# In-gel digestion for mass spectrometric characterization of proteins and proteomes

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**In-gel digestion of proteins isolated by gel electrophoresis is a cornerstone of mass spectrometry (MS)-driven proteomics. The 10-year-old recipe by Shevchenko *et al.* has been optimized to increase the speed and sensitivity of analysis. The protocol is for the in-gel digestion of both silver and Coomassie-stained protein spots or bands and can be followed by MALDI-MS or LC-MS/MS analysis to identify proteins at sensitivities better than a few femtomoles of protein starting material.**



## INTRODUCTION

Many proteomics efforts rely on the pre-separation of target proteins by one- or two-dimensional gel electrophoresis[1]. Identification of proteins from polyacrylamide gels offers a number of important advantages compared to gel-free approaches. Sequencing of sharp, molecular weight-separated protein bands increases the dynamic range of analysis of protein mixtures (ratio of lowest to highest abundance protein detectable) as peptides produced by in-gel tryptic cleavage of each band are sequenced in separate experiments. For complex mixture analysis, spreading out the proteome over 10–20 gel slices dramatically increases the depth of analysis, and hence the number of identified proteins and detected post-translational modifications. At the same time, gel electrophoresis removes low molecular weight impurities, including detergents and buffer components, which are often detrimental for mass spectrometric sequencing. Another advantage is that the polyacrylamide matrix is a safe container to handle, derivatize and archive femtomole quantities of proteins[2].

Heterophase digestion of proteins within a polyacrylamide matrix is controlled by enzyme diffusion[3,4] and, for optimal efficiency, requires carefully adjusted reaction conditions that, at the first glance, might not be in line with conventional protein chemistry procedures. Much higher concentration of the enzyme (usually trypsin) is required compared to in-solution digestion, which often results in a significant background of autolysis products. In addition, casting the gels and handling the excised spots (bands) of interest increase the risk of contaminating samples with human and sheep keratins and might enhance chemical noise in analyzed samples. (Measures to minimize this contamination are described in **Box 1**.)

The in-gel digestion protocol presented here was originally introduced in 1996 (ref. 5), and has been used thousands of times over the last 10 years in the authors' and other groups. Using the experience accumulated during this time, we now describe a protocol that has been optimized to balance the time of digestion (and hence the speed of the entire protein identification routine) with the yield of tryptic peptides. At the same time, the recipe is flexible and can easily be adapted to meet the specific requirements of any particular proteomics experiment[3] or integrated into a robotic sample processing pipeline. The method applies with no or minor adjustments to one- or two-dimensional gels stained with Coomassie brilliant blue R250 or G250 or with silver[5]. The in-gel digestion procedure is compatible with downstream MALDI-MS and nanoES MS/MS characterization of digests of isolated protein bands or spots. When complex protein mixtures are gel-separated and analyzed by LC MS/MS (termed "GeLCMS"), the in-gel digestion procedure enables the analysis of entire proteomes of organelles and the majority of proteins in cell lysates. By including $H_2{}^{18}O$ into the digestion buffer[6–8] or by mixing SILAC-labeled protein mixtures before separation[9,10], it enables quantification of the digestion products as well as accurate *de novo* interpretation of multiplexed tandem mass spectra of tryptic peptides[11]. Peptide mixtures can also be purified before LC MS/MS on STAGE (Stop and go extraction) tips before loading[12].

## MATERIALS

### REAGENTS

- Ammonium bicarbonate (Sigma)
- Dithiothreitol (DTT; Sigma)
- Iodoacetoamide (Sigma)
- Trypsin (porcine, sequencing grade, modified; Promega Corp.)
- Water (LiChrosolv grade; Merck or Fischer Scientific)
- Acetonitrile (HPLC gradient grade; Merck or Fischer Scientific)
- Formic acid (reagent grade, Merck)

## BOX 1 | REDUCING KERATIN AND CHEMICAL BACKGROUND

- Wear gloves at all times and rinse them occasionally as they readily accumulate static charge and attract dust and pieces of hair and wool.
- Perform all operations in a laminar flow hood and use a dedicated set of pipettes, tips, tubes that should be stored in the hood in a dust-free environment.
- Do not use polymeric detergents (Twin, Triton, etc.) for cleaning flasks and glass plates for electrophoresis.
- Always visually check flasks, tubes and pipette tips for contaminating particles.

- Trifluoroacetic acid (Uvasol grade, Merck)
- $H_2{}^{18}O$ (Sigma Chemicals), only used if subsequent manual *de novo* interpretation of spectra of tryptic peptides[13] or their relative quantification by this method[6] is intended

**EQUIPMENT**

- Laminar flow hood (Heraeus)
- Air-circulation thermostat (Memmert)
- Bench-top centrifuge 5415D (Eppendorff)
- 0.65 ml thin-walled PCR tubes (Sorenson BioScience)
- Vacuum centrifuge RC 1022 (ThermoElectron Corp.)

**REAGENT SETUP**

**Trypsin** Prepare a solution of 13 ng $\mu l^{-1}$ trypsin in 10 mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile. Dissolve the content of 20 µg vial in 1.5 ml of the buffer. Use $H_2{}^{18}O$ instead of normal water for peptide quantification or *de novo* sequencing. ▲ **CRITICAL** Make shortly before use; discard unused volume. If only a small volume of trypsin buffer is required, the lyophilized enzyme can be redissolved in 1 mM HCl and 10 µl aliquots stored at −20 °C before use. Note that after thawing frozen aliquots, pH should be adjusted by adding 1.5 µl of 50 mM ammonium bicarbonate shortly before use.

- 100 mM ammonium bicarbonate in water ▲ **CRITICAL** Make ammonium bicarbonate buffer daily in large (50–100 ml) volumes and discard after use.
- 10 mM DTT in 100 mM ammonium bicarbonate. ▲ **CRITICAL** Make shortly before use.
- 55 mM iodoacetamide in 100 mM ammonium bicarbonate. ▲ **CRITICAL** Make shortly before use.
- 5% formic acid in water (vol/vol)

**Processing of bands (spots) from one- or two-dimensional gels** Upon electrophoresis, proteins should be fixed within a polyacrylamide matrix by incubating the entire gel in 5% (vol/vol) acetic acid in 1:1 (vol/vol) water: methanol. Staining with Coomassie, at the same time, fixes proteins, whereas a separate fixation step should precede colloidal Coomassie or silver staining. The in-gel digestion procedure is, in principle, compatible with any convenient silver staining protocol. However, the reagents used to improve staining sensitivity and contrast must not modify proteins covalently. Thus, avoid treating gels with crosslinking reagents (such as glutaraldehyde) or strong oxidizers, such as chromates or permanganates. Note that the abundance of silver-stained spots (bands) strongly depends, among other factors, on the time of gel exposure to the developing solution. If possible, allocate two lanes on a one-dimensional gel for protein standards (e.g., 50 and 200 fmol of bovine serum albumin). Semiquantitative estimation of the amount of protein available for sequencing helps to choose optimal sample loading (Step 7). Do not start with silver staining of gels of unknown protein preparations. First stain them with Coomassie and then, if required, directly by silver without prior destaining.

Note that, especially in femtomole sequencing, controls (blank gel pieces excised and processed in parallel with the experimental bands or spots) are usually unreliable and do not accurately represent actual patterns of keratin peptides and related contaminations. Instead, contaminating precursors should be identified by database searching (see TROUBLESHOOTING section).

## PROCEDURE

### Excise protein bands (spots) ● TIMING ~5 min per band per spot

**1|** Rinse the entire slab of a one- or two-dimensional gel with water for a few hours, put a plastic tray with the gel onto a light box and excise bands (spots) of interest with a clean scalpel.

▲ **CRITICAL STEP** Take special care to prevent massive keratin contamination of the samples (**Box 1**).

**2|** Cut excised bands (spots) into cubes (ca. 1 × 1 mm). Note that smaller pieces could clog pipette tips.

**3|** Transfer gel pieces into a microcentrifuge tube and spin them down on a bench-top microcentrifuge.

### In-gel reduction, alkylation and destaining of proteins ● TIMING 60 min

**4|** Additional reduction/alkylation step is only performed for processing of silver-stained bands (spots) or to prepare samples for manual or automated *de novo* sequencing[14] (option A). If rapid identification of Coomassie-stained bands (spots) is intended, skip reduction/alkylation and proceed directly with the steps described in option B.

**(A) Processing silver-stained bands (spots) or samples for *de novo* sequencing**

(i) Add 500 µl of neat acetonitrile and incubate tubes for 10 min until gel pieces shrink (they become opaque and stick together).

(ii) Spin gel pieces down, remove all liquid.

(iii) Add 30–50 µl of the DTT solution to completely cover gel pieces. Incubate 30 min at 56 °C in an air thermostat.

(iv) Chill down the tubes to room temperature (ca. 22 °C), add 500 µl of acetonitrile, incubate for 10 min and then remove all liquid.

(v) Add 30–50 µl of the iodoacetamide solution (the volume should be sufficient to cover the gel pieces) and incubate for 20 min at room temperature in the dark.

(vi) Shrink gel pieces with acetonitrile and remove all liquid.

**(B) Destain gel pieces excised from Coomassie-stained gels** ● TIMING 30 min

(i) Add ca. 100 µl of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubate with occasional vortexing for 30 min, depending on the staining intensity.

(ii) Add 500 µl of neat acetonitrile and incubate at room temperature with occasional vortexing, until gel pieces become white and shrink and then remove acetonitrile. Although the bulk of Coomassie staining should be removed, it is not necessary to destain the gel pieces completely.

■ **PAUSE POINT** Samples are now ready for in-gel digestion. Alternatively, they can be stored at −20 °C for a few weeks.

### Saturate gel pieces with trypsin ● TIMING 120 min

**5|** Add enough trypsin buffer to cover the dry gel pieces (typically, 50 µl or more, depending on the volume of a gel matrix) and leave it in an ice bucket or a fridge.

**6|** After ca. 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer.

**7|** Leave gel pieces for another 90 min to saturate them with trypsin and then add 10–20 µl of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage.
▲ **CRITICAL STEP** Although after ca. 30 min dried gel pieces do not absorb any more buffer, the yield of tryptic peptides increases substantially while extending the incubation time, presumably because of slow diffusion of the enzyme into a polyacrylamide matrix[3].

**Digestion**

**8|** Place tubes with gel pieces into an air circulation thermostat and incubate samples overnight at 37 °C for analyses performed at the limit of instrument sensitivity, which require maximal peptide recovery. Otherwise (typically, for the rapid identification of Coomassie stainable spots (bands) by MALDI mass fingerprinting), the acceptable digestion yield—exceeding, on average, 75% of the yield of overnight cleavage—can be achieved in 30 min at 55 °C (ref. 3). If the protein digest needs to be further analyzed by MALDI TOF MS, proceed to Step 9; otherwise, proceed to Step 10 directly.
▲ **CRITICAL STEP** It is important to avoid a temperature gradient between the bottom and the lid of the tube to prevent condensation of water at the inner surface of the lid and, consequently, premature dehydration of the gel pieces.

**Withdraw an aliquot from the digest for the protein identification by MALDI peptide mass mapping**

**9|** Chill tubes to room temperature, spin down gel pieces using a microcentrifuge and withdraw 1–1.5 µl aliquots of the supernatant directly from the digest without further extracting the gel pieces[15]. As a typical volume of the digestion buffer is approximately 50 µl, this leaves ample peptide material for the subsequent MS/MS analysis, if required.
■ **PAUSE POINT** Non-extracted digests can be stored at −20 °C for a few months until it is decided if further LC MS/MS analysis is required.

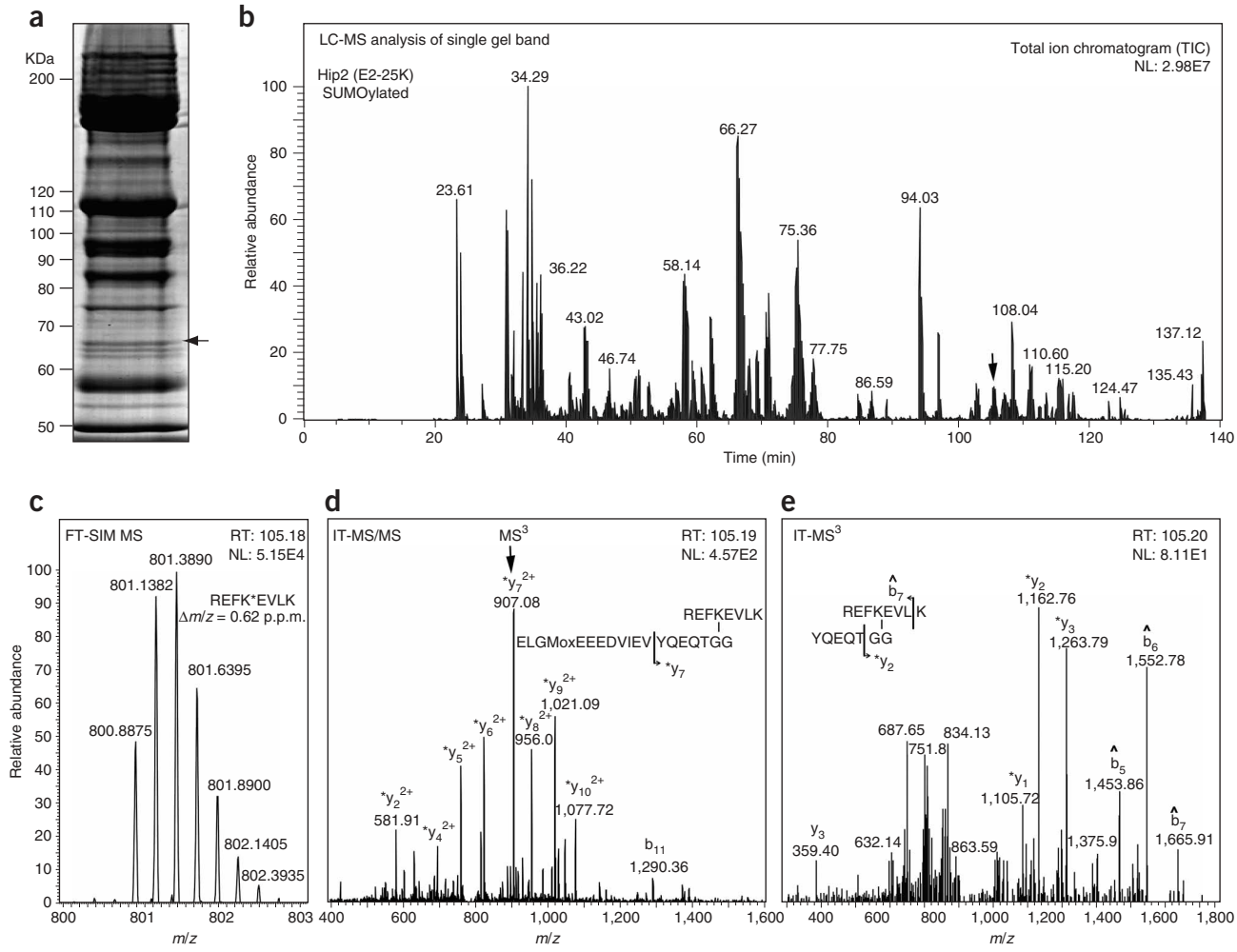


**Figure 1 |** Characterization of a modified protein by GeLCMS. (**a**) Coomassie-stained gel of a GST-SUMO-1 pull down. The lane marked with an arrow was excised, in-gel digested as described in this protocol and analyzed by LC-MS on an LTQ-FT instrument using two consecutive stages of tandem MS[22]. A total of 20 peptides identified Hip2 (Huntingtin interacting protein 2) in the stained band. (**b**) Total ion current (TIC) of Hip2 peptides eluting from the chromatography column. (**c–e**) Mass spectrum, MS/MS and MS/MS/MS spectrum of the SUMO-1-modified peptide, unambiguously localizing the SUMO modification site to K14 of Hip2. Inset shows the structure of the branched Hip2-Sumo-1 peptide as well as prominent cleavage products (see ref. 23 for an introduction to peptide sequencing).

**Extract peptide digestion products**

● TIMING **15 min**

**10|** Add 100 µl of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) to each tube and incubate for 15 min at 37 °C in a shaker. For samples with much larger (or smaller) volume of gel matrix, add the extraction buffer such that the approximate ratio of 1:2 between volumes of the digest and extraction is achieved.

▲ CRITICAL STEP To withdraw the supernatant, use a pipette with fine gel loader tip to prevent clogging the needle of autosampler injector or nanoLC MS/MS column. Collect the supernatant into a PCR tube, dry down in a vacuum centrifuge. (Do not discard extracted gel pieces.)

■ PAUSE POINT Dried extracts can be safely stored at −20 °C for a few months.

**Redissolve tryptic peptides for further analysis**

**11|** For further LC MS/MS analysis, add 10–20 µl of 0.1% (vol/vol) trifluoroacetic acid into the tube, vortex and/or incubate the tube for 2–5 min in the sonication bath and centrifuge for 15 min at 6.7*g* (10,000 r.p.m.) at the bench-top centrifuge and withdraw the appropriate aliquot for further analysis. Dry down the rest in a vacuum centrifuge and store at −20 °C as contingency.

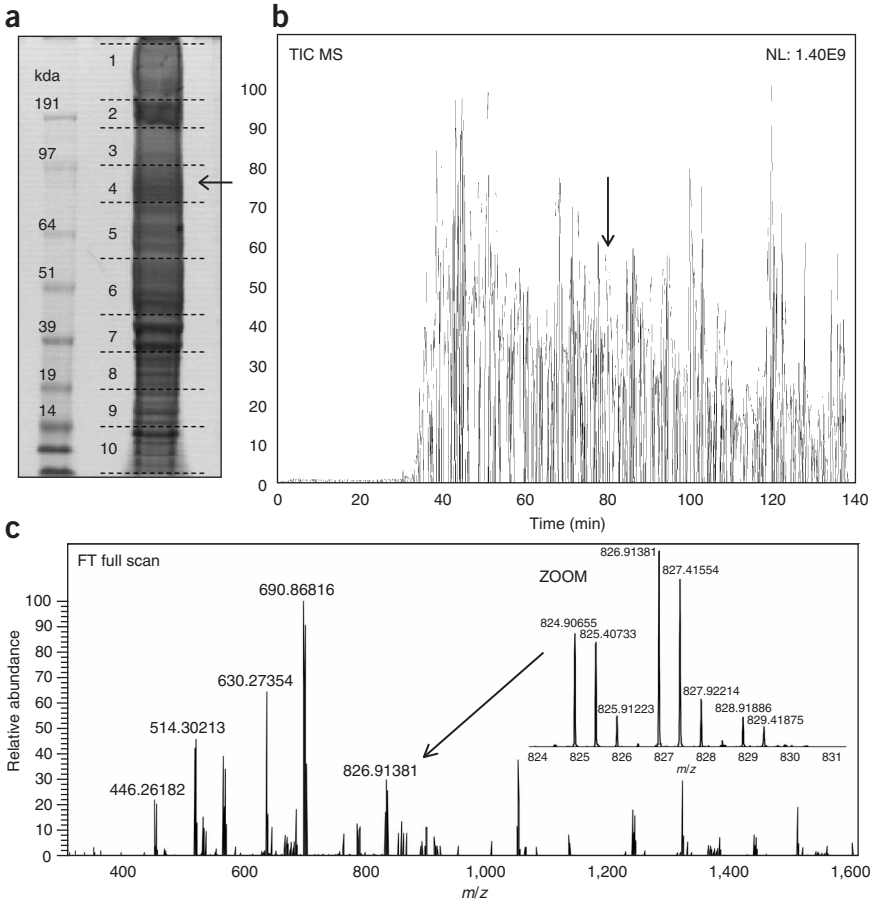


**Figure 2** | Characterization of a complex proteome. (**a**) Total cell lysate from HeLa cells was separated by 1D gel electrophoresis and sliced into ten parts. Each slice was in-gel-digested as described in this protocol. (**b**) TIC of the slice marked with an arrow in (**a**). Owing to the high number of eluting peptides no individual chromatographic peaks are visible. (**c**) Full-scan spectrum from the elution point marked by an arrow in (**b**). At least 30 co-eluting SILAC peptide triplets are present in the spectrum.

● TIMING

Excise protein bands (spots): ~5 min per band or spot
In-gel reduction and alkylation of proteins: 60 min
Destain gel pieces excised from Coomassie-stained gels: 30 min
Saturate gel pieces with trypsin: 120 min
Digestion: 30 min to overnight
Extract peptide digestion products: 15 min

? TROUBLESHOOTING

**Processing of protein bands or spots**

Although the measures described in **Box 1** can drastically reduce the abundance of background keratin peptides, it is almost impossible to remove them completely, and some will remain detectable while sequencing proteins at the low femtomole level. Peptide background originating from keratin and trypsin autolysis products should be considered as a possible (albeit not the major) source of false-positive identifications, especially when searches are performed against a species-specific sequence database. In any case, we recommend adding sequences of most common contaminating proteins (human and sheep keratins, trypsin, etc.) to the database used for searching the tandem mass spectra. Borderline assignments can additionally be validated via *de novo* interpretation of corresponding spectra[16].

**Extraction of peptide digestion products**

Do not discard extracted gel pieces: if, for any reason, the digestion failed, it can be repeated with the same gel pieces using the same enzyme (trypsin) or using another enzyme. Note that, in the latter case, strong peptide background may be encountered because of the digestion of residual intact trypsin.

## ANTICIPATED RESULTS

**Figure 1** shows the results of applying the protocol described here to the analysis of an isolated gel band obtained in the analysis of sumoylation sites[17]. As can be seen in the figure, many peptides were recovered yielding sequence coverage more than 90% by ES MS/MS, including unambiguous identification of the SUMO-1 modification site. The analysis of gel bands for protein identifications at very low amounts (low femtomole) or the characterization of post-translational modifications at somewhat higher amounts (100 fmol to 1 pmol) is feasible with modern MS instrumentation. Furthermore, combined with quantitative proteomics methods[18], all proteins within the dynamic range, sequencing speed and sensitivity constraints of the MS instrumentation used[19] in a complex protein mixture can be analyzed[19]. **Figure 2** shows the results of complex mixture analysis with this protocol. Thousands of peptides are extracted from a gel slice covering about one-tenth of the full protein mass range of a HeLa cell lysate, leading to the identification and quantification of more than 500 proteins in a single slice.

The protocol enables the efficient digestion of proteins fixed within a polyacrylamide gel matrix. Estimated peptide recovery at the picomole protein level is, on average, 70–90% compared to in-solution digests[3,4], but is strongly peptide-dependent. Overall, the benchmarked sensitivity of protein identification from isolated silver-stained bands is in the range of few femtomoles of protein starting material[20], although there is no reason why sensitivities in the attomole range or beyond should not be reachable in complex mixtures using this protocol. The same digestion procedure allows identification of proteins in a variety of ways, such as high throughput mass mapping by MALDI-TOF-MS or highly specific characterization of proteins by LC MS/MS. Similar routine can be applied to prepare gel-separated proteins for the digestion by other enzymes, such as LysC. We note, however, that the efficiency of in-gel digestion drops with increasing the MW of the employed enzyme[21].

**COMPETING INTERESTS STATEMENTS** The authors declare that they have no competing financial interests.

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