

TECHNICAL BULLETIN

# ISOQUANT<sup>®</sup> Isoaspartate Detection Kit

Instructions for Use of Product  
**MA1010**

# ISOQUANT<sup>®</sup> Isoaspartate Detection Kit

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## **1. Description**

### **1.A. Intended Use**

The ISOQUANT® Isoaspartate Detection Kit is intended for quantitative detection of isoaspartic acid residues in proteins and peptides. These isoaspartic acid (Isoasp) residues can result from the gradual nonenzymatic deamidation of asparagine or rearrangement of aspartic acid residues during storage or handling; however, only 70–85% of such rearrangements give rise to Isoasp residues. References 1 and 2 provide some general background information on protein deamidation.

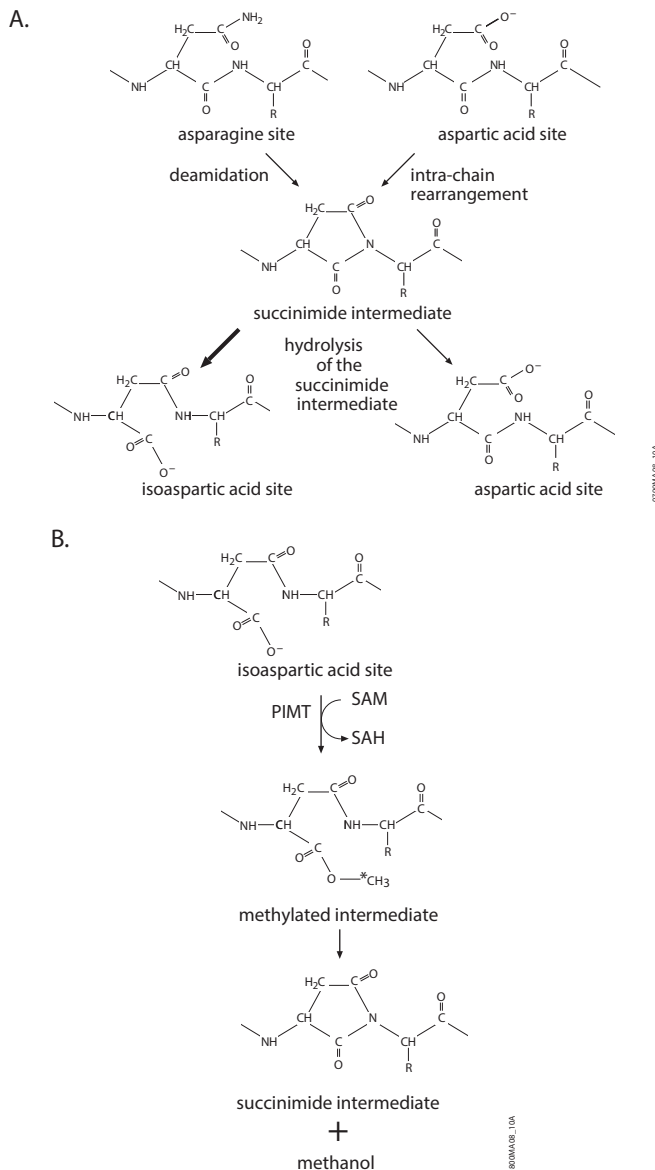
### **1.B. Assay Principle**

The deamidation of asparagine residues and rearrangement of aspartic acid residues is characterized by the formation of a succinimide intermediate that resolves to form a mixture of isoaspartic acid (typically 70–85%) and aspartic acid (3) (Figure 1, Panel A). The rate and extent of isoaspartic acid formation can vary widely among proteins, depending on the amino acid sequence and size of the target protein. Deamidation of Asn residues has been observed most frequently at Asn-Gly and Asn-Ser sites within proteins (4).

The ISOQUANT® Isoaspartate Detection Kit uses the enzyme Protein Isoaspartyl Methyltransferase (PIMT) to specifically detect the presence of isoaspartic acid residues in a target protein (5,6). PIMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to isoaspartic acid at the  $\alpha$ -carboxyl position, generating S-adenosyl homocysteine (SAH) in the process (Figure 1, Panel B). Spontaneous decomposition of this methylated intermediate results in the release of methanol and reformation of the succinimide intermediate.

While asparagine deamidation is the most frequently observed source of deamidation, glutamine has also been reported to undergo deamidation through the formation of a six-member ring (7). Glutamine deamidation occurs much less frequently than asparagine deamidation and aspartic acid rearrangement (8). The ISOQUANT® Kit will not detect glutamine deamidation.

In addition, the assay does not detect isoaspartic acid residues located at the N terminus of a peptide. The ISOQUANT® Kit is designed to provide information regarding the global formation of isoaspartic acid residues at Asn **and** Asp sites, not at each site separately. However, the PIMT enzyme supplied in the kit may be used in conjunction with another method to detect Isoasp at separate sites (4,9).



**Figure 1. Panel A.** Protein deamidation at asparagine and intrachain bond rearrangement at aspartic acid. The side chains of asparagine or aspartic acid residues can spontaneously form a succinimide ring intermediate with the peptide backbone, which rearranges to form isoaspartic acid (70–85%) and aspartic acid (15–30%). **Panel B.** Detection of isoaspartic acid via PIMT-catalyzed generation of SAH and methanol.

### 1.C. ISOQUANT® Isoaspartate Detection Kit

The ISOQUANT® Kit provides reagents for deamidation detection using an HPLC method. The SAH (S-Adenosyl Homocysteine) HPLC standard allows the quantitation of Isoasp in the unknown sample. Isoasp-DSIP may be used as a control.

The ISOQUANT® Kit protocol detects a coproduct of the methylation reaction, SAH. Since this is a relatively small molecule, it can usually be isolated from peptides and quantitated by reverse phase high pressure liquid chromatography (RP-HPLC). Results from test samples are compared to those from an SAH HPLC standard (provided).

**!** A coproduct of the methylation reaction, SAH, is quantitated by RP-HPLC.

### 1.D. Assay Sensitivity

Detection levels as low as 10pmol can be achieved. The sensitivity of the assay can be limited if the peptide being assayed co-elutes with the SAH. If this occurs, optimization of the elution conditions or pretreatment of the sample may be necessary to obtain maximal sensitivity.

Certain substances are known to interfere with the reaction (e.g., SDS) and are addressed in the sample requirements (Section 3). A simple test for interfering substances may be performed by adding a known quantity of Isoasp-DSIP into the same volume of storage buffer as that used for the highest volume of sample being tested. By comparing the results to those of a control, interfering materials in either the sample or the buffer can be detected.

## 2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
<b>ISOQUANT® Isoaspartate Detection Kit</b>	<b>100 assays</b>	<b>MA1010</b>

Each kit contains sufficient reagents to perform at least 100 determinations. Includes:

- 1ml Reaction 5X Buffer (1 × 1ml; blue tube cap)
- 500µl S-Adenosyl-L-Methionine (SAM, 1mM) (1 × 500µl; green tube cap)
- 1ml Protein Isoaspartyl Methyltransferase (1 × 1ml; red tube cap)
- 50µl Isoasp-DSIP\* (1 × 50µl; white tube cap)
- 300µl S-Adenosyl-Homocysteine\* (SAH) (1 × 300µl; yellow tube cap)
- 1ml Stop Solution NR (1 × 1ml)

**!** \*Check Product Information sheet for concentration of the SAH and Isoasp-DSIP supplied with the kit.

**Storage Conditions:** Store the entire ISOQUANT® Kit at –30°C to –10°C until use. After the first use, store the Stop Solution at room temperature and all other buffers and solutions at –30°C to –10°C. After thawing, warm to room temperature to avoid the formation of a precipitate in the Reaction 5X Buffer or Stop Solution. If this occurs, gently agitate the tube at room temperature until the precipitate dissolves.

### 3. HPLC Detection Protocol

This analytical method involves the detection of small amounts of SAH in the presence of significant quantities of other reaction components. Therefore, an HPLC system (instrument and software) capable of detecting and integrating less than 10pmol of SAH is required. An SAH standard is provided for the construction of a standard curve.

#### 3.A. Planning the Assay

We recommend performing each test sample, control, standard and blank reaction in duplicate at the least. Ample SAH is included in the kit to allow additional concentrations of the standard to be assayed, if desired.

##### Considerations

- The molecular weight of each protein or peptide substrate must be known in order to determine the molar ratio of isoaspartic acid to substrate (e.g., a 1mg/ml solution of a protein with a molecular weight of 50kDa would be 20μM).
- When working with an unfamiliar protein or peptide, it is best to assay two different amounts that are five- to tenfold apart to increase the chance of obtaining data in the linear range of the assay. If you are uncertain if isoaspartic acid is present, assay 75pmol and 375pmol of the sample (10μl each of 7.5μM and 37.5μM solutions).
- Some proteins, such as aged calmodulin (10,11), contain Isoasp residues in peptide sequences that make them poor substrates for methylation by PIMT. In such cases, the PIMT methylation reaction may not be complete, and as a consequence, the measured amount of Isoasp may be underestimated if it falls in the upper end of the assay range. To determine if this is the case, perform the assay with two amounts of the sample, one in the upper end of the assay range and the other at 1/3 to 1/2 this amount. If the higher amount yields a lower calculated value for the Isoasp:protein ratio, perform future assays of this protein only in the lower end of the assay range.

#### 3.B. Sample Preparation

Protein secondary structure may interfere with detection of isoaspartic acid residues in the protein substrate. To minimize this effect, polypeptides larger than 8kDa may need to be denatured and/or fragmented by proteolysis before they are used in the ISOQUANT® Kit. Review the assay considerations below, and then perform proteolysis, if necessary. Optional digestion protocols are described in Section 4.

##### Considerations

- To determine if proteolysis is necessary, assay a sample of native protein along with a digested or denatured sample. If the number of picomoles of isoaspartic acid measured in the intact protein is similar to the number of picomoles in the digested protein, the digestion step may be omitted in future assays.
- When performing protease digestions, keep the digestion time as short as possible. Digestion times should be optimized for each protein under study.
- If the protein substrate has been protease-digested, the sample buffer used for blank reactions should contain the same protease and buffer constituents present in the digestions but without the protein substrate. This will account for any isoaspartic acid residues present in the protease.

### 3.B. Sample Preparation (continued)

#### Sample Requirements

- 75–375pmol protein or peptide substrate per assay in a 10µl volume.

The following additives are **acceptable at or below the levels indicated**:

- DTT, 10mM; β-mercaptoethanol, 20mM; NaCl, 150mM; PMSF, 1.5mM; Triton® X-100, 1%; urea, 6M.

These additives are **not acceptable at any level**:

- SDS, guanidinium-HCl, SAH

### 3.C. HPLC Detection Test Preparation

**Note:** The sensitivity is 10pmol.

#### Materials to Be Supplied by the User

- NANOpure® water
- sample buffer (e.g., the buffer in which your sample protein is suspended or, if using protease-digested unknowns, the protease digestion reaction minus the sample protein)
- heating block or water bath at 30°C
- HPLC equipment
- Synergi™ Hydro-RP (Phenomenex Cat.# 00F-4375-EO) or comparable hydrophilic end-capped reverse phase resin
- HPLC-grade mobile phase A: 50mM potassium phosphate [pH 6.2]
- HPLC-grade mobile phase B: methanol, 100%
- HPLC autosampler vials, 100–300µl

#### Preparing the SAM Stock Solution

1. Calculate the total amount of SAM (1µl/reaction) and NANOpure® water (9µl/reaction) needed based on the number of reactions to be performed (see Table 1).
2. In a 1.5ml microcentrifuge tube, add the water and then the SAM. Mix by gentle vortexing, spin briefly to bring all liquid to the bottom of the tube and store on ice in the dark until needed.

**Table 1. Volumes Needed to Prepare SAM Stock Solution.**

Component	Volume per Reaction
1mM SAM	1µl
NANOpure® water	9µl
<b>total volume</b>	<b>10µl</b>

## Preparing the Isoasp-DSIP Control

The Isoasp-DSIP is provided as a measure of the PIMT reaction efficiency. Theoretically, 1pmol of Isoasp-DSIP should yield 1pmol SAH. In practice, the yield from the Isoasp-DSIP reactions will be 1pmol  $\pm$  10% as compared to the SAH standard.

**Note:** If the PIMT reaction efficiency is significantly reduced (below ~80% of the SAH standard), then a component of the sample buffer might be interfering with the reaction.

1. Thaw the Isoasp-DSIP, mix by gentle vortexing, then collect the liquid by brief centrifugation in a microcentrifuge.



It is critical to pipet accurately in Step 2.

2. **Check the Certificate of Analysis for the actual concentration of the Isoasp-DSIP supplied** (note there are two different concentrations listed: legacy concentration and corrected concentration). Legacy concentration is calculated using this equation:

$$\mu\text{M Isoasp-DSIP} = (\text{Average } A_{280} \times 10^6) \div 5,600\text{M}^{-1} \text{ cm}^{-1}$$

The equation does not take into account the Isoasp-DSIP purity, which can range from 92–100%. Peptide purity is determined chromatographically (HPLC). Use the legacy concentration if you need to compare your results with previous experiments that used this concentration for preparing the Isoasp-DSIP control.

The corrected concentration takes into account the Isoasp-DSIP peptide purity by using the equation:

$$\mu\text{M Isoasp-DSIP} = [(\text{Average } A_{280} \times 10^6) \div 5,600\text{M}^{-1} \text{ cm}^{-1}] \times \text{peptide purity}$$

Incorporating peptide purity into the calculation yields the true peptide concentration. Using the corrected concentration to set up the Isoasp-DSIP control will result in a slight increase in the quantity of Isoasp-DSIP peptide that is added to the reaction. Because of this increase, there will also be a slight increase in the amount of SAH generated by the reaction when compared to control reactions prepared using the legacy concentration value. This should be considered if these results are compared to data generated prior to this change. The intent of this change is to improve the performance of the ISOQUANT® Isoaspartate Detection Kit by reducing the lot-to-lot variability of Isoasp-DSIP.

Prepare the required amount of working solution by diluting the Isoasp-DSIP to 7.5μM in sample buffer (the same solution in which your test sample is suspended). Cap, mix by gentle vortexing, and store on ice.



### 3.C. HPLC Detection Test Preparation (continued)

#### Preparing the Unknown (Test) Samples

1. Duplicate reactions are recommended for each test sample. Each ISOQUANT® determination will require 10–50pmol of isoaspartate in a 10µl volume. Depending on your protein, this may translate to 75–375pmol of the sample protein. Based on these parameters, dilute the sample protein in sample buffer to the desired concentration.
2. Dilute the required amount of each test sample in sample buffer.

#### Preparing the SAH HPLC Standard

1. Thaw the SAH standard solution, mix by gentle vortexing, then collect the liquid by brief centrifugation in a microcentrifuge.
2. **Check the Certificate of Analysis for the exact concentration of the SAH standard supplied.** Dilute the SAH to **7.5µM** (7.5pmol/µl) in water or adjust for the concentration difference in the standard calculations. Mix by gentle vortexing, and store on ice.



It is important to be as accurate as possible when pipetting the SAH standard because it provides the basis for quantitation of the unknowns.

#### Preparing the Master Mix



Duplicate reactions are recommended for each test sample. Determine the total number of reactions required (samples, Isoasp-DSIP control and blanks).

In a 1.5ml microcentrifuge tube, prepare sufficient master mix for the number of reactions to be performed (Table 2). Add the components in the order listed, mix by vortexing and spin briefly to eliminate foaming. Store on ice in the dark.

**Table 2. Volumes Needed to Prepare Master Mix.**

Component	Volume per Reaction
NANOpure® water	10µl
Reaction 5X Buffer	10µl
SAM stock solution	10µl
PIMT (add last)	10µl

### 3.D. Reaction Protocol

1. In 0.5ml microcentrifuge tubes, assemble the blank, Isoasp-DSIP control and unknowns as shown in Table 3 at room temperature. For sample buffer, use the buffer in which your test sample is suspended, or if you are using protease-digested samples, use the protease digestion reaction (minus the sample protein).

Duplicate reactions are recommended for each test sample

**Table 3. Preparation of Blank, Reference Standard and Test Samples.**

	Blank	Control	Test Sample
sample buffer	10µl	—	—
Isoasp-DSIP	—	10µl	—
test sample	—	—	10µl
<b>total volume/tube</b>	<b>10µl</b>	<b>10µl</b>	<b>10µl</b>

2. Add 40µl of master mix to the tubes containing the blank, Isoasp-DSIP control or unknowns. Cap tightly, mix thoroughly and place at 30°C to start the reaction.
3. Incubate all reactions for 30 minutes in a 30°C heating block or water bath. (To incubate all reactions for the same amount of time, stagger the start and end of the 30°C incubation by a constant interval.)
4. After the proper time interval, add 10µl Stop Solution NR (0.3M phosphoric acid) to each tube.
5. Place the samples containing Stop Solution (from Step 4) on ice for 5–10 minutes to halt the reaction completely.
6. Centrifuge the tubes at 12,000–14,000 × *g* and 4°C for 8–10 minutes in a microcentrifuge to bring any precipitate to the bottom of the tube. Store in the dark at 4°C until analysis. If the samples will not be analyzed on the same day, store at –20°C.

### 3.E. Reverse Phase HPLC Analysis

#### Performing HPLC

1. Prepare duplicate SAH standard samples by adding 10µl of 7.5µM SAH standard to 50µl of NANOpure® water. This will give 50pmol in a 40µl injection volume.  
**Note:** If desired, prepare a standard curve by diluting the SAH standard in NANOpure® water to give samples containing 5–50pmol per injection.
2. Attach the column to the HPLC instrument, and equilibrate the resin with 10% mobile phase B (90% mobile phase A) at a flow rate of 1ml/minute.  
**Note:** This protocol has been optimized for the Synergi™ Hydro-RP column (Phenomenex Cat.# OOF-4375-EO) with guard attached. Different columns may require modifications to this protocol. Read the column manufacturer's instructions for proper use.

### 3.E. Reverse Phase HPLC Analysis (continued)

3. Although it is feasible to manually inject the samples using a 40µl loop, we recommend use of a temperature-controlled autosampler set at 4°C. Transfer 55µl of each reaction, taking care to avoid any precipitate that may have formed, to an appropriate autosampler vial (100–300µl capacity) and place in the autosampler tray. If a temperature-controlled autosampler is not available, stopped reactions can be stored at 4°C for up to 1 day or at –20°C for up to 7 days.



It is important to minimize exposure to light.

4. Program the HPLC system with the following method:
  - a. Initial parameters are 10% mobile phase B, 90% mobile phase A, at 1ml/minute, detection at 260nm.  
**Note:** Always use a 40µl water “blank” as the first injection.
  - b. Inject 40µl of sample.
  - c. Start a gradient to increase the mobile phase B to 30% (70% mobile phase A) over 5 minutes.
  - d. Reduce the mobile phase B from 30% to 10% (70% to 90% mobile phase A) over 30 seconds.
  - e. Hold the mobile phase B concentration at 10% (90% mobile phase A) for 7.5 minutes to equilibrate the column for the next sample.

#### Notes:

- a. We have found that no washes between samples is necessary with as many as 25 consecutive samples.
  - b. If more than 25 samples are being injected, wash the column as described below after the 25th injection. After the column wash, inject 40µl of water and run method before injecting additional samples.
5. The sample injection protocol shown here will not elute the detergent present in the reaction mix, which binds very tightly to the column resin. The following wash will help clean the column. All steps are at 1ml/minute.
  - a. Wash Column with 10% methanol/90% water for 16 minutes.
  - b. Increase methanol to 100% over 5 minutes. Hold at 100% methanol for 20 minutes.
  - c. Reduce methanol to 10% over 5 minutes. Hold at 10% methanol/90% water for 5 minutes.
  - d. Equilibrate column with 10% methanol/90% 50mM potassium phosphate (pH 6.2) for 20 minutes.
6. Integrate the SAH peaks at 260nm for each sample and plot the standard curve samples (in picomoles) versus peak area.

**Note:** The control, Isoasp-DSIP sample, is at 50pmol. The SAH peak produced from this reaction should be equivalent to 50pmol ( $\pm 10\%$ ) of the SAH standard.

### 3.F. Calculations, Interpretation of Results and Performance Characteristics

#### Calculations

Determine the average peak area per picomole from the 50pmol SAH standard. Divide the average peak area for the duplicate test samples, or the Isoasp-DSIP control, by the SAH peak area/mole. If an SAH standard curve was performed, plot the peak areas versus the appropriate amount (pmol) of standard. Use the linear regression line obtained from this plot to determine the SAH concentration in the experimental samples. Since the predicted amount of SAH produced has a 1:1 stoichiometry with the isoaspartic acid present, the result is expected to be equivalent to the molar quantities of isoaspartic acid.

#### Example Calculations (Based on Figure 2 Data)

SAH standard (50pmol)

Area = 42.06 mAU<sup>2</sup>

Area/pmol = 0.841 mAU<sup>2</sup>/pmol

Isoasp DSIP control

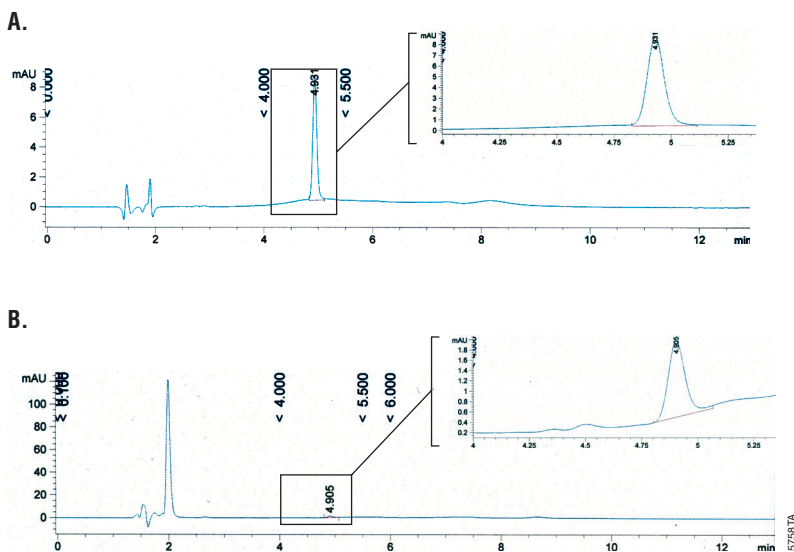
Area = 42.9 mAU<sup>2</sup>

pmol Isoasp in Control =  $42.9\text{mAU}^2 / (0.841\text{mAU}^2/\text{pmol}) = 51$

(This is within 2% of the expected value.)

A change in the integration parameter “slope sensitivity” is necessary in the new ISOQUANT® HPLC method because of the baseline drift. This baseline drift is caused by the increasing concentration of methanol running through the system. The baseline is set at the initial point of the method (methanol at 10%), and as the gradient ramps to 30% methanol, there is no baseline correction. Because the scale of the chromatograms are very small (<10mAU), small changes in the solvent system can cause the baseline to drift. This can also be seen inversely in the chromatograms when the method reduces the methanol from 30% back to 10% over 0.5 minutes. There is a sharp decrease in the baseline level after 5.75 minutes. The baseline drift is less noticeable when the peak areas are large (e.g., 10pmol Isoasp-DSIP compared to 50pmol SAH). Thus, the integration parameter of slope sensitivity should be changed to compensate for the baseline drift due to the increasing concentration of methanol.

### 3.F. Calculations, Interpretation of Results and Performance Characteristics (continued)



**Figure 2. RP-HPLC chromatograms of SAH standard and Isoasp-DSIP control reactions. Panel A.** Fifty picomoles of SAH standard. **Panel B.** Ten picomoles of Isoasp-DSIP control. Samples were prepared and analyzed following the standard protocol. RP-HPLC was performed using a Synergi™ Hydro-RP column with guard attached to an Agilent 1100. Samples were injected automatically at a flow rate of 1ml/minute. Elution was with 10% HPLC-grade mobile phase B, followed by a gradient to 30% B in 5 minutes before returning to 10% B in 30 seconds to equilibrate the column for 7.5 minutes before injecting the next sample. Chromatograms show peaks detected at 260nm.

#### Interpretation of Results

The picomoles of Isoasp measured per picomole of test sample is an indication of the Isoasp content of the overall protein. For example, a value of 0.21pmol Isoasp per picomole of sample may reflect a 21% Isoasp content at a single site or a lower percentage of Isoasp distributed over several sites. If the value obtained is greater than 1, this indicates the presence of more than 1pmol of Isoasp per picomole of sample. It is also important to remember that Isoasp residues are generated as a result of both Asn deamidation and Asp rearrangement and that only 70–85% of such rearrangements give rise to Isoasp residues. Thus, the measured amount of Isoasp in a protein does not give the exact level of Asn deamidation or Asp rearrangement that has occurred.

If the amount of isoaspartic acid estimated from the initial assay is greater than the highest SAH standard, adjust the amount of unknown added in subsequent assays to reduce the amount of isoaspartic acid to 10–50pmol per assay.

## Performance Characteristics

The precision and sensitivity of this method are dependent on pipetting accuracy and on the reverse phase HPLC system used (column, instrument and software).

For quantitation of isoaspartic acid groups in proteins, it is best to generate a signal between 10 and 50pmol per 40μl injection. The assay method has been shown to detect isoaspartate below 10pmol, but not all HPLC systems may be capable of this. The limit of quantitation (LOQ) can be determined from the following equation (12):

$$\text{LOQ} = \frac{10\sigma}{s}$$

where  $\sigma$  = standard deviation and  $s$  = slope of standard curve.

Since the blanks typically generate no signal above the baseline noise, it is not possible to calculate a standard deviation of the blank. Therefore, use the standard deviation of the lowest concentration in the standard curve.

## 4. Optional: Digestion Protocols

### 4.A. Trypsin Digestion Protocols

This general protocol will work for most proteins. Laboratories may substitute their own proteolysis protocol or use a variation of one of the protocols below.

It is important, however, to observe the following guidelines:

- The final concentration of the protein to be assayed should be known as accurately as possible. This is necessary for an accurate determination of isoaspartic acid content (pmol Isoasp/pmol protein).
- The final protein concentration after proteolysis should be >5μM (to give 75–375pmol/10μl for the assay [HPLC format]).
- Precipitation of proteins with acetone or TCA is not recommended. These methods may not give 100% recovery, which could lead to inaccurate isoaspartic acid/mole of protein determinations.
- For each protease digestion, perform a blank digestion in parallel, containing the protease, buffers and other constituents of the digest but minus the unknown. This control is necessary to determine the amount of background signal generated by constituents of the sample.
- Chemical cleavage methods are not recommended, as the harsh conditions may lead to isoaspartic acid formation in the test samples.
- Normal tryptic digestions using neutral pH may result in the introduction of artificial deamidation. The AccuMAP™ Low pH Protein Digestion Kits use low pH digestion conditions to suppress the introduction of artificial deamidation.

#### 4.B. Nondenaturing Digestion Protocol

##### Materials to Be Supplied by the User

- 1–3mg/ml Sequencing Grade Modified Trypsin (Cat.# V5111) stock in 50mM acetic acid diluted, if necessary, for use in digestions.
  - 100mM phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol
1. Use 0.5–1nmol of the protein sample for digestion. The protein should be at a concentration of at least 10 $\mu$ M (0.5nmol in 50 $\mu$ l). Prepare a blank reaction tube by adding to a second tube an equal amount of the buffer in which the protein sample is dissolved. Perform all subsequent manipulations on both tubes.
  2. For optimal trypsinization, the pH of the digestion reaction should be between 6.5 and 9.0.
  3. Add trypsin to achieve a 1:20 to 1:100 (w/w) ratio of protease:substrate. Incubate at 37°C for 1–2 hours.
  4. Stop the proteolysis by adding 0.01 volume of 100mM PMSF (final concentration = 1mM).
  5. Store the completed digests at 4°C or –20°C until needed.
  6. The effectiveness of the proteolysis may be monitored by HPLC or by running 2–5 $\mu$ g of the digested protein on SDS-PAGE (4–20% polyacrylamide) alongside an undigested sample.

#### 4.C. Denaturing Digestion Protocol

Certain proteins are more resistant to proteolysis in their native state and should be denatured prior to proteolysis. If the protein sample was not successfully digested using the nondenaturing method, try the denaturing digestion method below.

##### Materials to Be Supplied by the User

- 8M urea in 50mM Tris-HCl (pH 8.0)
  - 100mM PMSF in 100% ethanol
  - 50mM Tris-HCl (pH 8.0)
  - **optional:** 5mM DTT
1. Use 0.5–1nmol of the protein sample for digestion. The protein should be at a concentration of at least 10 $\mu$ M (0.5nmol in 50 $\mu$ l). Prepare a reaction “blank” by adding an equal amount of the buffer in which the protein sample is dissolved to a second tube. Perform all subsequent manipulations on both tubes. Dry the samples in a vacuum centrifuge.  
**Note:** Some proteins, especially small ones, may renature.
  2. Resuspend the samples in 20 $\mu$ l of 8M urea in 50mM Tris-HCl (pH 8.0). If the samples were already in a pH 6.5–9.0 buffer, the 8M urea solution can be prepared in NANOpure® water. If the protein contains disulfide bonds, these can be disrupted by adding DTT to 0.5mM final concentration. Vortex to resuspend all of the protein, then allow the sample to denature at room temperature for 5–10 minutes.
  3. Add 60 $\mu$ l of 50mM Tris-HCl (pH 8.0) to dilute the urea to 2M.
  4. Follow Steps 3–6 in the Nondenaturing Digestion Protocol, Section 4.B.

## 5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). Email: [techserv@promega.com](mailto:techserv@promega.com)

Symptoms	Causes and Comments
Variable results	<p>Column may be dirty. Strip the column using the protocol described in Section 3.E, Step 4.</p> <p>Inaccurate pipetting. Use a positive displacement pipette for volumes &lt;10µl.</p> <p>The protein may have sheltered or inaccessible Isoasp groups. Proteolyze the unknown protein according to one of the protocols in Section 4.</p>
SAH peak in 0pmol Isoasp-DSIP reaction	<p>Demethylation of SAM. Avoid subjecting the SAM to multiple freeze-thaw cycles.</p> <p>There may be contaminant in mobile phase A. Replace the solution.</p>
No SAH peak with the Isoasp-DSIP control	<p>One or more components of the reaction may be missing. Repeat reactions, making sure that all components have been added.</p> <p>Store PIMT at -30°C to -10°C.</p>
Poor peak resolution	<p>The RP-HPLC column may be dirty. Clean with high organic wash according to the manufacturer's instructions. A protocol to wash the Synergi™ Hydro-RP Column is provided in Section 3.E, Step 4.</p> <p>The guard column may be dirty. Change to a new guard column.</p>
Distorted or absent peaks	<p>Sample buffer may be interfering with PIMT in sample reaction or HPLC run. Add equivalent volume of sample buffer to DSIP control to test for interference, then change sample buffer if necessary.</p>



## 6. Composition of Buffers and Solutions

### Reaction 5X Buffer

0.5M sodium phosphate (pH 6.8)  
5mM EGTA  
0.02% sodium azide  
0.8% Triton® X-100

### Stop Solution NR

(6X solution) 0.3M phosphoric acid

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## 8. Summary of Changes

The following changes were made to the 12/24 revision of this document:

1. In Section 3.E, Step 3, storage conditions for stopped reactions have been updated.
2. Technical bulletin cover, template and font were updated.
3. Minor formatting changes were made, such as changing bullet points under “Notes:” to a., b., etc.

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