Bench Protocol to Perform TAILS v4

We have been performing TAILS for 12 years and this is now a very streamlined and highly optimized approach.

The one request we have is to follow this protocol exactly. Exactly. After you achieve good results then you may consider specific modifications that may optimize results for your particular samples. In particular, **do not even think of using aldehyde derivatized chromatography beads**, ever. Beads lead to massive peptide losses and contamination by non-specific binding. We tried for years to get these to work adequately and gave up, preferring to develop the polymer.

Genral notes re starting material:

• Ideally start with a total of 1 to 3 mg of proteome[1,2]. To reduce costs for TMT or iTRAQ one can use a minimum of 250 μg (iTRAQ 4-plex) or 200 μg (TMT 6-plex, 10-plex) per channel. For dimethylation labeling experiments we recommend 1 mg per channel. For any given experiment use no less than 1 mg *total* protein (spread over all channels).

[1] The reason is to avoid high peptide losses after the polymer pull out step of the internal tryptic peptides.

*[2] It is highly recommended labelling to begin with a precipitated protein sample. This initial precipitation step will remove small molecular weight primary amines (i.e. amino acids and nucleic acids) that otherwise seriously interfere with labeling. This is a <u>very</u> important consideration for tissue culture medium, which contains high levels of amino acids. This is the most common cause for poor labeling efficiency, which should be nearly 100%. If it is not then you are NOT following these instuctuctions exactly. Start with a gently dried protein pellet. Do not overdry as it is hard to dissolve and losses will always occur. Alternatively, for samples in solution, it is **crucial** that buffers do not contain primary amines. Measure and adjust protein concentrations and amounts before starting TAILS protocol. Remember that cell culture medium has high concentrations of free amino acids thus high concentrations of primary amines.*

• *During preparation of protein extracts it is crucial to include protease inhibitors, either in the form of commercial tablets, or individually added inhibitors against protease classes and chelators (e.g. EDTA).*

• *Ensure protein pellet is completely solubilized prior to beginning labelling, as incomplete labeling will occur if the sample is not in solution (i.e. avoid flakes and precipitates). Adding small incremental amounts of 1 M NaOH aids in protein solubilisation and can later be neutralized with acid and retain proteins in solution. Quick tip-probe sonication can help.*

• *Nucleic acids can interfere with any proteome preparation. If extracting proteomes from whole cell lysates or tissue extracts, ensure using tip-probe sonication for DNA shearing as well as DNAses + RNAses. Nucleic acids make solutions viscous and hard to solubilize. Nucleic acids also contain primary amines that interfere with labeling so eliminating them by these simple procedures is key.*

I. Proteome Extraction

Any proteome can be extracted using the following basic protocol:

Generic proteome preparation from murine or human tissues

- We recommend using a ULTRA-TURRAX (IKA) high-speed homogenizer for proteome extractions from murine or human tissues. Alternatively a Cell Crusher instrument (http://cellcrusher.com/) can be used. Both work well in our hands.
- Choose your favourite extraction buffer. Depending on the type of experiment and tissue we usually use either (i) 100 mM HEPES, 150 mM NaCl, 10 mM EDTA, 1x HALT inhibitor cocktail, (ii) T-PER Tissue Protein Extraction Buffer (Pierce), or (iii) 6 M guanidine hydrochloride. These can be used also sequentially, exactly in this order, to prepare soluble proteins first and then the membrane or insoluble protein fractions from the residual.
- Use 10x excess of extraction buffer to tissue weight. For higher extraction yields, perform two cycles with 10-fold excess each (total ratio: 20:1 v/w).
- Centrifuge samples for at least 10 min at >10,000g to pellet cell debris and insoluble proteins. Pool supernatants in blue capped BD Falcon tubes. Discard pellet or use it for subsequent extraction with a different buffer.
- Perform chloroform-methanol Precipitation (as described above)
  - Keep in mind: Final volume will be 10x protein sample volume
  - Resolubilize protein pellet in 6 M GuHCl. Keep volume as small as possible.
- Determine protein concentration using Bradford Analysis
  - Dilute samples at least 1:10 and make sure to not pipette less than 5 µL to avoid pipetting and dilution errors.
  - Use the appropriate amount of proteome for your TAILS analysis.

Generic proteome preparation from cell culture supernatants

1. Use 3 T150 flasks for each condition to analyze the conditioned medium
2. Grow cells in culture medium so that cells will be 80-90 % confluent at the time of medium collection.
3. Wash cells 3 times with warm PBS and incubate 1-2 hours in prewarmed <u>serum-free</u> culture medium to allow for residual serum proteins etc to be exchanged to medium from the cell surfaces. Discard this medium and replace with fresh 37 °C <u>serum free, phenol red</u>

free medium then incubate overnight collect if thi sis the protocol desired, or else if synchronizing cells by serum free incubation, then now:
4. Repeat extensive washing of cells with PBS at 37 °C and culture in serum-free, phenol-red free culture medium for time sourse desired e.g. 24-48 h before collection of conditioned medium and cell membranes (2-3 gentle washes using warm (at 37 °C) PBS or medium).
5. Harvest conditioned medium and immediately add protease inhibitors + 10 mM EDTA. Keep the conditioned medium cool at 4 °C or on ice from this point on.
6. Clarify conditioned medium by centrifugation to remove any cells (500 x g, 5 min) and filter through 0.2 μm pore size to remove particulate matter.
7. Concentration of Conditioned Medium with TCA/acetone method:
    7.1. Add 100% trichloroacetic acid (TCA) to conditioned media to a final of 15% (v/v).
    7.2. Sit on ice for 3-4 h.
    7.3. Spin at 4 °C at high speed (>9000g) for 15min.
    7.4. Decant supernatant.
    7.5. Add freezer cold (-20 °C) 100% acetone (approximately equal volume of the original starting amount.
    7.6. Spin as in 7.3.
    7.7. Decant and wash again (3 times)
    7.8. Resuspend the pellet in 200 mM HEPES pH 8 at a protein concentration ~1-2 mg/mL. See notes on protein solubilisation above for help (footnote 4).

Generic proteome preparation from cell lysates

1. Lyse cells with 1% SDS, 0.1M DTT in 200 mM HEPES, pH 8 + protease inhibitors (prepared fresh) and tip-probe sonicate 3 times for 1 minute at setting 3. Rest on ice between sonication rounds to maintain sample as cold as possible. Preferred volume is 1-2 mL, but ultimately dependent on preparation type[5].
Note: 1M DTT stock is 0.0154g / 100 μL.
2. Centrifuge at 16,000 g for 15 min at 4 °C and keep supernatant.
3. Incubate supernatant with 1 μ L of benzonase if available for 15 minutes at room temperature.

Thiol Blocking

4. Incubate lysate at 37 °C for 60 min to ensure complete reduction. During incubation prepare a 10x stock of N-ethylmaleimide (NEM) at a concentration of 100 mM. This is prepared by dissolving 0.1251 g/ mL.
5. Add NEM to a final concentration of 10 mM and incubate for 5 minutes. Then put on ice or add directly to buffers for precipitation (step 6).
6. Precipitate protein by method-of-choice (many prefer acetone methanol if using large volumes, see below).
7. Resuspend protein in 200 mM HEPES pH 8 and measure protein concentration. Note: solubilisation by tip-probe sonication or strong base might be required.

Acetone/methanol precipitation

1. Add 8 sample volumes of freezer cold acetone and 1 sample volume of freezer cold methanol in *chemically resistant tubes (e.g. blue-capped Falcon tubes) for this step to avoid leeching of polymeric compounds and plasticizers into the sample*
2. Mix well by vortexing and let precipitation occur at -80 °C for 2 hours
3. Centrifuge at maximum speed for 15 min
4. Carefully discard supernatant
5. Wash pellet thoroughly with freezer cold methanol. Break up pellet by pipetting gently up and down
6. Centrifuge, repeat wash
7. Let pellet air dry. Do not over-dry pellet since this complicates re-solubilization [4]

As mentioned before aim to start with a total of 1 to 3 mg of proteome. For any given experiment use no less than 1 mg *total* protein (spread over all channels).

A: DIMETHYL LABELING

Advantages: Low cost, perfect for two sample comparisons, perfect to learn TAILS. We extensively tried, but now do not use or recommend three-plex dimethylation due to generally much lower peptide numbers identified.

Day 1: Reduction, Alkylation and Dimethylation

1. Add 6 M GuHCl[3] to ≥3 M, final (starting protein concentration 1-2 mg/mL)
2. Add HEPES pH 7.5 to 100 mM, final
3. Check that pH = 7.5. *This is important. Use for example pH paper and a micropipette to minimize sample loss.*
4. Add fresh DTT (dithiothreitol) to 5 mM, final, and incubate for 60 min at 37 °C
5. Add IAA (iodoacetamide) to 15 mM, final, and incubate for 30 min at room temperature in the dark (make IAA fresh; e.g. as 0.5 M stock)
6. Add DTT to 15 mM, final (i.e. to be equal concentration to IAA) and incubate 20 min at room temperature to quench alkylation
7. IN FUMEHOOD: Prepare a 2 M working stock of CH_2O (light formaldehyde) and a 1 M working stock of $NaBH_3CN$ (light sodium cyanoborohydride; light channel)
8. Add CH_2O (light dimethylation) to 40 mM, final
9. Immediately afterwards add $NaBH_3CN$ to 20 mM, final
10. Adjust pH to 6-7 and incubate overnight at 37 °C [4, 5, 6]

[3] Do not use urea, not even deionized urea. This is to prevent carbamylation of proteins when heated at 37C.

[4] *Alternatively incubate at 50 °C for 1 h, add fresh $NaBH_3CN$ and incubate for an additional 1 hour (not recommended, but reduces protocol length considerably).*

[5] If using duplex dimethylation for quantitation add $^{13}CD_2O$ (heavy formaldehyde) and $NaBH_3CN$ (light sodium cyanoborohydride; heavy channel; +6) to the second sample. Note these conditions may differ from what you currently use for peptide labeling. Remember the key step in TAILS is labeling at the protein level and so conditions are different.

[6] If using triplex dimethylation for quantitation, add CD_2O (medium formaldehyde) + $NaBH_3CN$ (light sodium cyanoborohydride; medium channel; +4) and $^{13}CD_2O$ (heavy formaldehyde) + $NaBD_3CN$ (heavy sodium cyanoborohydride; super heavy channel; +8) appropriately, but triplex is NOT recommended.

Day 2: Precipitation and digestion

1. Add fresh formaldehyde (20 mM; either light, medium, or heavy) and *sodium cyanoborohydride* (10 mM; either light or heavy) and incubate 37 °C for 2 hours to ensure complete labeling
2. Quench reaction by adding 1 M Tris, pH 6.8 to 100 mM, final
3. Vortex sample and check that pH is 6-7
4. Incubate 37 °C for 1 hour
5. For quantitative experiments mix samples now[7]

[7] If using triplex dimethylation for quantitation, acidify sample to pH <2.5 in fume hood to eliminate residual $NaBH_3CN$ and $NaBD_3CN$ before mixing samples to avoid cross labeling between channels

Chloroform/Methanol precipitation

Transfer sample to chemically resistant tubes (*e.g.* blue-capped Falcon brand) to avoid leeching of polymeric compounds and plasticizers into the sample.
Note: maximum volume during chloroform/methanol precipitation will be 10x the starting volume.
Note: do not use plastic serological pipettes for dispensing chloroform or methanol; these are not chemically resistant and their use will lead to contamination and sample loss due to dissolved plastic.

6. Add 4 sample volumes freezer cold methanol, vortex
7. Add 2 samples volumes freezer cold chloroform, vortex
8. Add 3 sample volumes ice-cold water, vortex
9. Centrifuge >5,000 rcf and retain interphase containing the protein precipitate (2 min if using 1.5-2 mL Eppendorf tubes and 10 min if using 15 mL Falcon Tubes) **Note: It is advisable to leave some supernatant on top of the interphase to avoid disturbing the protein layer → this greatly improves yields.*
10. Add 750 μL freezer-cold methanol to the pellet and transfer to a 2 mL Eppendorf tube. Rinse Falcon tube with another 750 μL freezer cold methanol and combine. Centrifuge at >15,000 rcf and discard supernatant

11. Wash pellet 3-4x with 1.5 mL freezer cold methanol (initially vortex to ensure thorough washing and later gently lift pellet to minimize sample loss)
12. Invert tube and partially air-dry the pellet (very important: do not over dry or pellet will be difficult to re-solubilize)
13. Re-suspend in 100 mM NaOH. Start with 20 μL and add an additional 2-5 μL if necessary to re-suspend. If difficult to re-suspend, brief tip-probe sonication can help.
14. Immediately after re-solubilizing add water stepwise aiming for [protein] >1 mg/mL
15. Add 1 M HEPES pH 7.2 to 50 mM, final

[8] The initial success rate of this step is highly sample dependent. Stepwise, add more water and HEPES if necessary to maintain solubility. Aim to keep protein sample as concentrated as possible without precipitation

16. Check pH 7.5-8 [9]

[9] Retain 20 μg sample for parallel digestion with LysargiNase (for C termini identification by a separate shotgun analysis) or half of the sample for GluC as a second TAILS analysis to increase coverage of N-termini, if desired. If using a second enzyme for a parallel TAILS analysis, it is advised to increase the initial amount of protein per channel.

17. Add trypsin at a protease:protein ratio of 1:100 and gently pipette up and down to mix sample. Use LysargiNase or GluC in a 1:50 ratio.
18. Incubate overnight at 37 °C or for 2-4 hours at 42 °C to shorten the protocol length
19. Validate complete digestion by analyzing a small aliquot by 10% SDS-PAGE and by checking that there are tryptic peptides by MALDI-TOF-MS (optional)
20. After trypsin digestion retain 10-20 μg sample for pre-TAILS analysis (equivalent to a shotgun analysis). This is useful to assign N termini to protein isoforms by the Isoform Assignment Score (IAS), see auf dem Keller, U., Prudova, A., Gioia, M., Butler, G.S., and Overall, C.M. 2010. A Statistics Based Platform for Quantitative N-Terminome Analysis and Identification of Protease Cleavage Products. Molecular Cellular Proteomics 9, 912-927 and to use 2+ peptides per protein for identification if this is a concern.

Day 3: Negative Selection Using HPG-ALDII Polymer
Polymer can be purchased at http://www.flintbox.com/public/project/1948/

1. Thaw frozen polymer solution at room temperature (not on ice)
2. Add HPG-ALDII polymer at a polymer:peptide ratio of 5:1 (w/w; check that the polymer has been pre-washed)
3. Immediately add $NaBH_3CN$ to a 20 mM, final and mix gently
4. Check pH is 6-7
5. Incubate overnight at 37 °C [10]

[10] Day 3 and 4 can be combined following a shorter tryptic digest at 42 °C. On Day 3 check that the trypsin digestion has gone to completion (step 19).

Day 4: Sample Recovery

1. Add 1.0 M Tris pH 6.8 to 100 mM, final
2. Check that pH is 6-7
3. Incubate at 37 °C for 30 min
4. Pre-wash 10-kDa MWCO Amicon column (0.5 ml centrifugal filters):
   a) Add 400 μL 100 mM NaOH, centrifuge 10,000 rcf for 10 min and discard flow-through
   b) Add 400 μL dH_2O, centrifuge 10,000 rcf for 10 min and discard flow-through (repeat 2-3x)
5. Load the peptide-polymer mixture and spin 10,000 rcf for 15 min
6. Collect flow-through and transfer to a clean 1.5 mL tube
7. Wash filter with 400 μL dH_20 and spin 10,000 rcf for 15 min
8. Collect flow-through and combine into previous 1.5 mL tube
9. Pipette out the remaining polymer solution in the filter
10. Thoroughly rinse filter with 100 μL dH_2O
11. Reposition filter upside down in a new tube and collect fraction with a quick spin
12. Combine all flow-through fractions (this step dramatically inceases yield of hydrophobic peptides)
13. Refer to STAGE Tip protocol to desalt and store preTAILS and TAILS samples "on tip" prior to LC-MS

B: TANDEM MASS TAG (TMT) LABELING variant strategy

Day 1: Reduction, alkylation and labeling

1. Precipitate 200 μg protein per condition by chloroform-methanol precipitation as above (in 1.5 mL Eppendorf tube)
2. Re-dissolve pellet in 30 μL 6 M GuHCl
3. Add 50 μL nanopure water
4. Add 20 μL 1 M HEPES, pH 8.0 and mix
5. Add TCEP (not DTT or mercaptoethanol as thiols interfere with labeling) from TMT kit to 10 mM, final. If preparing TCEP stock solution in-house check pH after addition. Adjust to pH 8 if necessary.
6. Incubate for 30 min
7. Add iodoacetamide (IAA) to 25 mM, final
8. Incubate at 25 °C in the dark for 30 min
9. Dissolve TMT labels (0.8 mg each) in a volume of DMSO equal to the total reaction mix (~110-115 μL), so the labeling is performed in 50% v/v DMSO. *This is critical and is different to the protocol suggested by manufactures for peptide labeling. TAILS employs labeling at the protein level.*
10. Add TMT labels to appropriate samples (*e.g.* 1:1), mix well
11. Incubate at 25 °C in the dark for 1 hour

12. Add 25 µL 1 M ethanolamine (prepared fresh) and incubate for 30 min to quench unreacted TMT labels
13. Combine samples into a single tube. 6-plex samples fit into a 15 mL tube so aim for this. For 10-plex samples use a 50 mL Falcon tube, which the 2 mg of sample precipitates nicely as a visible pellet [15]
14. Clean up unreacted reagents by acetone precipitation described below
15. Continue from dimethylation protocol Day 2, step 13 for pellet re-solubilization in NaOH and continue from there

[15] For 10 plex you may try avoiding the 50 mL tube and transfer all sample into multiple 1.5 mL Eppendorf tubes after mixing for precipitation and then higher speed centrifugation is possible
[16] *This protocol has been proven effective for 10-plex TMT labeling of 200 µg sample per channel, for 6-plex TMT an increase in protein amount to 250 µg per channel may be necessary to achieve as satisfying results (i.e. 1.5 mg total starting protein)*

Acetone/methanol precipitation
1. Add 8 sample volumes of freezer cold acetone and 1 sample volume of freezer cold methanol [17]
2. Mix well by vortexing and let precipitation occur at -80 °C for 2 hours
3. Centrifuge at maximum speed for 15 min
4. Carefully discard supernatant
5. Wash pellet thoroughly with freezer cold methanol. Break up pellet by pipetting gently up and down
6. Centrifuge, repeat wash
7. Let pellet air dry. Do not over-dry pellet since this complicates re-solubilization

[17] *Use chemically resistant tubes (e.g. blue-capped Falcon tubes) for this step to avoid leeching of polymeric compounds and plasticizers into the sample*

[18] *Proper washing of the pellet with methanol is absolutely critical to avoid experimental induced N-terminal acetylation of tryptic peptides by residual acetone. This will compromise efficiency of the HPG polymer pull-out step resulting in carryover of acetylated and blocked tryptic peptides in the TAILS supernatant sample*