

## Product Information

### THROMBIN CleanCleave™ KIT

Product Number **RECOM-T**  
Technical Bulletin No. MB-445  
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## TECHNICAL BULLETIN

### Product Description

Sigma's Thrombin-Agarose is a 50% (v/v) suspension produced by immobilizing bovine thrombin (molecular weight of 37 kDa) and is designed for cleavage of recombinant fusion proteins. This format circumvents the need for removal of thrombin by chromatographic techniques. The ligand density used allows for fast and efficient cleavage; the resin can be reused multiple times with only minimal loss in cleavage efficiency. The resin can also be used over a wide pH and ionic strength range and in the presence of chaotropic agents such as urea, thus allowing for compatibility with an assortment of recombinant protein expression strategies.

Thrombin-agarose can be used to cleave any fusion protein containing a thrombin cleavage site. The optimal cleavage sites for thrombin<sup>1</sup> are:

- a) P4-P3-Pro-Arg/Lys • P1'-P2'  
P4 and P3 are hydrophobic residues, P1' and P2' are non-acidic residues, and Arg/Lys • P1' is the scissile bond.
- b) P2-Arg/Lys • P1'  
Where P2 or P1' is glycine and Arg/Lys • P1' is the scissile bond.

Cleavage of the fusion protein is performed in a batch format and removal of the resin is accomplished either by centrifugation or by collection of the resin in a column. The cleavage time may range from 2 hours to overnight depending upon reaction conditions, temperature, amount of resin used, and fusion protein.

### Properties

Thrombin-agarose has a wide range of activity.<sup>2</sup> The optimal pH range for cleavage of a fusion protein is between 8 and 9. Thrombin-agarose is active at a pH as low as 5.0, however, cleavage rates are reduced by

more than 40% when the pH of the reaction is dropped below 7. Cleavage was significant from 0 mM NaCl to 500 mM NaCl, with only a 20% activity change in cleavage over this NaCl range. Thrombin-agarose can be used over a variety of temperatures from 4 °C to 37 °C. Cleavage rates at 4 °C are significantly reduced (80%) compare to rates at room temperature. Minimal increase in cleavage rates are observed at 37 °C over those at 25 °C. Thrombin-agarose has been found to function well in the presence of 0.1% Triton X-100, 1 M urea, and 5 mM EDTA. This is by no means a comprehensive list. Thrombin contains disulfide bonds, so reducing agents such as dithiothreitol and  $\beta$ -mercaptoethanol should be avoided.

### Disclaimer

Sigma's Thrombin CleanCleave Kit is for laboratory use only. Not for drug, household or other uses.

### Materials Provided

- 10x Cleavage Buffer, Product No. T 9685 10 ml  
500 mM Tris-HCl, pH 8.0, 100 mM CaCl<sub>2</sub>
- Thrombin-Agarose, Product No. T 7151 1 ml  
50% suspension in 50% glycerol, 20 mM Tris-HCl, pH 8.2. Furnished as ml packed gel.
- Disposable column, Product No. C 4969 1 each

### Resin Characteristics

Matrix: 4% Beaded agarose

Ligand immobilized: Minimum 0.15 mg/ml resin

Molecular weight exclusion limit:  $2 \times 10^7$

Particle size: 45-165  $\mu$ m

Maximum linear flow rate: Approx. 11.5 cm/hr

Cleavage rate of synthetic substrate:

10  $\mu$ moles of N-benzoyl-Phe-Val-Arg-p-nitroanilide  
(Product No. B 7632) per micromole of bound  
thrombin per second.

#### Cleavage rate of fusion protein:

200  $\mu$ l of a 50 % suspension of resin will cleave >85% of 1 mg of fusion protein in 1 ml in 4 hours at room temperature.

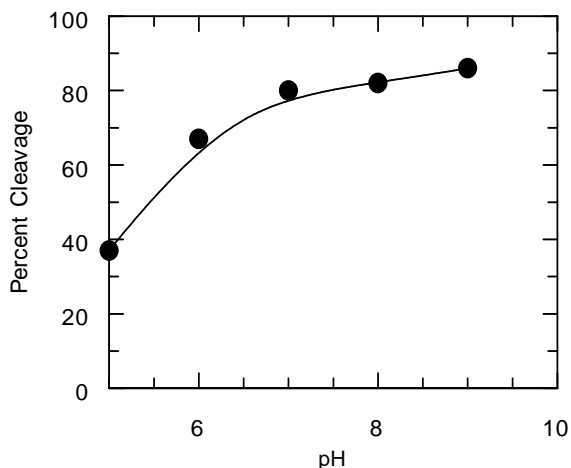
#### Stability of linker:

No detectable leakage of thrombin from thrombin-agarose after 1 month at 2-8  $^{\circ}$ C. Detection limit : <5 picomoles/ml packed gel

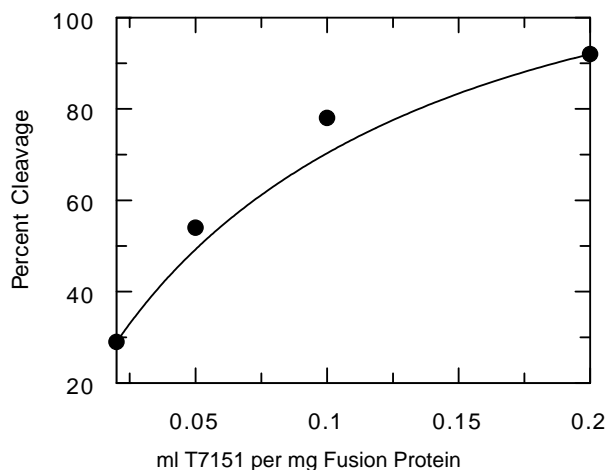
#### Storage

Store kit at -20  $^{\circ}$ C. For convenience, 10x cleavage buffer may be stored at 2-8  $^{\circ}$ C

Effect of pH on Fusion Protein Cleavage



Effects of Resin Amount on Fusion Protein Cleavage



#### Sample Preparation

Samples of fusion proteins should be purified prior to cleavage. Samples having a high viscosity due to the presence of chromosomal DNA or RNA will clog the resin. Purification is best accomplished by passing the sample over an affinity column specific for the fusion protein. The fusion protein should be exchanged into 50 mM Tris-HCl pH 8.0, 10 mM  $\text{CaCl}_2$ . Samples that contain particulates should either be centrifuged or filtered. Crude mixtures may reduce the reusability and reproducibility of the thrombin agarose. Samples containing proteases should be used with caution as these samples may also reduce the performance of the resin. Protease inhibitors may reduce the performance of the resin and should be avoided.

#### Procedure

##### Fusion Protein Cleavage

These guidelines are for cleaving of 1 mg of fusion protein in a final volume of 1 ml. Determining optimal cleavage conditions, temperature, the length of incubation and the stability of the fusion protein should

be done on pilot experiments to achieve maximum recovery prior to large scale reactions.

#### Washing Resin

1. Thoroughly resuspend the thrombin-agarose resin to make a homogeneous slurry.
2. Remove a 100  $\mu$ l aliquot of a 50% (v/v) suspension of resin and gently spin in a microcentrifuge at 500 x g (2500 rpm) to pellet the resin. Remove the supernatant.
3. Add 500  $\mu$ l of 1x cleavage buffer and gently resuspend by inverting the tube. Centrifuge at 500 x g and remove the supernatant.
4. Repeat step 3.

#### Cleavage Reactions

1. Add 100  $\mu$ l of 10x cleavage buffer to the centrifuged beads and gently resuspend the beads.
2. Add 1 mg of fusion protein and bring to a final volume of approximately 1 ml with water.

3. Incubate the cleavage reaction at room temperature or other appropriate temperature with gentle agitation to keep beads suspended.
4. Remove aliquots at 1, 2, 4, 6 and 24 hours and gently centrifuge to remove the resin and analyze supernatant for cleavage. Analysis of cleavage reaction may be done by SDS-PAGE.

#### Recovery of Fusion Protein

Select one of the two following procedures for elution.

#### Centrifugation Recovery

1. Centrifuge the mixture for 5 minutes at 500 x *g* remove the supernatant containing the cleaved fusion protein.
2. To increase recovery, resuspend the resin with an equal volume of 1 x cleavage buffer, centrifuge the mixture, remove supernatant and add to the first supernatant.

Note: The resin maybe centrifuged briefly at high *g* forces (10,000 x *g*) with minimal loss in cleavage efficiency. In order to prolong the effectiveness of the resin, however, a 5 minute centrifugation at 500 x *g* is recommended

#### Column Recovery

1. Transfer the cleavage reaction solution into the column.
2. Allow the resin to settle. Drain and collect the eluent.
3. Rinse the resin with 5 bed volumes of 1x cleavage buffer and collect the eluent.
4. Combine eluents.

#### Resin Regeneration

1. Rinse the resin with an equal volume of 50 mM Tris-HCl pH 8.0, 500 mM NaCl.
2. Rinse the resin with an equal volume of 50 mM Tris-HCl pH 8.0.
3. Repeat step 2 twice.
4. Store as a 50 % suspension in 50 % glycerol, 20 mM Tris-HCl pH 8.0 at -20 °C.

Note: Thrombin should be stored in the absence of NaCl or other salts to prolong the life of the resin.

The resin can be used a minimum of five times with little loss of effectiveness. Reusability will depend upon cleavage conditions, incubation time, temperature, sample preparation, the use of chaotropic or other extreme conditions and proper storage condition.

## Results

### Troubleshooting

No cleavage seen:

- Check sequence to verify that there is an appropriate thrombin binding site.
- Use optimal cleavage conditions.

Incomplete cleavage:

- Increase the resin to fusion protein ratio and increase the incubation time to overnight.
- Use optimal pH, salt and increase the temperature to 25 °C or higher if necessary.
- The addition of 1 M urea or other mild denaturants may be useful.

Secondary cleavage:

- A second thrombin site may exist.
- Reduce the incubation time and the resin to fusion protein ratio to minimize secondary cleavage.
- Use suboptimal cleavage conditions to slow the cleavage rate down.
- Reduce the temperature of the cleavage reaction to 2-8 °C.

## References

1. Chang, J.-Y, Eur. J. Biochem., **151**, 217 (1985)
2. Di Cera, E. *et al.*, Biochemistry, **30**, 7913-7924 (1991)

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