately 60 µl. Run on SDS-PAGE gel.

For both the His-tagged and the total proteins, probe for the presence of your proteins by 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

EZ Buffer™

0.06M Tris-HCl, pH 6.8
10% (v/v) glycerol
2% (w/v) SDS
5% (v/v) 2-mercaptoethanol