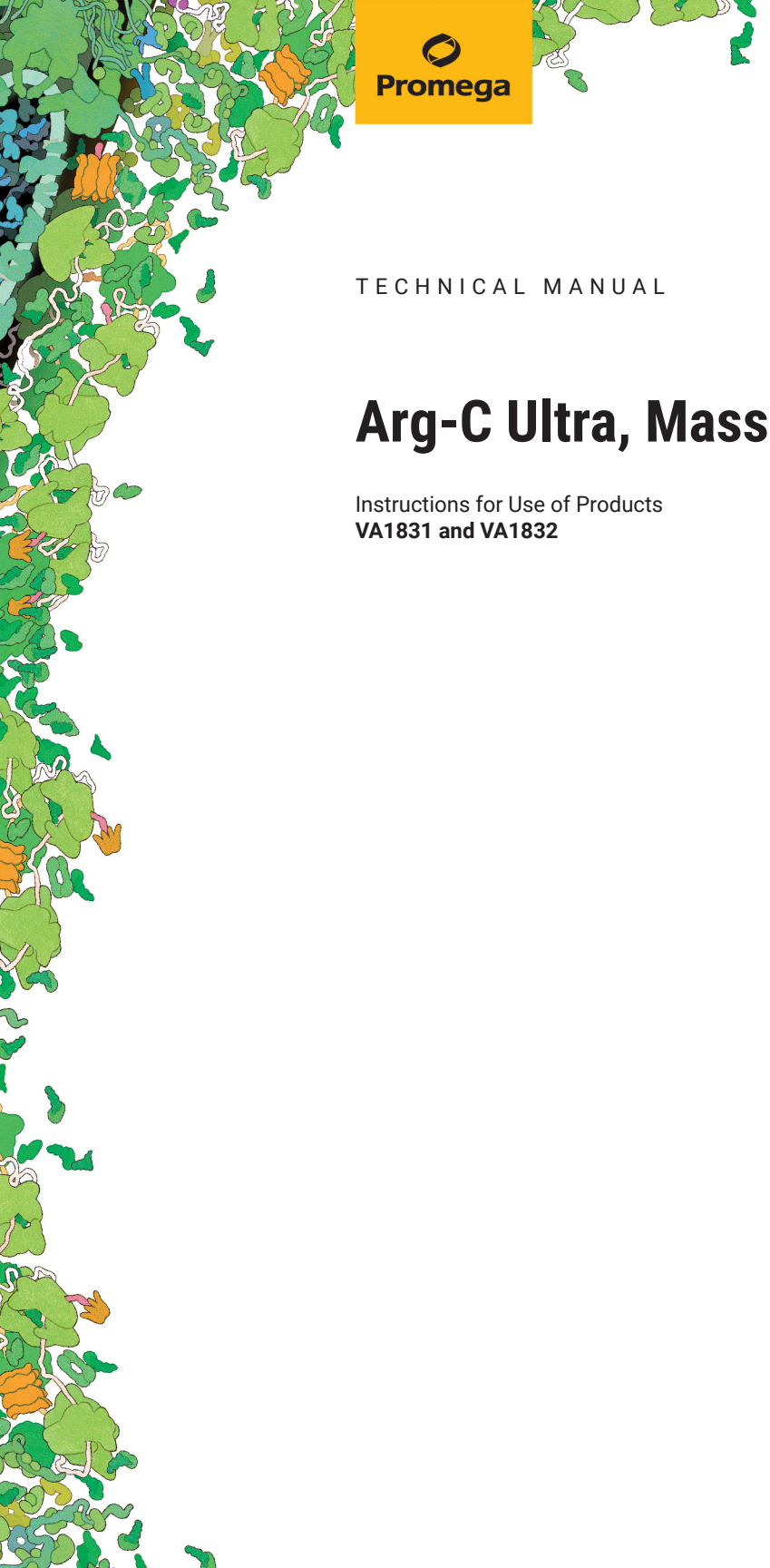

TECHNICAL MANUAL

Arg-C Ultra, Mass Spec Grade

Instructions for Use of Products
VA1831 and VA1832

Revised 5/25
TM754

Arg-C Ultra, Mass Spec Grade

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

Arg-C Ultra, Mass Spec Grade, is an endoprotease that preferentially cleaves proteins on the C-terminal side of arginine residues. The enzyme is a His-tagged cysteine protease, isolated and purified from *Phorphyromonas gingivalis*, and is also known as gingipain R2 or RgpB. It digests proteins under reducing conditions to produce peptides that are highly suitable for identification and characterization by mass spectrometry.

Arg-C Ultra, Mass Spec Grade is highly selective for cleavage only after arginine residues, in contrast to "traditional" Arg-C (also known as clostripain), which cleaves extensively at lysine as well as arginine residues (Figure 1).

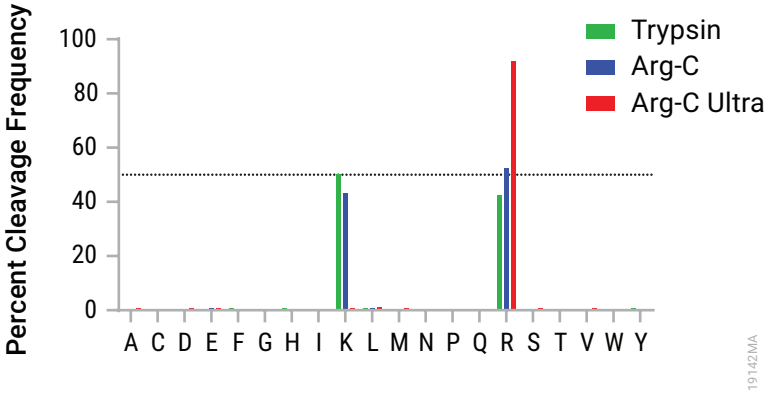

Figure 1. C-terminal cleavage specificity of various proteases that cleave after arginine. Human K562 extract was digested overnight at 37°C with the indicated proteases using a 1:50 enzyme:substrate ratio. Peptides were analyzed by LC-MS/MS on a Orbitrap Exploris® 240 (Thermo Fisher Scientific). Data analyses were conducted using Byonic software (Protein Metrics) with no enzyme specified.

In addition to the remarkable digestion specificity, Arg-C Ultra, Mass Spec Grade is also an extremely efficient enzyme that can be used to achieve nearly 100% digestion efficiency (e.g., approaching zero missed cleavages) under optimized conditions. Arg-C Ultra, Mass Spec Grade is superior to trypsin digestion in terms of digestion efficiency, and is nearly 100-fold more efficient than the Lys-C enzyme, as indicated by fewer missed cleavages from a 2-hour 1:500 digestion of K562 with Arg-C Ultra, Mass Spec Grade, compared to a 20-hour 1:50 digestion of K562 with Lys-C. Arg-C Ultra, Mass Spec Grade achieves this efficiency with minimal loss of digestion specificity (Figure 2).

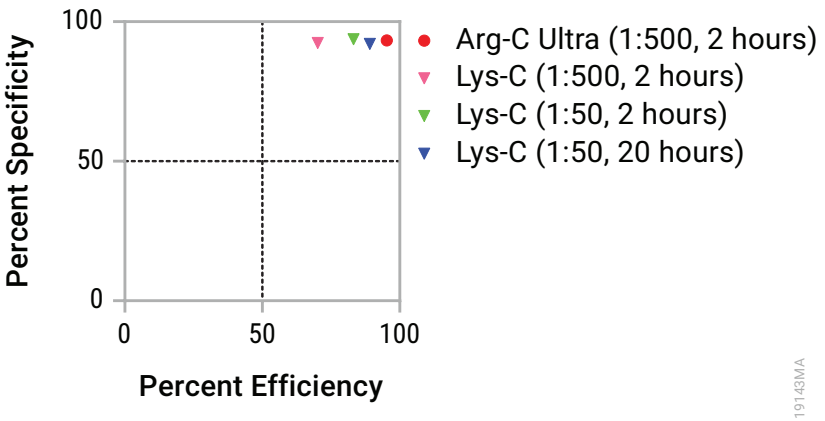

Figure 2. Arg-C Ultra, Mass Spec Grade is fast and efficient. Specificity-efficiency plot in which specificity represents the percent of cleavages at the preferred amino acid (arginine for Arg-C Ultra, Mass Spec Grade, lysine for Lys-C) and efficiency is the inverse of percentage of missed cleavages. Human K562 extract was digested with Arg-C Ultra or Lys-C under the indicated conditions. Peptides were analyzed by LC-MS/MS on a Orbitrap Exploris® 240 (Thermo Fisher Scientific). Data analyses were conducted with Byonic software (Protein Metrics) with no enzyme specified.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Arg-C Ultra, Mass Spec Grade	**5µg**	**VA1831**
	20µg	**VA1832**

Storage Conditions: Upon arrival, store frozen Arg-C Ultra, Mass Spec Grade at –30°C to –10°C. Prior to first use, thaw the frozen Arg-C Ultra, Mass Spec Grade solution quickly by hand and place on ice or at +2°C to +10°C. After the first thaw, Arg-C Ultra, Mass Spec Grade can be stored for 3 months at +2°C to +10°C. For long-term storage, store at or below –20°C. No loss of activity was detected after five freeze-thaw cycles.

Concentration: Arg-C Ultra, Mass Spec Grade is provided at 0.2µg/µl.

3. Important Considerations

Arg-C Ultra, Mass Spec Grade is a cysteine protease and thus requires the presence of a reducing agent during digestion. The following reducing agents have been shown to work with Arg-C Ultra, Mass Spec Grade:

- L-cysteine: 2–10mM (We recommend 10mM final concentration.)
- DTT or TCEP: ≥10mM (We recommend at least 10mM final concentration.)

Additionally, Arg-C Ultra, Mass Spec Grade:

- Is tolerant to at least 6M urea
- Is inhibited by low concentrations of guanidine hydrochloride (guanidine-HCl) due to the structural similarity of guanidine and the arginine-side chain. Avoid the use of guanidine-HCl.
- Is inhibited by cysteine-alkylating agents, such as iodoacetamide. Take care to neutralize such reagents prior to digestion. Alternatively, cysteines can be alkylated following digestion.
- Has been tested and shows excellent activity across a pH range of 5.5–9. We recommend starting at pH 8.0 and optimizing as needed.

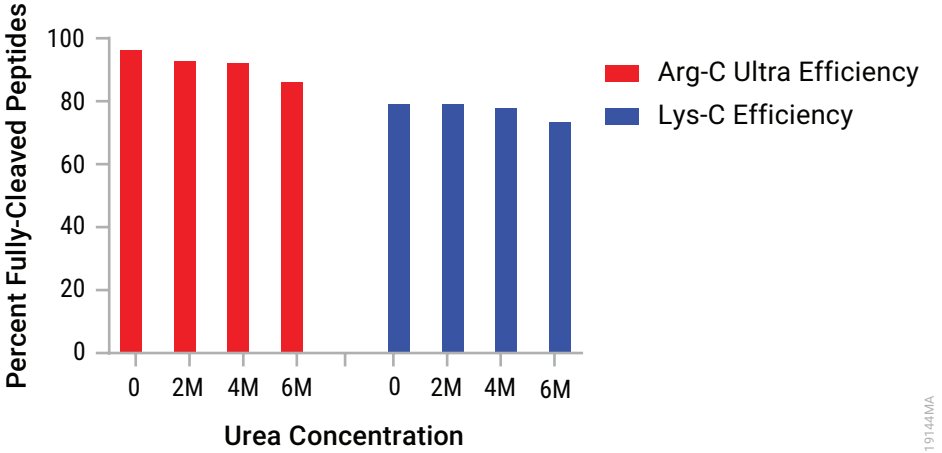

Figure 3. Effect of urea concentration on digestion efficiency of Arg-C Ultra, Mass Spec Grade and Lys-C. Human K562 extract was digested with Arg-C Ultra, Mass Spec Grade or Lys-C at 1:100 for 2 hours at 37°C at a variety of urea concentrations. Peptides were analyzed by LC-MS/MS on a Orbitrap Exploris® 240 (Thermo Fisher Scientific). Data analyses were conducted using Byonic software (Protein Metrics, Inc.) with no enzyme specified.

4. Preparing Samples

Materials to Be Supplied By the User

- NANOpure® water or equivalent
- reducing agent (e.g., L-cysteine, DTT, TCEP)
- denaturant (e.g., urea)
- digestion buffer (e.g., Tris, ammonium bicarbonate, etc.)
- alkylating agent (e.g., iodoacetamide)

4.A. Standard Protocol for Reduction and Alkylation

This protocol example uses MS Compatible Human Protein Extract, Intact (K562 cell extract; Cat.# V6941), which has a urea concentration of approximately 6.5M.

1. Thaw a vial of MS Compatible Human Protein Extract, Intact (Cat.# V6941; 100µl at 10µg/µl).
2. Reduce by adding 1µl of 0.5M DTT (final concentration 5mM).
3. Incubate at 37°C for 30 minutes.
4. Alkylate by addition of 1.5µl of 1M iodoacetamide (final concentration of 15mM).
5. Incubate in the dark at room temperature for 30–60 minutes.

   Notes:

   a. Reduced and alkylated samples are ready for digestion (Section 5), or can be stored at –70°C until ready to digest.

   b. Ensure samples are neutralized with excess reducing agent prior to adding Arg-C Ultra, Mass Spec Grade to avoid alkylating the active-site cysteine. This can be done during the digestion step by ensuring that reducing agent is present in the digestion reaction **prior** to adding enzyme. It's important to consider that both DTT and L-cysteine can be used to neutralize alkylating agents, but TCEP cannot.

4.B. Preparing L-Cysteine

Note: If using L-cysteine as the reducing agent in the digestion protocol, take precautions to ensure the pH is adjusted properly.

1. Prepare a stock of 1M L-cysteine in water. Store 90µl aliquots at or below –20°C.
2. Add 10µl of 5M NaOH to 90µl of 1M L-cysteine to neutralize the pH.
3. Add 800µl of water to make a working solution of 100mM L-cysteine. Use this solution immediately after preparing and discard unused solution.

5. Digestion Protocol

5.A. Digestion Conditions

- Enzyme-to-substrate (E:S) ratio: 1:10 to 1:500
- Digestion time: 30 minutes to 2 hours
- Buffer pH: 5.5–9.0
- Buffers: Sodium acetate, sodium citrate, ammonium acetate, Tris-HCl, Bis-Tris, ammonium bicarbonate
- Denaturant concentration: Excellent activity at 6M urea. Avoid guanidine-HCl.

5.B. Digesting Complex Protein Mixtures

The following protocol is a guideline. Protocols must be optimized and tailored to the substrate in use and desired experimental outcome.

In this example protocol, we digested 50µg of K562 extract with 0.5µg of enzyme (1:100) at 37°C (pH 7.5) in a final volume of 100µl. This 20-fold sample dilution yielded a final protein concentration of 0.5µg/µl and a final urea concentration of approximately 0.3M during digestion. You may wish to avoid diluting the sample.

1. Add 5µl (50µg) of reduced/alkylated K562 extract (prepared in Section 4.A) to a tube.
2. Add 10µl of 10X reducing agent stock (e.g., 10µl of 100mM DTT).
3. Add 10µl of 10X digestion buffer (e.g., 10µl of 0.2–0.5M Tris HCl, pH 7.5).
4. Add 72.5µl of NANOpure® water or equivalent.
5. Add 2.5µl of Arg-C Ultra, Mass Spec Grade (0.5µg).

   Note: Add the enzyme **after** adding the reducing agent to minimize the possibility of active site alkylation, when alkylating agent has been used.
6. Incubate at 37°C for 1–2 hours.
7. Terminate the digestion reaction by acidifying the mixture to pH 2 using TFA or formic acid.

5.C. Digesting Challenging Proteins

Challenging proteins, such as antibodies, are often denatured using guanidine-HCl; however, since Arg-C Ultra, Mass Spec Grade is inhibited by guanidine-HCl, we recommend using an alternative denaturant. High concentrations of urea can be used, although care should be taken to avoid heating and/or using Tris buffers to minimize possible carbamylation. Alternatively, approaches such as SP3® or PAC (2,3), can be employed to achieve on-bead denaturation. These approaches are compatible with proteolysis by Arg-C Ultra, Mass Spec Grade.

6. Combining Arg-C Ultra, Mass Spec Grade and Lys-C Digestion

Arg-C Ultra, Mass Spec Grade can be used in combination with Lys-C to produce "tryptic" peptides. Given the high activity of Lys-C and particularly Arg-C Ultra, Mass Spec Grade compared to trypsin, digestion efficiency with Arg-C Ultra, Mass Spec Grade and Lys-C can be superior to digestions with trypsin or trypsin/Lys-C.

6.A. Sequential Digestion

1. Digest the sample of interest under reducing conditions using Arg-C Ultra, Mass Spec Grade.

   Optimize the digestion with Arg-C Ultra, Mass Spec Grade to achieve a combination of desired efficiency and specificity. Typically, this involves adjusting the E:S ratio and digestion time. We recommend starting with a 1:50–1:200 E:S ratio and 1–2 hours of digestion time.

2. Terminate the Arg-C Ultra, Mass Spec Grade digestion by adding an alkylating agent (e.g., iodoacetamide or chloroacetamide). This method effectively inactivates the enzyme by targeting its active site cysteine, and we recommend it for optimal results.

   Alternatively, terminate the digestion by adding 250mM guanidine-HCl, keeping the final guanidine-HCl concentration below 1M to prevent inhibition of the Lys-C enzyme in Step 3.

3. Add either native Lys-C or recombinant Lys-C to complete the sequential digestion. Optimize the digestion conditions by varying the E:S ratio and digestion time. We recommend a 1:10–1:50 E:S ratio with 2–18 hours of digestion time.

4. Acidify the digestion reaction to pH 2 using TFA or formic acid to inhibit Lys-C activity and to prepare the sample for LC-MS analysis.

   Note: If you used guanidine-HCl to terminate the Arg-C Ultra, Mass Spec Grade enzyme digestion in Step 2, alkylate the sample before performing LC-MS.

6.B. Simultaneous Digestion

Urea concentrations higher than approximately 0.5M should be avoided during simultaneous digestion, since high urea concentrations may result in the two proteases digesting each other, thereby reducing digestion efficiency. The protocol example below uses MS Compatible Human Protein Extract, Intact (K562 cell extract, Cat. # V6941), which has an initial urea concentration of approximately 6.5M that is diluted to approximately 0.3M during digestion.

1. Add 5µl (50µg) of reduced/alkylated K562 extract (prepared in Section 4.A) to a tube.
2. Add 10µl of 10X reducing agent stock (e.g., 10µl of 100mM L-cysteine).
3. Add 10µl of 10X digestion buffer (e.g., 10µl of 0.2–0.5M Tris HCl, pH 8.0).
4. Add 72.5µl of NANOpure® water or equivalent.
5. Add 2.5µl of Arg-C Ultra, Mass Spec Grade (0.5µg).

   Note: Add the enzyme **after** adding the reducing agent to minimize the possibility of active site alkylation, when alkylating agent has been used.
6. Add approximately 1.0µg of native Lys-C (e.g., Cat. # VA1170 or equivalent).
7. Incubate at 37°C for 1–2 hours.
8. Terminate the digestion reaction by acidifying the mixture to pH 2 using TFA or formic acid to make the sample ready for LC-MS analysis.

7. Troubleshooting

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

Symptoms	Causes and Comments
Poor digestion efficiency	Ensure reducing agent has been added to the digestion (to ensure activation of active site cysteine).
	Ensure excess alkylating agent has been removed or neutralized (to avoid alkylating active site cysteine).
	Ensure guanidine-HCl is not present in the sample prior to digestion. Avoid guanidine-HCl entirely or use it in combination with methods such as SP3®/PAC that allow for extensive removal of buffer components. Very low mM concentrations of guanidine-HCl are enough to completely inhibit Arg-C Ultra.
	Ensure protein is soluble at digestion pH.
	Ensure protein is efficiently unfolded to give enzyme access to digestion sites (e.g., reduce, alkylate, add denaturants).
	Optimize digestion parameters (pH, enzyme amount, digestion time).

Symptoms	Causes and Comments
Low specificity	Reduce the amount of enzyme used.
	Reduce the digestion time.
	Effectively inhibit the enzyme after digestion via sample acidification (TFA or formic acid) or inhibition by an alkylating agent.

8. References

1. Curtis, M.A., *et al.* (1999) Molecular genetics and nomenclature of proteases of *Porphyromonas gingivalis*. *J. Periodontal Res.* **34**, 464–72.
2. Hughes, C.S., *et al.* (2019) Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nat. Protoc.* **14**, 68–85.
3. Batth, T.S., *et al.* (2019) Protein aggregation capture on microparticles enables multipurpose proteomics sample preparation. *Mol. Cell. Proteomics* **18**, 1027–35.

9. Related Products

Product	Size	Cat.#
Lys-C, Mass Spec Grade	20µg	VA1170
rLys-C, Mass Spec Grade	15µg	V1671
Arg-C, Sequencing Grade	10µg	V1881
Sequencing Grade Modified Trypsin (lyophilized)	5 × 20µg	V5111
Sequencing Grade Modified Trypsin (frozen)	5 × 20µg	V5113
Trypsin/Lys-C Mix, Mass Spec Grade	20µg	V5071
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
Trypsin Platinum, Mass Spectrometry Grade	100µg	VA9000
rAsp-N, Mass Spec Grade	10µg	V1160
Glu-C, Sequencing Grade	50µg	V1651
Chymotrypsin, Sequencing Grade	25µg	V1061
DTT, Molecular Grade (DL-Dithiothreitol)	5g	V3151
TCEP	15mg	VB1000
Iodoacetamide	15mg	VB1010

For Research Use Only. Not for Use in Diagnostic Procedures.

10. Summary of Changes

The following changes were made to the 5/25 revision of this document:

1. Updated attribution for two third party trademarks.
2. Corrected a typographical error.