Cat. # 7360

For Research Use

TaKaRa

HRV 3C Protease

Product Manual

v201612Da

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I. Description

HRV 3C Protease is a recombinant 3C protease derived from human Rhinovirus type 14 expressed in *E. coli*. This product is a highly purified 6xHN-tagged protein. The enzyme has the same activity as the native protein and cleaves a specific amino acid sequence (LeuGluValLeuPheGln ↓ GlyPro). It is useful for cleaving tag sequences from fusion proteins that contain a HRV 3C Protease cleavage site.

Because this product has a N-terminal 6xHN tag (which is a modified His-tag), it can be easily eliminated from the protease reaction solution through immobilized metal affinity chromatography (IMAC) using TALON® Metal Affinity Resin or His60 Ni Superflow Resin. Additionally, because the enzyme is active at 4℃, protein cleavage reactions can be performed under conditions that do not affect the activity or stability of the target protein.

This product includes a Cleavage Control Fusion Protein. The Control Protein has a N-terminal 6xHN tag. Digestion of the Control Protein with HRV 3C Protease results in a 52 kDa TF protein and a 24 kDa GST peptide. Successful cleavage of the Control Protein can be easily confirmed using SDS-PAGE.

II. Components

HRV 3C Protease (1 U/μl)	500 U
Cleavage Control Fusion Protein (1 μg/μl)	10 μg
10X HRV 3C Cleavage Buffer	10 ml

III. Storage

-20℃

IV. Composition

HRV 3C Protease Storage Buffer

50 mM	Tris-HCl (pH 8.0 at 25℃)
150 mM	NaCl
1 mM	EDTA
0.5 mM	Tris(3-hydroxypropyl)phosphine (THP)
50%	Glycerol

Cleavage Control Fusion Protein Storage Buffer

50 mM	Tris-HCl (pH 7.5 at 25℃)
100 mM	NaCl
10 mM	EDTA

10X HRV 3C Cleavage Buffer

500 mM	Tris-HCl (pH 7.5 at 25℃)
1.5 M	NaCl

V. Activity Definition

1 unit (U) is defined as the amount of enzyme that cleaves at least 95% of the Cleavage Control Fusion Protein (100 μg in 1X HRV 3C Cleavage Buffer) in 16 hours at 4℃.

VI. Protocol

HRV 3C Protease is useful for cleaving recombinant fusion proteins that contain the HRV 3C Protease cleavage site (e.g., for preparing untagged target protein). This cleavage is particularly useful in combination with *E. coli* expression vectors such as pCold™ TF DNA (Cat. #3365)*, pCold ProS2 DNA (Cat. #3371)*, and pCold GST DNA (Cat. #3372)* and other expression vectors containing an HRV 3C cleavage site. HRV 3C Protease can remove solubility-promoting tags (e.g., TF, ProS2, GST) from fusion proteins expressed using these vectors. These expression vectors have a HRV 3C Protease cleavage site upstream of the multiple cloning site, enabling removal of the tag(s). In addition, these vectors also contain a N-terminal His tag upstream of the HRV 3C Protease cleavage site. Therefore, after proteolysis, IMAC affinity chromatography with a resin such as TALON Metal Affinity Resin can be used to bind both the tag and HRV 3C Protease, and thereby remove them from the untagged target protein.

pCold I DNA (Cat. #3361)*, pCold II DNA (Cat. #3362), pCold III DNA (Cat. #3363), and pCold IV DNA (Cat. #3364) do not contain a HRV 3C Protease cleavage site. HRV 3C Protease can not be used for tag cleavage of fusion proteins expressed with these vectors.

* Not available in all geographic locations. Check for availability in your area.

Workflow for Tag Cleavage from Fusion Proteins using HRV 3C Protease

Purified Fusion Protein with N-terminal tag
↓
Tag cleavage using HRV 3C Protease
↓
Purification using TALON Metal Affinity Resin, etc.
↓
Removal of tags and HRV 3C Protease
↓
Target Protein

Notes:

- 1 U of HRV 3C Protease will cleave at least 95% of Cleavage Control Fusion Protein (100 μg) when the reaction is allowed to proceed at 4℃ for 16 hours in 50 μl of HRV 3C Cleavage Buffer. However, because the cleavage efficiency may differ based on the primary and secondary structure of the protein and the reaction buffer, preliminary testing should be performed to determine the optimal amount of HRV 3C Protease.
- HRV 3C Protease reactions can be performed in a buffer that is optimal for the target protein (see Table 1. Effect of Buffer Components on HRV 3C Protease Activity). When removing the cleaved tags and protease from the protease reaction with IMAC using a product such as TALON Metal Affinity Resin, do not include reducing agents (e.g., 2-mercaptoethanol) or chelating agents (e.g., EDTA) in the protease reaction. These agents cause the elution of chelated metal ions.*
  - * TALON Metal Affinity Resin is complexed with Co^{2+} ions, which are much more recalcitrant to reduction with 2-ME compared to resin complexed with Ni^{2+} ions. Purification of 6xHis tag fusion proteins on TALON Metal Affinity Resin is possible in the presence of 30 mM 2-ME.
- Reactions can be performed at 4 - 37℃, but 4℃ is recommended as the standard reaction temperature.

Table 1. Effect of Buffer Components on HRV 3C Protease Activity

Component	Activity (%)*	Component	Activity (%)*
(1X HRV 3C Cleavage Buffer)	100	0.5 mM DTT	100
0.2 M NaCl	100	1 mM DTT	100
0.8 M NaCl	< 100	2 mM DTT	100
1 mM $ZnCl_2$	100	0.5 mM THP	100
10 mM $ZnCl_2$	0	1 mM THP	100
100 mM $ZnCl_2$	0	2 mM THP	100
0.1% Triton	100	0.5 mM TCEP	100
1% Triton	100	1 mM TCEP	100
0.1% Tween 20	100	2 mM TCEP	100
1% Tween 20	100	1% Glycerol	100
0.1% Nonidet P40	100	5% Glycerol	< 90
1% Nonidet P40	100	10% Glycerol	< 90
1 mM PMSF	100	0.5 M Urea	0
5 mM PMSF	100	1 M Urea	0
8 mM PMSF	< 100	2 M Urea	0
0.1 mM Leupeptin	< 100	0.5 M Guanidine	0
0.5 mM Leupeptin	< 70	1 M Guanidine	0
0.75 mM Leupeptin	< 70	2 M Guanidine	0
1 mM EGTA	100	0.1 M Imidazole	0
20 mM EGTA	100	0.2 M Imidazole	0
50 mM EGTA	100	0.5 M Imidazole	0
1 mM EDTA	100	10 mM Na Phosphate Buffer	< 90
20 mM EDTA	100	50 mM Na Phosphate Buffer	< 90
50 mM EDTA	100	100 mM Na Phosphate Buffer	< 90

* Relative activity when the component is added to 1X HRV 3C Cleavage Buffer (activity with 1X HRV 3C Cleavage Buffer alone is defined as 100%).

< VI-a. Cleavage in Solution >

VI-a-(1). Optimization: Small-Scale Cleavage of Target Protein

1. Prepare the following reaction solutions in 1.5 ml microtubes.

Target protein*	10, 20, 50, and 100 μg each
10X HRV 3C Cleavage Buffer	5 μl
HRV 3C Protease	1 μl (1 U)
Purified water	to 50 μl

The ratio of HRV 3C Protease to target protein (unit/μg) are 1 : 10, 1 : 20, 1 : 50, and 1 : 100, respectively.

* For control reactions 1 - 10 μg of Cleavage Control Fusion Protein should be used.

2. Incubate the reaction at 4℃. The standard reaction time is 16 hours. If you desire a shorter reaction time, collect and assay samples at the following time points: collect 10 μl of the reaction solution after 1, 3, 6, and 16 hours and mix with 10 μl of 2X SDS Sample Buffer. Store samples at -20℃ until SDS-PAGE analysis.
3. Perform SDS-PAGE. Run a small amount of undigested target protein simultaneously for comparison. Determine the amount of enzyme and reaction time necessary to cleave the target protein based on the results of SDS-PAGE.

VI-a-(2). Scale-up and Recovery of the Target Protein

When a fusion protein has a N-terminal His or HN tag, it is possible to recover the target protein after cleavage using a metal chelating resin. An example in which TALON Metal Affinity Resin (Cat. #635501) is used is described below.

1. Scale up the reaction solution in VI-a-(1). Add the target protein and HRV 3C Protease to achieve the volume ratio determined by the optimization procedure described in Step VI-a-(1) above.
2. Perform the reaction at 4℃ for the optimal length of time.
3. Prepare 1X HRV 3C Cleavage Buffer by diluting the necessary volume of 10X HRV 3C Cleavage Buffer and place at 4℃. Perform all steps described below at 4℃.
4. Allow the appropriate amount of TALON Metal Affinity Resin* to equilibrate in 1X HRV 3C Cleavage Buffer to produce a 50% slurry suspension.

   * The protein-coupling capacity of TALON Metal Affinity Resin is 5 - 15 mg/ml resin.
5. Add the 50% slurry of TALON resin to the HRV 3C Protease reaction solution. Mix by inversion for 1 hour at 4℃.
6. Centrifuge the resin-containing solution at 700 X *g* for 5 minutes and recover the supernatant. The target protein will be in the supernatant. The HRV 3C Protease and the tags will be bound to the resin. Alternatively, the resin-containing solution can be transferred to a TALON 2 ml Disposable Gravity Column (Cat. #635606) and the eluate is recovered.
7. Analyze the purified protein in the eluate by SDS-PAGE.

< VI-b. Cleavage During Dialysis >

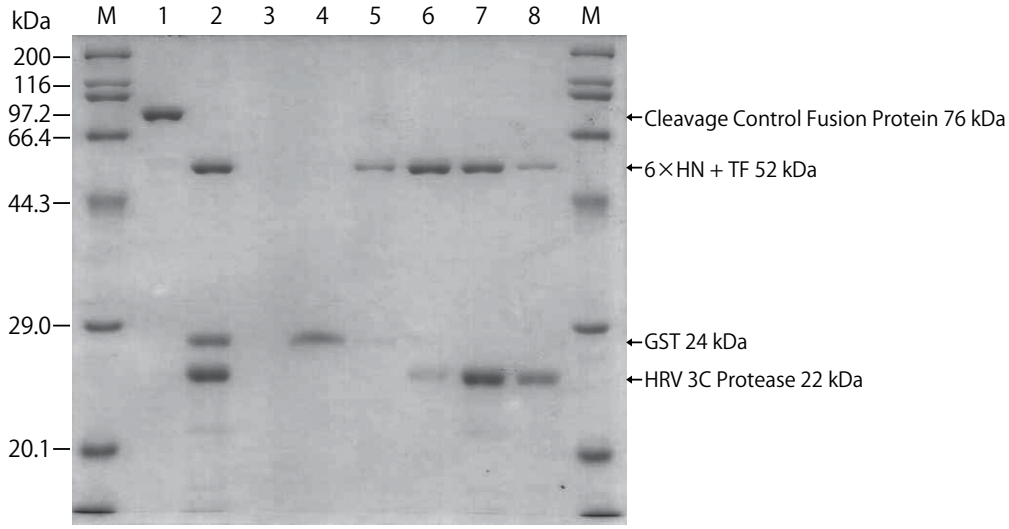
1. Prepare 1X HRV 3C Cleavage Buffer by diluting the necessary amount of 10X HRV 3C Cleavage Buffer and placing at 4℃. Perform all steps described below at 4℃ or on ice.
2. Add HRV 3C Protease to the target protein solution so that the HRV 3C Protease : target protein ratio (unit/μg) is 1 : 10. Transfer the mixture into a dialysis tube. We recommend using a dialysis membrane with a molecular weight cutoff value (MWCO) of 22 kDa or less.
3. Transfer the dialysis tube to a vessel containing approximately 100 - fold volume 1X HRV 3C Cleavage Buffer and perform dialysis at 4℃ for approximately 16 hours.
4. Remove the cleaved tags and the HRV 3C Protease by IMAC using TALON Metal Affinity Resin, etc. (see VI-a-(2).4-6).
5. Analyze the purified protein by SDS-PAGE.

< VI-c. Cleavage on a Metal Affinity Column >

1. Dilute the necessary amount of 10X HRV 3C Cleavage Buffer to prepare 1X HRV 3C Cleavage Buffer and leave at 4℃. Perform all steps described below at 4℃.
2. Transfer an appropriate amount of TALON Metal Affinity Resin* to a TALON 2 ml Disposable Gravity Column. Equilibrate with 10 bed volumes of Equilibration Buffer for TALON resin.
   * The protein binding capacity of TALON Metal Affinity Resin is 5 - 15 mg/ml resin.
3. Add the target protein to the TALON Resin.
4. Attach caps to the top and bottom of the column and mix gently by inversion for 1 hour to bind the target protein to the resin.
5. Wash unbound protein with 10 ml Equilibration Buffer (equal to 5 column volumes).
6. Equilibrate the column with 20 ml 1X HRV 3C Cleavage Buffer (equal to 10 column volumes).
7. Add an appropriate amount of HRV 3C Protease and mix gently by inversion for 16 hours at 4℃.
8. Elute the target protein with 10 ml Wash Buffer (Equilibration Buffer containing 5 mM imidazole) (equal to 5 column volumes). His tag sequences were cleaved by HRV 3C Protease, and therefore the target protein is present in this fraction.
9. If necessary, add Elution Buffer to the column to recover the uncleaved proteins and the His tags.
10. Analyze each fraction by SDS-PAGE.

VII. Experimental Example

10 μg of Cleavage Control Fusion Protein was incubated for 16 hours at 4℃ according to the protocol in Section VI-a. Cleavage in Solution. After the tag cleavage reaction, protein was purified using TALON Metal Affinity Resin and a TALON 2 ml Disposable Gravity Column. The flow-through, wash, and elution fractions were analyzed by SDS-PAGE. Cleavage of GST from the HN-tagged TF fusion protein and HRV 3C Protease were observed.

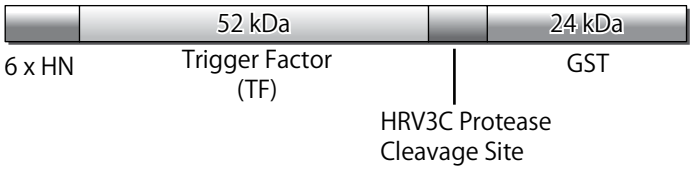


12% SDS-PAGE Stained with LabSafe GEL Blue (Cat. #786-35)*

M : Protein Molecular Weight Marker (Broad) (Cat. #3452)
Lane 1 : Cleavage Control Fusion Protein (76 kDa)
Lane 2 : Reaction solution after cleavage with HRV 3C Protease: Includes GST (24 kDa), HN tagged TF (52 kDa) and HRV 3C Protease (22 kDa).
Lane 3 : TALON Resin flow-through
Lane 4 : TALON Resin Wash Fraction: Includes GST.
Lane 5 - 8 : TALON Resin Elution Fraction: Includes HN tagged TF and HRV 3C Protease.

* Not available in all geographic locations. Check for availability in your area.

Structure of Cleavage Control Fusion Protein:



VIII. Related Products

pCold™ TF DNA (Cat. #3365)*
pCold™ ProS2 DNA (Cat. #3371)*
pCold™ GST DNA (Cat. #3372)*
TALON® Metal Affinity Resin (Cat. #635501-4/635652/635653)
TALON® 2 ml Disposable Gravity Column (Cat. #635606)

* Not available in all geographic locations. Check for availability in your area.