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Questions Answers

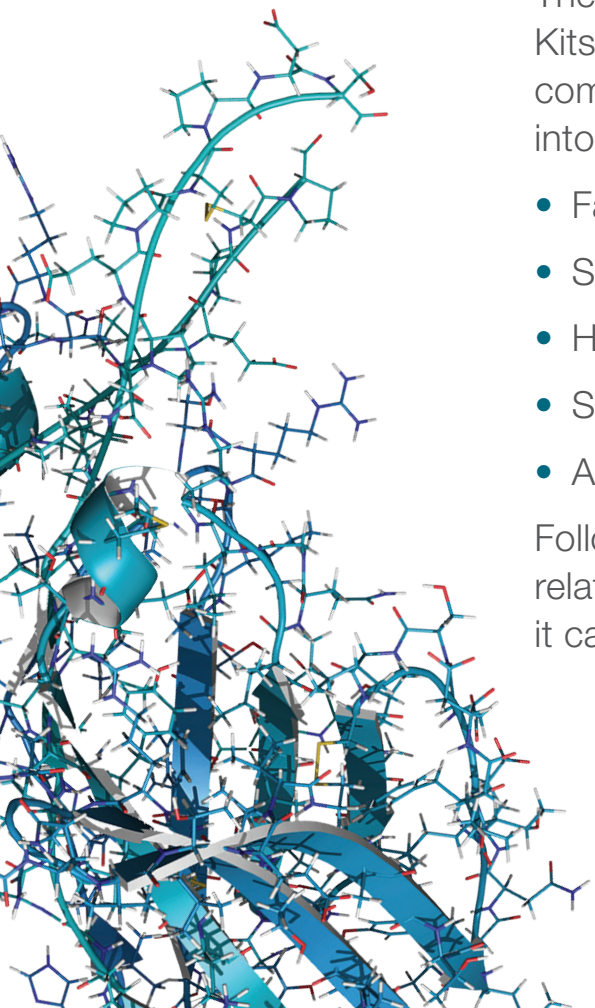
SMART Digest ImmunoAffinity (IA) Kits
Smarter protein preparation

The modern biopharmaceutical and protein research laboratory is tasked with providing high quality analytical results, often in high-throughput, regulated environments. One of the key areas which affects these requirements is sample preparation. Current technologies employed are subject to high levels of irreproducibility, poor sensitivity, and protracted methodologies that often require 24 hours.

The Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Kits remove these issues by providing a process that combines immunoaffintiy capture and digestion of proteins into a single well. The resulting protocol is:

- Fast
- Simple
- Highly reproducible
- Sensitive
- Amenable to automation

Following are some frequently asked questions relating to how the technology works and how it can be implemented.



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Questions Answers

Immunoaffinity Step

Question: What is the loading capacity of the beads?

Answer: We recommend using a 30 µL aliquot of beads per sample. The loading capacity for 30 µL of beads is at least 5 µg of antibody or more.

Question: Will my protein digest during the affinity step?

Answer: The digestion is temperature and buffer dependent. Because the affinity step is performed at lower temperatures (room temperature), in an optimal binding/suboptimal digestion buffer, no digestion is normally observed during the affinity step. Once the buffer is changed to the digestion buffer, which is designed to enhance digestion, and the sample is heated, digestion will proceed rapidly.

Question: What kind of biotin should I use to biotinylate my antibody (SMART Digest IA kit Streptavidin only)?

Answer: The literature shows that most biotinylation methods are effective, so long as the linker is of a reasonable length. We recommend the use of NHS-biotin. For additional details refer to the biotinylation procedure outlined in the product manual.

Question: Will biotin or biotin-like compounds in plasma limit loading capacity?

Answer: In tests where the resin was prewashed with plasma, compared to those were it was loaded with antibody first, no reduction in loading capacity was observed.

Question: What is the best method for cross-linking my antibody (SMART Digest IA kit, Protein A and G only)?

Answer: The literature shows that many cross-linking methods are effective. We recommend using a glutaraldehyde based cross-linking method.
For additional details refer to the cross-linking procedure outlined in the product manual.

Question: Can I use the Protein A and/or G versions to perform enrichment of all IgGs present in the sample?

Answer: Samples can be added directly to the SMART Digest IA kit, Protein A and G beads for bulk fractionation of immunoglobulins. For this workflow, no cross-linking is required. Be sure to take into account the capacity of the beads when developing a bulk fractionation workflow.

Question: What volume of materials can I process per well?

Answer: Volumes ranging from 5 to 1000 µL of sample per well are readily processed.

Digestion Step

Question: What heater shaker device should I use?

Answer: Uniform heating is key to sample reproducibility; shaking is a necessity to avoid any diffusion limitations. A heater shaker device with the following features is required:

- Heating block capable of uniformly heating samples to 70 ˚C
- Heated lid
- Shaking

Question: Can I use my standard PCR instrument?

Answer: Unfortunately, no. Shaking is a necessity to avoid any diffusion limitations.

Question: What kind of samples have you worked with?

Answer: To date we have successful applications in mouse, monkey, beagle and human plasmas. We also have successful applications in cell lysates, urine and cerebral spinal fluid.

Question: What is the volume range of materials that can be digested post enrichment?

Answer: Generally, 200 µL (50 µL sample post wash and 150 µL digestion buffer; see SMART Digest ImmunoAffinity Kit User Manual for additional information). Following the immunoaffinity enrichment and wash steps, the sample volume is generally reduced to 50 µL or less. For every 1 µL of sample remaining, 3 µL of digest buffer should be added. The minimum recommended digestion volume is 50 µL (12.5 µL sample post wash, 37.5 µL digestion buffer) and the maximum recommended volume is 400 µL.

Question: What amount of materials can be digested?

Answer: Up to 3.5 mg and as little as 200 pg.

Question: How much trypsin is there in each well?

Answer: Every 30 µL of beads contains 14 µg of immobilized trypsin.

Question: Can you vary the amount of trypsin used depending on protein load?

Answer: One of the benefits of using immobilized trypsin is that there is reduced autolytic activity. As such, there is no need to vary the amount trypsin used for any given sample.

Question: What is a typical digestion time?

Answer: All proteins vary with regards to digestion; adjust temperature and incubation time accordingly. A recommend strategy for screening digestion time is outlined below.

1. Create a method in your heater/shaker–set temperature to 70 ºC and RPM to1400.
2. Allow the temperature to reach equilibrium for at least 5 minutes.
3. Prepare eight identical samples using a relatively high known concentration of native analyte in the matrix of operation (dilute them to 50 µL with ultrapure water, if necessary) and add to individual SMART Digest wells.
4. Add 150 µL of SMART Digest IA buffer to each well and cap.
5. Place all wells firmly into the preheated heater/shaker.
6. Periodically (e.g. every 5 to 15 minutes) remove a sample from the strip.
7. Centrifuge, filter or perform an SPE process with a SOLAµ HRP plate (P/N 60209-001) then analyze the samples to determine the extent of digestion (see diagram and table below).

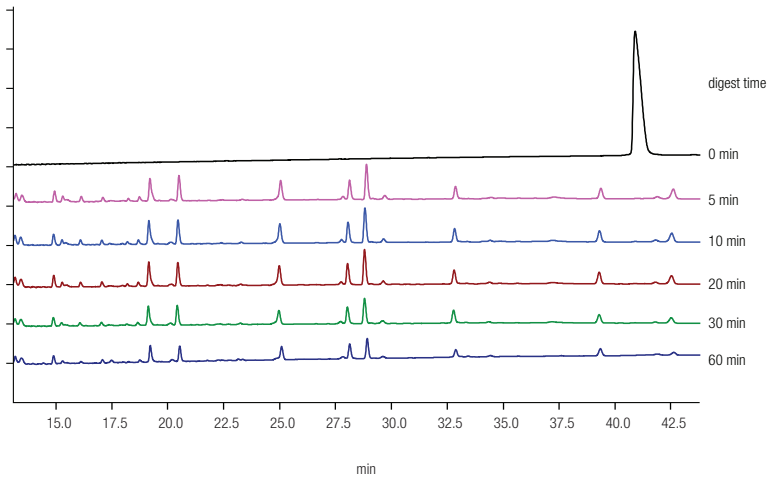
Trypsin digests within minutes

Recommended digestion starting conditions for known proteins

Protein	Digest Time (min)
Insulin	4
BSA	< 5
Carbonic anhydrase	< 5
Lysozyme	< 5
Apo-B	30
IgG	45
IgG in 50 µL plasma*	75
Ribonuclease A	150
Thyroglobulin	240
C-reactive protein	240

200 µL protein solution (100 µg/mL) at 70 °C
*IgG in plasma (17.5 mg/mL total protein) at 70 °C

Carbonic Anhydrase, 29 KDa



This diagram shows a time course experiment for the digestion of carbonic anhydrase.

By removing consecutive samples and monitoring the extent of digestion, the optimum digestion time can be determined.

In this case full digestion is complete in **5 minutes.**

Question: Do I have to use the SMART Digest IA buffer?

Answer: The SMART Digest IA buffer was optimized for maximum trypsin activity at elevated temperatures. Other buffers can be used, but their use may negatively impact trypsin activity. If your application requires the use of an alternative buffer, digestion time and temperature should be optimized accordingly.

Question: Are there salts in the SMART Digest IA buffer?

Answer: The SMART Digest buffer contains about 0.5M salts. These salts greatly assist in achieving rapid digestion at high temperatures. Desalting through the use of valve switching, or the use of Thermo Scientific™ SOLAμ™ SPE cleanup is advised.

Question: What is the pH of the SMART Digest IA buffer?

Answer: The pH is approximately 7.2.

Question: Do I have to reduce and alkylate my protein?

Answer: The SMART Digest IA kits were engineered to be thermally stable. When operated at high temperatures (e.g. 70 ºC), denaturation and digestion occurs simultaneously. Therefore, for many **quantitative** workflows, there is no need to perform the additional steps of denaturation, reduction and alkylation. However, during this process many disulfide bonds will remain intact. As a result for **characterization** workflows where maximum sequence coverage is required it is recommended that you perform reduction and alkylation after digestion. Denaturants and reducing reagents can negatively impact digestion using the SMART Digest IA kits.

Question: Will disulfide bonds scramble during digestion?

Answer: If there are free cysteines, it is possible for disulfides to scramble before, during or after digestion. We would therefore recommend performing alkylation prior to digestion.

Question: Does digestion using the SMART Digest IA kit at high temperatures result in an increase in post-translational modifications?

Answer: In comparison to in-solution digests a comparable number of PTMs have been observed when screening for deamidation, amidation, methylation and oxidation. No modifications to existing PTMs, such as phosphorylated sites, have been observed.

Question: Can I use surfactants with SMART Digest IA kits?

Answer: Many surfactants negatively impact not only digestion, but LC-MS performance as well. Of the surfactants we have screened, octylglucoside is the only MS compatible surfactant that does not negatively impact trypsin activity. It is not charged, so does not impact MS ionization and exists as one molecular weight, it therefore does not result in multiple background peaks.

Question: Is the trypsin mutated to be heat stable? Would this affect my sample?

Answer: During the immobilization process the trypsin is chemically modified in such a way that it is chemically stabilized while maintaining it's specificity. The selectivity of the cleavage site is not affected.

Question: What helps mitigate against trypsin self-digestion (autolysis)?

Answer: The immobilization of trypsin prevents it from attacking and digesting itself, contrary to what happens in an in-solution digestion.

Question: Is complete sample digestion achieved?

Answer: Yes, extensive studies have shown that complete sample digestion is achieved in as little as 5 minutes for simple mono-protein samples, to 3.5 hours for complex matrices such as plasma.

Question: What is the resin made of to which the trypsin is coupled in the SMART Digest IA kit?

Answer: 20 µm PS-DVB core made hydrophilic with a two-tailed coating.

Question: Why do I need to perform reduction and alkylation post digestion?

Answer: The reducing agent lowers digestion efficiency and adds extra steps unless you are specifically looking for disulphides.

Question: Is it compatible with isobaric tagging e.g. SILAC, ITRAQ etc….?

Answer: The current digest buffer contains amines. However, amine free buffers are available.
For more information contact Technical Support: www.thermofisher.com/chromexpert

Question: Is the SMART Digest IA kit compatible with gels?

Answer: No, as the beads are unable to penetrate gels and start digestion.

Question: Can you use the SMART Digest IA kit with Lys-C or other enzymes?

Answer: Yes, perform post digestion.

Question: Why do you not need to use urea to unfold the protein?

Answer: The reason urea is needed as a first step in an in-solution protocol is to disrupt the sample and partially unfold the proteins. The proteins need to be partially unfolded so that the trypsin enzyme can have better access to the internal amino acid chain, not just the surface of the protein of interest.

The reason the SMART Digest IA kit doesn't need urea is that it uses heat to unfold the protein.

Question: Why don't we need to quantitate the total amount of protein and titer the enzyme?

Answer: The SMART Digest IA kit contains an excess of enzyme capable of digesting between 200 pg and 3.5 mg of protein. As most samples will fall in this range it is not necessary to routinely quantitate protein concentration prior to digestion.

Question: What is the composition of the SMART Digest IA buffer?

Answer:

Chemical Name	CAS No.	EINECS No.	Kit Component	Weight %
Water	7732-18-5	231-791-2	2	50-95%
Glycerol	56-81-5	200-289-5	2	< 20%
Tris Base	77-86-1	201-064-4	2	< 10%
Tris-HCl	1185-53-1	214-684-5	2	< 10%
Calcium Chloride	10043-52-4	233-140-8	2	< 10%
Sodium Azide	26628-22-8	247-852-1	2	< 0.1%

Question: How much urea can be used with the SMART Digest IA kit?

Answer: The SMART Digest kit is not affected by concentrations of up to 0.5 M of urea. If the concentration is higher than this we recommend that the sample is diluted to <0.5 M of urea, prior to beginning digestion using the SMART Digest kit.

Question: How compatible is the SMART Digest IA kit with detergents e.g. CHAPS, OGS, TWEEN and RIPA?

Answer:

- CHAPS – Reduction ~ 30% in digest efficiency.
- OGS – no reduction in digestion efficiency.
- TWEEN – no reduction in digestion efficiency.
- RIPA – ~The addition of RIPA, for ribonuclease A digestion, results in a concentration-dependent effect, where initial enzyme inhibition is overcome by improved substrate solubilization at higher concentrations only. 20% reduction in digestion efficiency.
  Compensate for losses in digestion efficiency by extending the digestion time accordingly.

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The **SMART Digest IA kit** is simple to implement and satisfies the analytical workflow demands of the biopharmaceutical industry.

It offers significant benefits over existing conventional in-solution digest protocols.

- Significantly faster
- High sensitivity
- Simple protocol
- Amenable to automation
- More reproducible

Ordering Information

Part Number	Description
Streptavidin	
60110-101	SMART Digest IA Kit, Streptavidin (Av) non-magnetic
60110-102	SMART Digest IA Kit, Av non-magnetic with SOLAμ SPE and collection plate
60110-103	SMART Digest IA Kit, Av magnetic with SOLAμ SPE and collection plate
60110-104	SMART Digest IA Kit, Av magnetic
Protein A	
60111-101	SMART Digest IA Kit, Protein A non-magnetic
60111-102	SMART Digest IA Kit, Protein A non-magnetic with SOLAμ SPE and collection plate
60111-103	SMART Digest IA Kit, Protein A magnetic with SOLAμ SPE and collection plate
60111-104	SMART Digest IA Kit, Protein A magnetic
Protein G	
60112-101	SMART Digest IA Kit, Protein G non-magnetic
60112-102	SMART Digest IA Kit, Protein G non-magnetic with SOLAμ SPE and collection plate
60112-103	SMART Digest IA Kit, Protein G magnetic with SOLAμ SPE and collection plate
60112-104	SMART Digest IA Kit, Protein G magnetic

Complementary Products

Part Number	Description
60103-351	Thermo Scientific™ 96 well vacuum manifold
60104-243	Thermo Scientific™ vacuum pump (NA)
60104-241	Thermo Scientific™ vacuum pump (EU)
60209-001	SOLAμ HRP SPE plate

Find out more at **thermofisher.com/SMARTdigest**

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