Thrombin Kits

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Description

Thrombin is an endoprotease that naturally functions as a blood clotting factor to convert fibrinogen to fibrin. Human thrombin is one of the most active site-specific proteases, which is an advantage because a very low mass ratio of enzyme to target protein is needed for efficient cleavage. Although no single consensus sequence describes the specificity of thrombin cutting, cleavage frequently occurs after the proline-arginine residue pair when properly exposed in a three-dimensional structure.

Novagen's Biotinylated Thrombin and Restriction Grade Thrombin are functionally tested for specific cleavage of the sequence LeuValProArg↓GlySer in recombinant fusion proteins. This thrombin recognition sequence is encoded by many popular expression vectors, including a variety of Novagen's vectors. The cleavage site is usually located between the cloning sites and other vector-encoded fusion peptide sequences, which allows proteolytic removal of the fused peptide from the expressed target protein.

Biotinylated Thrombin is similar to Restriction Grade Thrombin, but it has biotin covalently attached for easy removal of the enzyme from cleavage reactions using immobilized streptavidin. Both thrombin products are rigorously tested for specific cleavage of a recombinant fusion protein and are functionally free of contaminating protease activity. The Thrombin Cleavage Capture Kit contains Biotinylated Thrombin, Streptavidin Agarose and other reagents that enable cleavage of fusion proteins followed by convenient, quantitative removal of thrombin.

Restriction Grade Thrombin and Biotinylated Thrombin are supplied ready for use in a stabilized storage buffer and are prepared from plasma that tests negative for HBsAg and HIV antibodies. A Dilution/Storage Buffer and 10X reaction buffer are included in the kits. For convenience, one thrombin unit is defined as that amount of enzyme required to cleave 1 mg of a test protein when incubated in standard digest buffer at 20°C for 16 hours. Each vial contains 50 units of human thrombin at a concentration of approximately 1.0 units/µl (may vary from 0.5–1.5 units/µl; refer to the Certificate of Analysis for the exact concentration of the lot you purchase). A Cleavage Control Protein is also included for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions.

Components

Thrombin Cleavage Capture Kit

The kit contains enough components to treat up to 50 mg of recombinant protein.

- 50 U Biotinylated Thrombin (approx. 1 U/µl in 50 mM sodium citrate, pH 6.5, 200 mM NaCl, 0.1% PEG-8000, 50% glycerol)
- 2 ml Thrombin Dilution/Storage Buffer (50 mM sodium citrate, pH 6.5, 200 mM NaCl, 0.1% PEG-8000, 50% glycerol)
- 5 ml 10X Thrombin Cleavage Buffer (200 mM Tris-HCl pH 8.4, 1.5 M NaCl, 25 mM $CaCl_2$)
- 2 × 0.4 ml Streptavidin Agarose (50% slurry in phosphate buffer, pH 7.5, 0.02% sodium azide)
- 10 µg Cleavage Control Protein (in 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 20 mM EDTA, 50% glycerol)
- 10 Spin Filters, 2-ml capacity

Storage: Store Biotinylated Thrombin, Dilution/Storage Buffer, 10X Thrombin Cleavage Buffer, and Cleavage Control Protein at –20°C. Store Streptavidin Agarose and Spin Filters at 4°C. *Do not freeze Streptavidin Agarose!*

Biotinylated Thrombin and Restriction Grade Thrombin Kits

The kits contain enough enzyme and buffer to digest up to 50 mg of recombinant protein.

- 50 U Biotinylated Thrombin (approx. 1 U/µl in 50 mM sodium citrate, pH 6.5, 200 mM NaCl, 0.1% PEG-8000, 50% glycerol)

  or

  50 U Restriction Grade Thrombin (approx. 1 U/µl in 50 mM sodium citrate, pH 6.5, 200 mM NaCl, 0.1% PEG-8000, 50% glycerol)
- 2 ml Thrombin Dilution/Storage Buffer (50 mM sodium citrate, pH 6.5, 200 mM NaCl, 0.1% PEG-8000, 50% glycerol)
- 1 ml 10X Thrombin Cleavage Buffer (200 mM Tris-HCl, pH 8.4, 1.5 M NaCl, 25 mM $CaCl_2$)
- 10 µg Cleavage Control Protein (in 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 20 mM EDTA, 50% glycerol)

Storage: Store all components at –20°C.

Related products/available separately	Size	Cat. No.
Cleavage Control Protein	10 µg	69069
Streptavidin Agarose	5 ml	69203-3
S-protein HRP Conjugate	50 µl	69047-3
S-protein AP Conjugate	50 µl	69598-3
S•Tag™ AP Western Blot Kit (colorimetric)	25 blots	69213-3
S•Tag HRP LumiBlot™ Kit	25 blots	69058-3
S•Tag AP LumiBlot Kit	25 blots	69099-3
Spin Filters, 2-ml capacity	pkg/10	69072-3
Spin Filters, 5-ml capacity	pkg/2	69074-3
Perfect Protein™ Markers, 15–150 kDa	100 lanes	69149-3
Perfect Protein Markers. 10–225 kDa	100 lanes	69079-3
Perfect Protein Western Markers	25 lanes	69959-3
S•Tag Thrombin Purification Kit		69232-3

Thrombin Cleavage

Thrombin is a site-specific protease that exhibits very low non-specific cleavage under many conditions. A 1:2000 wt:wt ratio of thrombin to target protein (equivalent to one unit per milligram of target protein) is generally sufficient for cleavage in 1X Thrombin Cleavage/Capture Buffer at 20°C for 16 h. However, because each target protein presents the cleavage site somewhat differently, it is recommended to test several thrombin concentrations, temperatures and/or incubation times to optimize specificity and efficiency of cleavage. Note that excess thrombin may result in unwanted proteolysis at secondary sites. Incubation temperatures ranging from 4°C to 37°C can be used, although we recommend 20°C as the starting point for most proteins. Testing can be carried out in small scale reactions, which can be scaled up proportionately when optimal conditions are found.

Avoid the presence of serine protease inhibitors during cleavage (for example, thrombin is effectively inhibited by 1 mM PMSF) When thrombin cleavage of immobilized protein is desired, higher amounts of thrombin may be required than for solution-based reactions.

Small scale optimization

For small scale digestions the Thrombin is first diluted in Thrombin Dilution/Storage Buffer. The dilutions can be stored in this buffer at –20°C for several weeks; however, to avoid loss of activity we do not recommend extended storage of dilutions. The following protocol is an example of a simple optimization experiment designed to estimate the appropriate range of enzyme:target protein.

1. Make 1:25, 1:50, 1:100 and 1:200 serial dilutions of Thrombin in Thrombin Dilution/Storage Buffer. The dilutions will contain approximately 0.04, 0.02, 0.01, and 0.005 U enzyme per µl.

2. Assemble the following components in a series of 5 labeled tubes.

5 µl	10X Thrombin Cleavage/Capture Buffer
10 µg	target protein
1 µl	Diluted Thrombin (each tube receives 1 µl of a different enzyme dilution. The fifth tube receives 1 µl Dilution/Storage Buffer only as a negative control)
x µl	deionized water
50 µl	total volume

3. Incubate the reactions at room temperature (20–21°C), taking 10 µl aliquots into 10 µl 2X SDS sample buffer after 2, 4, 8 and 16 h.
4. Determine the extent of cleavage of the samples by SDS-PAGE analysis.

Scale-up

When a satisfactory condition is found, scale up the reaction proportionately. Note that if the reaction volume is kept in proportion from the above small scale example, a relatively large volume (5 ml) would be used for a 1 mg digestion. If desired, another preliminary experiment can be performed in which the reaction volume is varied while keeping the enzyme:target protein ratio and incubation conditions constant, which will determine appropriate adjustments needed for higher concentrations of enzyme and target protein.

Factors that affect thrombin activity

Many variables can be introduced into the cleavage reaction, depending on the buffer used with the target protein and other conditions. To test the effect of a given component or buffer, pilot digests can be performed with the target protein or Cleavage Control Protein supplied in the kit. We have analyzed the effect of a number of conditions and components commonly used with recombinant proteins on thrombin activity. The following table shows the relative amount of Biotinylated Thrombin needed to achieve > 95% cleavage of the Cleavage Control Protein under various conditions.

Condition or Component	Biotinylated Thrombin (for > 95% cleavage*)
1X Thrombin Cleavage Buffer	1
4 °C	2
37 °C	1
without $CaCl_2$	1
10 mM βME	1
10 mM DTT	3
1 mM glutathione	1
1% Triton X-100	1
1% Tween-20	1
1% NP-40	1
10 µM heparin	< 1‡
10 mM EDTA	1
1X His•Bind® Bind Buffer	1
1X His•Bind Wash Buffer	3
1X His•Bind Elute Buffer	4
0.5 M NaCl	2
1.0 M NaCl	2
20 mM MES pH 5.0	2
20 mM MES, pH 6.0	2
1X PBS pH 7.5	1
1X PBS pH 8.4	1

* 1 = > 95% cleavage with 1X enzyme,
2 = up to 2X more enzyme required
3 = 2–5X more enzyme required
4 = > 5X more enzyme required.

‡ Addition of heparin can enhance activity; the amount of thrombin is reduced by up to 50%.

βME = β-mercaptoethanol,
DTT = dithiothreitol,
PBS = phosphate buffered saline

To enhance cleavage of some recombinant proteins, it is possible to carry out thrombin digestion in the presence of protein denaturants, which may expose the cleavage site to the enzyme more effectively and/or keep proteins in solution. The following table shows the effect of several urea concentrations and 0.01% SDS on thrombin activity after 2 h and 16 h of incubation at 20°C.

Thrombin Activity Remaining After Incubation

	after 2 hours	after 16 hours
cleavage buffer	100%	95–100%
cleavage buffer + 1 M urea	100%	93%
cleavage buffer + 2 M urea	100%	73%
cleavage buffer + 3 M urea	69%	45%
cleavage buffer + 0.01% SDS	71%	not determined

Monitoring cleavage

Cleavage can be easily monitored by including a parallel reaction using the supplied Cleavage Control Protein in the same buffer system as the target protein. The Cleavage Control Protein is converted from a single 48 kDa band to two bands of 35 kDa and 13 kDa following thrombin cleavage. Inclusion of a control digest enables confirmation of enzyme activity and cleavage specificity, which is especially important when cleavage conditions have been modified. Whereas sufficient control protein is provided to allow Coomassie detection of cleavage products, smaller scale reactions (< 0.5 µg) can be monitored by Western blotting. The Cleavage Control Protein has an S•Tag peptide on the amino-terminal side of the thrombin recognition sequence. The 13 kDa cleavage product can be detected using either S-protein AP Conjugate or S-protein HRP Conjugate. High sensitivity chemiluminescent detection of S•Tag fusion proteins can be performed using the S•Tag AP or HRP LumiBlot Kits. Also included in the S•Tag LumiBlot Kits is a convenient set of Perfect Protein Western Blot Markers to confirm the molecular weight of cleavage reaction products.

Thrombin cleavage can also be monitored by testing for the removal of peptides from fusion proteins upstream of the cleavage site. For example, S•Tag fusion proteins can be blotted with and without thrombin treatment and detected with any of the S•Tag Western Blot Kits. Successful cleavage results in removal of the S•Tag peptide and no band will be evident at the size corresponding to the target protein. A similar strategy can be used with T7•Tag®, CBD•Tag™, or other peptides that may be cleaved from fusion proteins and for which a detection reagent is available.

Biotinylated Thrombin Capture

After the cleavage reaction, Biotinylated Thrombin can be quantitatively removed with Streptavidin Agarose (supplied in the Thrombin Cleavage/Capture Kit) using a ratio of 16 µl settled resin (32 µl of the 50% slurry) per unit of enzyme. Following the proteolytic digestion of the target protein, the Biotinylated Thrombin is bound batchwise to Streptavidin Agarose and the target protein recovered by spin-filtration. In 1X Thrombin Cleavage buffer, > 99% of the enzymatic activity is captured by Streptavidin Agarose in a 30 min incubation. Streptavidin Agarose is supplied as a 50% slurry that can be added directly to the cleavage reaction.

Caution: *Streptavidin Agarose is stored in a buffer containing 0.02% sodium azide and should be handled with caution. Wear gloves and appropriate laboratory clothing.*

1. Ensure that the Streptavidin Agarose beads are evenly suspended by gently mixing by inversion before removing an aliquot.
2. Transfer the desired amount of agarose to the reaction. Use a wide-bore pipet tip. We recommend using a minimum of 25 µl Streptavidin Agarose slurry because smaller resin volumes are difficult to manipulate.
3. Incubate at room temperature for 30 min with gentle shaking.
4. Transfer the entire reaction to the sample cup of a Spin Filter.
5. Centrifuge at 500 × g for 5 minutes. The filtrate in the collection tube contains the cleaved protein, free of Biotinylated Thrombin.

References

Chang, J.-Y., (1985) *Eur. J. Biochem.* **151**, 217–224.

Chow, B.K., Morrow, G.W., Ho, M., Pedlerson, R.A., McIntosh, C.H., Brown, J.C., and MacGillivray, R.T. (1990) *Peptides* **11**, 1069–1074.

Lottenberg, R., Christensen, U., Jackson, C. M., and Coleman, P. L. (1981) *Meth. Enzymol.* **80**, 341–361.

McKenzie, K.R., Adams, E., Britton, W.J., Garcia, R.J., and Basten, A. (1991) *J. Immunol.* **147**, 312–319.

Sankar, S. and Porter, A.G. (1991) *J. Virol.* **65**, 2993–3000.