

Paul Gershon

In-gel digestion procedure (in depth version)

Read “Tips for avoiding keratin contamination” at the end of this document

Reagents

Use “MilliQ”, or “nano-pure” water (or better: HPLC or MS grade water purchased bottled).

All organic reagents should be HPLC grade:

Milli-Q water

Acetonitrile (CH₃CN): A996-4 FISHER

Ammonium bicarbonate: A643-500 a FISHER

Formic Acid (FA): 0128-01 J.T. Baker

TFA: Small ampoules, high purity (I have details somewhere in the lab)

Prepare solutions fresh:

Solution	Recipe
0.1% formic acid (FA)	10 µL FA + 10 ml water
60% ACN/5% FA	30mL ACN + 2.5 mL FA + 17.5 mL H ₂ O
60% ACN/0.1% FA	30 mL ACN + 50 µL FA + 40 mL H ₂ O
25 mM NH ₄ HCO ₃	0.1 g NH ₄ HCO ₃ + 50 mL H ₂ O *
50% CH ₃ CN/25 mM NH ₄ HCO ₃	25 mL ACN + 0.1 g NH ₄ HCO ₃ + 25mL H ₂ O
0.02 µg/µl Trypsin in 25 mM NH ₄ HCO ₃	1 ml 25 mM NH ₄ HCO ₃ + 20 µg trypsin

All % solutions (v/v)

* Should be pH 7.8 (check)

• 0.05 to 0.1 mg/ml trypsin (recombinant, Roche P/N 708985, or Promega, modified, porcine, sequencing grade, P/N V5111, or Trypsin Gold, Mass Spectrometry Grade, P/N V5280) in 25 mM ammonium bicarbonate, 1 mM Ca²⁺, pH 7.8 (often works even better in presence of 10% acetonitrile or 10% 1-propanol (not 2-propanol))

• 5% (v/v) trifluoroacetic acid (TFA)/50% (v/v) CH₃CN

Use glass pipet/glass containers only for handling concentrated acids (!)

For reduction/alkylation only:

- 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate with ~10% organic solvent (acetonitrile or 1-propanol) (DTT solutions oxidize and do not keep well, prepare fresh. Beware mercaptoethanol and DTT, they may contain keratin). TCEP (tris-2-carboxyethylphosphine) may be preferable.
- 55 mM iodoacetamide in 25 mM ammonium bicarbonate (iodoacetamide is light-sensitive; prepare fresh.)

Consumables/equipment

Gloves

Powder-free nitrile gloves (never use latex gloves, as natural rubber contains significant amounts of keratin and other proteinaceous materials).

Microfuge tubes:

• 0.2 mL or 0.5 mL or 1.5 mL microcentrifuge tubes (Eppendorf brand will minimize contamination, others have recommended BioRad: 0.5 ml (223-9480) or 1.5 ml (223-9480), or National Scientific # CN 0659-GTS retailed as Fisher #11-842-1).

Alternatively, microfuge tubes can be pre-washed by filling with either:

- 50% CH₃CN/0.1% trifluoroacetic acid (TFA))
- 50% pure methanol in pure water,
- 60% ACN/5%FA.
- 60% acetonitrile/0.1% FA

Then dry the stock of prewashed tubes before use

. Do not use coated DNase/RNase-free or silanized tubes, or tubes with an O-ring.

Pipet tips

DO NOT FILL PIPET TIP BOXES WITH YOUR BARE HANDS!!! Wear gloves – or better yet, buy pre-filled boxes.

Other:

- Vacuum centrifuge (Speedvac)
- 37°C and 56°C water baths (f you are doing sample reduction)
- Vortex mixer, preferably with an attachment that allows you to attach tubes then walk away while samples are vortexing.

Millipore ZipTips, micro-C18, part no. ZTC18M096 (available from Fisher) or Omix C18 tips from Varian (we have been using A5700310).

EITHER Hamilton syringes, 1701N (10 µL) and 1710N (100 µL) (or 1701RNR and 1710RNR) (blunt needles) OR the equivalent pipettors.

Source material

The most frequent sample contaminants after keratin are serum proteins and cytoskeletal proteins. If working from cultured cells, use serum-free medium if possible.

SDS PAGE

- Try to use a 1 mm thick gel.
- * Try to run a long resolving gel (like 30 cm or more) if possible.
- * If available, try purchasing precast gels, pre-made electrophoresis running buffer, and a staining kit.

If casting your own gels:

- * Before use, bleach the gel plates, comb, spacers and gel-staining tray.
 - * Handle the plates by the edges and wear nitrile gloves to avoid transfer of keratin from your hands to the gel plates. Before preparing solutions, wash the glassware well to remove any dust that might have accumulated during storage. After the acrylamide and buffers have been combined (and before the polymerization catalysts have been added), filter the solution to remove dust.
 - * If making your own SDS PAGE gel, try to prepare it ~ 18 – 24 hours ahead of time to allow maximal polymerization.
- It is highly recommended to electrophorese a positive control such as BSA or some other known protein that stains to approximately the same level as your unknown protein of interest.

Gel staining, band and excision and washing

The efficiency of protein digestion and peptide extraction may depend on properties of the specific protein (hydrophobicity, etc.). In general, picomole amounts are more likely to be successful than lower amounts, and greater amounts of target protein are nearly always better.

Staining:

The sensitivity of colloidal coomassie staining may be 2 – 3x greater than that of silver stain.

If you use conventional Coomassie, stain and destain the gel with <10% acetic acid (higher concentrations may interfere with trypsin digestion). For colloidal Coomassie stain, Invitrogen Colloidal Blue stain (P/N LC6025) should be fine.

Try to disassemble the gel sandwich and setup staining under a laminar flow hood.

During staining, keep the staining container closed to keep dust out.

Band excision:

- A single concentrated gel band is better than several more dilute bands. Greater numbers of gel pieces are not necessarily better.

- Band cutting is an important step for performance in a laminar flow hood.

- Before starting, wipe the outside of all pipettors with an ethanol-soaked kimwipe to remove dust and other contaminants. If a pipettor becomes contaminated at any point in the protocol, wipe it off again.

(1) Using a clean scalpel or razor blade (you may wish to first sonicate the blade for 5 min CH₃CN or ethanol in a glass beaker), excise protein bands/spots of interest from the stained polyacrylamide gel. Excise only the darkest part(s) of the band, leaving the lighter edges behind.

Cut each gel slice into small pieces (~1 mm³) and transfer the pieces to a clean, sterile 0.5 - 1.5 mL pre-rinsed microcentrifuge tube. Write the number of cubes somewhere (like on the tube).

- Also excise and dice a gel piece from a protein-free region of the gel in parallel, as a control, and your positive control (BSA) if you ran it.

Washing:

- (1) Wash the gel pieces with 0.5 mL of 25 mM NH_4HCO_3 . Vortex for 1 hr, then discard the wash using a gel-loading pipet tip for the final portion.
- (2) Add ~100 μL (or enough to cover the gel pieces) of 25 mM ammonium bicarbonate/50% CH_3CN and vortex for 10 min. Discard the wash (pale blue - this step should start to release coomassie from the gel pieces) using a gel-loading pipet tip.
- (3) Repeat this wash/dehydration step ~2 times. If gel pieces are still very blue after the 2nd wash, you can rehydrate the gel with 25 mM NH_4HCO_3 then repeat the washes. This color criterion should be used to determine whether or not to perform additional washes.
- (4) Add ~100 μL (or enough to cover the gel) of 100% CH_3CN and vortex for 10 min, discard the wash. The gel piece should have shrunk and become visibly opaque/white.
- (5) SpeedVac the gel pieces **to complete dryness** (~10 - 30 min in the SpeedVac). Complete dehydration of the gel slices is important.

Reduction and alkylation after excision (optional)

This should be done if the protein is rich in disulfides. However, this procedure inevitably results in some sample loss, and is often unnecessary. We generally avoid it.

If doing reduction/alkylation, some people recommend doing it prior to SDS-PAGE, and I would concur.

However, if alkylating at the gel-slice step, do it as follows:

- (1) Add sufficient 10 mM DTT solution to cover the gel pieces, and incubate for 1 hr at 56°C.
- (2) Cool to room temperature and replace the DTT solution with roughly the same volume of 55 mM iodoacetamide solution. Incubate for 45 min at room temperature in the dark with occasional vortexing.

If in-gel:

- (3) Wash gel pieces (rehydrate) with ~100 μL of 25 mM ammonium bicarbonate, pH 8, for 10 min while vortexing, and dehydrate with ~100 μL of 25 mM ammonium bicarbonate/50% acetonitrile. Repeat rehydration and dehydration.

(4) Remove the liquid phase and dry the gel pieces in a vacuum centrifuge for 20 min.

Digestion

Do not add more solution than can be absorbed by the gel pieces, since non-absorbed trypsin will autolyse, and trypsin autolytic products may dominate subsequent mass spectra.

The enzyme:substrate ratio employed for in-gel digestions (>1:10) is greater than for in-solution digestions due to the hindered enzyme access to the protein substrate within the gel. The relatively low buffer concentration (25 mM) minimizes the possibility of subsequent salt interference with ionization in the mass spectrometer.

(1) The required volume of 0.05 - 0.1 mg/mL (2 - 4 μ M) trypsin solution can be estimated by calculating the total gel volume excised (e.g., 2 mm x 8 mm x 1 mm = 16 mm³ = 16 μ L).

(2) Pre-cool the gel pieces and trypsin solution to 0 °C (on ice). This will help minimize trypsin autolysis before the trypsin has entered the gel slice.

(3) Rehydrate (re-swell) the gel pieces by adding one volume of the trypsin of the solution.

(4) Incubate in ice (or at 4 °C) for 30 min. If trypsin solution remains in the sample after 30 min., **pipette it off**.

(5) If necessary, overlay the rehydrated gel pieces with a minimum amount of 25 mM NH₄HCO₃ to keep them immersed throughout digestion.

(6) Spin briefly and incubate in a shaker at 37 °C overnight (6 - 20 hrs).

Peptide recovery

(1) Briefly vortex and spin the digest. Add a volume of water equal to twice the volume of the gel piece, vortex for 10 min, spin, sonicate 5 min. Use a gel-loading tip to remove the digest solution (not the gel) and transfer it to a fresh 0.5 mL tube (**tube A**) containing 5 μ l of 60% CH₃CN/5% FA (or 50% CH₃CN/5% TFA).

(2) To the gel pieces, add 50 μ L (or enough to cover) of 60% ACN/5%FA (or 50% CH₃CN/5% TFA). Vortex 10 min, spin, sonicate 5 min. Pool extracted peptides together in **tube A**.

(3) Repeat step (2) 2X.

(4) Vortex the extracted digests, spin and Speed Vac to reduce volume to 5 - 10 μ L and lower the CH₃CN concentration to <5% (some people speedvac @ 45 °C, and mention that this step may take ~60 min, but in my lab the SpeedVac and vacuum system seems very efficient at room temperature, so **check frequently to prevent the digest from running dry**).

Evaporating the solution to complete dryness will result in some loss of peptides. If it runs dry, reconstitute in 5 - 20 μ L of 5% CH₃CN/0.1% FA with 5 min. sonicate. Proceed to ziptip cleanup).

(5) Add 20 μ L of 0.1% FA to **tube A**. Vortex, spin, sonicate 5 min.

At this point, samples can be stored at +4, -20 or -80°C. (CH₃CN and water may separate into two phases at -20°C, but for these small samples, it doesn't seem to matter.). However, storage should be minimized.

ZipTip cleanup of samples

From this point, because the trypsin is dead, you don't need to be quite so worried about keratin.

Use Millipore ZipTips, micro-C18, part no. ZTC18M096, or Omix C18 tips from Varian (we have been using A5700310).

You can try using Hamilton syringes, 1701N (10 μ L) and 1710N (100 μ L) (or 1701RNR and 1710RNR) (blunt needles, as used in HPLC) for smooth flushing of ziptips. We generally don't bother with syringes (since Omix tips don't fit the Hamilton syringe needle, just pipet carefully with a good pipettor. We fit the Omix tips on the end of a regular yellow pipet tip ("stacked tips"), and put this on a P-200 pipettor, thereby getting the smooth flows with larger wash volumes mentioned below.

You will need the following solutions:

(1) 0.1% TFA or FA in good quality water

(2) 50/50 mixture of solution 1 and acetonitrile OR: If FA was used in (1), use 60% CH₃CN/0.1% FA for (2). The resulting solution is referred to as "50/50". 10 mg/ml CHCA MALDI matrix can be included in the 50/50.

Procedure:

(1) Condition the tip: Rinse the tip with 10 μ l of 50/50. If using a 100 μ l syringe, you can do a single, smooth 100 μ L rinse. Before the start of the rinse, tap the syringe plunger and squirt through a bit of solvent to get rid of air bubbles.

(2) Equilibrate the tip: Rinse either 5x with 10 μ l of 0.1% TFA (or 0.1% FA) in water (if you are using a ziptip with pipettor) or, if using a syringe or "stacked tips", do a single 100 μ L rinse in a single smooth motion.

(3) Adsorb: Slowly load the peptides from **tube A** into the Ziptip *via* repeat pipetting up/down (draw the sample solution **slowly** back and forth through the ZipTip at least 10x - even 30x to be safe). **Be careful to not let the tip run dry and to not introduce air bubbles into the tip.** *A worry in using the same syringe for all samples at the adsorption step, is the possibility of cross-contamination. Omix stacked-tips gets around this.*

(4) Rinse: Flush 0.1% TFA or 0.1% FA slowly through the tip to rinse out salts and other water-soluble impurities. You can flush 3X with 10 μ L (combining the 1st wash into **tube A**). If using a syringe, do this by emptying a 100 μ l syringe (or P-200 w/ stacked tips) through the tip.

Elute: There are two ways to elute the ziptip with 50/50. **Be careful not to let the tip run dry or introduce air bubbles into the tip.**

(1) **Pipetter:** Put 1 - 3 μ L of 50/50 (**containing matrix**) into a 200 μ L PCR tube or better, on the spot of a MALDI target plate. With a 10 μ L pipetter or syringe, draw the solution slowly back and forth through the C18 resin (**without introducing air into the resin**) at least 20 - 30 times for maximal recovery. Deposit finally on fresh spots of the target plate (see "Spotting", below).

For the most concentrated sample try putting just 1 microliter of matrix-containing 50/50 into top of the ziptip bed using a very fine tip (gel loader or finer), then remount the pipettor and push the 1 microliter right through, directly onto the plate, maybe in a couple of spots if possible (see "Spotting", below).

(2) **Syringe:** Put 4 μ L of 50:50 (**containing matrix**) in a 10 μ L syringe. Put the tip on the end of the syringe and elute slowly. Discard the first one μ L, then deposit the rest in small drops on a MALDI target plate (see "Spotting", below). Do the elution slowly to allow desorption to take place.

If not eluting directly onto a target plate but instead into a microtube, samples **should be analyzed as soon as possible after elution, because peptides tend to become irreversibly adsorbed to the tube walls or otherwise vanish.**

Spotting

The key to good MALDI-MS is a discrete spot with an **opaque, thick layer of matrix**. This can be obtained from a **concentrated sample** with **minimal impurities**, in which **matrix has crystallized well** during spot drying.

Spot size

In general, aim to generate **small** spots, by spotting less than 0.5 microliters of material (0.3 microliters if possible). The small volume helps prevent the spot spreading too much on the plate.

Use of a freshly polished plate may also help prevent spreading (though some may argue that the polish may contain impurities, so polishing the plate in my lab is optional, we do it only once every few cleanings).

General spotting technique

Just quickly dip the pipet tip to the plate but don't hold it there and don't touch the plate with the tip, if possible just bring it close enough to the surface for the small drop to transfer. 0.2 – 0.5 microliters should be fine. This way, the spot does not spread too much and does not “dome” (form a convex shape) too much in the middle, leading to slow drying in the middle and depositing a thin layer of crystals around the edges only. It just forms a fairly flat, rapidly drying spot. This way, you may end up with several replicate, small spots, but I don't recharge for how many spots you generate, only for how many get analyzed. Probably only one of each replicate set will be analyzed, if the crystals look good – maybe the best looking one.

Plasticware used (especially during/after mixing with matrix)

Matrix crystallizes well if the spot dries rapidly and evenly in the **absence** of residual surface-active agents used during manufacture, which prevent spots from ever drying.

Other impurities leached from plastic tubes (eg. residual plasticizer, or silanol coating from low binding or DNase/RNase-free tubes) may leech into the sample (in the acidic solutions that we use for ziptipping for example), leading to overwhelming PEG-like polymer signals in spectra.

Impurities can be minimized by minimizing the use of plasticware, and being careful what brands are used. **Never use silanized (low adsorption) tips or tubes**. Plain Eppendorf brand polypropylene microtubes seem to be the best for consumable plastics. Teflon tubes would be the very best but may be unrealistically expensive as consumables, though good for storing pure acids for example.

If you mix your final sample with matrix in a microtube, **first rinse the tube by pipetting 30 microliters CH₃CN, vortexing briefly then dumping out the CH₃CN and allowing to dry.**

Obtaining a concentrated sample

If using a Ziptip or Omics tip, a concentrated sample can be obtained by eluting the tip in a small volume of 50/50 eluent that **already contains matrix.**

For the most concentrated sample try putting just 1 microliter of matrix solution into top of ziptip bed using a very fine tip (gel loader or finer), then eluting as described above.

As mentioned above, if storing peptides in solution in a microtube, **peptides may become irreversibly adsorbed to the tube walls or otherwise vanish.**

Maximizing the probability of success (recap)

1. Commit as much protein as you can.
2. Use a 1.0 mm thick gel.
3. **Avoid excess gel piece w/o proteins.**
4. Excise your band tightly (only the heart of the band).
5. Don't over-stain your gel after the band becomes visible.
6. Destain your gel thoroughly.
7. If storing prior to initiating this protocol, keep the gel pieces moist at -20 oC.
8. Always digest a blank piece of gel and a known protein band as control.

Avoiding Keratin Contamination (tips/recap)

Keratin is abundant in hair, nails, horn and hoof. Remember your skin is your largest organ, it is made largely of protein (keratin), has a huge surface area/volume ratio and it turns over at a very high rate with dead skin becoming (or adhering to) dust particles. Fingerprints and woolen clothing are also rich sources of keratin. We walk around each day with a veritable keratin fountain spewing from our heads. If these statements seem improbable or unreasonable, read "The Secret Life of Dust" by Hannah Holmes (Wiley, John & Sons, Inc., August 2001). This keratin-polluted environment leads to contamination of experimental samples and is a significant problem for the analysis of low abundance proteins.

The key to protein ID in the context of environmental keratins is keratin:target protein ratio. Since the mass spectrometer detects proteins at femtomolar levels, the slightest amount of dust in a sample can result in keratin being identified as the major sample protein. To avoid or minimize contamination with human keratins, cleanliness is very important. If working from sypro- or silver-stained amounts of target protein, the importance of avoiding keratin is proportionately greater.

A key factor in avoiding keratin is to avoid contamination with dust particles. Start with a scrupulously clean work surface. If possible, for best dust control during sample prep work such as cutting gel bands or performing in-gel trypsin digestion, try working in a HEPA-filtered laminar-airflow hood. If you do the latter, keep exclusive-use packs of plasticware after first wiping out the inside of the hood with ethanol, and wiping the surfaces of the packs of plasticware with ethanol.

Another key: DO NOT HANDLE ANYTHING THAT WILL COME INTO CONTACT WITH YOUR PROTEIN WITH YOUR BARE HANDS!!! Always wear powder-free nitrile gloves (never use latex gloves, as natural rubber contains significant amounts of

keratin and other proteinaceous materials). It may be necessary to wear protective clothing during all phases of sample preparation and to cover your head.

Paper and cardboard containers are rich sources of dust. If environmental keratin from your gel running buffer or gel plates spreads keratin at a relatively low background concentration throughout your gel slice or over its surface, minimizing slice volume gains in importance. The relatively large surface area:volume ratio of an exposed gel surface can be a dust trap. Handle gels as though they are keratin-magnets.

Try filtering electrophoresis buffers, gel mixes etc. through nitrocellulose filters. Better still, to keep keratin out, try purchasing precast gels, pre-made electrophoresis running buffer. For electrophoresis/staining, work from kits as much as you can.

If you provide us initially with your less critical samples (eg. controls), we can work out contamination and other issues before progressing to the more important samples.

When casting gels:

Clean the gel plates well with a soapy sponge, rinsing well with ethanol (or bleach overnight). Handle the plates by the edges and wear gloves to avoid transfer of keratin from your hands to the gel plates. Before preparing solutions, wash the glassware well to remove any dust that might have accumulated during storage. After the acrylamide and buffers have been combined (and before the polymerization catalysts have been added), filter the solution to remove dust.

When staining gels:

Rinse the staining container well before beginning. Also, rinse the gel thoroughly at each stage. Keep the container closed to keep dust out.

When cutting gel bands:

This is the step where keratin contamination is most likely to occur. If possible, work in a laminar flow hood. Before starting, wipe down the work area and any tools that will contact the gel band with an ethanol-soaked Kimwipe.

When doing enzymatic digestions or other pre-analysis assays:

A basic rule of thumb is to not touch the inside of the microcentrifuge tube with anything but the pipet tip, because it is very easy to transfer keratins from the pipet to the digest solution. Before starting, wipe the outside of all pipets with an ethanol-soaked kimwipe to remove dust and other contaminants. If a pipet becomes contaminated at any point in the protocol, wipe it off again. Another especially important tip: DO NOT FILL PIPET TIP BOXES WITH YOUR BARE HANDS!!! Wear gloves – or better yet, buy pre-filled boxes. This way, there's a much smaller chance

that any undesired contaminants from the outside of the pipets will make it into your protein solution.

Perhaps most importantly, don't leave any microfuge tube open for a prolonged period, this would allow dust from the air (and therefore keratin) to enter.