HRV 3C Protease

Table of Contents

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About the Kit

HRV 3C Protease	500 U	71493-3

Description

HRV 3C Protease is a recombinant form of the 3C protease from human rhinovirus type 14. The enzyme is a highly purified His•Tag® fusion protein. HRV 3C Protease site-specific cleavage allows production of recombinant proteins virtually free of vector-encoded sequences. A number of factors, including small size (22 kDa), robust activity at 4°C, high specific activity, and His•Tag fusion, make HRV 3C Protease an ideal choice for rapid removal of His•Tag fusions, as well as the protease, by digestion followed with immobilized metal affinity chromatography (IMAC) (1–3). HRV 3C Protease recognizes the cleavage site: LeuGluValLeuPheGln/GlyPro (3).

HRV 3C Cleavage Control Protein is included for conducting control digests in parallel with experimental samples, or to test cleavage under customized buffer conditions. HRV 3C Protease cleaves the 53.1-kDa control protein into two fragments with apparent molecular weights of 14.0 kDa and 39.1 kDa, which are easily visualized by standard SDS-PAGE and Coomassie blue staining.

Components

- 500 U HRV 3C Protease (2000 U/ml in 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM THP, 50% glycerol, pH 8.0)
- 10 µg HRV 3C Cleavage Control Protein (in 100 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 50% glycerol, pH 7.4)
- 10 ml 10X HRV 3C Protease Cleavage Buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5)

Storage

Store HRV 3C Protease at –20°C. Store HRV 3C Cleavage Control Protein and Protease Cleavage Buffer at –20°C or 4°C.

HRV 3C Protease

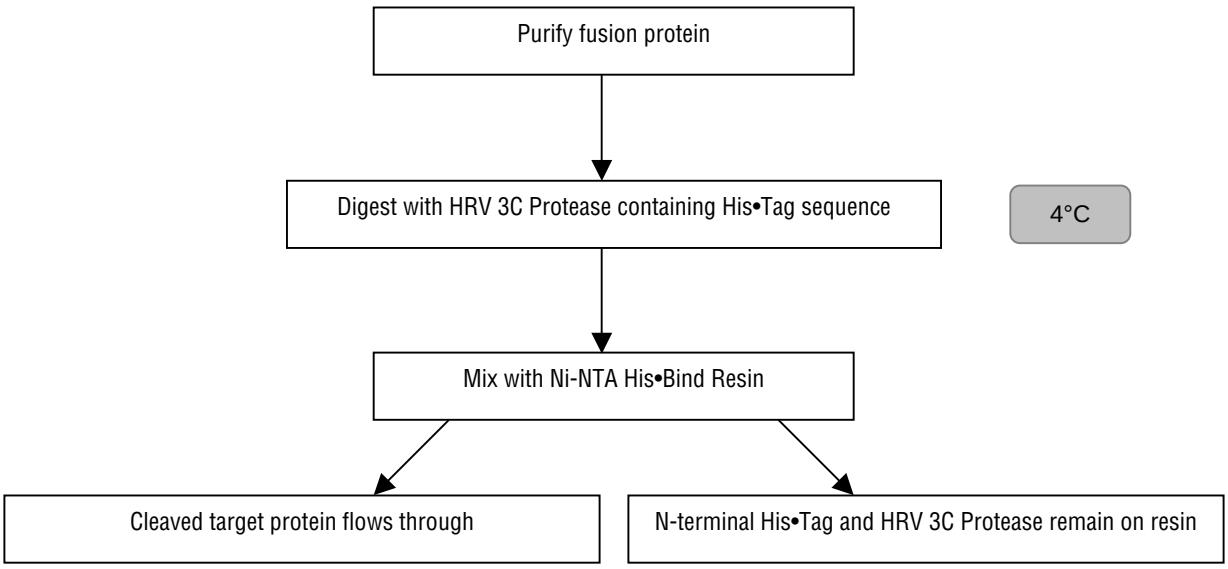
HRV 3C Protease is a restriction grade protease with a specific activity of 1800–2000 U/mg. One unit of HRV 3C Protease is defined as the amount of enzyme that will cleave > 95% of 100 µg HRV 3C Cleavage Control Protein in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5 at 4°C for 16 h. However, because each target protein presents the cleavage site somewhat differently, we recommend testing several enzyme-to-target protein ratios, concentrations, temperatures, and/or incubation times to optimize the efficiency of cleavage. Incubation temperatures ranging from 4–37°C can be used; we recommend 4°C as the starting point for most proteins.

When cleavage of immobilized proteins is desired, higher amounts of HRV 3C Protease may be required than for solution-based reactions.

The His•Tag® fusion protease is designed to specifically remove tags from proteins and to be removed from the digest by IMAC. The digest is put on a column, the protease binds, and the cleaved target protein flows through. If the cleaved tag is a His•Tag sequence, the tag also binds to the IMAC column, providing an efficient method to remove tags and protease from target protein.

There are situations in which the target protein is not readily recovered from the Ni-NTA His•Bind® Resin; for example, metal-binding proteins. Other instances in which a target protein would remain bound to the Ni-NTA His•Bind Resin include proteins with a C-terminal His•Tag sequence, or proteins containing an N-terminal His•Tag sequence downstream of the HRV 3C Protease cleavage site. These cases can be controlled through vector and cloning choices.

Experimental outline



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Factors that influence HRV 3C activity

Depending on the buffers used and their chemical components, HRV 3C Protease cleavage efficiency may be affected. Perform pilot digests with the target protein or the HRV 3C Cleavage Control Protein supplied in the kit to evaluate the effect of a given component or buffer. The following table shows the relative activity of HRV 3C Protease under various conditions.

Table 1

Condition or Component	Relative HRV 3C Protease activity (%)
1X HRV 3C Cleavage Buffer	100
0.8 M NaCl	150
0.2 M NaCl	110
0.75 mM Leupeptin*	50
8 mM PMSF*	50
> 1 mM TLCK*	50
10% glycerol (4)	114
100 mM $ZnCl_2$	50
1X Ni-NTA His•Bind® Wash Buffer	100
1X Ni-NTA His•Bind Elute Buffer	100
0.75X Ni-NTA His•Bind Elute Buffer	100
0.5X Ni-NTA His•Bind Elute Buffer	100
0.25X Ni-NTA His•Bind Elute Buffer	100
0.13X Ni-NTA His•Bind Elute Buffer	100
3 M Urea	0
2 M Urea	0
1 M Urea	40
3 M Guanadine	0
2 M Guanadine	0
1 M Guanadine	0
1 mM DTT*	100
50 mM EDTA**	100
50 mM EGTA**	100
0.1% Triton™ X-100	> 100
0.1% Tween™ 20	> 100
0.1% Nonidet™ P-40	> 100
1% Triton X-100	100
1% Tween 20	100
1% Nonidet P-40	100

*(5)

**While the concentration of these reagents is compatible with HRV 3C Protease activity, compatibility with IMAC may be affected.

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Cleavage

Cleavage in solution

Small scale optimization

The following protocol is an example of a simple optimization experiment designed to estimate the appropriate ratio of enzyme:target protein. In this approach, a constant amount of enzyme is added to three different amounts of target protein. Samples are analyzed at increasing incubation times. This example represents HRV 3C Protease:target protein ratios (unit/µg) of 1:5, 1:25, and 1:50.

1. Assemble the following components in separate 1.5-ml tubes.

4 µl	10X HRV 3C Protease Cleavage Buffer
10, 50, 100 µg	target protein
1 µl	HRV 3C Protease
X µl	deionized water
50 µl	total volume

2. Incubate the reactions at 4°C. Remove 10 µl samples after 1, 3, 6, and 16 h. Add 10 µl 2X SDS sample buffer to each 10 µl sample. Store at -20°C until SDS-PAGE analysis.
3. Determine the extent of cleavage of the samples by SDS-PAGE analysis. Run an undigested sample of the target protein as a control.

Note: For most applications, a HRV 3C Protease:target protein ratio (unit/µg) of 1:100–1:5 incubated at 4°C for 16 h is appropriate.

Scale up

When a satisfactory condition for the small-scale reaction is found, increase the size of the reaction proportionately. Specific instructions for purification of His•Tag® fusion proteins using Ni-NTA His•Bind® Resin can be found in User Protocol TB273.

1. Prepare 1X HRV 3C Cleavage Buffer by diluting the supplied 10X stock with sterile water. Chill at 4°C prior to use.
2. Add HRV 3C Protease to fusion protein at the desired protease:protein ratio.
3. Incubate at 4°C for the desired time.
4. Equilibrate desired amount Ni-NTA His•Bind Resin as 50% slurry with 1X HRV 3C Cleavage Buffer.

Note Ni-NTA His•Bind Resin has a binding capacity of 5–10 mg protein per ml settled resin.

5. Add digest to equilibrated 50% slurry Ni-NTA His•Bind Resin. Mix by gentle tumbling or agitation for 20 min at 4°C.
6. Load onto a spin filter column.
7. Centrifuge at 1000 × g for 1 min and collect protein of interest in the flow through.
8. **Optional:** Proteins adsorbed by Ni-NTA His•Bind Resin can be eluted separately with 1X Ni-NTA Elute Buffer.
9. If desired, analyze samples using SDS-PAGE analysis.

Cleavage in dialysis

1. Collect eluate from purification of fusion protein.
2. Add HRV 3C Protease to achieve a HRV 3C Protease:target protein ratio (unit/µg) of 1:10.
3. Dialyze against 100 vol HRV 3C Protease Cleavage Buffer for 16 h at 4°C.

Note: We recommend dialysis membrane pore size < 22,000 kDa MWCO.

4. Remove the His•Tag portion of the fusion protein and the HRV 3C Protease by IMAC.
5. If desired, analyze samples using SDS-PAGE analysis.

On-column cleavage

HRV 3C Protease can be added to Ni-NTA His•Bind® Resin to which a His•Tag® fusion protein has been bound. Specific instructions for purification of His•Tag fusion proteins using Ni-NTA His•Bind Resin can be found in User Protocol TB273. The following protocol describes on-column purification and cleavage. The purification and cleavage could also be performed by batch method, collecting the resin by centrifugation at 500 × g for 5 min. The on-column cleavage method captures the fusion tag and HRV 3C Protease, while the protein of interest is released and flows through the column.

1. Prepare 1X HRV 3C Protease Cleavage Buffer by diluting the supplied 10X stock with sterile water. Chill to 4°C prior to use.
2. Equilibrate desired amount Ni-NTA His•Bind Resin as a 50% slurry with 1X Ni-NTA Bind Buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0).
3. Add bacterial lysate to desired amount of Ni-NTA His•Bind Resin.

Note *Ni-NTA His•Bind Resin has a binding capacity of 5–10 mg protein per ml settled resin.*

4. Mix by gentle tumbling or agitation for 30–60 min at 4°C.
5. Load lysate/Ni-NTA His•Bind mixture on a column with the bottom outlet capped.
6. Remove bottom cap and collect column flow-through.
7. Wash 3 times each with 15–20 settled resin vol 1X Ni-NTA Wash Buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0).
8. Wash 2 times each with 15–20 settled resin vol 1X HRV 3C Protease Cleavage Buffer.
9. Replace bottom cap.
10. Prepare 2 settled resin vol 1X HRV 3C Protease Cleavage Buffer + HRV 3C Protease by adding 400 U HRV 3C Protease to 2 ml 1X HRV 3C Cleavage Buffer (prepared in Step 1) per ml settled Ni-NTA His•Bind Resin.
11. Add 2 settled resin vol 1X HRV 3C Cleavage Buffer + HRV 3C Protease (prepared in Step 10) per ml settled resin to the column.

Note: *More rapid cleavage may be achieved by adding a larger quantity of HRV 3C Protease.*

12. Pipet up and down to mix.
13. Incubate at 4°C for 16 h.

Note: *Cleavage may be complete in less than 16 h.*

14. Remove bottom cap and collect eluate as it flows from the Ni-NTA His•Bind column. The eluate should contain the protein of interest, while the His•Tag portion of the fusion protein and the HRV 3C Protease should remain bound to the Ni-NTA His•Bind Resin.
15. Additional 1X HRV 3C Protease Cleavage Buffer (0.5–1.0 settled resin vol) may be used to wash void volume containing protein of interest from the column. Collect this dilute sample separately.
16. If the eluate contains HRV 3C Protease, the residual protease can be removed by passing the sample over an equilibrated Ni-NTA His•Bind Resin.
17. **Optional:** Proteins adsorbed by Ni-NTA His•Bind Resin can be eluted separately with 1X Ni-NTA Elute Buffer.
18. If desired, analyze samples using SDS-PAGE analysis.

Removal of HRV 3C Protease

IMAC removal step is greater than 95% efficient in protease capture (6). A colorimetric assay can be used to determine the amount of residual HRV 3C Protease present (5).

Troubleshooting Guide

Table 2

Problem	Probable cause	Solution
Incomplete cleavage	Suboptimal HRV 3C Protease to fusion protein ratio	Confirm the amount of fusion protein in the digestion. Adjust the amount of HRV 3C Protease added to at least 10 U/mg fusion protein.
	Insufficient incubation period	Increase reaction time.
	HRV 3C Protease recognition site not present or has been altered during the course of cloning.	Verify presence of optimal HRV 3C Protease cleavage sequence.
	HRV 3C Protease recognition site is not accessible.	Reversibly denature protein with non-ionic detergents, denaturants (see Table 1).
	HRV 3C Protease inhibitors present (see Table 1)	Dialyze the fusion protein against Cleavage Buffer before cleaving with HRV 3C Protease.
	On-column cleavage less than optimal.	Increase HRV 3C Protease concentration.
		Perform cleavage in solution or dialysis.
HRV 3C Protease contamination after purification with Ni-NTA His•Bind® resin	Ni-NTA His•Bind Resin was saturated with His•Tag® fusion protein or fusion tag. Removal of HRV 3C Protease by the resin was incomplete.	Increase amount of Ni-NTA His•Bind Resin or decrease amount of extract loaded on Ni-NTA His•Bind Resin.
		Pass sample over freshly prepared or regenerated Ni-NTA His•Bind Resin.
Multiple bands present on SDS-PAGE Gel following cleavage by HRV 3C Protease	Similar secondary recognition sequences in protein of interest (1–3)	Adjust reaction conditions to minimize exposure of secondary cleavage sites (e.g., salt concentration, time, temperature).
	Proteolysis at secondary sites due to excess HRV 3C Protease	Reduce HRV 3C Protease concentration.
	Proteolysis in bacterial host	Use protease-deficient strain (e.g., *lon* or *ompT*), such as *E. coli* BL21(DE3).

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