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PROTEOMICS

Montage In-Gel Digest$_{96}$ Kit User Guide

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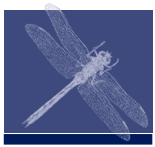
P36379, Rev. –, 09/01

This kit was developed in partnership with

Contents



Introduction

In-gel digestion of proteins resolved via 1D or 2D gel electrophoresis is a common technique used in Proteomics prior to obtaining protein structural information by Mass Spectrometry (MS).

The In-Gel Digest$_{96}$ Kit provides a fast, convenient and reproducible method to digest and purify up to 96 polyacrylamide gel slices simultaneously for MS analysis. This protocol is adapted from published protocols (see Parker, et al., [1998] Electrophoresis 19: 1920–1932). The procedure is optimized for Coomassie™ blue, Colloidal Coomassie, or SYPRO® Ruby SDS polyacrylamide stained gels of a 1–1.5 mm thickness.

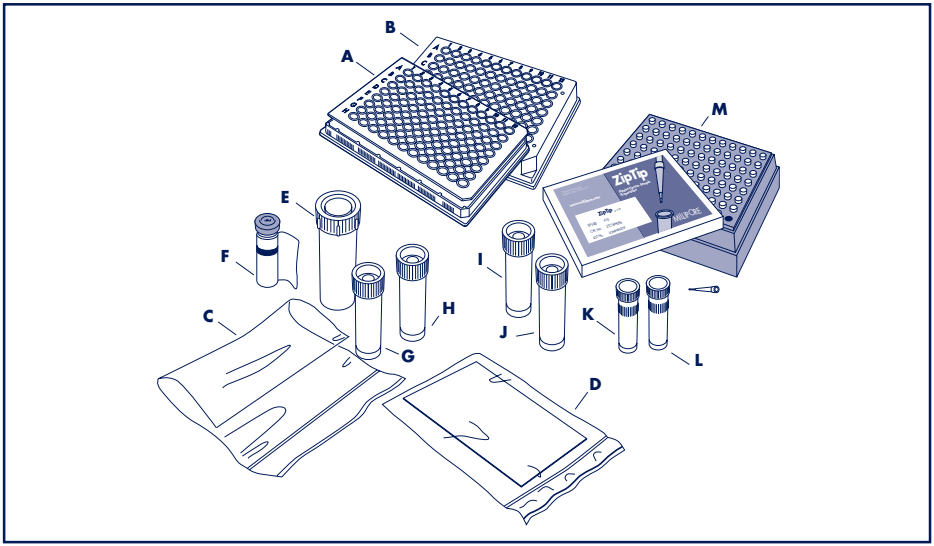
Millipore Corporation has developed two versions of the In-Gel Digest$_{96}$ Kit in partnership with Proteome Systems Incorporated:

- Millipore Cat. No. LSKG DZT 96 —
  In-Gel Digest$_{96}$ Kit **with** ZipTip® Purification and Concentration
- Millipore Cat. No. LSKG D00 96 —
  In-Gel Digest$_{96}$ Kit **without** ZipTip Purification and Concentration

Introduction, continued

NOTE: Silver stained gels will require optimization including an additional destaining step before digestion. The recovery from nondestructive silver stained gels has been shown to be significantly lower than Coomassie blue or SYPRO Ruby stained gels (see Lopez, et al., [2000] Electrophoresis 21:3673–3683).

Kit Components



Letter	Part	Function
A	MultiScreen$_{96}$ plate	Retains gel piece
B	V-bottom collection plate	Holds extracted digest
C	Incubation bag	Reduces evaporation during digestion

Kit Components, continued

Letter	Part	Function
D	Sealing tape	Blocks unused MultiScreen$_{96}$ plate wells, ensuring proper vacuum for wells in use
E	Destaining Solution	Removes stain
F	Trypsin Enzyme	Protein digestion enzyme
G	Trypsin Resuspension Solution	Dissolves trypsin powder
H	Extraction Solution	Recovers digest from gel piece
I	ZipTip Wetting Solution (LSKG DZT 96 only)	Conditions ZipTip pipette tip for use
J	ZipTip Washing Solution (LSKG DZT 96 only)	Removes contaminants
K	ZipTip Elution Solution (LSKG DZT 96 only)	Recovers peptides from ZipTip pipette tip
L	MALDI-TOF Matrix (LSKG DZT 96 only)	
M	ZipTip pipette tips (LSKG DZT 96 only)	Purifies and concentrates peptides

Additional Equipment Required

In order to perform the protocol, obtain the following equipment not included in the kit:

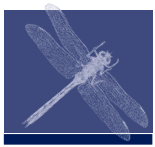
- MultiScreen Vacuum Manifold (Millipore Cat. No. MAVM 096 0R)
- Forceps (Millipore Cat. No. XX62 000 06)
- Vacuum Pump (Millipore Cat. No. XX55 000 00)
- Millex® FG_{50} Vacuum Pump Filter Guard (Millipore Cat. No. SLFG 050 10)

Storage Conditions

All solutions, once prepared, should be stored at 2–8 °C and used within two weeks, with the exception of:

- Trypsin Resuspension Solution (Vial 3) — Use immediately or aliquot in 1mMHCL and store at -20 °C for a maximum of 2 weeks. Once thawed, do not refreeze this solution.
- MALDI-TOF Matrix Solutions (Vial 8) — Use immediately or aliquot and store at -20 °C for a maximum of two weeks. Once thawed, do not refreeze this solution.

Refer to the “Preparation of Solutions” section for more information.



Procedure for In-Gel Digestion

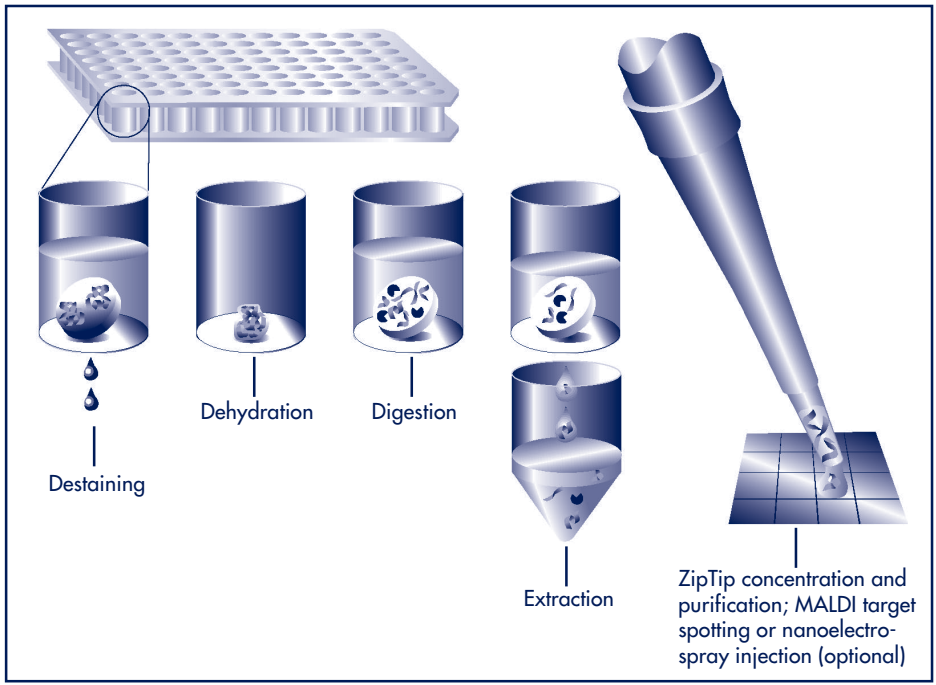
Please read the entire protocol before processing your samples.

Protocol steps include:

- Excision of the gel piece
- Destaining
- Dehydration
- Digestion
- Extraction
- Evaporation and re-suspension
- ZipTip adsorption (optional)
- MALDI target spotting of purified, concentrated digest in presence of matrix (optional)

NOTE: This protocol does not contain reduction and alkylation steps. Most 1D and 2D gel electrophoresis protocols include either reduction or reduction/alkylation of the sample prior to running the gel.

Overview of Procedure



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 Multi-Screen plate (referred to as the underdrain).
- Keep the trypsin cold at all times, especially after being reconstituted.
- If the kit is to be used multiple times (all 96 wells are not used at once), the trypsin enzyme should be resuspended in 300 µL of 1 mM HCL instead of the trypsin resuspension solution provided in the kit. Once the enzyme is dissolved, prepare 100 µL aliquots of the enzyme solution and store at -20 °C for a maximum of two weeks. Refer to the "Preparation of Solutions" section for more information.
- A trypsin autodigestion calibration peak is usually observed at 842 daltons. The 2211 daltons peak seen with other trypsin sources is not normally observed with the trypsin included in this kit.
- Take care when collecting the extracted peptides from the MultiScreen plate. The vacuum manifold must be adjusted to 4–6" Hg vacuum prior to using to ensure the quantitative collection of the peptides into the V-bottom plate for subsequent analysis.

Preparation of Solutions

All reagents are supplied with the exception of acetonitrile, hydrochloric acid, and water, which should be supplied by the user. A high purity HPLC grade of acetonitrile and Milli-Q® grade water or equivalent are recommended for use with this kit.

NOTE: All solutions, once prepared, should be stored at 4 ºC and used within two weeks with the exception of the Trypsin (Vial 3) and MALDI-TOF Matrix (Vial 8) solutions, which should be used immediately.

CAUTION: TFA is highly corrosive and volatile. Prepare TFA solutions in a fume hood.

Destaining Solution - Vial 1 (acetonitrile/ammonium bicarbonate)

1. Add 20 mL of water to ammonium bicarbonate solid in Vial 1.
2. Add an additional 20 mL of acetonitrile.
3. Mix to dissolve contents.

Trypsin Resuspension Solution - Vial 2 (ammonium bicarbonate)

1. Add 6 mL water to ammonium bicarbonate solid in Vial 2.
2. Mix by vortexing to dissolve contents.

Trypsin Enzyme - Vial 3 (porcine trypsin in ammonium bicarbonate)

1. Gently tap Vial 3 on a lab bench to dislodge any powder clinging to the sides of the vial so that all powder collects at the bottom of the vial.
2. Add 1 mL of trypsin resuspension solution from Vial 2 to trypsin enzyme in Vial 3.
3. Mix thoroughly to dissolve contents.
4. Transfer entire contents of Vial 3 into Vial 2. This solution must be kept at 4 °C or on ice at all times and should be used as quickly as possible after being reconstituted. Use immediately.
5. Discard Vial 3.

For multiple samples

If 96 samples are not run simultaneously and the kit is to be used on different days, the trypsin must be dissolved in an inactive form prior to aliquoting and freezing (storage). Follow the steps below to ensure proper stability of the enzyme.

1. Add 300 µL of 1 mM HCL (not included in the kit) to Vial 3.
2. Mix thoroughly to dissolve contents.
3. Prepare three 100 µL aliquots in 2 mL vials and store at -20 °C for a maximum of two weeks.
4. When ready to use, thaw aliquot and add 1.9 mL of trypsin resuspension solution from Vial 2.
5. Mix thoroughly. This solution must be kept at 4 °C or on ice at all times and should be used immediately.

Extraction Solution - Vial 4 (acetonitrile/TFA)

1. Add 2.5 mL of water to TFA solution in Vial 4.
2. Add 5.0 mL of acetonitrile to TFA solution in Vial 4.
3. Mix contents.

NOTE: The In-Gel Digest$_{96}$ Kit without ZipTip pipette tips will NOT contain Vials 5–8.

ZipTip Wetting Solution - Vial 5 (acetonitrile in water)

1. Dispense 4 mL of water into empty Vial 5.
2. Add 4 mL of acetonitrile to Vial 5.
3. Mix contents.

ZipTip Washing Solution - Vial 6 (TFA in water)

The solution in Vial 6 is ready to use.

ZipTip Elution Solution - Vial 7 (acetonitrile/TFA)

1. Add 1.0 mL of acetonitrile to TFA solution in Vial 7.
2. Mix contents.

MALDI-TOF Matrix - Vial 8 (α-Cyano-4-hydroxycinnamic acid in acetonitrile/TFA)

1. Add ZipTip elution solution in Vial 7 to α-Cyano-4-hydroxycinnamic acid (CHCA) solid in Vial 8.
2. Invert several times to dissolve CHCA. Allow solution to sit at room temperature for at least 10 minutes.

NOTE: The CHCA crystals may appear yellow and may not dissolve completely. Neither of these qualities will affect performance. Use immediately or store at -20 °C for a maximum of two weeks (upon thawing, use immediately).

Preparation of MultiScreen Vacuum Manifold

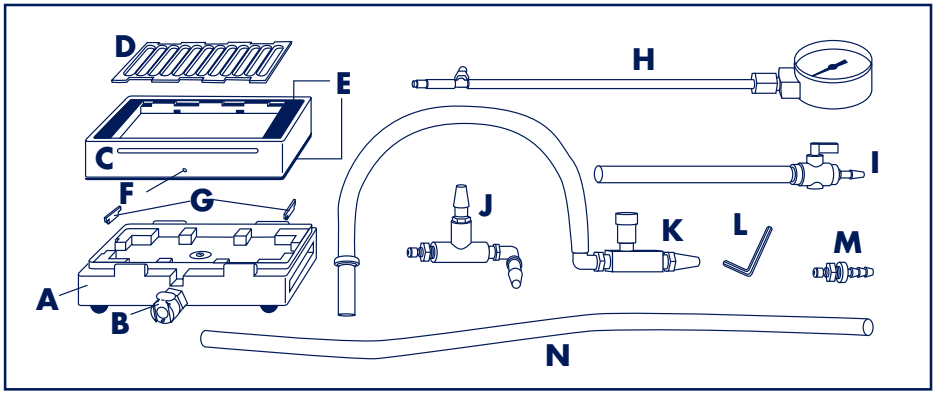
Before using the MultiScreen manifold, unpack the components and set them up according to the procedures outlined in this section.

Unpacking the Vacuum Manifold

Unpack and ensure that you have the following parts of the vacuum manifold:

- Vacuum manifold base with quick disconnect body
- Ring assembly with gaskets, bleeder valve, and support grid
- Straight connector with quick disconnect coupling insert
- 3-way connector with quick disconnect coupling insert
- Vacuum ON/OFF valve with tubing
- Vacuum control valve with tubing
- Vacuum pressure gauge with tubing
- FEP-lined PVC tubing
- Plate alignment tabs
- Hex key wrench

Parts and Functions of the Vacuum Manifold



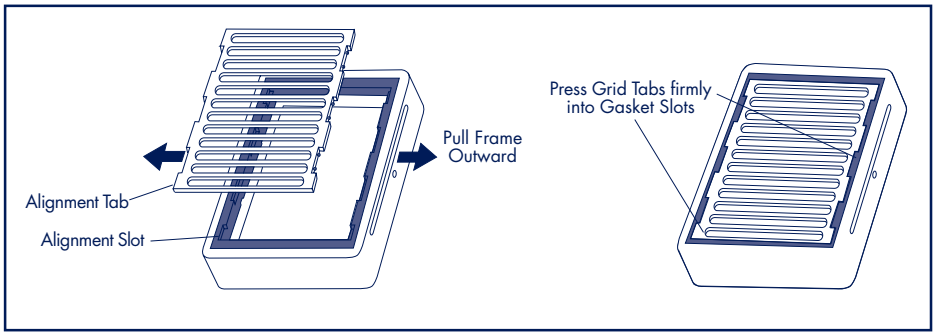
Letter	Part	Function
A	Vacuum Manifold Base	Supports standard or deep well ring.
B	Quick Disconnect Body	Allows straight or 3-way connector to be attached to manifold base.
C	Plastic Ring, standard well	Holds gaskets and manifold support grid above the manifold basin to allow the use of standard receiver plates.
D	Manifold Support Grid	Supports plate during filtration. Must be used.
E	Vacuum Manifold Gaskets	Seal manifold support grid and ring to prevent leakage.
F	Bleeder Valve	Fits into ring when pressure gauge is not in use.

Parts and Functions of the Vacuum Manifold, continued

Letter	Part	Function
G	Plate Alignment Tabs	Allows the correct alignment of undersized receiver plates.
H	Vacuum Pressure Gauge	Measures vacuum pressure in plenum.
I	On/Off Valve	Opens or closes valve to vacuum source.
J	Three-Way Connector	Replaces straight connector to allow use of both the on/off valve and the vacuum control valve.
K	Vacuum Control Valve	Controls amount of vacuum pressure.
L	Hex Key	Removes or replaces the bleeder valve.
M	Straight Connector	Connects manifold to vacuum source using the on/off valve and tubing.
N	FEP-lined PVC Tubing, 1/4" I.D.	Connects assemblies to vacuum pressure pump or vacuum source.

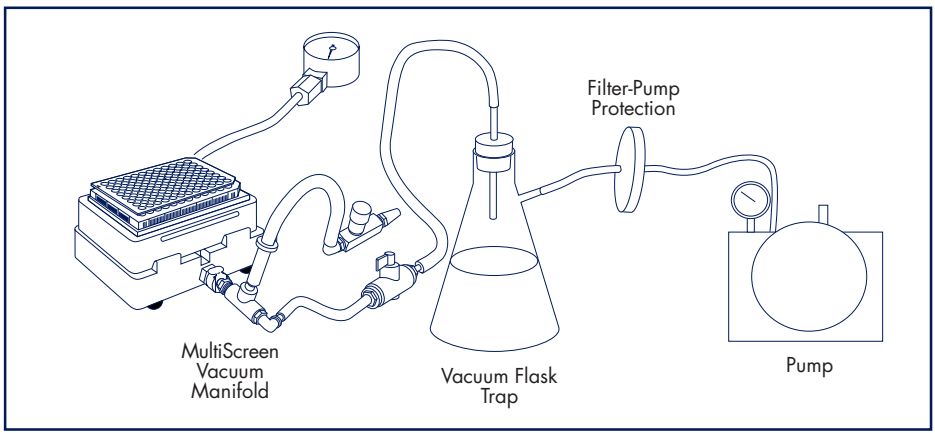
Installing the Support Grid

The stainless steel support grid provides both alignment and support for the MultiScreen plate during vacuum filtration procedures. The vacuum manifold kit includes the support grid already assembled in the ring. However, if the grid becomes dislodged, you must install it in the gasket. The support grid and the top gasket have complementary tabs/slots that align the two parts properly. In addition, the grid has an "up side" label stamped on one side. When correctly oriented, this label should be facing the operator when looking down at the assembled manifold. Pull out on the sides of the ring while simultaneously pressing down on the grid. When properly installed, the grid and gasket mate tightly, and remain in place during routine procedures.



Assembling the Vacuum Manifold

1. Push the coupling insert on the end of the three-way connector into the quick disconnect body on the side of the manifold base until it clicks.
2. Push the end of the ON/OFF valve tubing as far as it will go onto the end fitting of the three-way connector.
3. Push the end of the vacuum control valve tubing as far as it will go onto the top fitting of the three-way connector.

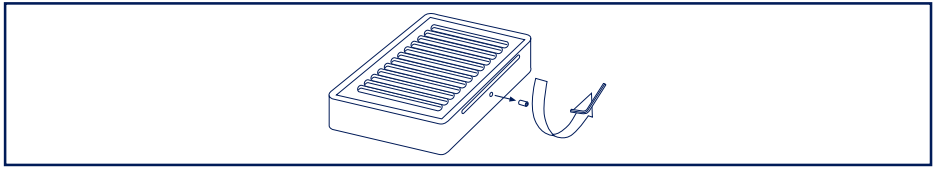


4. Place the ring assembly onto the top of the manifold base.

Installing the Vacuum Pressure Gauge

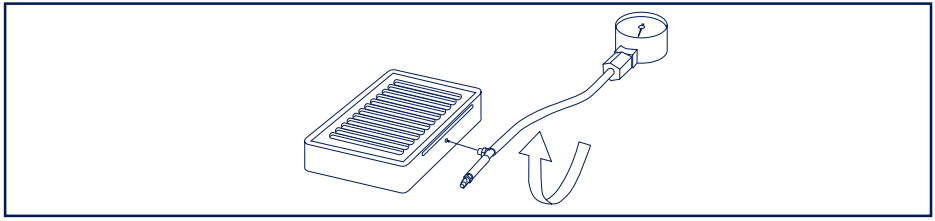
1. Remove the bleeder valve from the side of the ring using the hex key wrench provided.

   NOTE: Do not lose the bleeder valve because the manifold will not operate correctly without it when the vacuum pressure gauge is removed.



2. Attach the pressure gauge by screwing its connector into the ring. Use the connector that projects perpendicular from the pressure gauge tube. The built-in vacuum release valve at the end of the tube will hiss during operation.

   NOTE: Do not overtighten the connection. No more than four rotations are required.



Configuring the MultiScreen Vacuum Manifold System

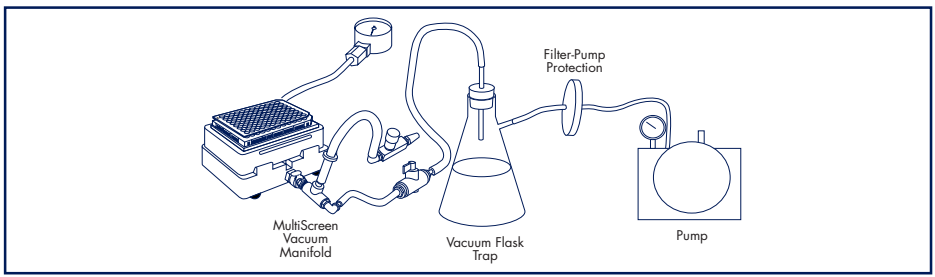
1. Place the vacuum manifold on a lab bench in a stable area unaffected by vibrations from the pump (or any type of shaker).

   NOTE: Do not place vacuum pump on the same surface as the manifold.

2. Connect your laboratory vacuum source to the vacuum manifold using the tubing provided.

   NOTE: Allow plenty of room to set up the system to avoid crimping the tubing, which would reduce air flow. Crimping can also cause the lining of the tubing to crack, leading to the loss of solvent resistance. If your tubing does crimp, cut it off below the crimp and reconnect.

3. Place a Millex-FG_{50} filter and a vacuum flask trap in the vacuum line to protect the vacuum source from contamination. Your configuration should look like this:

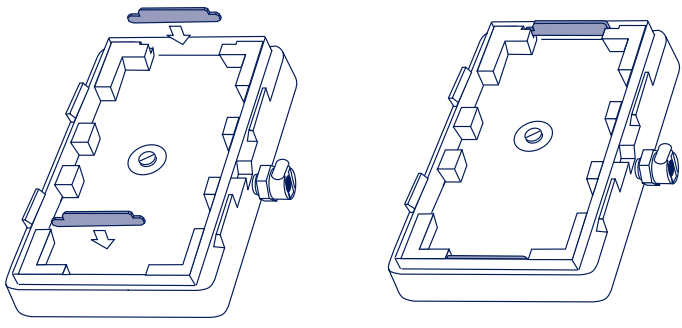


Aligning Collection Plates

The MultiScreen vacuum manifold comes with two plate alignment tabs that will need to be installed. These tabs align the wells of the filtration plate with the wells of the collection plate. Install the plate alignment tabs as follows:

1. Remove the two alignment tabs from the shipping bag.
2. Position the tabs with the shorter sides pointing up.

3. Insert the tabs in the manifold base by gently pressing them into the tab slots.



Digestion Protocol

NOTE: The vacuum manifold pressure should be adjusted to 4–6" Hg.

1. Cut the protein spots or bands (2–3 mm diameter) precisely out of the gel using a scalpel or razor blade; do not include any unstained acrylamide gel. Using unserrated flat tipped forceps, place the gel pieces into the wells of the MultiScreen plate.

   NOTE: If some wells are not used, the self-adhesive plastic sealing tape (supplied in kit) should be used to cover them to ensure adequate vacuum generation.

   CAUTION: To avoid keratin contamination, do not touch the bottom of the MultiScreen plate (known as the underdrain). The underdrain of the MultiScreen plate should not be removed.

2. Place the MultiScreen plate on top of the vacuum manifold system.
3. Add 100 µL of Destaining Solution from Vial 1 to each well containing a gel piece, replace the plate cover, and incubate for 20 minutes.
4. Remove plate cover and apply vacuum to draw the Destaining Solution from the wells. Firmly press on the MultiScreen plate to initiate vacuum. Release the vacuum once all the solution has been emptied from the wells.
5. Repeat steps 3 and 4, three times.
6. Add 200 µL of 100% acetonitrile (not provided in the kit) to each well containing gel pieces, replace the plate cover, and incubate for 10 minutes.

Digestion Protocol, continued

7. Remove plate cover and apply vacuum for 60 seconds to draw the acetonitrile from the wells. Release the vacuum.

   NOTE: At this point, the membrane filters in the MultiScreen plate will revert to an opaque white color and the gel piece is reduced in size.

8. Remove the MultiScreen plate from the manifold and place it on top of the supplied 96 well V-bottom polypropylene collection plate.
9. Add 50 µL of the prepared Trypsin Solution from Vial 2 (or from resuspended aliquot vial) to each well containing a gel piece. Replace the plate cover.
10. Place the plate assembly into the supplied self-sealing plastic bag.
11. Incubate using either of the following conditions:
    a) 6 hours at 37 °C
    b) overnight at 30 °C.

Extraction Protocol

1. Remove the plastic ring from the top of the vacuum manifold and place the collection plate into the bottom of the manifold. Replace the plastic ring and center the MultiScreen plate on the manifold support grid.
2. Add 50 µL of Extraction Solution from Vial 4 to each well containing a gel piece.
3. Replace the plate cover and incubate for 30 minutes.
4. Remove the plate cover and apply vacuum by firmly pressing down on the MultiScreen plate to initiate transfer of the digest to the collection plate.

NOTE: If using the kit containing ZipTip pipette tips, continue to the next section. If not using ZipTip pipette tips and a reduction of sample volume is desired, insert the collection plate into a suitable centrifugal vacuum concentrator containing a microtiter plate rotor (SpeedVac™ or equivalent) to dry down the extracted peptide solution.

ZipTip$_{\mu\text{-C18}}$ Purification and Concentration

NOTE: At this point, a sample volume reduction to < 5 µL is required.

1. Insert the collection plate containing the extracted peptides into a suitable centrifugal vacuum concentrator containing a microtiter plate rotor (SpeedVac or equivalent) to dry down the extracted peptide solution to < 5 µL.
2. Resuspend the peptides by adding 10 µL of ZipTip Washing Solution from Vial 6 to each well in the collection plate containing digest.
3. Attach a ZipTip$_{\mu\text{-C18}}$ pipette tip to a 10 µL pipettor and set the volume to 10 µL.
4. Prewet the tip by aspirating ZipTip Wetting Solution from Vial 5. Dispense to waste. Repeat two times.
5. Equilibrate the ZipTip pipette tip by aspirating ZipTip Washing Solution in Vial 6. Dispense to waste. Repeat two times.
6. Bind peptides by aspirating and dispensing (cycling) the resuspended digest through the tip five times.
7. Wash the tip by aspirating ZipTip Washing Solution from Vial 6. Dispense to waste. Repeat two times.

For Direct Spotting onto a MALDI-TOF MS Target

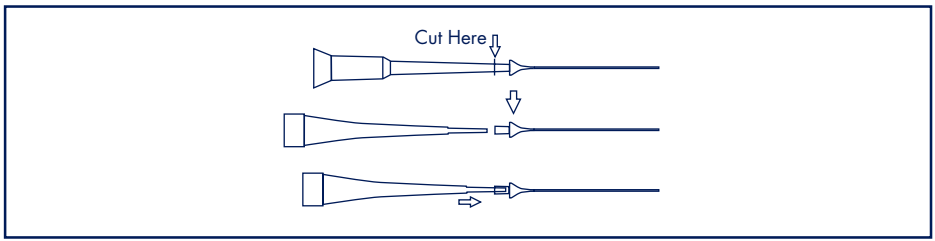
8. Elute the purified peptide digest by aspirating 2 µL of MALDI matrix from Vial 8 into the ZipTip pipette tip. Dispense directly onto MALDI plate. For optimal results, draw the eluted peptide/matrix elution solution up and down through the tip two or three times directly on the MALDI target prior to a final dispense.
9. Allow spots to dry and acquire spectra.

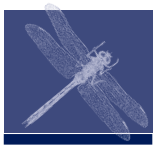
For Nanoelectrospray

Sample can be eluted from the ZipTip pipette tip by cycling 1–4 µL of 50% methanol in either 0.1% acetic acid or 0.1% formic acid/water through the tip. The sample can be eluted directly into the nanospray needle using a GELoader™ tip (Eppendorf cat. no. 0030 001 222) and following the procedure below:

1. Cut the GELoader tip about 2–3 mm above where the tip is fused to its capillary end.
2. Before the final dispense, firmly press the cut-down GELoader tip onto the ZipTip pipette tip with a slight twisting motion.

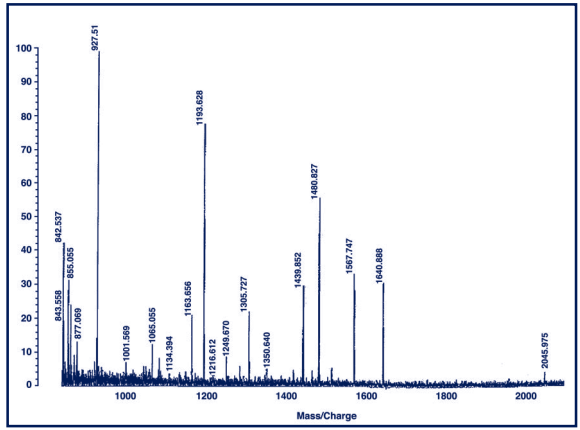
The leak-free fit allows elution directly into a nanospray needle.



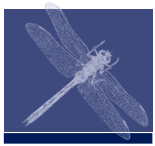


Product Performance

The following MS Spectra demonstrates typical results when performing in-gel digestion using the Montage In-Gel Digest$_{96}$ Kit.



Bovine Serum Albumin Digestion. *250 fmole of purified Bovine Serum Albumin (BSA) was loaded on a 10% Tris-Tricine/SDS precast gel and stained with colloidal Coomassie blue. The protein band was excised and processed with the Montage In-Gel Digest$_{96}$ Kit. The sample was then analyzed on a Kratos Axima-CFR MALDI-TOF MS in reflectron mode. Subsequent database search of the resulting peptides using ProFound correctly identified the protein with 21% coverage. Each lot of In-Gel Digest$_{96}$ Kits are subjected to this quality control test, ensuring sensitivity and reproducibility. Data courtesy of Alla Bogdanova, Proteome Systems, Inc.*



Troubleshooting

This section outlines how to troubleshoot poor mass spectra results that may be encountered when using the Montage In-Gel Digest$_{96}$ Kit.

Visit www.millipore.com/montage for an up-to-date listing of troubleshooting procedures and Frequently Asked Questions.

Problem	Possible Causes	Suggestions
Degradation of chemicals	Chemicals not stored at specified conditions	Refer to Storage Conditions section of manual for proper storage conditions
Enzyme deactivation	HCL was not added to enzyme prior to aliquoting (pertains to users that are using the kit multiple times)	Refer to Preparation of Solutions section of the manual
Low protein concentration	Protein concentration in the gel piece is below the detection level of the kit	Digest gel pieces with >250 fmole of protein loaded on the electrophoresis gel
Low peptide concentration	Digest sample is too dilute	Use ZipTip to purify and concentrate

Troubleshooting, continued

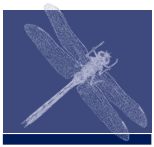
Problem	Possible Causes	Suggestions
Poor protein retention in gel slice prior to digestion step	Gel slice was macerated upon excision and protein has diffused out of gel during destaining and wash steps	Do not macerate gel
Incomplete digestion	Sample was not adequately alkylated and reduced prior to digestion procedure	Alkylate and reduce sample prior to running gel
Insufficient removal of wash solutions during vacuum filtration steps	Incomplete vacuum seal	■ Check that vacuum is set to 4-6" Hg. ■ Push down on plate to ensure proper seal ■ Ensure that sealing tape is covering unused wells of plate if 96 samples are not being run simultaneously
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

Troubleshooting, continued

Problem	Possible Causes	Suggestions
Incomplete ZipTip binding of peptides	C18 beads dewetted before sample was applied. The hydrophobic beads can de-wet in less than a minute.	After wetting with ACN, flush the tip with 0.1% TFA and leave the plug immersed in liquid until immediately before sample binding.
	Sample was not sufficiently acidified with TFA. The pH should be below 4.	Spike sample with a few microliters of 0.5-1% TFA.
	Sample not freely soluble	Add Guanidine HCl to the sample to achieve a final concentration between 1-4M. Guanidine actually enhances binding by helping to wet the hydrophobic surface and reducing polypeptide secondary structure.

Troubleshooting, continued

Problem	Possible Causes	Suggestions
Incomplete elution of peptides from ZipTip	Sample tenaciously adsorbed to the C18 particles.	Increase acetonitrile content of desorption solution to a maximum of 75–90% ACN (v/v) in 0.1% TFA.
	Sample not freely soluble in ACN.	Decrease ACN concentration to 20–40% in a 0.1% TFA or suitable ion-pairing agent.

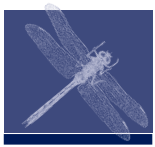


Product Specifications

In-Gel Digest$_{96}$ Kit with ZipTip pipette tips:
Generation of peptide fragment coverage, from 250 fmole of BSA loaded and separated on a 1D SDS polyacrylamide gel, enabling protein identification following MALDI-TOF MS (Reflectron mode) analysis.

Trypsin, Proteomics Sequencing Grade:
Porcine Trypsin purified and lysine residues modified by reductive alkylation to produce a stable product, resistant to autolysis. In addition, it has been TPCK treated to remove chymotryptic activity. For more details, see Sigma® Technical Bulletin.

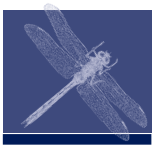
ZipTip$_{\mu\text{-C18}}$ pipette tips:
Pipette tip (material of construction): polypropylene
Media: C18 spherical silica, 15 µm, 200 Å pore size
Tip capacity: 10 µL
Adsorption bed: 0.2 µL
Length: 31 mm
Capacity (when used with saturating amounts of analyte): 2.0 µg
Working pH range: 2 to 13



Ordering Information

This section lists catalogue numbers for the Montage In-Gel Digest$_{96}$ Kit. See "Technical Assistance" for information about contacting Millipore. You can also buy Millipore products on-line at www.millipore.com/purecommerce.

Product	Catalogue No.	Samples/Kit
Montage In-Gel Digest$_{96}$ Kit **with** ZipTip pipette tips	LSKG DZT 96	96/kit
Montage In-Gel Digest$_{96}$ Kit **without** ZipTip pipette tips	LSKG D00 96	96/kit



Technical Assistance

For more information, contact the Millipore office nearest you. In the U.S., call **1-800-MILLIPORE** (1-800-645-5476). Outside the U.S., see your Millipore laboratory catalogue for the phone number of the office nearest you. Or, visit our web site at www.millipore.com/techservice.

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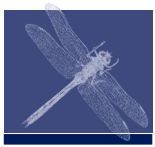

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