

Profiling ICAT Label Procedure for Budding Yeast Whole Proteome

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Protein Isolation, Reduction, ICAT Labeling

- A. Resuspend pellets from 25 ml cultures (e.g. 25 ml experimental timepoint sample and 25 ml control asynchronous sample, each at OD600 = 0.5) in 400 μ l PBS + PMSF (1X) – optional step to remove trace media
- B. Spin down pellets in 1.7 ml tubes, remove supernatant
- C. Add 1.5 ml 10% TCA to each sample
- D. Place tubes on ice for at least 1 hour
- E. Spin at 4°C, 10 min
- F. Perform two 90% acetone rinses for each sample (spin as in step E each time)
- G. Remove supernatant thoroughly with Hamilton syringe
- H. Resuspend each sample in 0.5 ml labeling buffer (200 mM Tris 8.3, 6M urea, 0.05% SDS, 5 mM EDTA)
For 10 ml ⊗ 3.6 g urea
 100 μ l 0.5 M EDTA
 50 μ l 10% SDS
 2 ml 1 M Tris pH 8.3
 water to 10 ml, mix well
- I. Make sure fully white material well resuspended – facilitate resuspension with pipette tip
- J. Sonicate in bath sonicator for 5 min – solution will still be a tad milky
- K. Analyze protein concentration via BCA assay
*aim to label 2 mg of experimental sample and 2 mg for control for a total of 4 mg – yields ~1500 proteins in final analysis
- L. Pull out 5 μ l from each sample for PAGE gel analysis (“pre-label” sample)
- M. Add TCEP to final concentration of 5 mM TCEP in 0.5 ml for each sample (e.g. 10 μ l 0.25 M stock)
- N. Incubate for 60 min in 37 bath – agitate often
- O. Add ICAT – add 10 tubes worth of ICAT to each 0.5 ml sample (20 tubes/analysis)
[10 X 100 μ g (175 nmole) = 1750 nmol/1ml = ~3.4 mM]
- P. Incubate 3 hrs in 37 bath in light-tight tube (pre-label light exposure kills ICAT reagent)
- Q. Quench by adding DTT to final concentration of 12 mM (12 μ l 0.5 M stock)
- R. Agitate and let sit for 5 min at RT
- S. Pull out 5 μ l from each sample for PAGE gel analysis (“post-label” sample)
- T. Combine samples, add 13 ml 20 mM Tris pH 8.3, 5 mM EDTA to dilute urea and SDS
- U. Digest with trypsin overnight (20 ng/ μ l, ~20 tubes 20 μ g/tube modified trypsin) in 37°C water bath
- V. Mix in sediment, acidify with ~700 μ l 5% phosphoric acid, and pull out 100 μ l for PAGE gel analysis
- W. Quick spin down to pellet sediment 2000g for 5 min – decant supernatant – this step prevents clogging of SCX column

SCX Cation Exchange (Integral HPLC Instrument or alternatively use Vision Instrument that does both cation exchange and avidin automatically)

- A. Acidify standards (pH 2), sample (pH 2), and buffers A and B (pH 2.9) with 5% phosphoric acid
- B. Check all solvents to ensure adequate volumes are made
- C. Pressurize all solvents (check He) – leave on low pressure for run
- D. Check HPLC plumbing
 - 1) 2 ml sample loop on #2,5
 - 2) #3 goes to waste
 - 3) #1 syringe pump (in from water, out to #4)
 - 4) Clean reagent pump with water and then buffer A on 'Inject'
 - 5) Sample Table: 'Inject' (load sample on loop, loop off-line from column)
'Load' (100%A for 10 min at least 5', loop in-line)
Repeat Inject and Load as necessary for multiple injections
'Inject' (run dogleg method)
- E. Prime system with 100% buffer A on 'Inject' (don't forget to hit SET for buffer choice!)
- F. Check 'Forward' setting for column
- G. Attach conditioned PolyLC SCX Polysulfoethyl A 4.6 X 200 mm + appropriate Javelin guard column
- H. Run quick clean gradient to clean column
- I. Run 5 nm 3-5 peptide standard (e.g. bradykinin, angiotensin, etc.; acidify standard to pH~2 before loading)
- J. Acidify sample to pH~2 if not already done
- K. Load sample with mechanical autoloader – set Sample Table to 'Load'; use manual syringe loader or programmed multimethod for slow load at low pressure (<30% column vol. ~ 1 ml; 0.5 ml/min)
- L. Load up 1.7 ml tubes in sample collector (set collector at 1 min interval for 0.8 ml/fraction) – collect ~60 fractions during gradient
- M. Run a version of method 'Dogleg 800' for gradient run with Sample Table on 'Inject' (e.g. 0-25% buffer B over 30 min; 25-100% buffer B over 20 minutes)
- N. Label and freeze samples at -20°C
- O. Print out trace (Group Analysis, open .bio file, Page Setup, print to Arbor or Balsa)

Avidin Chromatography (manual method with syringe)

- A. Dry down samples for 10-20' in Speed-Vac to remove ACN (choose ~30 peak samples)
- B. Load up ABI avidin cartridge in ABI syringe adaptor, ringstand (1 avidin cartridge sufficient for ~30 purifications)
- C. Clean syringe with water
- D. Inject 1000 µl Affinity Buffer Elute onto column
- E. Inject 2000 µl of Affinity Buffer Load onto column
- F. Add 500 µl of Affinity Buffer Load to sample + 100 µl 1 M ammonium bicarbonate
- G. Check pH with paper to make sure column and sample are neutral
- H. Prepare tube to collect flow-through
- I. Load neutralized sample onto column slowly (~ 1 drop/sec or slower), and collect flow-through
- J. Reload sample slowly and save flow-through
- K. Inject 500 µl Affinity Buffer Load onto column
- L. Inject 1000 µl Affinity Buffer Wash 1 onto column
- M. Inject 1000 µl Affinity Buffer Wash 2 to column
- N. Prepare glass (TFA depolymerizes plastic tubes) tube to collect eluate
- O. Inject 800 µl Affinity Buffer Elute onto column SLOWLY and collect eluate
- P. Inject 2000 affinity buffer elute to column
- Q. Inject 2000 µl Affinity Buffer Load to column
- R. Go back to step F for next sample and so on
- S. Dry down all samples to complete dryness in Speed-Vac (~2 hours)
- T. Acid cleave in TFA as per ABI protocol