TECHNICAL MANUAL

Rapid Digestion—Trypsin and Rapid Digestion—Trypsin/Lys-C Kits

Instructions for Use of Products
VA1060 and VA1061

Revised 11/17
TM500

Rapid Digestion—Trypsin and Rapid Digestion—Trypsin/Lys-C Kits

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Rapid Digestion—Trypsin and Rapid Digestion—Trypsin/Lys-C Kits are designed to shorten protein digestion times to 60 minutes versus the typical 4–18 hours. Both kits contain three components: i) Protease (Rapid Trypsin or Rapid Trypsin/Lys-C Mix); ii) Resuspension Buffer, and; iii) Rapid Digest Buffer optimized for rapid digestion.

Protein digestion with these kits follows a simple-to-use protocol that is both fast and efficient. The protocol is flexible, can accommodate a large range of sample volumes and protein concentrations and requires no special laboratory equipment or off-line desalting. The entire sample preparation procedure is performed in less than 60 minutes. A general schematic for this workflow is shown in Figure 1.

1. Description (continued)

While many proteases are used in bottom-up mass spectrometric (MS) analysis (1–3), trypsin (4,5) is the de facto protease of choice for most applications. There are several reasons for this: Trypsin is highly efficient, active and specific. Tryptic peptides produced after proteolysis are ideally suited, in terms of both size (350–1,600 Daltons) and charge (+2 to +4), for MS analysis. For more than two decades, trypsin has found a large number of applications in bottom-up proteomics workflows involving in-gel digestion (6), analysis of samples from immunoprecipitation and affinity enrichment (7,8), large-scale fractionation studies (9,10), peptide mapping of biopharmaceuticals (11), chemoproteomics (12,13) and studies of drug metabolism (14). Trypsin is also used for quantitative applications, particularly those involving biomarkers (15–17). Alternative proteases certainly complement proteomics analysis; however, the majority of proteomics studies begin with digestion using trypsin.

One significant drawback to trypsin digestion is the long sample preparation times, which typically range from 4 hours to overnight for most protocols. Achieving efficient digestion usually requires that protein substrates first be unfolded either with surfactants or denaturants such as urea or guanidine. These chemical additives can have negative effects, including protein modification, inhibition of trypsin or incompatibility with downstream LC-MS/MS. Accordingly, additional steps are typically required to remove these compounds prior to analysis.

In an effort to shorten the time required to prepare samples for LC-MS/MS analysis, we have developed a specialized trypsin preparation that supports rapid and efficient digestion at temperatures as high as 70°C. There are several benefits to this approach. First, proteolytic reaction times are dramatically shortened. Second, because no chemical denaturants have been added, off-line sample cleanup is not necessary, leading to shorter preparation times and diminished sample losses.

The Rapid Digestion Kits are highly flexible. They can accommodate a variety of additives including reducing and alkylating agents. There are no restrictions on sample volume or substrate concentrations with these kits. Furthermore, the protocol is simple to follow and requires no laboratory equipment beyond a heat block. Digestion is achieved completely using an in-solution approach, and since the enzyme is not immobilized on beads, the protocol does not have strict requirements for rapid shaking and off-line filtering to remove beads.

In addition to the benefits of this flexibility, we also developed a Rapid Digestion—Trypsin/Lys-C mixture. Like the Trypsin/Lys-C Mix previously developed to prepare maximally efficiently proteolytic digests, particularly for complex mixtures, Rapid Digestion—Trypsin/Lys C is ideally suited for studies that require improved reproducibility across samples.

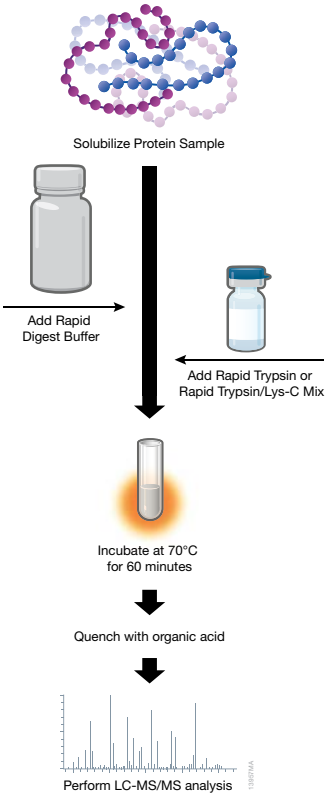

Figure 1. The Rapid Digestion Kit workflow. After solubilizing the protein substrate, add Rapid Digest Buffer (add 3X the protein substrate sample volume) along with enzyme (Rapid Trypsin or Rapid Trypsin/Lys-C). Heat at 70°C (typically ~60 minutes) to rapidly digest the protein substrate. Quench the digest with organic acid (i.e., neat formic acid) then perform direct LC-MS/MS analysis.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Rapid Digestion Kit–Trypsin	**1 each**	**VA1060**

The system contains sufficient reagents to perform up to 100 reactions. Includes:

- 30ml Rapid Digest Buffer
- 0.5ml Resuspension Buffer
- 100µg Rapid Trypsin Gold, MS Grade

PRODUCT	SIZE	CAT.#
Rapid Digestion Kit–Trypsin/Lys-C	**1 each**	**VA1061**

The system contains sufficient reagents to perform up to 100 reactions. Includes:

- 30ml Rapid Digest Buffer
- 0.5ml Resuspension Buffer
- 100µg Rapid Trypsin/Lys-C Mix, MS Grade

Storage Conditions: Store the Rapid Trypsin protease, Rapid Trypsin Lys-C protease, Resuspension Buffer and Rapid Digest Buffer at –10°C to –30°C. After reconstitution of the protease in Resuspension Buffer, store at –70°C for up to 1 month. Minimize freeze-thaw cycles of all reagents.

Note: Resuspension Buffer is 50mM acetic acid. The low pH of this buffer reversibly inhibits proteases, minimizing proteolysis during storage.

3. Protein Preparation Before Digestion

We recommend the use of protein samples (50µl–1ml) resuspended in pure water, mildly-buffered solutions (Tris buffers at a concentration of 25mM or lower, pH 7.0–8.0), or those that have been eluted following affinity purification (with organic acid solutions like 0.1% TFA) for best compatibility with the Rapid Trypsin Digestion workflow (see Figure 1). Dissolve samples in the appropriate buffer, then perform the digestion protocol. Protein concentrations should range from 0.1–1mg/ml.

Avoid the use of phosphate or carbonate buffers.

Materials to Be Supplied by the User

- NANOpure® (or equivalent grade) water
- formic or trifluoroacetic acid (neat)
- TCEP (if performing reduction/alkylation)
- iodoacetamide (if performing reduction/alkylation)
- Axygen Maximal recovery tubes (Axygen Cat.# MCT-060-L-C)

4. Digestion Protocols

The following protocols are meant to produce protein digests, typically within 30–60 minutes. Some samples, such as complex mixtures or substrates, which might be more difficult to digest (i.e., disulfide rich or membrane proteins) may take longer (usually no longer than 3 hours). We recommend, when optimizing the protocol, to perform a timecourse to identify optimized conditions. Titration with several enzyme/substrate (E/S) ratios is also beneficial.

While reduction and alkylation is often needed for applications like peptide mapping, where all of the protein needs to be characterized, many applications like quantitative analysis of complex mixtures or biomarker studies don't always require reduction or alkylation. Because the samples are heated (and therefore denatured), the protocols do not require (or recommend) denaturants. Here we provide protocols for proteolysis with and without reducing/alkylating agents.

4.A. Proteolysis without Reduction and Alkylation

1. Set a water bath, oven or heat block (such as an Eppendorf Thermomixer®) to 70°C.
2. Add 3X volume of Rapid Digest Buffer. For example, to 50µl of protein substrate add 150µl of Rapid Digest Buffer.
3. Resuspend 100µg of the protease (Rapid Trypsin or Rapid Trypsin/Lys-C Mix) in 100µl of Resuspension Buffer to make a concentration of 1mg/ml. Store the resuspended material on ice until ready for use.
4. Add enzyme in a 1:10 E/S ratio. For example, add 2µl of the resuspended Rapid Trypsin (2µg of protease for every 20µg of substrate for Rapid Trypsin) or 4µl of the resuspended Rapid Trypsin/Lys-C Mix (4µg of the Rapid Trypsin/Lys-C for every 20µg of substrate). **Note:** The E/S ratio can be optimized to suit specific assays. We recommend, if optimization is required, that you titrate this ratio. For more information see Section 6., Troubleshooting.
5. Incubate for up to 60 minutes at 70°C.

   Note: Increase digestion time to as long as 3 hours for more difficult-to-digest proteins or complex samples
   Optional: Shake at 450–600rpm on a Thermomixer®.
6. Terminate the reaction with 5µl of formic acid for every 200µl of digestion reaction. The final concentration of the organic acid should be 0.1–2%.
7. Freeze the samples at –20°C or below or proceed directly to LC-MS/MS analysis.
8. For LC-MS/MS analysis we recommend using either a capillary column (75µM × 10–50cm) for nanoflow applications (0.25–0.6 microliters per minute) or a 1mm ID column for higher flow applications (1–100µl per minute, typically).

   Note: An on-line C_{18} trapping column or off-line desalting procedure is strongly recommended.

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4.B. Proteolysis After Reduction and Alkylation

Note: Reduction and alkylation (Steps 2–5) can be performed concurrently. Simply add all reagents sequentially, prior to proteolysis (no incubations between steps necessary), and proceed with the heated digestion step (Step 6).

1. Add 3X volume of Rapid Digest Buffer. For example, to 50µl of protein substrate add 150µl of Rapid Digest Buffer.
2. Add TCEP to a final concentration of 2mM. For example, add 4µl of 100mM TCEP. (We recommend evaluating concentrations from 1–10mM. However, 2mM is our recommendation for a starting concentration.)
3. Incubate the reaction at 37°C for 45 minutes. (Temperatures up to 50°C are permissible, but disulfide-rich proteins can precipitate, so precipitation should be monitored.)
4. Allow the mixture to cool to room temperature, then add iodoacetamide to a concentration 2.5X above the TCEP concentration. For the example provided in Step 2, add 10µl of 100mM iodoacetamide.
5. Incubate for 60 minutes at room temperature.
6. Set a water bath, oven or heat block (such as an Eppendorf Thermomixer®) to 70°C.
7. Resuspend 100µg of the protease (Rapid Trypsin or Rapid Trypsin/Lys-C Mix) in 100µl of Resuspension Buffer to make a concentration of 1mg/ml. Store the resuspended material on ice until ready for use.
8. Add enzyme in a 1:10 E/S ratio. For example, add 2µl of the resuspended Rapid Trypsin (2µg of protease for every 20µg of substrate for Rapid Trypsin) or 4µl of the resuspended Rapid Trypsin/Lys-C Mix (4µg of the Rapid Trypsin/Lys-C for every 20µg of substrate). **Note:** The E/S ratio can be optimized to suit specific assays. We recommend, if optimization is required, that you titrate this ratio. For more information see Section 6., Troubleshooting.
9. Incubate for up to 60 minutes at 70°C.

   Note: Increase digestion time to as long as 3 hours for more difficult-to-digest proteins or complex samples **Optional:** Shake at 450–600rpm on a Thermomixer®.
10. Terminate the reaction with 5µl of formic acid for every 200µl of digestion reaction. The final concentration of the organic acid should be 0.1–2%.
11. Freeze the samples at –20°C or below or proceed directly to LC-MS/MS analysis.
12. For LC-MS/MS analysis we recommend using either a capillary column (75µM × 10–50cm) for nanoflow applications (0.25–0.6 microliters per minute) or a 1mm ID column for higher flow applications (1–100µl per minute, typically).

    Note: An on-line C_{18} trapping column or off-line desalting procedure is strongly recommended.

5. Affinity Purification Protocol

1. Set a water bath, oven or heat block (such as an Eppendorf Thermomixer®) to 70°C.
2. Enrich the protein as required by your affinity purification protocol. IgG enrichment usually requires either Protein A, Protein G or streptavidin beads coated with a biotinylated capture reagent (i.e., goat anti-human IgG). Avoid phosphate or carbonate buffers.
3. Elute the target protein with 0.1% TFA. The following steps correspond to elution volumes of 50µl. If your elution volume is >50µl, use the appropriate scaling, and remember that for every 50µl of sample volume, a 3X volume of Rapid Digest Buffer and an enzyme/substrate ratio of at least 1:10 (1:5 for Rapid Trypsin/Lys-C) should be used.
4. Resuspend 100µg of the protease (Rapid Trypsin or Rapid Trypsin/Lys-C Mix) in 100µl of Resuspension Buffer to make a concentration of 1mg/ml. Store the resuspended material on ice until ready for use.
5. Add enzyme in a 1:10 E/S ratio. For example, add 2µl of the resuspended Rapid Trypsin (2µg of protease for every 20µg of substrate for Rapid Trypsin) or 4µl of the resuspended Rapid Trypsin/Lys-C Mix (4µg of the Rapid Trypsin/Lys-C for every 20µg of substrate). **Note:** The E/S ratio can be optimized to suit specific assays. We recommend, if optimization is required, that you titrate this ratio. For more information see Section 6., Troubleshooting.
6. Incubate for up to 60 minutes at 70°C.

   Note: Increase digestion time to as long as 3 hours for more difficult-to-digest proteins or complex samples
   Optional: Shake at 450–600rpm on a Thermomixer®.
7. Terminate the reaction with 5µl of formic acid for every 200µl of digestion reaction. The final concentration of the organic acid should be 0.1–2%.
8. Freeze the samples at –20°C or below or proceed directly to LC-MS/MS analysis.
9. For LC-MS/MS analysis we recommend using either a capillary column (75µM × 10–50cm) for nanoflow applications (0.25–0.6 microliters per minute) or a 1mm ID column for higher flow applications (1–100µl per minute, typically).

   Note: An on-line C_{18} trapping column or off-line desalting procedure is strongly recommended.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**.

Symptoms	Causes and Comments
Protein precipitated	Too much reducing agent. Lower the concentration of reducing agent or create a range of lower concentrations.
	Heating temperature was too high. Evaluate multiple temperatures (i.e., 37°–55°C).
	Proteolysis time was too short. Perform a time-course reaction.
	Phosphate or carbonate buffer was used in the protein substrate solution. Change the composition of the buffer for the protein substrate solution.
Large number of missed cleavages	Inadequate E/S ratio or too short a digestion time. The amount of enzyme added will correlate with time required to complete digestion. (**Note:** Complete digestion implies that there is no further increase in sequence coverage, total spectra or peptide peak area). The number of missed cleavages is not always correlated with a complete reaction. For example, a complete digestion could still have 20% missed cleavages. Many factors influence digestion efficiency.
	Pre-incubate with Rapid Trypsin/Lys-C before high-temperature incubation. (You can incubate for 30 minutes at 25°–37°C to allow the Lys-C extra time to digest, but this is not required.)
	Check to see if protease inhibitors are present.
Low enzyme activity	Enzyme has lost activity. If using a reconstituted enzyme that was aliquoted and stored frozen, go back to the original enzyme tube and reconstitute fresh enzyme.
Large number of artificial PTMs, such as deamidation or disulfide-bond scrambling	High temperature induces artificial PTMs. Lower the temperature or shorten proteolysis time to minimize PTMs.
Large number of semi-tryptic peptides	Digestion time might be too long or the E/S ratio is too high. Shorten digestion time and optimize the E/S ratio.
Low sequence coverage	Too little sample is loaded onto the LC column for analysis or reduction/alkylation is required. Try loading more sample onto the LC column or add a reducing/alkylating agent.
Low analytical precision	Digestion efficiency is not optimal. Use Rapid Trypsin/Lys-C. Incubate at room temperature for 30 minutes to allow extra time for the Trypsin/Lys-C to digest.

7. References

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8. Related Products

Product	Size	Cat.#
Goat Anti-Human Biotinylated IgG	4ml	V7830
High Capacity Magne® Streptavidin Beads	3ml	V7820
PolyATtract® System 1000 Magnetic Separation Stand	1 each	Z5410
Magne® Protein A Beads, 20% Slurry	1ml	G8781
	5ml (5 ×1ml)	G8782
	50ml	G8783
Magne® Protein G Beads, 20% Slurry	1ml	G7471
	5ml (5 ×1ml)	G7472
	50ml	G7473

Proteases and Mass Spec Reagents

Product	Size	Cat.#
Trypsin Gold Mass Spec Grade	100µg	V5280
Trypsin/Lys-C Mix Mass Spec Grade	20µg	V5071
	100µg (5 × 20µg)	V5073
rLys-C, Mass Spec Grade	15µg	V1671
ProteaseMAX™ Surfactant, Trypsin Enhancer	1mg	V2071
	5 × 1mg	V2072
IdeS Protease	5,000 units	V7511
	2 5,000 units	V7515
IdeS Protease, Frozen	2,000 units	V7512
PNGase F	500 units	V4831
6 × 5 LC-MS/MS Peptide Reference Mix	50µl	V7491
	200pmol	V7495

9. Summary of Change

The following change was made to the 11/17 revision of this document:

1. Updated Storage Conditions, changing the temperature for Rapid Digest Buffer.

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