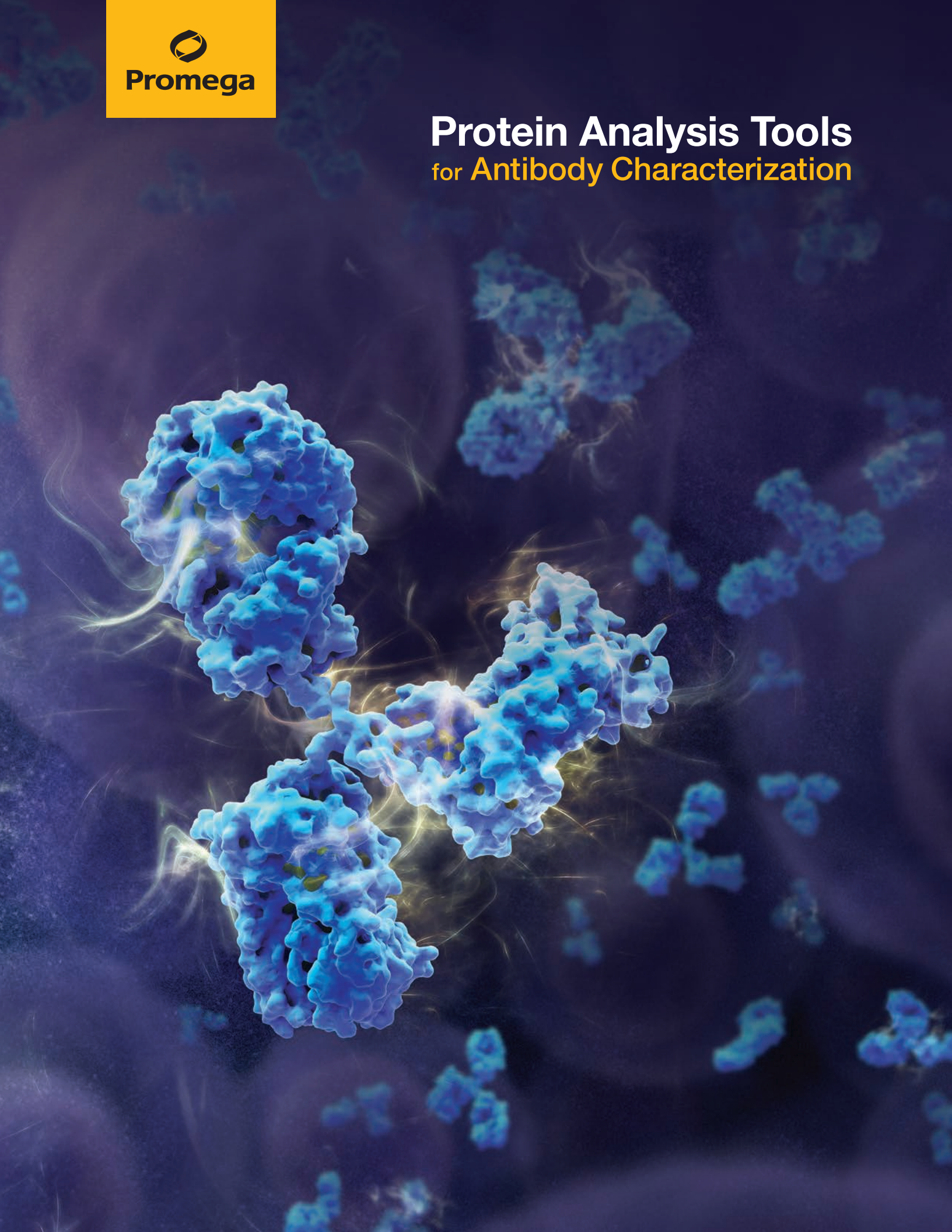
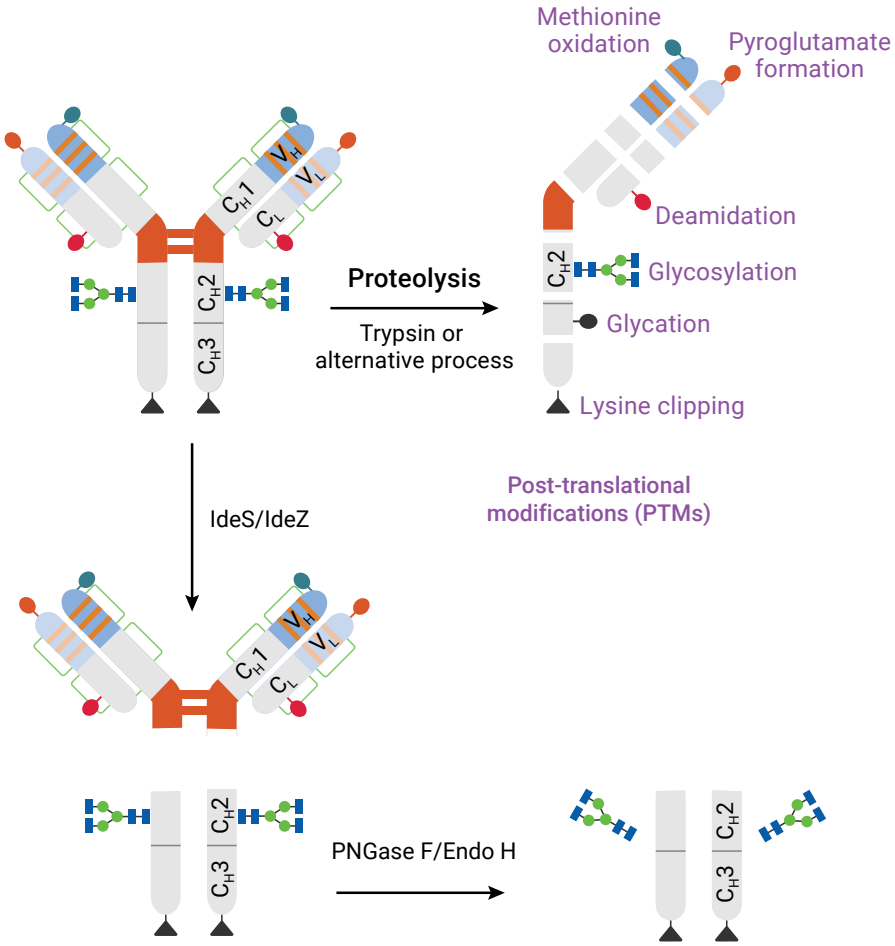
Promega

# Protein Analysis Tools
## for Antibody Characterization



## Common Antibody Characterization Methods




# Introduction

Therapeutic monoclonal antibodies are large, complex molecules that undergo numerous post-translational modifications (PTMs). Nonenzymatic PTMs spontaneously occur in biotherapeutic proteins during manufacturing and storage. The major nonenzymatic PTMs are deamidation, disulfide bond scrambling and oxidation. These modifications can affect efficacy and stability of biotherapeutic proteins and are the subject of careful monitoring. Nonenzymatic PTMs can also be induced during protein sample preparation for peptide mapping and can compromise analysis. Major causes of nonenzymatic PTMs induced during sample preparation include alkaline pH as well as excipients and impurities that have protein-oxidizing activity.

Additional PTMs, both enzymatic and nonenzymatic, can occur in vivo after therapeutic administration of a monoclonal antibody. These modifications include deamidation, oxidation, glycation, glycosylation, alteration of disulfide and trisulfide bonds, and removal of both N-terminal and C-terminal residues.

Because these PTMs can affect the efficacy and safety profile of the drug, the structural variants must be assessed and quantified. In-depth characterization of antibody PTMs remains a significant hurdle because their large size (~150kDa) makes conventional mass spectrometry analysis extremely challenging. Therefore, specialized antibody purification and characterization methods have been developed.

## Antibody Purification

Since monoclonal antibodies are produced by specific B cells that are clonally related, these antibodies are the same isotype and have identical specificity for the antigen epitope. Depending on the starting material used, various antibody purification strategies exist, including classical chromatography (e.g., ion exchange), affinity-purification (e.g., on immobilized protein A and protein G beads) and antigen-specific affinity purification.

## Antibody Characterization

Antibody characterization tools include proteases and other protein analysis reagents for biologics testing, including antibody-specific proteases such as IdeS and IdeZ, and general proteases (e.g., trypsin, Lys-C and trypsin/Lys-C) and glycosidases. Antibody internalization can be studied with pH-reactive dyes.

## Protein Analysis Reagent Benefits:

- Highly specific mass spec grade proteases in both native and recombinant formats
- High-capacity, magnetic bead-based purification
- Kits designed to suppress PTMs and artificial deamidation
- Unique protease blends
- Rapid digestion formats

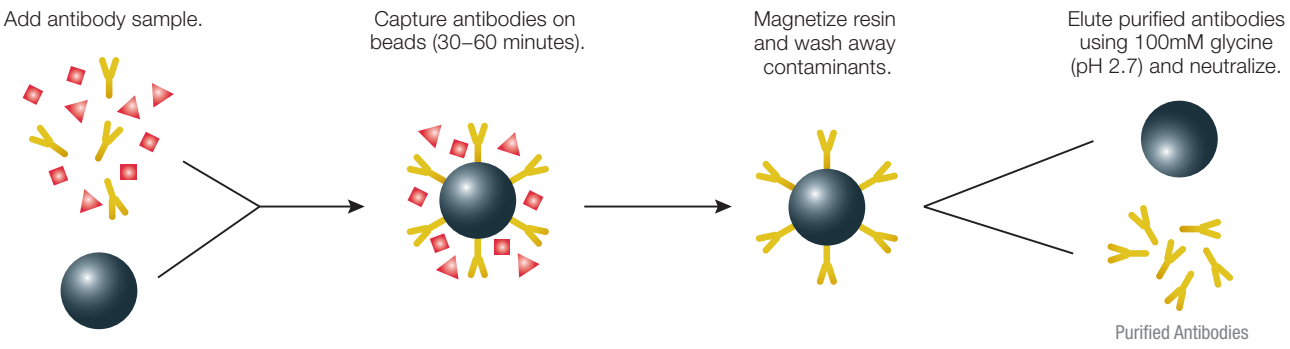
## Magne® Protein A and Protein G Beads

- High binding capacity: Up to 25mg of antibody per milliliter of settled beads
- Simple: Easy to handle beads with fast magnetic response
- High purity: Low nonspecific binding
- Flexible: Accommodate sample volumes from 20µl to 50ml

Magne® Protein A Beads and Magne® Protein G Beads are magnetic affinity beads with high specificity and high capacity for binding antibodies from cell culture supernatant, ascites fluid and serum samples. Antibody purification can be performed easily from a single sample or multiple samples in parallel, or in a high-throughput automated fashion.

Recombinant Protein A from *Staphylococcus* and recombinant Protein G from *Streptococcus* are covalently attached in an oriented fashion to magnetic beads. Biological samples are added, and antibodies are captured by the beads. Using magnetic devices, beads are attached and unbound material is washed away. Finally, antibodies are eluted using lower pH buffer and the solution is neutralized.

The magnetic method minimizes antibody losses encountered during the column chromatography, dialysis and centrifugation concentration steps found in traditional antibody purification protocols. The beads enable high-yield, high-purity antibody purification without using expensive liquid chromatography equipment.




**Antibody purification using Magne® Protein A or Magne® Protein G Beads.**

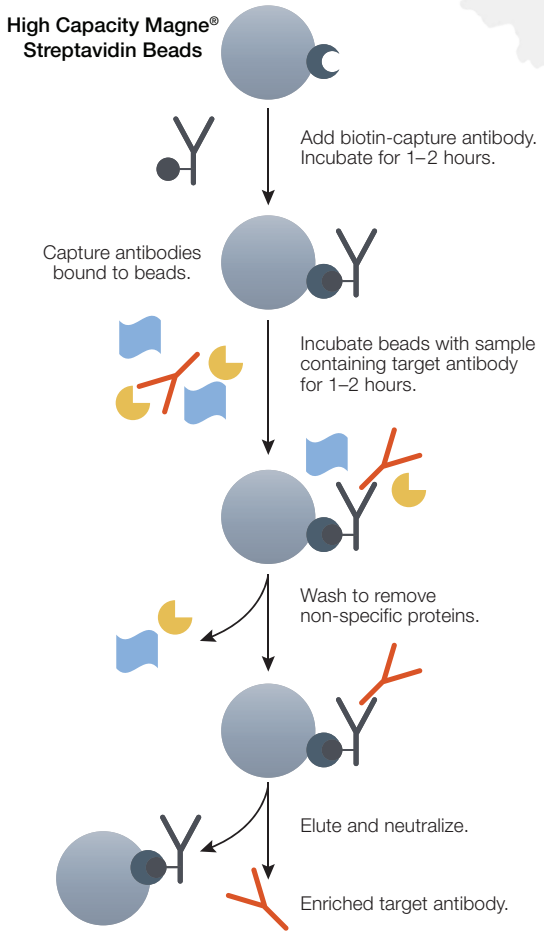
# High Capacity Magne® Streptavidin Beads

- Minimize contaminants: Highly specific binding results in greater purity of isolated antibody
- Automation-compatible: Rapid magnetic response enables use with high-throughput formats
- Characterize large dynamic range: High binding capacity

High Capacity Magne® Streptavidin Beads are magnetic affinity beads with high specificity and high capacity for binding biotinylated antibodies and proteins. The magnetic beads are composed of iron oxide encapsulated by macroporous cellulose, resulting in low nonspecific binding, making them ideal for use with complex biological samples. The beads also have excellent magnetic properties for rapid and efficient capture using magnetic stands.

The high affinity of biotin for streptavidin ($K_d$ = $10^{-15}$) means the biotin-streptavidin interaction cannot be reversed under nondenaturing conditions.

High Capacity Magne® Streptavidin Beads are well-suited for pharmacokinetic studies of therapeutic antibodies during preclinical trials. For example, biotinylated anti-human IgG bound to High Capacity Magne® Streptavidin Beads can be used for enrichment of human IgG from sera or plasma samples of nonprimate animals and analyzed using mass spectrometry. The high binding capacity of these beads enables enrichment of antibodies over a wide range of concentrations using a small amount of beads. Enrichment can be automated for high throughput and scaled up to handle a variety of sample volumes.

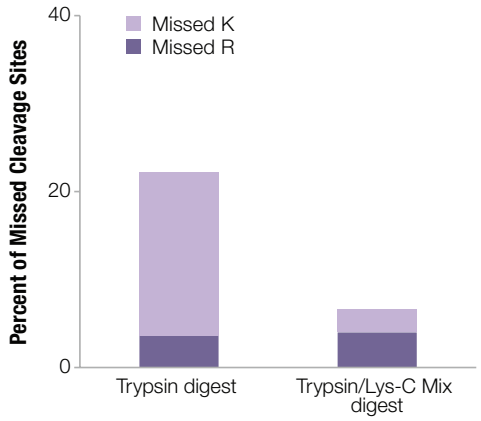



**Antibody purification using High Capacity Magne® Streptavidin Beads.**

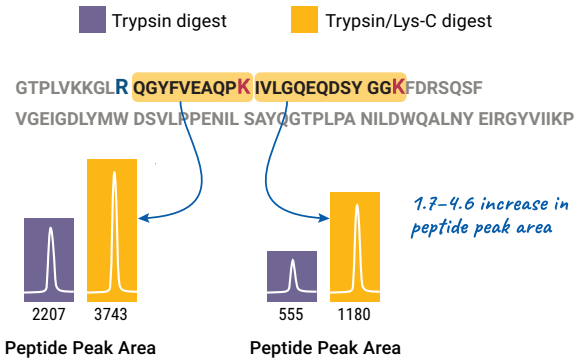
## Trypsin/Lys-C Mix, Mass Spec Grade

- Complete digestion at most cleavage sites: Increased reproducibility between samples and more accurate quantitative data
- Retains activity under strong denaturing conditions: Digest trypsin-resistant proteins
- More peptides per sample: Increased protein coverage
- Tolerates trypsin-inhibiting contaminants: Generate mass spectrometry data from poor quality sample material

Trypsin/Lys-C Mix, Mass Spec Grade, is a mixture of Trypsin Gold, Mass Spectrometry Grade, and rLys-C, Mass Spec Grade. The Trypsin/Lys-C Mix is designed to improve in-solution digestion of proteins or protein mixtures. Trypsin/Lys-C Mix is recommended for in-solution digestion of standard and proteolytically resistant proteins.




**Benefits of Trypsin/LysC Mix compared to standard tryptic digestion.** Using the Trypsin/Lys-C Mix resulted in high-efficiency digestion compared to trypsin alone.



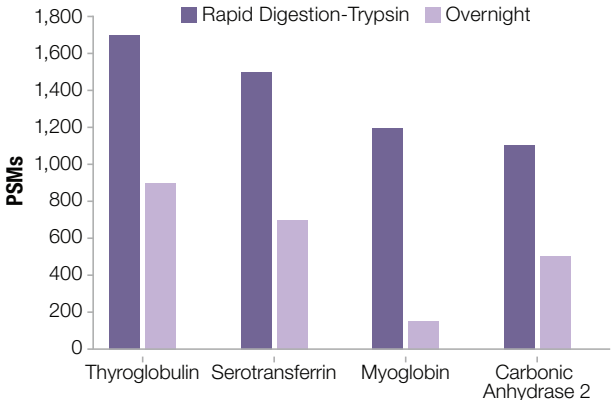

**Trypsin/LysC Mix improves peptide mapping for mass spectrometry.** Incomplete digestion with trypsin was evident after comparing peptide peak intensities in trypsin and Trypsin/Lys-C Mix digests.

# Rapid Digestion—Trypsin/Lys-C Kit

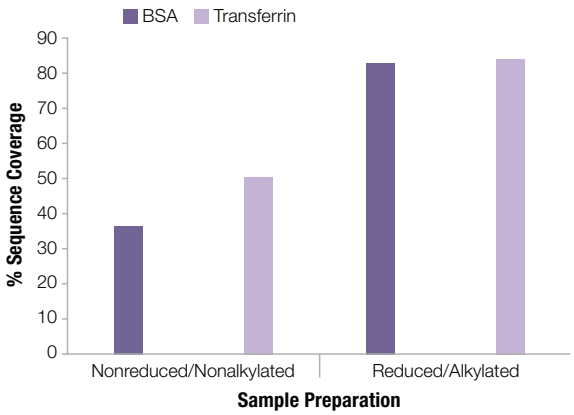
- **Digestions at 70°C:** No chemical denaturants needed, accelerated proteolysis
- **Scale up or adjust protease/substrate ratio:** Optimize reaction conditions as required
- **No alkylation or reduction required:** Minimizes protocol steps
- **No desalting or filtration required prior to MS analysis:** Minimize sample loss

One significant drawback to trypsin digestion is long sample preparation times, which range from 4 hours to overnight. Achieving efficient digestion requires that protein substrates first be unfolded using surfactants or denaturants, such as urea or guanidine. However, these chemicals can have negative side-effects and are usually removed prior to analysis.

The original Trypsin/Lys-C Mix was developed for maximally efficient proteolytic digestion of samples that require improved reproducibility. The new Rapid Digestion—Trypsin/Lys-C Kit enables enhanced digestion of Trypsin/Lys-C in less time. In this optimized protocol, rapid and efficient digestion is achieved at temperatures as high as 70°C. Using this approach, proteolysis incubation times are dramatically shortened and no chemical denaturants or off-line sample cleanup steps are needed.




**Benefits of Rapid Trypsin/LysC Mix compared to overnight tryptic digestion.** Comparison of peptide spectral matches (PSMs) with Rapid Digestion versus overnight digestion showed maximally efficient digestion in a much shorter time.




**Improved sequence coverage with reduction and alkylation steps.** Both Rapid Digestion–Trypsin and Rapid Digestion–Trypsin/Lys-C (35-minute digestions) are compatible with reduction and alkylation steps, resulting in increased sequence coverage.

## rAsp-N, Mass Spec Grade

- Less expensive alternative to native Asp-N
- Larger volume (5X more protease) for more consistent resuspension

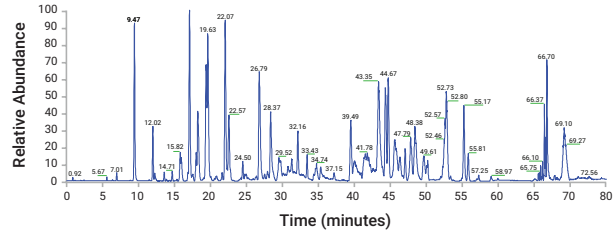
rAsp-N, Mass Spec Grade, is a recombinant protease that was cloned from *Stenotrophomonas maltophilia* and purified from *E. coli*. rAsp-N is a highly active protease suitable for proteomic analysis of complex mixtures as well as peptide mapping of purified proteins, such as therapeutic monoclonal antibodies. The protease is provided in 10µg aliquots in a conical vial for easy and consistent resuspension.

## rLys-C, Mass Spec Grade

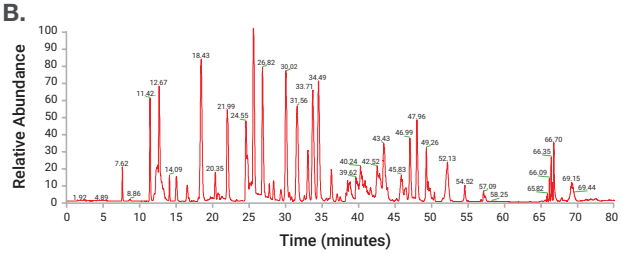
- Optimal activity in the pH range of 8–9
- Retains activity in denaturing conditions such as 8M urea

rLys-C, Mass Spec Grade, is a recombinant Lys-C expressed in *E. coli*. The sequence origin of rLys-C is Protease IV from *Pseudomonas aeruginosa*. Similar to a native Lys-C, rLys-C cleaves at the carboxyl side of lysine residues with exceptional specificity. rLys-C retains proteolytic activity under protein denaturing conditions such as 8M urea, which is used to improve digestion of proteolytically resistant proteins.

**A.**




**B.**




**C.**

| | Sequence Coverage (%) | |
|---|---|---|
| mAb Fragment | rAsp-N Digest | Trypsin/Lys-C Digest |
| Heavy Chain | 100 | 92 |
| Light Chain | 100 | 90 |

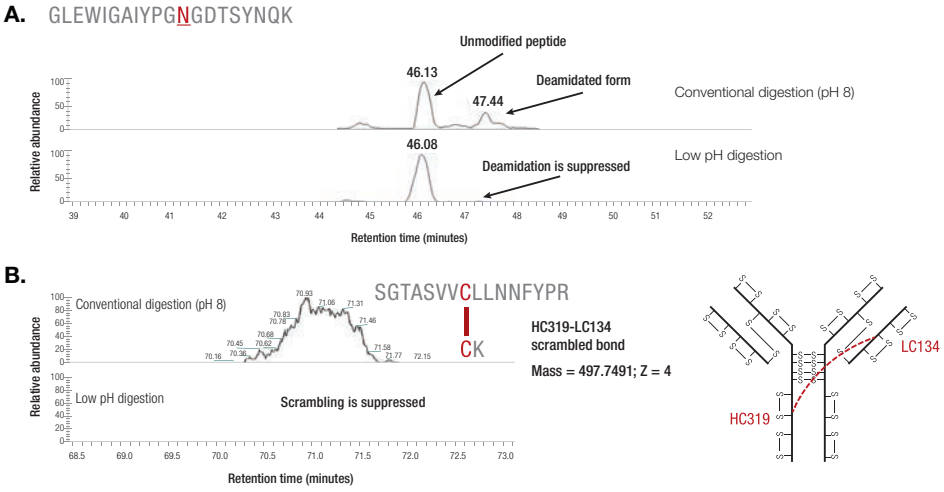
**Peptide mapping with rAsp-N or Trypsin/Lys-C.** Base peak chromatograms showing digestion of NISTmAb for 18 hours with rAsp-N (**Panel A**; 50:1 w:w) or with Trypsin/Lys-C (**Panel B**; 25:1 w:w). Digests were analyzed with a Q Exactive™ Plus mass spectrometer (Thermo Fisher Scientific). Sequence coverage from each digest is shown in the table (**Panel C**).

# AccuMap™ Low pH Protein Digestion Kits

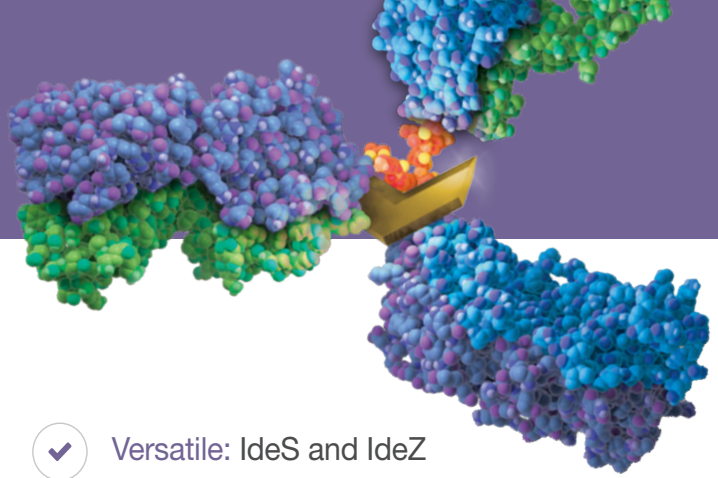
- **Reaction at low pH:** Suppresses artificial deamidation, disulfide bond scrambling and oxidation over the course of sample preparation
- **High digestion efficiency:** Sequence coverage comparable to standard digestion conditions
- **Optimized trypsin digestion:** Minimizes baseline noise by reducing over-digestion
- **Flexible protocol:** Use under nonreducing or reducing conditions, with either guanidine HCl or urea as denaturing reagents

Nonenzymatic post-translational modifications (PTMs) spontaneously occur in biotherapeutic proteins during manufacturing and storage. The major nonenzymatic PTMs are deamidation, disulfide bond scrambling and oxidation. These modifications can affect efficacy and stability of biotherapeutic proteins and are the subject of careful monitoring.

Nonenzymatic PTMs are also induced during protein preparation for peptide mapping, compromising analysis. Major causes of nonenzymatic PTMs induced during sample preparation include alkaline pH as well as excipients and impurities with protein oxidizing activity. With the AccuMAP™ Low pH Protein Digestion Kits, artificial nonenzymatic PTMs are suppressed, since sample preparation is performed at low pH using either reducing or nonreducing conditions.




**Complete suppression of deamidation and disulfide bond scrambling in IgG digested with the AccuMAP™ Low pH Protein Digestion Kit.** An extracted ion chromatogram of a GLEWIGAIYPGNGDTSYNQK peptide from Rituximab antibody, digested under conventional conditions (pH 8; **Panel A**) and at low pH (with AccuMAP™ Low pH Protein Digestion Kit; **Panel B**). The data show suppression of deamidation in Panel A and the complete suppression of disulfide bond scrambling in Panel B.

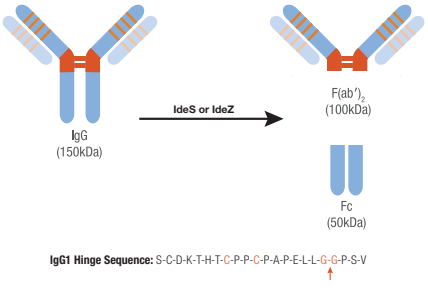


## IdeS and IdeZ Proteases

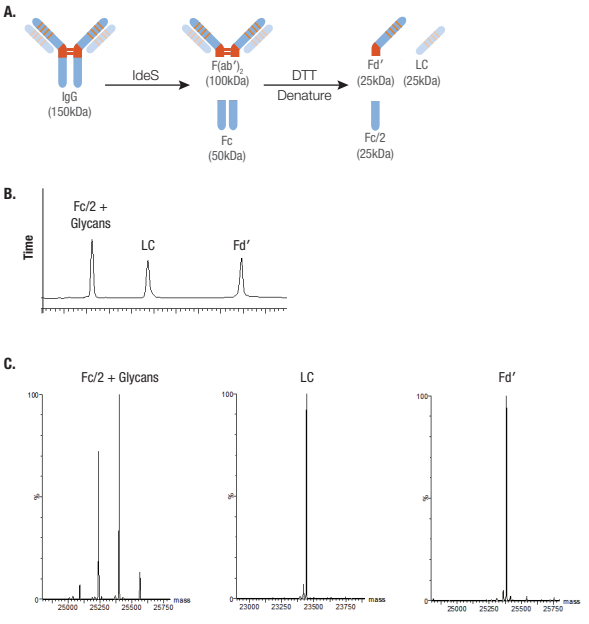
- Fast and easy: Digestion in 30 minutes with no optimization required
- Highly specific and reproducible: Cleave exclusively at a single site below the antibody hinge to produce F(ab′)$_2$ and Fc fragments
- High performance: Essentially complete digestion
- Versatile: IdeS and IdeZ both effectively cleave human IgG1, IgG2, IgG3 and IgG4, monkey, sheep, rabbit, humanized and chimeric IgGs as well as Fc-fusion proteins. However, IdeZ Protease cleaves mouse IgG2a and IgG3 significantly better than does IdeS Protease

IdeS Protease is an immunoglobulin-degrading enzyme from *Streptococcus pyogenes*. It is an engineered recombinant protease overexpressed in *E. coli* that cleaves Immunoglobulin G (IgG) with high specificity at a single site below the hinge region, yielding F(ab′)$_2$ and Fc fragments.

IdeZ Protease is an immunoglobulin-degrading enzyme from *Streptococcus equi* subspecies zooepidemicus. It is an engineered recombinant protease overexpressed in *E. coli*. Like IdeS Protease, IdeZ Protease specifically cleaves IgG molecules below the hinge region to yield F(ab′)$_2$ and Fc fragments. However, IdeZ Protease has significantly improved activity against mouse IgG2a and IgG3 subclasses compared to IdeS Protease.




**Above: Schematic diagram showing cleavage specificity of IdeS and IdeZ Proteases.**

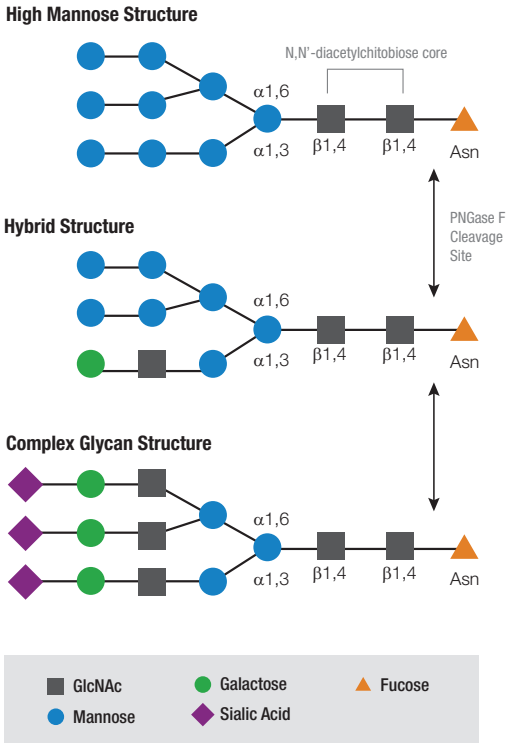



**Right: Characterization of therapeutic antibodies. Panel A.** Digestion of IgG with IdeS, followed by reduction, produces three 25kDa fragments. **Panel B.** Example of IdeS digestion products separated on HPLC. **Panel C.** Mass spectrometry results of the three IdeS digestion products.

## PNGase F

Determine protein glycosylation location and structure

PNGase F is a recombinant glycosidase cloned from *Elizabethkingia miricola* and overexpressed in *E. coli*. It has a molecular weight of 36kDa. PNGase F catalyzes the cleavage of N-linked oligosaccharides between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. PNGase F will not remove oligosaccharides containing alpha-(1,3)-linked core fucose commonly found on plant glycoproteins.




**Cleavage specificity of PNGase F on N-glycans.**

## Endoglycosidase H

Characterize glycan location and structure

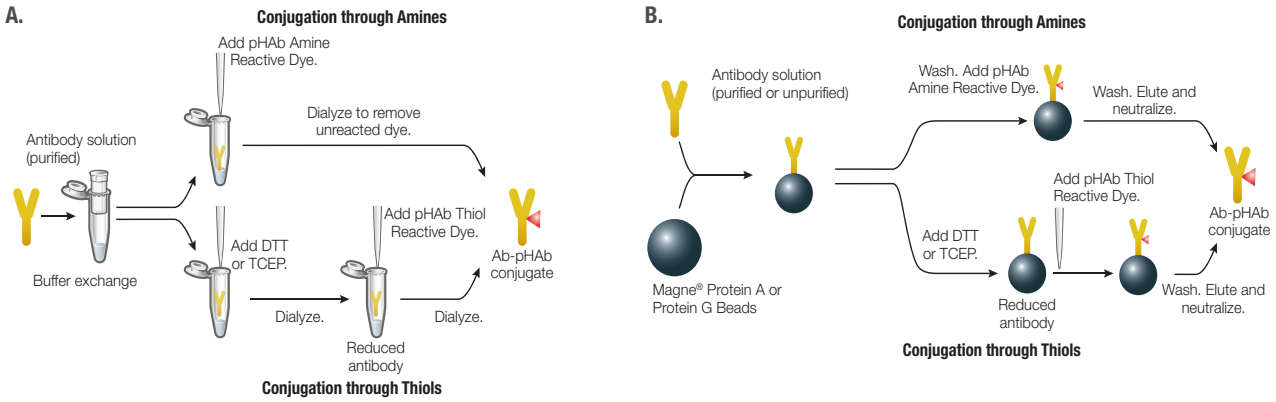
Endoglycosidase H (Endo H) is a recombinant glycosidase cloned from *Streptomyces plicatus* and overexpressed in *E. coli*. Endo H cleaves the chitobiose core of high mannose and a limited number of hybrid oligosaccharides from N-linked glycoproteins. It does not cleave complex glycans. Enzymatic cleavage is between the two N-acetylglucosamine residues in the diacetylchitobiose core of the oligosaccharide, leaving one N-acetylglucosamine residue on the asparagine. This is in contrast to PNGase F, which cleaves all asparagine-linked oligosaccharides.

## pHAb Amine and Thiol Reactive Dyes

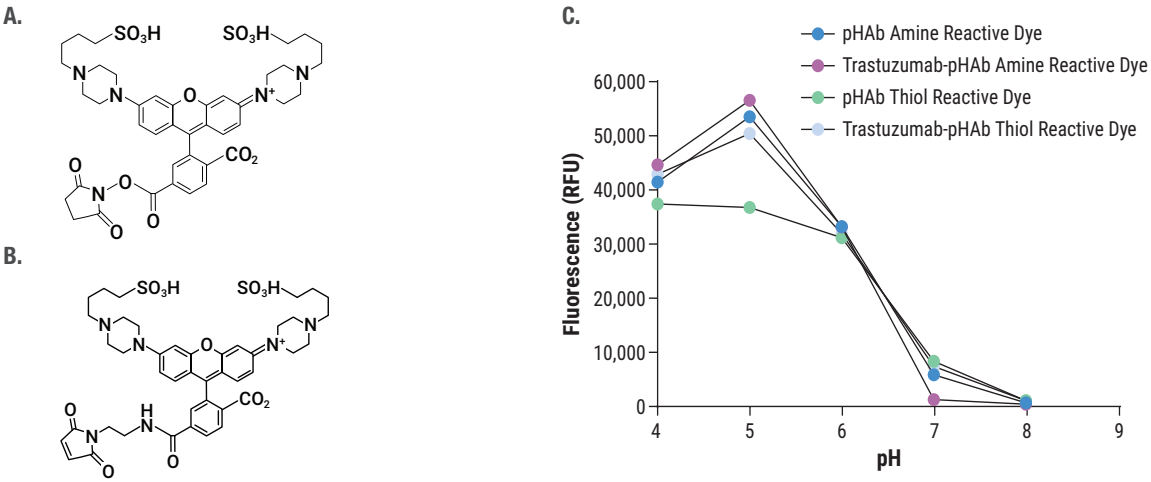
- Measure antibody internalization in real time
- Compatible with 96-well, plate-based assays
- High signal-to-background ratios
- On-bead conjugation

pHAb Reactive Dyes are pH sensor dyes that have very low fluorescence at pH >7 and a dramatic increase in fluorescence as the pH of the solution becomes acidic. pHAb Dyes have excitation maxima (Ex) at 532nm and emission maxima (Em) at 560nm.

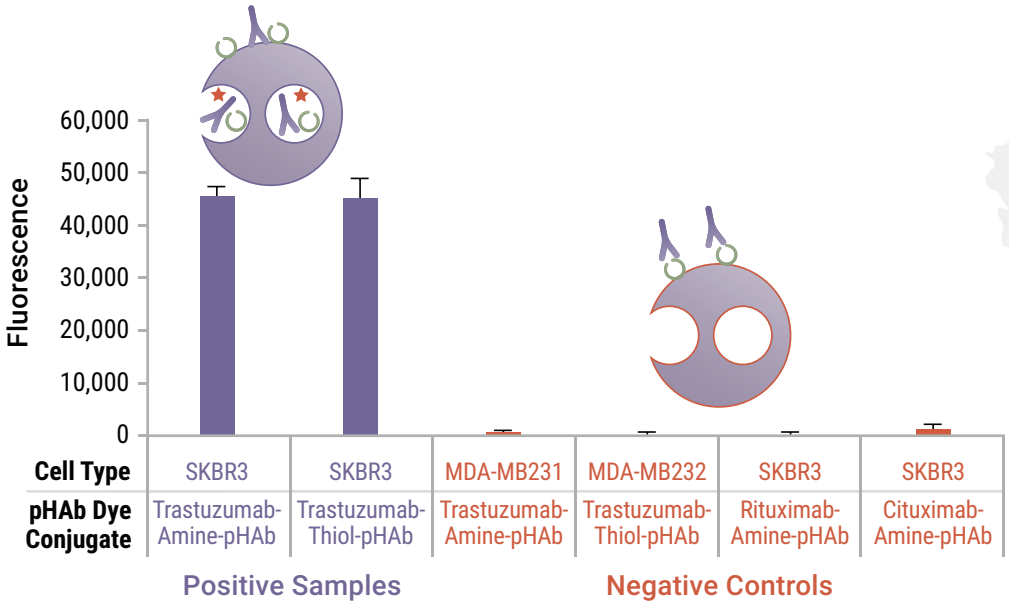
pHAb Reactive Dyes are designed specifically for antibody labeling and can be used for high-throughput antibody internalization screening assays.




**Schematic diagram showing two workflows by which pHAb Amine and Thiol Reactive Dyes can be conjugated to antibodies. Panel A.** Traditional in-solution conjugation of antibody. **Panel B.** On-bead conjugation of antibody using Magne® Protein A Beads or Magne® Protein G Beads.




**pHAb Reactive Dyes. Panel A.** pHAb Amine Reactive Dye for labeling amines of lysine residues on antibodies. **Panel B.** pHAb Thiol Reactive Dye for labeling thiols from reduced cysteines in the antibody hinge region. **Panel C.** Fluorescence is shown as a function of pH for Trastuzumab labeled with pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye compared to pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye alone.




**pHAb Reactive Dyes retain fluorescent response to pH after conjugation.** SKBR3, HER2-positive cells and MDA-MB231, HER2-negative cells were plated in a 96-well black, clear-bottom plate overnight. Trastuzumab and IgG (negative antibody control) were conjugated with pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye using on-bead conjugation.

# Ordering Information

| Product | Size | Cat.# |
|---|---|---|
| Magne® Protein A Beads | 1ml | G8781 |
| | 5ml (5 × 1ml) | G8782 |
| | 50ml | G8783 |
| Magne® Protein G Beads | 1ml | G7471 |
| | 5ml (5 × 1ml) | G7472 |
| | 50ml | G7473 |
| High Capacity Magne® Streptavidin Beads | 3ml | V7820 |
| Trypsin/Lys-C Mix, Mass Spec Grade | 20µg | V5071 |
| | 5 × 20µg | V5073 |
| | 1 × 100µg | V5072 |
| Rapid Digestion—Trypsin/Lys-C Kit | 1 × 100µg | VA1061 |
| rAsp-N, Mass Spec Grade | 10µg | VA1160 |
| rLys-C, Mass Spec Grade | 15µg | V1671 |
| AccuMap™ Low pH Protein Digestion Kits | 10 reactions | VA1040 |
| | 100 reactions | VA1050 |
| IdeS Protease, Lyophilized | 5,000U | V7511 |
| | 25,000U | V7515 |
| IdeZ Protease, Lyophilized | 5,000U | V8341 |
| | 25,000U | V8345 |
| PNGase F | 500U | V4831 |
| Endoglycosidase H | 10,000U | V4871 |
| | 50,000U | V4875 |
| pHAb Amine Reactive Dye | 1 × 250µg | G9841 |
| | 4 × 250µg | G9845 |
| pHAb Thiol Reactive Dye | 1 × 250µg | G9831 |
| | 4 × 250µg | G9835 |

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**www.promega.com/Proteomics**

# Custom Assay Services

Let's TALK CUSTOM

## Customizable reagents for protein analysis

- **High-Quality Proteases**
  Specifically developed for mass spectrometry applications
- **Flexible Manufacturing**
  Custom product sizes, format and packaging to meet your needs
- **OEM Supplier**
  Bulk or private-labeled reagents

Promega has the tools, expert staff and state-of-the-art facilities to support complete custom solutions for protein analysis. We provide all the post-delivery support to ensure your reagents work in your hands.

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For product information, visit:
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To learn more about Promega protein analysis, visit:

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