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Tandem Mass Tag (TMT) 10-plex Labeling of Yeast Peptides

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Lewis Lab | Yeast Protocols, Tools, a...

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Protocol status: Working
We use this protocol and it's working

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Guidelines

SAMPLE COLLECTION

- Make sure all chemicals are of the highest purity (MS grade).
- For yeast, collect at least 100 ml of cells in mid-exponential phase (OD_{600} ~0.5 in a Unico 1100RS spectrophotometer). Record the OD_{600} for each sample.
- Calculate the total OD units for each sample (OD_{600} x volume in ml; e.g. 0.5 × 100 ml = 50 OD units).
- This protocol is written for sample sizes ~50 OD_{600} units.

o Before starting protocol, determine if any samples are significantly larger than 50 OD units (60-70 is okay, but above that, the lysis step will not work well).

o If any samples are too large, they will need to be split into two or three samples. Take note of samples that require splitting and see the additional instructions for splitting samples at step 1.

Materials

MATERIALS

Pierce Quantitative Colorimetric Peptide Assay **Thermo Fisher Scientific Catalog #**23275

TMT10plex™ Isobaric Label Reagent Set **Thermo Fisher Scientific Catalog #**90406

Oak Ridge Centrifuge Tubes **Fisher Scientific Catalog #**05-563-10G

Troubleshooting

Safety warnings

Guanidine hydrochoride is a strong irritant to skin and mucous membranes, and can cause severe eye injury. Wear personal protective equipment (gloves, lab coat, safety glasses). For skin contact, immediately wash with water and soap and rinse thoroughly. In case of contact with eyes, rinse immediately with plenty of water for 15 minutes and seek medical advice. If accidentally ingested immediately contact a physician.

Urea is an irritant. In case of of skin contact, get medical aid. Immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. In case of eye contact, immediately flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. Get medical aid. In case of accidental ingestion, do NOT induce vomiting. If conscious and alert, rinse mouth and drink 2-4 cupfuls of milk or water. Seek medical attention.

Trifluoroacetic acid (TFA) warnings from supplier: Causes severe burns by all exposure routes. Inhalation may cause central nervous system effects. Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment. Hygroscopic. Use personal protective equipment. Use only under a chemical fume hood. Wash off immediately with plenty of water for at least 15 minutes. Immediate medical attention is required. Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. Move to fresh air. If breathing is difficult, give oxygen. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance.

Acetonitrile warnings from supplier: Highly flammable liquid and vapor. Harmful if swallowed, in contact with skin or if inhaled. Causes serious eye irritation. Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. Wear protective gloves/protective clothing/eye protection/face protection. In case of contact with eyes flush immediately with plenty of flowing water for 10 to 15 minutes holding eyelids apart and consult an ophthalmologist. Protect uninjured eye. Remove contact lenses, if present and easy to do. Continue rinsing. After contact with skin, wash immediately with plenty of water and soap. Remove contaminated, saturated clothing immediately. In case of skin reactions, consult a physician. If accidentally swallowed rinse the mouth with plenty of water (only if the person is conscious) and obtain immediate medical attention. Do NOT induce vomiting. Give nothing to eat or drink. IF exposed or concerned: Immediately call a POISON CENTER/doctor

Before start

BEFORE BEGINNING PROTOCOL

- All steps should be done at room temperature with room temperature reagents.
- Make fresh guanidine hydrochoride and urea solutions right before thawing cells. Dissolving both compounds is endothermic (heat absorbing), so it takes some time to get them into solution. DO NOT heat them to speed it up—this will cause carbamylation of peptides.
- Make sure a heat block is at 100°C immediately before thawing cells. Keep checking water level while preparing cells—the water will boil off quickly. Make sure there is water in wells when samples are ready to be boiled.
- Make sure the incubator for trypsin digest is set to 25°C.

DAY 1: CELL LYSIS and TRYPSIN DIGEST

1 Thaw frozen cells (collected in 50-ml conical tubes) at room temperature, making sure not to leave cells thawed longer than necessary. Centrifuge at max speed (4,696 rcf) in a swinging bucket rotor for 2 minutes. Carefully remove remaining media by pipetting. Suspend in 1 ml water and transfer to 1.5-ml microfuge tube.

Note

For any samples you need to split, add more water. For example, if one sample is 100 OD units, resuspend in 2 ml water and transfer 1 ml to each of two tubes to split the sample in half.

2 Centrifuge at 10,000 rcf for 1 min, then carefully remove the water by pipetting.

3 Repeat centrifugation at 10,000 rcf for 1 min, and again carefully remove the remaining water by pipetting.

4 Resuspend cells in 150 µl 6M guanidine hydrochoride 100 mM Tris-HCl pH 8 solution.

4.1 To make guanidine hydrochoride–Tris solution, add 2.87g guanidine hydrochoride to 5 ml 100mM Tris-HCl pH 8. Always make fresh just before thawing cells (see Guidelines & Warnings)

5 Boil samples for 5 min in 100°C heat block (make sure the water has not evaporated). Rest samples for 5 min by removing the tubes from the heat block and placing in a rack at room temperature.

6 Boil sample again for 5 min.

7 Precipitate proteins by adding methanol to a final concentration of 90%. Briefly vortex.

7.1 E.g. add 1.35 ml of 100% methanol to 150 µl of sample = 90%.

8 Centrifuge samples at 9,000 rcf for 5 min. Decant the supernatant by carefully pouring and then then pipetting off as much residual liquid as possible.

9 Air dry pellet for 5 min in a Biosafety Cabinet.

10 Resuspend pellet in 1ml 8M urea - 100 mM Tris solution.

Note

Peptide concentration should be <3 mg/ml at this point.

10.1 Make urea – Tris solution by dissolving 4.8g urea in 10ml 100mM Tris-HCl pH 8. Make solution fresh before thawing cells.

10.2 Resuspend by gently stirring with pipette. Pipette up and down once chunks are small. Pipette SLOWLY, trying not to introduce bubbles. Probe sonicate if necessary. The solution will be cloudy but should not have visible chunks. Because the pellets are difficult to resuspend, it works best to pipette buffer into all tubes to start the dissolution, and then work to resuspend each sample by pipetting.

Note

If samples over 50 OD units were split in step 1, they can be re-combined here by resuspending all of the split samples serially using the same 1 ml of urea – Tris buffer.

11 Transfer samples to Oak Ridge tubes.

12 Dilute urea to 2M with 100 mM Tris-HCl pH 8.0.

12.1 Add 3ml Tris to 1ml sample, then add an extra 0.5 ml tris to make sure urea is below 2M (or it will interfere with Bradford Assay).

13 Quantitate with Bradford Assay, no dilution of samples is necessary.

14 Add tris(2-carboxyethyl)phosphine (TCEP) to 2.5 mM and chloroacetamide to 10 mM.

Note

Make 100x stock by dissolving 0.36g TCEP and 0.47g chloroacetamide in 5ml of 100mM Tris-HCl pH 8. Requires mild heat (37°C) to fully dissolve. Store at -20°C.

14.1 Add 10µl 100x stock per 1ml sample.

15 Add trypsin at a ratio of 1:50 (1 mg trypsin per 50 mg protein) and incubate overnight at 25°C on a rotating mixer.

Note

Prepare trypsin stock fresh just before use at 1mg/ml dissolved in 50 mM acetic acid.

DAY 2: DESALTING AND LYOPHILIZATION OF PEPTIDES

16 Quench digestion by adding trifluoroacetic acid (TFA) to 0.6% final concentration.

Note

100% TFA should be stored at 4°C. TFA is toxic and should only be opened in a chemical hood.

17 Check that pH is <2.0.

17.1 Pipette 10 µl of sample onto pH indicator strips to test pH

17.2 If pH is > 2.0, add more TFA until pH is < 2.0.

Note

If pH is >2.0, protein will not bind to desalting column

18 Centrifuge 9,000 rcf for 5 min in large floor centrifuge.

Note

Pour off supernatant immediately after samples stop spinning. If you are not ready for them, spin a little longer, but don't let pellets sit in buffer. They will quickly begin to re-dissolve and break apart.

19 Desalt peptides (see protocol Peptide Desalting with a Vacuum Manifold), and freeze samples in liquid nitrogen.

20 Dry down or lyophilize peptides to remove acetonitrile.

Note

Due to the acetonitrile in the samples, lyophilization must be done in a lyophilizer capable of maintining -70°C or lower.

DAY 3: TMT LABELING

21 Suspend peptides in 200 mM tetraethylammonium bromide (TEAB) to ~8 µg/µl.

Note

Use concentrations calculated previously by Bradford Assay to determine how many total mg protein went into trypsin digest.

Note

Before resuspending peptides, make sure there is liquid nitrogen available. If you cannot acquire liquid nitrogen, store lyophilized peptides at -80°C.

21.1 To make 200 mM TEAB, dilute from1 M stock solution using ultrapure water.

21.2 To resupend lyophilized peptides:

1. Tap tubes to make sure powder is knocked off kimwipe lids before removing them.
2. Add the TEAB. Powder should go right into solution – can do a very quick pulse on the vortex if necessary.

3. Centrifuge at max speed in table top centrifuge for 2 min to make sure any peptides stuck to sides of tube go into liquid and to collect the liquid in the bottom of the tube.

22 Quantitate with Pierce Quantitative Colorimetric Peptide Assay according to manufacturer's instructions.

Note

Generally need to dilute samples 1:50 to be in linear range of assay.

23 Dilute peptides to 5 µg/µl with 200mM TEAB.

24 Prepare TMT vials

24.1 Pull one set of TMT vials out of the -20°C freezer and equilibrate to room temperature.

24.2 Do a quick spin in the centrifuge to collect TMT reagents at bottom of tubes before opening.

24.3 Suspend each 0.8 mg TMT vial with 50µl anhydrous acetonitrile (pipette acetonitrile in chemical hood).

24.4 Incubate for 5 minutes at room temp with occasional vortexing. Briefly centrifuge to collect sample at bottom of tube before opening.

25 Transfer 100 µl of samples to appropriate TMT tubes (=500µg of peptide per tube) and incubate for 1hr at room temperature.

Note

RECORD WHICH SAMPLE IS LABELED WITH WHICH TMT REAGENT

25.1 Keep remaining sample as unlabeled sample control. Store at -80°C

26 Add 8 µl of 5% hydroxylamine and incubate for 15 min to quench reaction.

27 Transfer all 150 µl samples to a single 1.7 ml tube and pipette up and down to mix.

27.1 Transfer 10 µl of pooled sample to a separate tube and freeze at -80°C. Analyze this sample for peptide labeling efficiency.

28 Desalt peptides (see protocol Peptide Desalting with a Vacuum Manifold), freeze samples in liquid nitrogen, and lyophilize. Samples can be stored at -80°C until ready for MS.