Recombinant Enterokinase Kits

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Description

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

Recombinant Enterokinase is a highly purified preparation of the catalytic subunit of bovine enterokinase, which recognizes the identical cleavage site (AspAspAspAspLys↓) as the native enzyme and has similar enzymatic activity. rEK exhibits superior rates of cleavage of fusion proteins containing the recognition sequence when compared to native enzyme (1). Novagen's rEK is purified to near homogeneity and, unlike some preparations of native bovine enterokinase, exhibits no secondary cleavage arising from contaminating proteases. When analyzed by SDS-PAGE on a 4–20% gradient gel under reducing conditions, rEK migrates as a single band with apparent molecular weight of 26 kDa. One unit of rEK cleaves 50 µg fusion protein in 16 h at 20°C in a buffer containing 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$.

A Cleavage Control Protein is included for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions. The 48 kDa control protein is cleaved by rEK into two proteolytic fragments of 32 kDa and 16 kDa which are easily visualized by standard SDS-PAGE.

Components

Enterokinase Cleavage Capture Kit

The kit contains enough components to treat up to 2.5 mg of recombinant protein.

- 50 U Recombinant Enterokinase (in 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 2 mM $CaCl_2$, 50% glycerol)
- 10 µg Cleavage Control Protein (in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 20 mM EDTA, 50% glycerol)
- 2 ml 1X rEK Dilution/Storage Buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 2 mM $CaCl_2$, 50% glycerol)
- 5 ml 10X rEK Cleavage/Capture Buffer (200 mM Tris-HCl pH 7.4, 500 mM NaCl, 20 mM $CaCl_2$)
- 1.5 ml EKapture Agarose (3 ml of a 50% slurry in phosphate buffer, pH 7.3, 0.5 M NaCl, 0.02% Thimerosal)
- 10 Spin Filters, 2-ml capacity

Storage: Store Spin Filters and EKapture Agarose at 4°C (Spin Filters can also be stored at room temperature). *Do not freeze EKapture Agarose.* Store other kit components at –20°C.

Recombinant Enterokinase Kit

The kit contains enough components to treat up to 2.5 mg of recombinant protein.

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

Storage: Store kit components at –20°C.

Related products/available separately	Size	Cat. No.
Cleavage Control Protein	10 µg	69069
EKapture™ Agarose	1.5 ml	69068-3
S-protein HRP Conjugate	50 µl	69047-3
S-protein AP Conjugate	50 µl	69598-3
S-protein FITC Conjugate	200 µl	69060-3
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
CDP-Star™ Substrate	40 ml	69086-3
SuperSignal™ Substrate	50 ml	69059-3
Spin Filters, 2-ml capacity	pkg/10	69072-3
Spin Filters, 5-ml capacity	pkg/2	69074-3

rEK Cleavage

Recombinant Enterokinase is a site-specific protease that exhibits very low non-specific cleavage under many conditions. One unit of rEK is generally sufficient for cleavage of 50 µg target protein in 1X rEK 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 rEK concentrations, temperatures and/or incubation times to optimize specificity and efficiency of cleavage. Note that excess rEK 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, rEK is effectively inhibited by 1 mM PMSF).

Small scale optimization

For small scale digestions the rEK can be diluted in rEK 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 3 serial dilutions of rEK in rEK Dilution/Storage Buffer to produce solutions having 0.1, 0.2, and 0.5 U enzyme per µl.
2. Assemble the following components in a series of 4 labeled tubes.

5 µl	10X rEK Cleavage/Capture Buffer
10 µg	target protein
1 µl	Diluted rEK (each tube receives 1 µl of a different enzyme dilution. The fourth 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.

The highest reaction rates and best cleavage efficiencies are demonstrated when the target protein is maintained at a concentration of 2.5 µM or above. Based on this target concentration, 10 µg of a target protein with a molecular weight of 50 kDa should be cleaved in a total volume of 80 µl or less. If the target protein is too dilute, pilot digestions of the control protein can be performed to determine the amount of rEK necessary to achieve cleavage, or the target protein can be concentrated. Examples of methods for concentrating proteins include:

1. Place the sample in dialysis tubing with an exclusion limit of 3500 MW and concentrate by sprinkling solid polyethylene glycol (15,000–20,000 molecular weight) or Sephadex G50 (Pharmacia) on the dialysis tubing. Leave the solid in contact with the tubing until the desired volume is reached, replacing it with fresh solid as necessary.
2. Use plastic disposable micro-concentrator units (e.g., Centricon, Amicon) as directed by the manufacturer to both desalt and concentrate the sample by ultrafiltration.

Notes:

a) The optimal enzyme specificity is achieved using the lowest amount of protease necessary to achieve complete cleavage.

b) Salt sensitivity: rEK is inhibited by imidazole or NaCl concentrations above 250 mM.

c) Denaturants or chaotropes: activity of the enzyme in solutions containing urea or guanidine should be confirmed with pilot digestions. rEK is especially sensitive to urea concentrations above 2 M.

d) Detergents: when digests are performed with as little as 0.0625% (w/v) SDS, significant secondary cleavage is seen. For this reason, it is recommended that SDS be avoided. rEK will tolerate Triton X-100 at concentrations of 1% without affecting specificity or activity.

e) Incubation temperature: rEK has higher activity at 21°C than at 37°C.

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 32 kDa and 16 kDa following rEK 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 rEK recognition sequence. The 16 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.

rEK 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 rEK 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.

rEK Capture

After the cleavage reaction, rEK can be effectively removed with EKapture Agarose (supplied in the rEK Cleavage/Capture Kit). Following cleavage of the target protein, the rEK is bound batchwise to EKapture Agarose and the target protein recovered by spin-filtration. When using 1X rEK Cleavage/Capture Buffer, a ratio of 50 µl settled resin (100 µl of the 50% slurry) per 2 units of enzyme will bind > 99% of the enzymatic activity in a 5 min incubation. Recovery of cleaved target protein is simplified by use of supplied Spin Filters, which enable efficient separation of the liquid phase of the reaction from the EKapture Agarose. For demanding applications where more stringent removal of rEK is required, two or more capture steps are recommended. Alternatively, any remaining rEK can be inactivated as described later in this section.

1. Determine the required amount of EKapture Agarose necessary to capture the rEK present in the cleavage reaction. The EKapture Agarose has sufficient capacity to bind 2 units rEK per 50 µl bed volume (100 µl slurry) in 1X rEK Cleavage/Capture Buffer. (If using a buffer other than the supplied rEK Cleavage/Capture Buffer, see "Capture buffer considerations" below). We recommend using a minimum of 25 µl EKapture Agarose slurry because smaller resin volumes are difficult to manipulate.

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

For maximum capture efficiency, EKapture Agarose should be pre-equilibrated in 1X rEK Cleavage/Capture buffer prior to use (Steps 2–6).

2. Prepare 1X rEK Cleavage/Capture Buffer by diluting the supplied 10X stock with sterile deionized water. You will need an amount of buffer corresponding to approximately 11 bed volumes of EKapture Agarose (as determined in Step 1).
3. Mix the EKapture Agarose (supplied as a 50% slurry) by inversion until fully resuspended. Using a wide mouth pipette, transfer the required amount of slurry into a clean centrifuge tube (transfer twice the required bed volume to account for the buffer).
4. Centrifuge at 1000 × g for 5 min and carefully remove and discard the supernatant.
5. Resuspend the agarose in ten bed volumes of 1X rEK Cleavage/Capture Buffer, centrifuge again, and remove and discard the supernatant.
6. Add one bed volume of 1X rEK Cleavage/Capture Buffer and fully resuspend. The EKapture Agarose is now equilibrated and ready to use.
7. Transfer the prepared EKapture Agarose from Step 6 to the sample cup of a 2-ml Spin Filter (included with the kit). Add the entire volume of the cleavage reaction to the prepared EKapture Agarose. For total volumes in excess of 2 ml, use a centrifuge tube or larger Spin Filter. Mix gently to resuspend the agarose. Do not vortex.
8. Incubate at room temperature for 5 min.
9. Centrifuge the reaction at 1000 × g for 5 min to remove the EKapture Agarose. Bound rEK is retained in the sample cup, and the cleaved target protein flows into the filtrate tube during centrifugation.

Capture buffer considerations

The binding of rEK to EKapture Agarose is sensitive to some buffer conditions in which rEK is fully active. If buffers other than the rEK Cleavage/Capture Buffer are required to maintain target protein solubility or activity, it is important to consider the impact such changes have on the affinity capture step. For example, the presence of 2M urea will reduce the capture efficiency of EKapture Agarose approximately 60%. Likewise, the presence of 0.25X His•Bind® Elution Buffer or other salts will reduce capture by 20-50%. EKapture binding is unaffected by DTT at concentrations up to 100 mM, and Triton X-100 up to 1%. The supplied rEK Cleavage/Capture Buffer is compatible with both cleavage and capture steps without any loss of efficiency of either step.

Monitoring rEK capture

To determine capture efficiency, perform pilot capture reactions under the modified buffer conditions and assay the unretained fractions for rEK activity, A simple, rapid fluorometric

peptide based assay using the substrate GlySer(Asp)$_4$Lys β-naphthylamide (Sigma) has been described (1, 2) which allows for rapid evaluation of buffer or dilution effects on capture efficiency. The assay requires a fluorometer or fluorescent spectrophotometer and has been successfully performed using a DNA Fluorometer (TKO-100; Hoefer). Whenever modified buffer conditions are tested, it is important to include an uncaptured rEK control to distinguish rEK capture from buffer mediated rEK inhibition. The results of the test can be used to adjust the amount of EKapture Agarose used for capture to compensate for any loss of binding efficiency.

Inactivation of rEK

While the use of EKapture Agarose under the described conditions will remove > 99% of the rEK activity, inactivation of any residual activity may be desired in some applications. Serine protease inhibitors will inactivate enterokinase. APMSF (a suicide substrate; Calbiochem) is a water soluble derivative of PMSF which effectively 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.

Caution: *APMSF and all protease inhibitors should be used with caution due to their toxicity. Please follow the manufacturer's recommendations for use. Note that treatment may also affect the target protein.*

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.