Recombinant Enterokinase

About the Kits

Recombinant Enterokinase	50 U	69066-3
Enterokinase Cleavage Capture Kit	50 U	69067-3

Description

Recombinant Enterokinase (rEK) is a highly purified preparation of the catalytic subunit of bovine enterokinase, which recognizes the identical cleavage site as the native enzyme, AspAspAspAspLys↓, and has similar enzymatic activity. rEK exhibits superior rates of cleavage of fusion proteins containing the recognition sequence when compared to the native enzyme (1). Novagen rEK is purified to near homogeneity and, unlike some preparations of native bovine enterokinase, exhibits no secondary cleavage arising from contaminating proteases. The preparation is also functionally tested for activity with fusion proteins, and is an ideal choice for rapid removal of N-terminal fusions and production of recombinant proteins virtually free of vector-encoded sequences.

The Enterokinase Cleavage Capture Kit is designed for highly specific cleavage of fusion proteins, followed by the rapid, affinity-based capture and removal of rEK. Following cleavage of the target protein, rEK is removed from the reaction with > 99% efficiency by affinity capture on EKapture™ Agarose. The EKapture Agarose is then removed by spin-filtration. No buffer changes are necessary as the same buffer conditions are used for both cleavage and capture procedures.

A Cleavage Control Protein is included in each kit for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions. rEK cleaves the 48-kDa Cleavage Control Protein into two proteolytic fragments of 32 kDa and 16 kDa, each of which are easily visualized by standard SDS-PAGE followed by Coomassie blue staining. The Cleavage Control Protein also features an amino terminal S•Tag™ sequence enabling sensitive detection of the 16 kDa proteolytic product with Western blot reagents.

USA and Canada
Tel (800) 526-7319
novatech@novagen.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
Freefone 0800 622935
Toll Free 1800 409445
customer.service@merckbiosciences.co.uk

All Other Countries
www.novagen.com
novatech@novagen.com

A Brand of EMD Biosciences, Inc., an Affiliate of Merck KGaA, Darmstadt, Germany
www.novagen.com

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

Components

Each kit contains enough components to treat up to approximately 2.5 mg recombinant protein.

Recombinant Enterokinase

- 50 U Recombinant Enterokinase (in 200 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 7.4)
- 10 µg Cleavage Control Protein (in 200 mM NaCl, 20 mM Tris-HCl, 20 mM EDTA, 50% glycerol, pH 7.5)
- 2 ml 1X rEK Dilution/Storage Buffer (200 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 7.4)
- 1 ml 10X rEK Cleavage Buffer (500 mM NaCl, 200 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.4)

Enterokinase Cleavage Capture Kit

- 50 U Recombinant Enterokinase (in 200 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 7.4)
- 10 µg Cleavage Control Protein (in 200 mM NaCl, 20 mM Tris-HCl, 20 mM EDTA, 50% glycerol, pH 7.5)
- 2 ml 1X rEK Dilution/Storage Buffer (200 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 7.4)
- 5 ml 10X rEK Cleavage/Capture Buffer (500 mM NaCl, 200 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.4)
- 1.5 ml EKapture™ Agarose (3 ml 50% slurry in phosphate buffer, 500 mM NaCl, 0.02% Sodium Azide, pH 7.3)
- 10 Spin Filters, 2-ml capacity

Storage

Store Recombinant Enterokinase (rEK), Cleavage Control Protein, and 1X rEK Dilution/Storage Buffer at –20°C. Store 10X rEK Cleavage/Capture Buffer at –20°C or 4°C. Store EKapture Agarose at 4°C. *Do not freeze EKapture Agarose.* Store Spin Filters at room temperature.

Caution: EKapture Agarose is supplied in a buffer containing 0.02% Sodium Azide. Handle with caution. Wear gloves and appropriate laboratory clothing.

Factors that influence rEK activity

Depending on the buffer used and its chemical components, rEK cleavage efficiency may be affected. Perform pilot digests with the Cleavage Control Protein to evaluate the effect of the buffer used. Table 1 shows how rEK activity is influenced by various conditions and components.

Table 1

Condition or Component	Effect on rEK activity
1X rEK Cleavage/Capture Buffer	None
1X His•Bind® Elute Buffer	Inhibitory
0.5X His•Bind Elute Buffer	Inhibitory
0.25X His•Bind Elute Buffer	Partially Inhibitory
0.125X His•Bind Elute Buffer	Partially Inhibitory
pH 5–9	None
< 0.5% Triton X-100	None
< 0.5% TWEEN 20	None
< 100 mM DTT	None
> 250 mM NaCl	Inhibitory
> 250 mM Imidazole	Inhibitory
> 1 mM PMSF	Inhibitory
> 2 M Urea	Inhibitory
> 2 M Guanadine	Inhibitory
0.0625% SDS	Secondary cleavage

rEK Cleavage

One unit of Recombinant Enterokinase (rEK) is defined as the amount of enzyme that cleaves > 95% of 50 µg Cleavage Control Protein in 50 mM NaCl, 20 mM Tris-HCl, 2mM $CaCl_2$, pH 7.4 at room temperature in 16 h. Because each target protein presents the cleavage site somewhat differently, we recommend testing several enzyme-to-target protein ratios, concentrations, temperatures, and incubation times to optimize the efficiency of cleavage. Incubation temperatures ranging from 4–37°C can be used. We recommend starting at room temperature (20–23°C), as rEK is most active at this temperature. Optimal specificity is achieved by using the lowest amount of rEK necessary to achieve complete cleavage. Excess rEK may result in unwanted proteolysis at secondary sites.

Small scale optimization

The following protocol is an example of a simple optimization experiment designed to estimate the appropriate ratio of rEK to target protein. In this approach, a constant amount of protease is added to three different amounts of target protein. Samples are analyzed at increasing incubation times. This example represents rEK:target protein ratios (unit/µg) of 1:20, 1:50, and 1:100.

Note: An rEK:target protein ratio (unit/µg) of 1:50 incubated at room temperature for 16 h is appropriate for most applications.

1. Dilute rEK in 1X rEK Dilution/Storage Buffer to a concentration of 1 unit rEK per 1 µl. Dilutions can be stored in this buffer at –20°C for several weeks. The concentration of rEK (units/µl) is provided on the tube label.
2. Assemble the following components in three separate 1.5-ml tubes:

5 µl	10X rEK Cleavage/Capture Buffer
20, 50, 100 µg	Target protein
1 µl	Diluted rEK (1 unit/µl)
X µl	Deionized water
50 µl	Total volume

3. Incubate reactions at room temperature. Remove 10 µl samples after 2, 4, 8, and 16 h. Add 10 µl 2X SDS sample buffer to each 10 µl sample. Store at –20°C until SDS-PAGE analysis.
4. Determine the extent of cleavage of the samples by SDS-PAGE analysis. Run an undigested sample of the target protein as a control.

Scale-up

When a satisfactory condition for the small scale reaction is found, increase the size of the reaction proportionately. If the reaction volume is kept in proportion to the example above, a relatively large volume will be required for digestion of 1 mg of target protein.

1. Prepare 1X Cleavage/Capture Buffer by diluting 10X Cleavage/Capture Buffer with sterile water. Chill at 4°C prior to use.
2. Add rEK to target protein at the desired rEK:target protein ratio and incubate at room temperature for the desired time.
3. Analyze samples by SDS-PAGE analysis.

rEK Capture

rEK can be removed from the sample using EKapture™ Agarose. rEK is bound batch-wise to EKapture Agarose, and the target protein recovered by spin-filtration. For demanding applications where more stringent removal of rEK is required, multiple capture steps are recommended. Alternatively, any residual rEK can be inactivated (see page 5).

In the following capture protocol, one volume (1 vol) is equivalent to the settled bed volume (e.g., 100 µl slurry yields 50 µl EKapture Agarose for a settled bed volume of 50 µl).

Pre-equilibration

1. Determine the required amount of EKapture Agarose necessary to capture the rEK present in the cleavage reaction. A ratio of 50 µl settled resin (100 µl of the 50% slurry) per 2 units rEK will bind > 99% of the enzymatic activity in a 5 min incubation in the 1X rEK Cleavage/Capture Buffer. Use at least 25 µl EKapture Agarose as smaller volumes are difficult to manipulate.

Note: If using a buffer other than the supplied 10X rEK Cleavage/Capture Buffer, see "Capture buffer considerations" below.

2. Prepare 1X rEK Cleavage/Capture Buffer by diluting the supplied 10X stock with sterile water sufficient for approximately 11 vol EKapture Agarose. Chill at 4°C prior to use.
3. Mix EKapture Agarose slurry by inversion until fully resuspended. Using a wide mouth pipette, transfer slurry into a clean centrifuge tube; transfer twice required vol to account for buffer.
4. Centrifuge at 1000 × g for 5 min. Carefully remove the supernatant and discard.
5. Resuspend EKapture Agarose in 10 vol 1X rEK Cleavage/Capture Buffer, and centrifuge again at 1000 × g for 5 min. Carefully remove the supernatant and discard.
6. Add 1 vol 1X rEK Cleavage/Capture Buffer and resuspend completely.

Capture

1. Transfer the pre-equilibrated EKapture Agarose to a 2-ml Spin Filter (supplied).
2. Add total volume of cleavage reaction to the Spin Filter sample cup. For volumes > 2 ml, use a centrifuge tube or 5-ml Spin Filter (Cat. No. 69074).
3. Mix gently to resuspend the agarose. *Do not vortex*. Incubate at room temperature for 5 min.
4. Centrifuge reaction at 1000 × g for 5 min to remove EKapture Agarose. Bound rEK is retained in the sample cup; the cleaved target protein and N-terminal fusion will flow into the filtrate tube during centrifugation.

Target protein isolation

1. Isolate and remove the N-terminal fusion from the target protein by IMAC (e.g., His•Bind® Resin, see User Protocol TB054) if applicable, or by size exclusion methods.
2. If desired, analyze samples by SDS-PAGE analysis.

Capture buffer considerations

Buffers other than 1X rEK Cleavage/Capture Buffer can be used with EKapture Agarose. Note, 2M urea will reduce the capture efficiency of the agarose approximately 60%. 0.25X His•Bind Elution Buffer or other salts will reduce capture by 20–50%. EKapture Agarose is unaffected by DTT at concentrations up to 100 mM, and Triton X-100 up to 1%.

Monitoring rEK capture

A simple, rapid fluorometric peptide-based assay using the substrate Gly(Asp)$_4$Lys β-naphthylamide (Sigma) has been described (2) which allows for rapid evaluation of buffer or dilution effects on EKapture Agarose capture efficiency. The assay requires a fluorometer or fluorescent spectrophotometer. Note, it is important to include an uncaptured rEK control to distinguish rEK capture from buffer-mediated rEK inhibition. Results from capture efficiency tests can be helpful in determining the optimal amount of EKapture Agarose needed.

Inactivation of rEK

Inactivation of any residual rEK activity (< 1%) may be desired in some applications. Serine protease inhibitors will inactivate rEK. For example, APMSF (Calbiochem) is a water-soluble suicide substrate derived from PMSF that inactivates rEK by covalent attachment to the active site serine residue. A working stock can be prepared in water at 50 mM (500X), and is stable for 1–3 months.

Note: Protease inhibitors are very toxic. Please use caution with these products and carefully follow the manufacturer's recommendations for use.

Troubleshooting Guide

Table 2

Problem	Probable cause	Solution
Incomplete cleavage	Suboptimal rEK to target protein ratio	Confirm the amount of fusion protein in the digestion. Adjust the amount of rEK added to at least 20 units/mg fusion protein.
	Insufficient incubation period	Increase reaction time.
	rEK recognition site not present or has been altered during the course of cloning	Verify presence of the rEK cleavage sequence (i.e., AspAspAspAspLys).
	rEK recognition site is not accessible	Reversibly denature protein with non-ionic detergents, denaturants (see Table 1).
	Inhibitors present (see Table 1)	Dialyze the fusion protein against rEK Cleavage/Capture Buffer before cleaving with rEK.
Incorrect banding pattern (e.g., multiple bands present) on SDS-PAGE gel following cleavage by rEK	Similar secondary recognition sequences in protein of interest	Adjust reaction conditions to minimize exposure of secondary cleavage sites (e.g., salt concentration, time, temperature).
	Proteolysis at secondary sites due to excess rEK	Reduce rEK concentration.
	Proteolysis in bacterial host	Use protease-deficient strain (e.g., *lon* or *ompT*), such as *E. coli* BL21(DE3).
	Premature truncation due to rare codons	Use codon-supplementing strain, such as *E. coli* Rosetta™ 2(DE3).

References

1. Collins-Racie, L. A., McColgan, J. M., Grant, K. L., DiBlasio-Smith, E. A., McCoy, J. M., and LaVallie, E. R. (1995) *Bio/Technology* 13, 982-987.

2. Antonowicz, I., Hesford, F. J., Green, J. R., Grogg, P., and Hadorn, B. (1980) *Clinica Chimica Acta* 101, 69-76.