

# ZipPlate<sup>®</sup> micro-SPE Plate

## In-Gel Digestion Protocol

### Introduction

In-gel digestion of proteins resolved via 1D or 2D gel electrophoresis is a common technique used in proteomics prior to obtaining protein sequence information by mass spectrometry (MS). The procedure is optimized for polyacrylamide stained gels of a 1–1.5 mm thickness.

### Required Equipment and Materials

See Product Ordering Information for catalogue numbers.

- ZipPlate micro-SPE Plate
- MultiScreen<sup>®</sup> Vacuum Manifold (Millipore catalogue number MAVM 096 0R). Refer to [www.millipore.com/zpmfld](http://www.millipore.com/zpmfld) for vacuum manifold set up instructions.
- 96-well V-bottom polypropylene microtiter collection plate
- Centrifuge equipped with a swing-out microtiter plate rotor, if performing centrifugal elution
- MALDIspot<sup>™</sup> Kit (if performing direct elution onto targets compatible with an Applied Biosystems Voyager-DE Workstation or QSTAR<sup>®</sup> System with oMALDI Source)
- Self-adhesive plastic sealing tape, if running partial plates
- Acetonitrile (ACN)
- Buffer 1: 25 mM ammonium bicarbonate/5% ACN
- Buffer 2: 25 mM ammonium bicarbonate/50% ACN
- Trypsin Resuspension Solution: 1 mM hydrochloric acid
- Trypsin Buffer: 11 µg/mL in 25 mM ammonium bicarbonate
- Promega Sequencing Grade Modified Trypsin: 20 µg/vial for use in preparing Trypsin Enzyme Solution
- Extraction/Wash Solution: 0.2% TFA
- Elution Solution: 50% ACN/0.1% TFA

**NOTE:** For ESI MS, substitute 1–10% formic acid for TFA.

### Trypsin Enzyme Solution Preparation Guidelines

#### Running an Entire Plate at Once

1. Gently tap the trypsin vial on a lab bench to dislodge any powder clinging to the sides of the vial.
2. Add 0.2 mL of 25 mM ammonium bicarbonate to the vial.
3. Mix thoroughly to dissolve the contents.
4. Transfer contents into a 2 mL tube.
5. Add 1.6 mL of 25 mM ammonium bicarbonate. Total volume of solution should be 1.8 mL. This solution must be kept on ice at all times and should be used as quickly as possible after being reconstituted.

#### Running a Partial Plate

1. Gently tap the trypsin vial on a lab bench to dislodge any powder clinging to the sides of the vial.
2. Add 100 µL of 1 mM hydrochloric acid to the vial.
3. Mix thoroughly to dissolve the contents.
4. Dispense twelve 8 µL aliquots into microcentrifuge tubes and store at -20 °C.
5. When ready to use, thaw an aliquot and add 136 µL of 25 mM ammonium bicarbonate.
6. Mix thoroughly. This solution must be kept on ice at all times and should be used immediately.

### Procedure for In-Gel Digestion

Please read the entire protocol before processing your samples. For vacuum manifold set-up instructions, refer to [www.millipore.com/zpmfld](http://www.millipore.com/zpmfld).

#### Protocol Guidelines

- Wear gloves and lab coats during all procedures.
- Use great care when performing all protocol steps to minimize keratin contamination from dust and handling. To minimize keratin contamination, do not touch the bottom of the ZipPlate device.
- 96 samples can be processed simultaneously in the ZipPlate 96 well micro-SPE plate. If fewer wells are being used, cover all unused (dry) wells with adhesive sealing tape to prevent contamination. Do not re-use a well once it's been used to process a sample.
- If running a partial plate, store the ZipPlate device on top of a 96 well microtiter plate. Place this assembly in a self-sealing plastic bag.
- Keep the trypsin cold at all times, especially after reconstitution.
- Use the volume of enzyme solution specified in the protocol to ensure proper digestion and optimal solution composition for resin wetting and subsequent peptide binding.
- Proper operation of ZipPlate device involves two vacuum settings: full vacuum (15–20" Hg) and low vacuum (5–8" Hg).
- Millipore recommends running standard controls as part of each digest procedure.
- The binding capacity of the C18 is typically 4–6 µg/well of an average-sized peptide.
- This kit can also be used with a centrifuge by replacing each vacuum step of the protocol with a one-minute centrifugation at 3000 rpm (1750 × g).

## Digestion Protocol

1. Cut the protein spots (2-D gels, 1–2 mm diameter) or bands (1-D gels, 1 × 3 mm) precisely out of the gel using a scalpel or razor blade; do not include any unstained acrylamide gel. Place the gel pieces into the ZipPlate wells.  
**NOTE:** If some wells are not used, self-adhesive plastic sealing tape may be used to cover them to prevent contamination.
2. Place the ZipPlate device on top of the vacuum manifold system. Adjust for a full vacuum (15–20" Hg) setting by turning the vacuum control valve clockwise to the closed position. Open the vacuum on/off valve and press lightly on the ZipPlate device to initiate vacuum. Apply full vacuum (15–20" Hg) to remove any buffer that may have been transferred into the plate wells along with the gel pieces. Turn off the vacuum.  
**NOTE:** If silver stained gels are to be processed, refer to Millipore Technical Note TN5721EN00.
3. Add 100  $\mu\text{L}$  of Buffer 1 to each well containing a gel piece, replace the plate cover, and incubate for 30 minutes.
4. Remove the plate cover. Apply full vacuum (15–20" Hg). Release the vacuum once all the solution has been emptied from the wells.  
**NOTE:** Occasionally, small “hanging” drops may be observed on the underside of the wells after vacuum removal of solutions. These drops will not affect the results and do not need to be removed until the elution step.
5. Add 100  $\mu\text{L}$  of Buffer 2 to each well containing a gel piece, replace the plate cover, and incubate for 30 minutes.
6. Remove the plate cover and apply full vacuum to draw the solution from the wells. Release the vacuum once all the solution has been emptied from the wells.
7. Repeat steps 5 and 6.  
**NOTE:** Most gel pieces will be destained at the end of this step. Some intense Coomassie blue spots may still be slightly blue.
8. Add 200  $\mu\text{L}$  of 100% acetonitrile to each well containing gel pieces, replace the plate cover, and incubate for 10 minutes.
9. Remove the plate cover and apply full vacuum for 2 minutes to completely remove acetonitrile from the wells. Release the vacuum.
10. Remove the ZipPlate device from the manifold and place it on top of a 96 well microtiter plate.
11. Add 15  $\mu\text{L}$  of the prepared Trypsin Enzyme Solution to each well containing a gel piece. Replace the plate cover.  
**NOTE:** 15  $\mu\text{L}$  of enzyme solution corresponds to 166 ng of trypsin/well. For low abundance proteins, a lower concentration of trypsin may be used provided the volume of solution remains 15  $\mu\text{L}$ .
12. Place the plate assembly into a self-sealing plastic incubation bag or controlled humidity incubator.  
**NOTE:** The use of heating blocks or tiles is not recommended. It may be helpful to place a dish with water in the incubator to prevent gel pieces from drying.
13. Incubate using either of the following conditions:
  - a) 3 hours at 37 °C.
  - b) overnight at 30 °C.**NOTE:** Gel pieces should not be completely dry after incubation.
14. Remove the plate assembly from the incubation bag. Remove the plate cover and pipette 8  $\mu\text{L}$  of 100% acetonitrile directly onto the resin or the bottom of the well. Do not add acetonitrile directly to the gel piece.  
**NOTE:** The addition of acetonitrile wets out the hydrophobic resin. If this step is omitted, peptides will not bind to the resin.
15. Replace the plate cover and place the plate assembly back into the incubation bag. Incubate for 15 minutes at 37 °C.  
**NOTE:** It is not recommended to incubate longer than 15 minutes.
16. Remove the plate cover and add 130  $\mu\text{L}$  of Extraction/Wash Solution to each well containing a gel piece. Replace the plate cover and incubate at room temperature for 30 minutes.  
**NOTE for ESI MS:** Extract with 130  $\mu\text{L}$  of 1.0% formic acid.

## Digestion Protocol, continued

17. Place the ZipPlate device on the vacuum manifold. Adjust for a low vacuum setting by turning the vacuum control valve counter clockwise to the open position. Open the vacuum on/off valve. Lightly press on the plate while slowly turning the vacuum control valve clockwise until the vacuum gauge reads 7–8" Hg. Stop the vacuum after the slowest wells are emptied. If the solution has not been removed after 10 minutes, adjust the manifold to full vacuum and release the vacuum once the solution has been emptied from all the wells.

**NOTE:** Drying peptides on the resin does not affect the recovery.

18. Add 100  $\mu\text{L}$  of Extraction/Wash Solution. Apply full vacuum (15–20" Hg). Release the vacuum once all the solution has been emptied from the wells.

**NOTE for ESI MS:** Wash with 1.0% formic acid.

19. Repeat step 18. Maintain vacuum for 5 additional minutes after all the wells are empty.

**NOTE:** Any drops remaining inside or on the underside of the wells at this point will interfere with peptide recovery. Remove drops by blotting the plate on an absorbent paper.

## Peptide Elution Procedures

There are three elution procedures:

Elution Procedure	Elution Volume
Vacuum	$\geq 20 \mu\text{L}$
Centrifugal	$\geq 5 \mu\text{L}$
MALDIspot	1–2 $\mu\text{L}$

Vacuum elution is not recommended for samples under 1 pmol.

For ESI MS: Substitute elution solution with 1.0% formic acid in 50–60% methanol.

The Centrifugal Elution Protocol and the Vacuum Elution Protocol procedures are outlined in the following sections of this user guide. For the MALDIspot Elution protocol, see Millipore publication number P36519 on Millipore's web site ([www.millipore.com/maldispot](http://www.millipore.com/maldispot)).

## Centrifugal Elution Protocol

Centrifugal Elution is required for elution volumes < 20  $\mu\text{L}$ .

**NOTE:** A loss of approximately 2  $\mu\text{L}$  can be expected due to a combination of evaporation and retention (hold-up volume) within the C18 resin.

1. Remove the ZipPlate micro-SPE plate from the vacuum manifold and blot the bottom. Place the ZipPlate device on top of a 96 well microtiter plate.
2. Dispense 5  $\mu\text{L}$  of Elution Solution directly onto the resin or the bottom of the well. Do not add the solution directly to the gel piece. Replace the plate cover.

**NOTE:** Use of a multichannel pipettor is recommended to rapidly dispense the Elution Solution, minimizing evaporation.

3. Centrifuge for 15 seconds at 3000 rpm (1750  $\times$  g).  
**NOTE:** Balance centrifuge accordingly.
4. Remove the plate assembly from the centrifuge and separate the plates. Cover the 96 well microtiter plate immediately using a ZipPlate cover.
5. Spot the samples from the 96 well microtiter plate onto the MALDI-TOF MS target. Overlay with matrix.  
**NOTE:** The entire sample must be spotted to obtain maximum MS signal.

If full plates are processed it is recommended to elute half of the plate, spot, and then elute the second half of the plate to minimize sample evaporation.

## Vacuum Elution Protocol

A minimum volume of 20  $\mu\text{L}$  is required for vacuum elution.

1. Remove the ZipPlate device from the vacuum manifold.
2. Remove the collar of the vacuum manifold. Place a 96 well microtiter plate into the manifold base. Remove the metal support grid from the collar. Replace the collar on top of the manifold base and place the ZipPlate device on the manifold.  
**NOTE:** Check that alignment tabs have been properly installed to correctly align the receiver plate with the ZipPlate device.
3. Dispense 20  $\mu\text{L}$  Elution Solution into the center of the wells containing gel pieces.
4. Adjust manifold vacuum to 5–7" Hg.
5. Apply vacuum to elute the peptides into a microtiter plate.
6. Spot samples from the 96 well microtiter plate onto the MALDI-TOF MS target. Dry and overlay with matrix.

**NOTE:** The entire sample must be spotted to obtain maximum MS signal. This can be accomplished by spotting, allowing the spots to dry, and overlaying. Repeat this procedure until all of the sample has been transferred.

## Troubleshooting

This section outlines how to troubleshoot poor mass spectra results that may be encountered when using the ZipPlate micro-SPE Plate. Visit [www.millipore.com/zipplate](http://www.millipore.com/zipplate) for an up-to-date listing of troubleshooting procedures and Frequently Asked Questions.

Problem	Possible Causes	Suggestions
Enzyme deactivation	HCl was not added to enzyme prior to aliquoting (if using partial plates).	Refer to Trypsin Solution Preparation Guidelines section of this protocol.
Poor protein retention in gel slice prior to digestion step	Gel slice was macerated upon excision and protein has diffused out of gel during destaining and wash steps.	Do not macerate gel.
Incomplete digestion	Improper digestion conditions	Enzyme was not properly reconstituted. Refer to Trypsin Solution Preparation Guidelines section of this protocol. Incubate gel pieces with trypsin for at least 3 hours at 37 °C. Verify that the pH of the digestion mixture is >6.
Insufficient removal of wash solutions during vacuum filtration steps	Incomplete vacuum seal	Check that vacuum is set properly.  Push down on plate to ensure proper seal. Check that all the wells are cleared before proceeding to subsequent protocol steps. Blot the underdrain of the ZipPlate device prior to the peptide elution step.
Peptides not extracted from gel following digestion	Silver stain procedure used contains fixatives.	Use a silver staining procedure that does not contain fixatives or stain gel with Coomassie Blue, colloidal Coomassie or Sypro® Ruby stains.
Incomplete binding of peptides	For efficient binding of peptides to C18 resin, the acetonitrile concentration should not exceed 5%.  Peptides are passed through the C18 resin too fast.	Check acetonitrile concentration.  Verify that vacuum manifold settings are 7–8 inches Hg during digest binding step.
Incomplete elution of peptides	Peptides are eluted from the C18 resin too fast.  Concentration of acetonitrile for elution is too low.	Verify that peptides are eluted at 5–7 inches Hg.  Add extra acetonitrile to the Elution Solution. Up to 70% acetonitrile may be necessary to elute all the peptides.

## Product Ordering Information

Description	Qty/Pack	Catalogue No.
ZipPlate micro-SPE plate	10	ZPC1 800 10
Montage® In-Gel Digest <sub>zp</sub> Kit	1	LSKG DZP 96
MALDIspot Kit	1	LSKZ PMS 96
MultiScreen Vacuum Manifold	1	MAVM 096 0R
Vacuum Pump (220 V)	1	WP61 220 50
Vacuum Pump (110 V)	1	WP61 115 60
Millex®-FG <sub>50</sub> Filter Unit, for vacuum line protection	10	SLFG 050 10
Stopper, No. 8, perforated, silicone	1	XX10 047 08 1
Vacuum Filtering Flask, 1L	1	XX10 047 05 1
Tubing, for vacuum use; silicone, 3/16" I.D., 1.4 m	1	XX71 000 04 1
Promega Sequencing Grade Modified Trypsin, 20 µg/vial	1	Promega V5111
V-bottom Polypropylene Microtiter Collection Plate	10	Greiner 651201
Self-adhesive Plastic Sealing Tape	100	Marsh Bio Products SP100

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