Video Article

Quantitative Phosphoproteomics in Fatty Acid Stimulated *Saccharomyces cerevisiae*

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Abstract

This protocol describes the growth and stimulation, with the fatty acid oleate, of isotopically heavy and light S. cerevisiae cells. Cells are ground using a cryolysis procedure in a ball mill grinder and the resulting grindate brought into solution by urea solubilization. This procedure allows for the lysis of the cells in a metabolically inactive state, preserving phosphorylation and preventing reorientation of the phosphoproteome during cell lysis. Following reduction, alkylation, trypsin digestion of the proteins, the samples are desalted on C18 columns and the sample complexity reduced by fractionation using hydrophilic interaction chromatography (HILIC). HILIC columns preferentially retain hydrophilic molecules which is well suited for phosphoproteomics. Phosphorylated peptides tend to elute later in the chromatographic profile than the non phosphorylated counterparts. After fractionation, phosphopeptides are enriched using immobilized metal chromatography, which relies on charge-based affinities for phosphopeptide enrichment. At the end of this procedure the samples are ready to be quantitatively analyzed by mass spectrometry.

Video Link

The video component of this article can be found at http://www.jove.com/video/1474/

Protocol

Cell growth and media

1. A single colony of *BY4742Δarg4Δlys1* cells overnight in 100 mL rich media to an OD_{600} of 1.0 then seed into two 1 liter cultures of a minimal yeast medium (0.17% Yeast Nitrogen Base without Ammonium Sulfate or Amino Acids, 0.5% Ammonium sulfate) containing a full complement of amino acids, supplemented with 20mg/L of isotopically normal or heavy arginine ($^{13}C_6$ $^{15}N_4$; Isotec) and lysine ($^{13}C_6$ $^{15}N_2$; Isotec).
2. The cells were grown for 18 hours, to an OD_{600} of 1.8. It is critical that the cells go through at least 9 generations to achieve full incorporation of the labeled isotopes. The light sample was pelleted, washed with sterile water, reseeded into an oleate containing medium (isotopically normal arginine and lysine, 0.2% Oleate (Sigma Chemicals) and 0.5% Tween 40 (Sigma Chemicals)) and stimulated for another 85 minutes. This yields an isotopically light, with respect to arginine and lysine, oleate-stimulated sample and an isotopically heavy glucose-grown reference sample.

Cell Lysis, Isolation and Fractionation of Peptides

3. Weigh centrifuge bottle. Harvest samples by centrifugation for 3 minutes. Remove media and aspirate excess liquid. Weigh pellet and bottle (will typically yield between 1-2 grams) then flash freeze in liquid nitrogen. Add a volume equivalent to pellet weight of 'grinding' buffer to the frozen pellets in liquid nitrogen (Phosphate Buffered Saline (PBS, Gibco), 10% glycerol, Protease Inhibitors (SigmaFAST Protease Inhibitor Tablets, Sigma) and HALT Phosphatase inhibitors (Thermo Scientific)).
4. Freeze the grinding vessel in liquid nitrogen. Wait until the liquid nitrogen ceases boiling. Transfer the pellet to the grinding vessel, with frozen ball bearings.
5. Grind at 600 rpm with a 1 min 20 sec cycle with a direction reversal for 3 minutes. Refreeze the grinding vessel in liquid nitrogen. Repeat 4 more times for 15 minutes total grinding time.
6. Collect the frozen grindate into a 50ml Falcon tube place dry ice. Store at -80 °C until ready to use.
7. Make a 10ml solution of 8M urea, 0.1M ammonium bicarbonate, 0.1M Tris pH 8.6. Add 3 volumes of the urea buffer to 1 volume of the frozen grindate (for example 3mls of the urea buffer added to a 1 gram pellet with 1 ml of PBS buffer). Immediately sonicate the mixture with a probe tip sonicator (2 - 10 second pulses). Keep the tip near the bottom of the tube to prevent foaming of the solution. The grindate should immediately go into solution.
8. Clear the lysate by centrifugation for 5 minutes at 4°C.
9. Transfer supernatant to fresh tubes.

10. Make a fresh aliquot of 0.5M Tris[2-carboxyethyl] phosphine (TCEP). Add to sample at 1:100 for a 5mM final concentration. Incubate at 37°C for 1 h.
11. Alkylation of reduced cysteines. Allow to cool to room temperature. Make a fresh aliquot of 1M iodoacetamide. Keep in the dark (we wrap the tube in foil). Add to a final concentration of 20mM. Incubate in the dark for 1 h at room temperature.
12. Quench the iodoacetamide with 20mM DTT from a 1M stock at room temperature for 1 h.
13. Quantify the protein with a standard Bradford or BCA assay (not described). Take a sample for analysis by SDS PAGE below.
14. To validate incorporation of the heavy isotopes, use 100 µl of the reduced and alkylated lysate. Dilute 1:4 with dH_2O, sonicate and add trypsin at 50:1 protein to trypsin. Digest for a minimum of 4 hours, dry sample down in a speed vac and then desalt with a C18 Ultramicrospin columns (The Nest Group).
    1. Hydrate column with 100 µl of Acetonitrile
    2. Equilibrate with 200 µl 0.1% TFA.
    3. Resuspend dry sample in 200 µl 0.1% TFA. Load onto column. Centrifuge at ~200 x g or the minimal speed required for flow through the column.
    4. Wash twice with 200 µl 0.1% TFA.
    5. Elute with twice with 50 µl 60% Acetonitrile, 0.1% TFA.
    6. Dry sample in a speed vac.
    7. Resuspend sample in 20 µl 0.1% Formic acid. Run 2 µl on a mass spectrometer. Check incorporation of isotopically heavy arginine and lysine by a 10 and 8 Dalton shift in the respective m/z spectra. The incorporation for each amino acid should be between 96 - 98%.
15. Mix equivalent amounts (mg of proteins) of the isotopically heavy and light samples.
16. Dilute sample 1:4 with 20% methanol.
17. Add trypsin at 1:200. Incubate overnight at 37°C.
18. Check that digestion has gone to completion by SDS PAGE. Run 2 and 4 µl of the predigestion sample and 8 and 16 µl of the post trypsin digest. Visualize by Coomassie staining that digestion is complete. If it is not complete, sonicate the sample with a probe tip sonicator briefly and digest again with trypsin.
19. Dry the sample down in a speed vac. When dry add 200 µl of filtered dH_2O and vortex till the pellet goes into solution. Dry sample down in a speed vac. Resuspend again with dH_2O. Dry sample again.
20. Resuspend pellet in 0.1% Trifluoracetic Acid (TFA; Sigma). Check that the pH is around 3 using pH strips. If needed acidify with 10% TFA.
21. Pellet out any precipitate.
22. Desalt the samples on a Waters Sep-Pak Vac 500 mg C18 column. Do not overload these columns. The maximum amount is 5 mg but it's better to load a little less. Divide the sample between multiple columns if necessary.
    1. Hydrate the column with 5 ml 100% acetonitrile. Centrifuge at ~200 x g or the minimal speed required for flow through the column.
    2. Equilibrate column with 5ml 0.1% TFA. Load sample. Collect flow and load again.
    3. Wash with 5 ml 0.1% TFA. Repeat.
    4. Elute with 2ml 60% Acetonitrile, 0.1% TFA.
    5. Dry sample down in a speed vac.

Peptide Fractionation and Isolation of Phosphopeptides

1. Peptides are fractionated on a HILIC column. We use a TOSOH TSK-Gel Amide-80 4.6 mm x 25 cm analytical column with a guard column. Take your time with this step, run the column for 2 hours at 90% A then run two blank gradients before loading your sample. The maximum load on a column this size is thought to be around XX, we typically do not load more than 5 mg per run.
2. Solvent A - 98% ACN/0.1% TFA

   Solvent B - 2% ACN/0.1% TFA

   Load in 90% A over 20', 90% A to 85% A over 5', 85% A to 60% A over 40', 60% A to 0% A over 5', 0% A to 90% A over 5'.
3. The flow rate is 1ml/min and fractions were collected at 2 minutes intervals beginning at the 85% A to 60% A gradient.
4. Combine fractions to reduce the number of fractions to 10. Increased fractions will likely yield greater coverage of the phosphoproteome.

Enrichment of phosphopeptides

1. Phosphopeptides are enriched using PHOS-Select Iron Affinity Gel. We aliquot our IMAC resin to avoid repeated freeze thawing. Make 50 mls of a 250 mM acetic acid, 30% ACN load solution. Bring the pH to 2.7 with HCl. Resuspend pellets in 500 µl of load solution. Check pH, adjust with HCl or NaOH as necessary.
2. Prewash 200 µl of IMAC beads in an appropriate column. We use Molbiotec spin columns.

   Add 15 µl of a 50% slurry (gel to load solution) to each fraction, and incubate the samples for 30' with end over end rotation. Keep the flow for analysis.
3. Wash samples 3 times with load solution and once with 500 µl filtered dH_2O.
4. Elute peptides with 2- 3 min with 400 µl of 50 mM Na_2HPO_4 (pH 8.4) buffer. Transfer to a glass vial.
5. Acidify to pH 3 with 5 µl 100% formic acid, flash freeze in liquid nitrogen, and dried sample down in a speed vac.
6. Resuspend phosphopeptides in 200 µl of 0.1% TFA and C18 clean as described above. Dry down eluted samples in a speed vac.
7. Resuspend peptides in 20 µl of 0.1% formic acid. Use 2 µl per mass spectrometer analysis or as needed.

Notes

For much of this protocol you will be working 'blind'. The cell lysis can be monitored by western blotting and Bradford or BCA assays, and the trypsin digestion can also be readily assessed. The HILIC column will gave a trace similar to this one, or as seen by others [1, 2] depending on the liquid chromatography machine that is used. Samples are monitored by mass spectrometry; test your samples on inexpensive machines such as LCQs or MALDIs before going to the high cost analyses on a high mass accuracy, high resolution machine. Finally if you a drying down a sample with acid in it, use glass.

Discussion

This method will yield an enrichment of phosphopeptides that can be quantitatively analyzed. A number of parameters can be altered in this protocol, but the most important aspect to remember is to preserve your phosphorylation. Preparation of cells for quantification can be achieved in a number of different ways, for example if you cannot label your cells *in vivo*, tags such as ICAT[3] or ITRAC [4] can be used post extraction to differentially label two cultures for quantification purposes.

For fractionation the most common methods are gel fractionation by SDS PAGE [5, 6], SCX chromatography [7], and HILIC based chromatography[1, 2]. We chose HILIC because it retains the peptides until the end of the gradient, rather eluting at the front of the gradient as in SCX but other groups have had good success with other methods.

When using the IMAC you may need to increase or decrease the amount of resin used, time of incubation or number of washes. There have also been studies looking at altering the initial chemistry of the load solution that we present here [8]. This combination of methods should allow the isolation of samples that are between 60% to 95% phosphopeptides. Once you have established a method for your particular system you should be able optimize for higher yield of phosphopeptides.

Enrichment of phosphopeptides can also be achieved with TiO_2 [9, 10] or phosphoramidite chemistry [11, 12], and studies have indicated that each method will yield overlapping yet distinct portions of the phosphoproteome [13].

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