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About the Kits

Factor Xa Cleavage Capture Kit	69037-3
Factor Xa, Restriction Grade	69036-3

Description

The Factor Xa Cleavage Capture Kit is designed for highly specific cleavage of fusion proteins followed by convenient affinity-based capture and removal of Factor Xa. After cleavage of the target protein, Factor Xa is removed with greater than 99% efficiency from the reaction by affinity capture on Xarrest™ Agarose. Following capture of Factor Xa, the agarose is removed by spin-filtration. No buffer changes are necessary, because the same buffer conditions are used for both cleavage and capture.

The protease is a highly purified preparation isolated from bovine plasma and activated with Russell's viper venom. Novagen's Factor Xa preparation is purified to near homogeneity and exhibits no secondary cleavage arising from contaminating proteases. The preferred cleavage site is IleGluGlyArg↓ (1). When analyzed by SDS-PAGE on a 4–20% gradient gel under reducing conditions, Factor Xa migrates as two bands with apparent molecular weights of 34 kDa and 29 kDa. One unit of Factor Xa Protease cleaves 50 µg fusion protein in 16 h at 20°C in a buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM CaCl₂.

A Cleavage Control Protein is included for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions. The 49 kDa control protein is cleaved by Xa into two fragments with apparent molecular weights of 32 kDa and 17 kDa, which are easily visualized by standard SDS-PAGE.

Components

Factor Xa Cleavage Capture Kit

The kit contains enough components to treat up to 20 mg of recombinant protein.

- 400 U Factor Xa, Restriction Grade (in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM CaCl₂, 50% glycerol)
- 10 µg Xa Cleavage Control Protein (in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 50% glycerol)
- 2 ml 1X Xa Dilution/Storage Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM CaCl₂, 50% glycerol)
- 5 ml 10X Factor Xa Cleavage/Capture Buffer (500 mM Tris-HCl pH 8.0, 1 M NaCl, 50 mM CaCl₂)
- 5 ml Xarrest Agarose (10 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 Xarrest Agarose at 4°C (Spin Filters can also be stored at room temperature). *Do not freeze Xarrest Agarose.* Store other kit components at –20°C.

Restriction Grade Factor Xa Kit

The kit contains enough components to treat up to 20 mg of recombinant protein.

- 400 U Factor Xa, Restriction Grade (in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM CaCl₂, 50% glycerol)
- 10 µg Xa Cleavage Control Protein (in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 50% glycerol)
- 2 ml 1X Xa Dilution/Storage Buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM CaCl₂, 50% glycerol)
- 1 ml 10X Factor Xa Cleavage/Capture Buffer (500 mM Tris-HCl pH 8.0, 1 M NaCl, 50 mM CaCl₂)

Storage: Store kit components at –20°C.



Related products/available separately	Size	Cat. No.
Xarrest Agarose	5 ml	69038-3
Xa Cleavage Control Protein	10 µg	69051
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

Factor Xa Cleavage

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

Small scale optimization

For small scale digestions the Factor Xa can be diluted in Xa 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 Factor Xa in Xa 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 Factor Xa Cleavage/Capture Buffer
10 µg	target protein
1 µl	Diluted Factor Xa (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 Factor Xa 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: Factor Xa 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. Factor Xa is sensitive to urea, guanidine-HCl and guanidine thiocyanate at or above concentrations of approximately 250 mM.
- d) Detergents: when digests are performed with as little as 0.001% (w/v) SDS, significant secondary cleavage is seen. For this reason, it is recommended that SDS be avoided. Factor Xa will tolerate Triton X-100 or N-lauroylsarcosine at concentrations of up to 1% without significantly affecting specificity or activity.
- e) Incubation temperature: Factor Xa is more active at 37°C than at 21°C and is stable for more than 2 months at 37°C under standard digestion conditions. However, room temperature is recommended as a starting point to minimize exposure of secondary cleavage sites.
- f) It has been reported that Factor Xa recognizes the cleavage site P2-arg↓P1', where P2 is usually gly. P1' is non-specific, but Ile and Thr are preferred (2).
- g) A method has been described for the elimination of non-specific cleavage of fusion proteins by Factor Xa (3). Non-specific cleavage sites were blocked by reversible acylation of target protein by 3, 4, 5, 6-tetrahydrophthalic anhydride prior to cleavage. After cleavage, the acyl groups were removed quantitatively by exposure to slightly acidic conditions.
- h) Factor Xa has been reported to cut poorly when hydrophobic amino acids are present on the C-terminal side of the cleavage site (4).

Monitoring cleavage

Cleavage can be monitored easily by including a parallel reaction using the supplied Xa Cleavage Control Protein in the same buffer system as the target protein. The Xa Cleavage Control Protein is converted from a single 49 kDa band to two bands of 17 kDa and 32 kDa following Factor Xa cleavage. Inclusion of a control digest enables confirmation of enzyme activity and cleavage specificity, which is especially important when cleavage conditions have been modified. While sufficient control protein is provided to enable Coomassie blue detection of cleavage products, smaller scale reactions (< 0.5 μg) can be monitored by Western blotting. The Xa Cleavage Control Protein has an S•Tag peptide on the amino-terminal side of the Factor Xa recognition sequence. The 17 kDa cleavage product can be detected using either S-protein AP or HRP Conjugate. High sensitivity chemiluminescent detection of S•Tag fusion proteins can be performed using the S•Tag AP or HRP LumiBlot Blot 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.



Factor Xa 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 Factor Xa 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.

Factor Xa Capture

After the cleavage reaction, Factor Xa can be quantitatively removed with Xarrest Agarose (supplied in the Factor Xa Cleavage/Capture Kit). Following cleavage of the target protein, the Factor Xa is bound batchwise to Xarrest Agarose and the target protein recovered by spin-filtration. When using 1X Factor Xa Cleavage/Capture Buffer, a ratio of 50 μ l settled resin (100 μ l of the 50% slurry) per 4 units of enzyme will remove > 99% of the enzymatic activity is captured by Xarrest Agarose 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 Xarrest Agarose. For demanding applications where more stringent removal of Factor Xa is required, two or more capture steps are recommended. Alternatively, any remaining Factor Xa can be inactivated as described later in this section.

1. Determine the required amount of Xarrest Agarose necessary to capture the Factor Xa present in the cleavage reaction. The supplied Xarrest Agarose has sufficient capacity to bind 4 units Factor Xa per 50 μ l bed volume (100 μ l slurry) in 1X Factor Xa Cleavage/Capture Buffer. (If using a buffer other than the supplied Factor Xa Cleavage/Capture Buffer, see "Capture buffer considerations" below). We recommend using a minimum of 25 μ l Xarrest Agarose slurry because smaller resin volumes are difficult to manipulate.

Caution: The Xarrest 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, Xarrest Agarose should be pre-equilibrated in 1X Factor Xa Cleavage/Capture buffer prior to use (Steps 2–6).

2. Prepare 1X Factor Xa 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 Xarrest Agarose (as determined in Step 1).
3. Mix the Xarrest 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 \times g for 5 min and carefully remove and discard the supernatant.
5. Resuspend the agarose in ten bed volumes of 1X Factor Xa Cleavage/Capture Buffer, centrifuge again, and remove and discard the supernatant.
6. Add one bed volume of 1X Factor Xa Cleavage/Capture Buffer and fully resuspend. The Xarrest Agarose is now equilibrated and ready to use.
7. Transfer the prepared Xarrest 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 Xarrest 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 five min.
9. Centrifuge the reaction at 1000 \times g for 5 min to remove the Xarrest Agarose. Bound Factor Xa is retained in the sample cup, and the cleaved target protein flows into the filtrate tube during centrifugation.

Capture buffer considerations

The binding of Factor Xa to Xarrest Agarose is sensitive to some buffer conditions in which Factor Xa is fully active. If buffers other than the Factor Xa 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 0.25X His•Bind[®] Elution Buffer or other salts will reduce capture by 20–50%. Xarrest capture is unaffected by DTT at concentrations up to 100 mM, and Triton X-100 up to 1%. The supplied Factor Xa



Cleavage/Capture Buffer is compatible with both cleavage and capture steps without any loss of efficiency of either step.

Monitoring Factor Xa capture

To determine capture efficiency, perform pilot capture reactions under the modified buffer conditions and assay the unretained fractions for Factor Xa activity. A simple, rapid colorimetric peptide-based assay for Factor Xa activity using the substrate benzoyl-ile-glu-gly-arg-p-nitroanalide (Sigma) has been described (4), which enables rapid evaluation of buffer or dilution effects on capture efficiency. The assay requires a spectrophotometer equipped with kinetic measurement capability. Whenever modified buffer conditions are tested, it is important to include an uncaptured Factor Xa control to distinguish Factor Xa capture from buffer-mediated Factor Xa inhibition. The results of the test can be used to adjust the amount of Xarrest Agarose used for capture to compensate for any loss of binding efficiency.

Inactivation of Factor Xa

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

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