Factor Xa Kits

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About the Kits

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

Description

Restriction Grade Factor Xa is a highly purified enzyme isolated from bovine plasma and activated with Russell's viper venom. The Novagen preparation is purified to near homogeneity and shows no secondary cleavage 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.

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 95% 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 kit also includes a Cleavage Control Protein for conducting control digestions in parallel with experimental samples, or to test cleavage under customized buffer conditions.

The 53.1-kDa Factor Xa Cleavage Control Protein is cleaved into two proteolytic fragments of 35.8 kDa and 17.3 kDa, each of which is easily visualized by standard SDS-PAGE followed by Coomassie™ blue staining. The Xa Cleavage Control Protein also features an amino terminal S•Tag™ sequence enabling sensitive detection of the 17.3-kDa proteolytic product with Western blot detection reagents.

Components

Factor Xa Cleavage Capture Kit

- 400 U Factor Xa, Restriction Grade (in 500 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0)
- 10 µg Xa Cleavage Control Protein (in 100 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 50% glycerol, pH 7.4)
- 2 ml Factor Xa Dilution/Storage Buffer (500 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0)
- 5 ml 10X Factor Xa Cleavage/Capture Buffer (1 M NaCl, 500 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0)
- 2 × 2.5 ml Xarrest™ Agarose (10 ml of a 50% slurry in 0.5 M NaCl, 0.02% Sodium Azide)
- 10 Spin Filters, 2-ml capacity

Restriction Grade Factor Xa Kit

- 400 U Factor Xa, Restriction Grade (in 500 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0)
- 10 µg Xa Cleavage Control Protein (in 100 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 50% glycerol, pH 7.4)
- 2 ml Factor Xa Dilution/Storage Buffer (500 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0)
- 1 ml 10X Factor Xa Cleavage Buffer (1 M NaCl, 500 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0)

Storage

Store Spin Filters at room temperature or 4°C. Store Xarrest Agarose at 4°C. Store all other components at –20°C.

Note: *Do not freeze Xarrest Agarose.*

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

Factors That Influence Factor Xa Activity

Depending on the buffers used and their chemical components, Factor Xa Protease cleavage efficiency may be affected. Perform pilot digests with the target protein or the Factor Xa Cleavage Control Protein supplied in the kit to evaluate the effect of a given component or buffer. The following table shows how the activity of Factor Xa Protease is influenced by various conditions and components.

Table 1

Condition or Component	Effect on Factor Xa Protease activity
1X Factor Xa Cleavage Buffer	None
1 mM PMSF	Inhibitory
0.1 mM APMSF	Inhibitory
1 mM AEBSF	Inhibitory
≥ 100 mM DTT	Partially inhibitory
≥ 0.1% SDS	Inhibitory
≥ 0.001% SDS	Secondary cleavage
≥ 25% Ethylene Glycol	Inhibitory
1–10% Ethylene Glycol	Partially inhibitory
0.25X His•Bind® Elute Buffer	Inhibitory
0.125X His•Bind Elute Buffer	Partially inhibitory
≥ 250 mM Urea	Partially inhibitory
≥ 3% N-Lauroylsarcosine	Partially inhibitory
≥ 1% Tween™ 20	Partially inhibitory
≥ 1% Nonidet™ P-40	Partially inhibitory
≥ 1% Triton™ X-100	Partially inhibitory
≥ 500 mM Guanidine hydrochloride	Inhibitory
≥ 250 mM Guanidine hydrochloride	Partially inhibitory
≥ 500 mM Guanidine thiocyanate	Inhibitory
≥ 250 mM Guanidine thiocyanate	Partially inhibitory
≥ 250 mM Imidazole	Inhibitory
≥ 250 mM NaCl	Inhibitory

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 cleaves 50 µg of Cleavage Control Protein to > 95% completion in 16 hours at room temperature (20–21°C) in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0. However, because each target protein presents the cleavage site somewhat differently, we recommend testing several enzyme-to-target protein ratios, concentrations, temperatures, and/or incubation times to optimize specificity and efficiency of cleavage. Incubation temperatures ranging from 4°C to 37°C can be used, although we recommend 20°C as the starting point for most proteins.

Notes:

a) Optimal enzyme specificity is achieved using the lowest amount of protease necessary for complete cleavage. Excess Factor Xa may result in unwanted proteolysis at secondary sites.

b) Denaturants or chaotropes: Activity of the enzyme in a solution 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.

c) Detergents: When digests are performed with as little as 0.001% (w/v) SDS, significant secondary cleavage is seen. For this reason, we recommend 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.

d) 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 (20–21°C) is recommended as a starting point to minimize exposure of secondary cleavage sites.

e) 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).

f) A method has been described in which 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 (3).

g) Factor Xa has been reported to cleave poorly when hydrophobic amino acids are present on the C-terminal side of the cleavage site (4).

Small scale optimization

The following protocol is an example of a simple optimization experiment designed to estimate the appropriate range of enzyme:target protein. Samples are analyzed at increasing incubation times. This example represents Factor Xa:target protein ratios (unit:µg) of 1:100, 1:50, and 1:20.

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.

1. Make three 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 four separate, labeled 1.5-ml 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). Remove 10 µl samples after 2, 4, 8, and 16 h. Add 10 µl 2X SDS sample buffer. Store at –20°C until SDS-PAGE analysis.
4. Determine the extent of cleavage of samples by SDS-PAGE analysis.

Scale-up

When a satisfactory condition is found, increase the size of the reaction proportionately. If the reaction volume is kept in proportion with the small scale example above, a relatively large volume (5 ml) would be used for a 1-mg digestion. If desired, perform another preliminary experiment by maintaining constant enzyme:target protein ratio and incubation conditions while varying the reaction volume. Determine appropriate adjustments necessary for higher concentrations of enzyme and target protein.

The fastest 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 concentration is too dilute, pilot digestions of the control protein can be performed to determine the amount of Factor Xa necessary to achieve cleavage. Alternatively, the target protein solution can be concentrated. Examples of methods for concentrating proteins follow.

1. D-Tube™ Dialyzers are ideally suited for concentrating dilute samples by evaporation because of the dual membranes and large surface area. For more information see User Protocol TB422.
2. Place sample in dialysis tubing with an exclusion limit of 3,500 MW and concentrate by sprinkling solid polyethylene glycol (15,000–20,000) or Sephadex™ G50 (Pharmacia) on dialysis tubing. Leave solid in contact with tubing until desired volume is reached, replacing with fresh solid as necessary.
3. Use plastic disposable microconcentrator units (e.g., Centricon™, Amicon) as directed by the manufacturer to both desalt and concentrate sample by ultrafiltration.

Monitoring cleavage

Cleavage efficiency 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 53.1-kDa band to two bands of 17.3 kDa and 35.8 kDa following Factor Xa cleavage. Inclusion of a positive control for the digestion 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 17.3-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®, or other peptides that may be cleaved from fusion proteins and for which a detection reagent is available.

Troubleshooting Guide

Table 2

Problem	Probable cause	Solution
Incomplete cleavage	Suboptimal Factor Xa to fusion protein ratio	Confirm the amount of fusion protein in the digestion. Adjust the amount of Factor Xa added to at least 20 U/mg fusion protein.
		Confirm the target protein is at a concentration of at least 2.5 µM.
	Insufficient incubation period	Increase reaction time.
	Factor Xa recognition site not present or has been altered during the course of cloning.	Verify presence of optimal Factor Xa cleavage sequence.
	Factor Xa recognition site is not accessible.	Reversibly denature protein with non-ionic detergents, denaturants (see Table 1).
	Factor Xa inhibitors present (see Table 1)	Dialyze the fusion protein against Cleavage Buffer before cleaving with Factor Xa.
Multiple bands present on SDS-PAGE Gel following cleavage by Factor Xa	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 Factor Xa.	Reduce Factor Xa concentration.
	Proteolysis in bacterial host	Use protease-deficient strain (e.g., *lon* or *ompT*), such as *E. coli* BL21(DE3).

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 µl settled resin (100 µl of the 50% slurry) per 4 units of enzyme will remove > 95% 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 amount of Xarrest Agarose necessary to capture Factor Xa present in cleavage reaction. The supplied Xarrest Agarose has sufficient capacity to bind 4 units Factor Xa per 50 µl settled resin vol (100 µl 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 µl Xarrest Agarose slurry because smaller resin volumes are difficult to manipulate.
2. Prepare 1X Factor Xa Cleavage/Capture Buffer by diluting supplied 10X stock with sterile deionized water. You will need an amount of buffer corresponding to approximately 11 settled resin vol of Xarrest Agarose (as determined in Step 1).
3. Mix Xarrest Agarose (supplied as 50% slurry) by gentle tumbling or agitation until fully resuspended. Using wide mouth pipette, transfer 2 settled resin vol slurry to clean centrifuge tube.
4. Centrifuge at 1000 × g for 5 min. Carefully remove and discard supernatant.
5. Resuspend agarose in 10 settled resin vol 1X Factor Xa Cleavage/Capture Buffer.
6. Centrifuge at 1000 × g for 5 min. Remove and discard supernatant.
7. Add 1 settled resin vol 1X Factor Xa Cleavage/Capture Buffer and fully resuspend. The Xarrest Agarose is now equilibrated and ready to use.
8. Transfer prepared Xarrest Agarose from Step 6 to sample cup of 2-ml Spin Filter (included with kit). Add entire volume of cleavage reaction to prepared Xarrest Agarose. For total volumes in excess of 2 ml, use centrifuge tube or larger Spin Filter. Mix gently to resuspend agarose. Do not vortex.
9. Incubate at room temperature for 5 min.
10. Centrifuge reaction at 1000 × g for 5 min to remove the Xarrest Agarose. Bound Factor Xa is retained in 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 at 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 > 95% of the Factor Xa activity, inactivation of any residual activity may be desired in some instances. Serine protease inhibitors (i.e., PMSF, APMSF, AEBSF) will inactivate Factor Xa. We recommend AEBSF (Cat. No. 101500), a more stable and non-toxic alternative to PMSF. A working stock can be prepared in water at 100 mM (100X) and is stable for 1–2 months, at 4°C, pH 7. AEBSF Will slowly hydrolyze at pH > 8.

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