User Instructions

X-Lyz™ Proteolysis Assay

To Monitor Proteolysis Activity and
Determine Degradation Extent

Catalog # IC286103-Trial

Features:

- ***15min quick assay***
- ***Wide linear analytical range***
- ***Compatible with reducing agents***
- ***Suitable for microplates and cuvettes***

For safely handling the reagents, refer to the MSDS sheets online:
www.x-lyz.com

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1 Product Description

Proteolysis is an important process involved in many biological pathways and proteomics workflows. It is often necessary to evaluate the activity of proteases and monitor the progress of proteolytic reactions. In the case of peptide mapping, tryptic digestion is routinely carried out before the quantitative analysis by LC-MS/MS. However, the validity of the proteomics data thus obtained largely relies on the assumption that digestion has been fully driven to completion, but not yet over-hydrolyzed. Monitoring the proteolytic degradation is critical for the downstream operation.

The X-Lyz™ Proteolysis Assay Kit is designed to measure the growing amount of amino groups in peptides/amino acids generated during the proteolytic reaction. The water-compatible reagent reacts with the primary amines to form a sensitive chromophore; its UV absorbance at 350nm is linearly proportional to amine concentrations over a wide linear range (25-6000nmol/mL). By simply reading the changes in absorbance, the assay may be used to monitor the progress of hydrolysis as the substrate protein is broken down into smaller pieces. Furthermore the number of cleavages (degradation extent) may also be quantitatively calculated by dividing the increased amount of amines over the substrate concentration. The analysis is easy to operate compared to other methods, such as trinitrobenzenesulfonic acid (TNBS), known as a dangerous explosive substance. The rapid color development of the X-Lyz™ reagent is complete after 15min incubation. The method is compatible with commonly used materials in the laboratory, such as reducing agents, detergents, alcohols, carbohydrates, and inorganic salts. It is suitable for assays both in microplates and test tube (cuvettes).

The X-Lyz™ Proteolysis Assay Kit, Catalog # IC286103-Trial, is sufficient for up to 400 assays in microplates and 60 assays in test tubes. The contents are specified in the table below.

Table 1: Contents in X-Lyz™ Proteolysis Assay Kit (Cat No. IC286103-Trial)

Items	Cat No.	Size	Contents	Store	Ship
Reagent A	IC286091	25mL	Containing Reagent A in aqueous solution, pH ~10.5	Room Temp	Room Temp
Reagent B	IC286092	5mL	Containing Reagent B in organic mixture	4ºC	
Norvaline Standard	IC286097	1mL	Containing norvaline 50µmol/mL in 10mM HCl	4ºC	
Instructions	Pub286104	N/A	Instructions for X-Lyz™ Proteolysis Assay	N/A	N/A

All reagents expire one year from the date of purchase.

The assay procedure simply involves mixing the Working Reagent (WR, prepared by combining reagents A and B at a ratio of 5/1) with samples, incubating for 15min and reading UV absorbance at 350nm.

2 Preparation of Standards and Blank

Blank and diluted Norvaline standards will be used to generate a standard curve. Use Table 2 & 3 as a guide to prepare the Blank and diluted Norvaline standards. Starting with the provided Norvaline standard (50µmol/mL, Cat No. IC286097), dilute into the desired concentrations, preferably using the same diluent (buffer) as the sample(s). The Blank contains only the diluent without Norvaline. The volumes shown in the tables are sufficient for three replications of each dilution.

Table 2: Preparation of Diluted Norvaline Standards for WR/Sample Ratio 10/1 Assays

Vials	Diluent (µL)	Norvaline Sources		Diluted Norvaline Standards	
		Sources	Volume (µL)	Concentration (nmol/mL)	Volume (µL)
1	550	50µmol/mL	75	6000	361
2	264	Vial 1	264	3000	355
3	346	Vial 2	173	1000	360
4	371	Vial 3	159	300	355
5	175	Vial 4	175	150	350
Blank	350	Vial 5	0	0	350

Table 3: Preparation of Diluted Norvaline Standards for WR/Sample Ratio 1/1 Assays

Vials	Diluent (µL)	Norvaline Sources		Diluted Norvaline Standards	
		Sources	Volume (µL)	Concentration (nmol/mL)	Volume (µL)
1	2940	50µmol/mL	60	1000	1600
2	1400	Vial 1	1400	500	1600
3	1200	Vial 2	1200	250	1600
4	1200	Vial 3	800	100	1600
5	1200	Vial 4	400	25	1600
Blank	1600	Vial 5	0	0	1600

3 Preparation of Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   Total Volume of WR Required = (Blank + # of Standards + # of Unknowns) x (Volume of WR per Replicate) x (# of Replicates) x 1.05 (5% extra)

2. Prepare WR by mixing five parts of Reagent A and one part of Reagent B. Use the WR as soon as possible; sitting too long may cause loss of activity.

   For example, to conduct an assay of one blank, five standards and three unknown samples, if 250µL WR is required each in three replicates,

   Total Volume of WR Required = (1 + 5 + 3) x 250µL x 3 x 1.05 = 7088µL

   A sufficient amount of WR may be prepared by mixing 6mL Reagent A and 1.2mL Reagent B in the case.

4 Absorbance Measurement

WR/Sample ratio of 10/1 or 1/1 may be used. The 10/1 ratio gives a wider analytical range, while 1/1 is a more sensitive procedure. The assay in the following procedure is conducted with a microplate reader; a spectrophotometer may also be used in a similar manner.

4.1.1 WR/Sample 10/1

The procedure has a linear range of 150-6000nmol/mL (primary amine concentration)

1. Pipette 25µL of each sample into a well in three or more replicates.

   Note: Different sample sizes may be used as long as the WR/Sample is 10/1. If the sample size is small, e.g. 10µL, it is recommended to use half-area microplates to maintain the detection sensitivity.

2. Add 250µL WR into each well and shake gently. Cover the plate with parafilm.
3. Incubate for at least 15min at temperature between 25-37ºC.
4. Remove parafilm. Measure and record absorbance at 350nm in a plate reader.

4.1.2 WR/Sample 1/1

The procedure has a linear range of 25-1000nmol/mL (primary amine concentration)

1. Pipette 75µL of each sample into a well in three or more replicates.

   Note: Different sample sizes may be used, e.g. 125µL, as long as the WR/Sample is 1/1.

2. Add 75µL WR into each well and shake gently. Cover the plate with parafilm.
3. Incubate for at least 15min at temperature between 25-37ºC.
4. Remove parafilm. Measure and record absorbance at 350nm in a plate reader.

5 Analysis of Proteolysis

5.1 Reaction

Buffer (Blank): Prepare a proper amount of buffer.
Substrate Solution: Prepare the solution of protein substrate in Buffer.
Digested Solution: Add a proper amount of protease to the Substrate Solution. Incubate under a proper condition. Read absorbance to monitor progress, or to determine the number of proteolytic cleavage.
Protease Control: Add a proper amount of protease to buffer to obtain the same concentration as in Digested Solution.

Note: The solutions must be free of any primary amine-containing substances other than that in the proteins, such as TRIS, free amino acids or ammonium ions. Non-primary amine buffers may be used instead, e.g. HEPES, BICINE, BIS-TRIS, BIS-TRIS propane, and BIS-TRIS methane.

5.2 Monitor Progress

1. Take samples from the Digested Solution at different time points during the incubation
2. Follow the procedure in the section Absorbance Measurement to read absorbance

3. Record the reading and reaction time. As the proteolysis proceeds and substrate protein is degraded into small peptides and/or amino acids; the intensity of absorbance should increase and level off towards the end.

5.3 Estimate Number of Cleavages

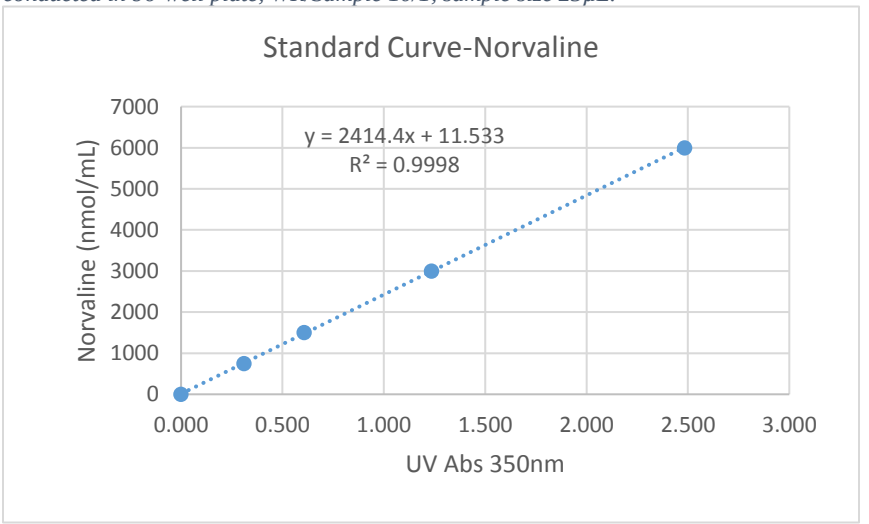
1. Take samples from each of Buffer (Blank), Norvaline Standards, Digested Solution, and Protease Control. Follow the procedure in the section Absorbance Measurement to read their absorbance.
2. Subtract the average absorbance of Blank from that of all other readings to obtain Corrected Absorbance (CA) of Norvaline Standards, Digested Solution, and Protease Control.
3. Generate a standard curve by plotting each Norvaline Standard CA vs. its concentration (e.g. Figure 1). Calculate each of the amine concentrations corresponding to Digested Solution CA and Protease Control CA.
4. Calculate Number of Cleavages in the formula below.

Number of Cleavages = Increased Amine Concentration ÷ Substrate Concentration

= [Digested - (Lys + N-Termini) x SC - Protease] ÷ SC

Where
Number of Cleavages: Average number of cleavages per protein molecule resulting from the reaction;
Digested: Total amine concentration in nmol/mL in Digested Solution after the reaction;
Lys: Number of unblocked lysine in the substrate protein;
N-Termini: Number of unblocked N-termini in the substrate protein;
SC: Substrate protein concentration in nmol/mL in Digested Solution;
Protease: Amine concentration in nmol/mL in Protease Control

Figure 1: Standard Curve of Norvaline Concentration vs. Absorbance. The assay was conducted in 96-well plate, WR/Sample 10/1, sample size 25µL.



6 Compatibility

The X-Lyz™ Conjugation Density Assay is compatible with reducing agents, common detergents, alcohols, urea, guanidine, metal chelators, and inorganic salts.

Primary amine-containing substances, such as amino acids, ammonia, and TRIS buffer interfere with the assay and is not suitable for the method. Non-primary amine buffers may be used to replace TRIS, such as HEPES, BICINE, BIS-TRIS, BIS-TRIS propane, and BIS-TRIS methane.

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