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Protein Detection:A Method for Measuring CRISPR Cas12a trans-cleavage Activity

蛋白检测 CRISPR Cas12a 蛋白反式切割活性检测方法

（*English Translation*）

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Forward

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Protein detection: A method for Measuring CRISPR Cas12a protein trans-cleavage activity

1 Scope

This document describes the principle, reagents and materials, equipment, preparation of reagent samples, experimental steps, and data processing of the method for measuring CRISPR Cas12a protein trans-cleavage activity.

This document is applicable to the detection of CRISPR Cas12a protein trans-cleavage activity.

2 Normative references

The content of the following documents constitutes indispensable provisions of this document through normative references in the text. Where a document is cited with a date, only the version corresponding to that date applies to this document; where a document is cited without a date, only the latest version (including all changed orders) applies to this document.

GB/T 6682 Primary water without ribonuclease, molecular water

3 Terms and Definitions

The following terms and definitions are applied to this document.

3.1

Clustered Regularly Interspaced Short Palindromic Repeats

The CRISPR sequence is a regular, short-spaced palindromic sequence derived from a segment of bacteriophage DNA infecting prokaryotic organisms, consisting of the leader sequence that is rich in AT and the interval sequence that is separated by the short repeat sequence. The leader sequence contains a promoter used to initiate transcription of the short repeat sequence and the interval sequence. The short repeat sequence generally consists of 28-37 base pairs, and the interval sequence generally consists of 32-38 base pairs, used to anchor the exogenous target DNA segments.

3.2

Cas12a protein

The Cas12a protein belongs to class 2, type V of the CRISPR Cas proteins families, which contains the RuvC nuclease domain. It can cleave target double-stranded DNA and target single-stranded DNA and can also trans-cleave non-target single-stranded DNA.

3.3

CRISPR-derived RNA

crRNA is an RNA sequence that is complementary to the exogenous target sequence, guiding the Cas12a protein to cleave the exogenous target sequence.

3.4

Trans-cleavage activity of Cas12a protein

After the Cas12a protein forms a ternary complex with crRNA and target DNA, the tenary complex exhibits ribonuclease activity independent of the target nucleic acid sequences, capable of cleaving single-stranded DNA of any sequences in the reaction system, which is known as the Cas12a protein trans-cleavage activity.

3.5

Trans-cleavage unit of Cas12a protein

The trans-cleavage unit of Cas12a protein is referred to the amount of Cas12a protein needed to cleave 1 pmol single-stranded DNA fluorescent reporter within 1 minute at 37 ℃ in a 20 μL reaction system, which is abbreviated as *trans*-U.

4 Principle

After the formation of a ternary complex of CRISPR Cas12a protein with crRNA and target DNA, the single-stranded DNA reporter in the reaction system can be trans-cleaved. Due to the fluorescence quencher and fluorophore labelled at both ends of the reporter sequence, the fluorescence signal is quenched while the reporter is intact. Whereas when the reporter is cleaved, the distance between the quencher and fluorophore increases, leading to the loss of quenching effect and the generation of the fluorescence signal. In this reaction, the trans-cleavage activity of the low concentration of CRISPR Cas12a protein is directly proportional to the fluorescence signal increase rate. Therefore, through continually collecting the fluorescence intensities of the trans-cleavage reaction system within 30 minutes and converting them into the maximum reaction rates of Cas12a trans-cleavage reaction using the fluorescence standard curve equation, linear fitting of different concentrations of Cas12a protein against the corresponding maximum reaction rates is carried out. Finally, quantitative determination of the concentration of Cas12a protein and the corresponding Cas12a protein trans-cleavage units in the reaction system are achieved by detecting the variation in the fluorescence signal intensities.

5 Reagents and materials

5.1 Water

Water of primary grade and molecular grade conforming to GB/T 6882 without ribonuclease.

5.2 10×Assay Buffer

Weigh precisely 0.29 g spermidine, 6.30 g tris hydroxymethyl aminomethane (Tris), 0.57 g magnesium chloride, 0.15 g dithiothreitol (DTT), 3.00 g glycine, and 5.08 g polyethylene glycol 20000 (PEG 20000) by using a ten-thousandth electronic balance. Pipette accurately 10 μL of Triton® X-100 by using a 0.5–10 μL adjustable micropipette, then dissolve all components in nuclease-free Grade I water. Adjust the pH to 8.5 at 25 °C with dilute hydrochloric acid, mix thoroughly, and bring to a final volume of 100 mL.

5.3 10×Annealing Buffer

Weigh precisely 0.13 g ammonium sulfate, 0.15 g potassium chloride, 0.02 g magnesium sulfate, and 0.32 g tris hydroxymethyl aminomethane (Tris) by using a ten-thousandth electronic balance. Dissolve them in nuclease-free grade I water, adjust the pH to 8.3 at 25°C with dilute hydrochloric acid, mix thoroughly, and bring to a final volume of 100 mL.

5.4 1 μmol/L Cas12a protein working solution

Pipette accurately 10 μL of 10 μmol/L Cas12a protein solution by using a 0.5-10 μL adjustable micropipette and mix with 90 μL of 1×assay buffer.

5.5 1 μmol/L crRNA solution

Pipette accurately 2 μL of 100 μmol/L crRNA solution by using a 0.5-10 μL adjustable micropipette and dilute it with 198 μL of nuclease-free Grade I water.

The crRNA sequence: 5'-AAUUUCUACUCUUGUAGAUUUAUCGCAACUUUCUACUGAAUU-3'.

5.6 100 nmol/L target double-stranded DNA solution

Pipette accurately 50 μL of 10 μmol/L forward primer and 10 μL of 10 μmol/L reverse primer by using a 0.5-10 μL adjustable micropipette. Anneal in 1×annealing buffer at 95 ℃ for 2 minutes, then slowly ramp down to 25 ℃ at a rate of 0.1 ℃/second. Dilute it with nuclease-free Grade I water to a final concentration of 100 nmol/L.

Forward primer sequence: 5'-GTTGTAAAACGACGGCCAGTTTTGTTATCGCAACTTTCTACTGAATTCGG-3'.
Reverse primer sequence: 5'-CCGAATTCAGTAGAAAGTTGCGATAACAAAACTGGCCGTCGTTTTACAAC-3'.

5.7 5 μmol/L single-stranded DNA fluorescent reporter solution

Pipette accurately 10 μL of 100 μmol/L single-stranded DNA fluorescent reporter solution by using a 0.5-10 μL adjustable micropipette into 190 μL nuclease-free Grade I water and mix thoroughly.

The sequence of the single-stranded DNA fluorescent reporter: 5'(6)-FAM-CCCCCCCC-3'-BHQ1.

6 Instruments and Equipment

Real-time fluorescence quantitative PCR detector (FQD-96A, Bioer Technology, China), Vortex mixer (VORTEX3 S025, IKA, Germany), Mini centrifuge (S1010E, SCILOGEX, USA), a ten-thousandth electronic balance (ME403, METTLER TOLEDO, Switzerland), 0.5-10μL adjustable micropipette (Research plus, Eppendorf, Germany), 10-100μL adjustable micropipette (Research plus, Eppendorf, Germany), 20-200μL adjustable micropipette (Research plus, Eppendorf, Germany), 100-1000μL adjustable micropipette (Research plus, Eppendorf, Germany).

7 Testing procedures

7.1 Establishment of a standard curve for Cas12a protein trans-cleavage activity

7.1.1 Prepare a serial 2-fold dilution of the single-stranded DNA fluorescent reporter stock solution according to Table 1, generating eight working solution gradients with concentrations of 2500.00 nmol/L, 1250.00 nmol/L, 625.00 nmol/L, 312.50 nmol/L, 156.25 nmol/L, 78.13 nmol/L, 39.06 nmol/L, and 19.53 nmol/L. Use an equal volume of nuclease-free Grade I water as blank controls.

Table 1. Concentration gradient dilution scheme for single-stranded DNA fluorescent reporter working solutions

Single-stranded DNA fluorescent reporter solution concentration (1 nmol/L, code)	**Preparation Method**
2500.00 (S_1)	Take 100 μL 5 μmol/L single-stranded DNA fluorescent reporter solution, add 100 μL of nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
1250.00 (S_2)	Take 100 μL S_1, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
625.00 (S_3)	Take 100 μL S_2, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
312.50 (S_4)	Take 100 μL S_3, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
156.25 (S_5)	Take 100 μL S_4, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
78.13 (S_6)	Take 100 μL S_5, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
39.06 (S_7)	Take 100 μL S_6, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
19.53 (S_8)	Take 100 μL S_7, add 100 μL nuclease-free Grade I water, vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
0 (NC)	nuclease-free Grade I water

7.1.2 Prepare the trans-cleavage activity assay reaction systems in PCR tubes on ice according to the component volumes specified in Table 2. The assay is divided into two groups：

Cleavage groups: All reactions contain target double-stranded DNA (dsDNA) solution, including reactions with each concentration of single-stranded DNA (ssDNA) fluorescent reporter working solutions and blank controls.

Non-cleavage groups: All reactions lack target dsDNA solution, including reactions with each concentration of ssDNA fluorescent reporter working solutions and blank controls.

Each concentration was tested with three technical replicates. After preparation, vortex-mix the reactions for 10 seconds and briefly centrifuge in a mini centrifuge to collect all liquid at the tube bottom.

Table 2. Component composition in the Cas12a protein trans-cleavage standard curve reaction system

Component	Specification	Cleavage Reaction System 1reaction volume/μL	Non-cleavage Reaction System 1 reaction volume/μL
Assay reaction buffer	10×	2	2
crRNA stock solution	1 μmol/L	0.8	0.8
Cas12a stock solution	1 μmol/L	0.4	0.4
Target double-stranded DNA stock solution	0.1 μmol/L	8	0
Single-stranded DNA fluorescent reporter working solution	Multiple concentrations	8	8
Nuclease-free Grade I water	——	0.8	8.8
Total volume	——	20	20

7.1.3 Place the PCR tubes containing the reaction solution on the real-time quantitative PCR instrument, set the reaction temperature to 37 °C, and collect fluorescence signals every 15 seconds for a continuous duration of 30 minutes.

7.2 Assay for trans-cleavage activity of Cas12a protein

7.2.1 According to the preparation method in Table 3, performing a serial 2-fold dilution of the Cas12a protein using 1 × assay buffer to obtain six working solution with concentrations of 10.000 nmol/L, 5.000 nmol/L, 2.500 nmol/L, 1.250 nmol/L, 0.625 nmol/L, and 0.313 nmol/L. Each concentration was tested in three technical replicates. Use an equal volume of nuclease-free Grade I water as blank controls.

Table 3. Serial Dilution Scheme for Target Protein

Enzyme concentration (nmol/L, code)	Preparation method
10.000 (E_1)	Take 2 μL 1 μmol/L Cas12a protein working solution, add 198 μL 1 × assay buffer, gently vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
5.000 (E_2)	Take 100 μL E_1, add 100 μL 1 × assay buffer, gently vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
2.500 (E_3)	Take 100 μL E_2, add 100 μL 1 × assay buffer, gently vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
1.250 (E_4)	Take 100 μL E_3, add 100 μL 1 × assay buffer, gently vortex-mix for 10 secs, then quick-spin in a mini centrifuge.

Continuation of Table 3

0.625 (E5)	Take 100 μL E_4, add 100 μL 1 × assay buffer, gently vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
0.313 (E6)	Take 100 μL E_5, add 100 μL 1 × assay buffer, gently vortex-mix for 10 secs, then quick-spin in a mini centrifuge.
0 (NC)	Nuclease-free Grade I water

7.2.2 Prepare the trans-cleavage activity assay reaction systems in PCR tubes on ice, following the component volumes specified in Table 4.

Table 4. Composition of the Cleavage Reaction Solution

Components of the reaction solution	Specifications	Amount per reaction/μL
Assay reaction buffer	10×	2
crRNA stock solution	1 μmol/L	0.04
Cas12a stock solution	Multiple concentrations	8
Target double-stranded DNA stock solution	0.1 μmol/L	0.4
Single-stranded DNA fluorescent reporter working solution	10 μmol/L	2
Nuclease-free water	——	7.56
Total volume	——	20

7.2.3 Place the PCR tubes containing the reaction solution into the real-time quantitative PCR instrument, set the reaction temperature to 37°C, and collect fluorescence signals every 15 seconds for a continuous duration of 30 minutes.

8 Data processing

8.1 Standard curve preparation for trans-cleavage activity of Cas12a protein

8.1.1 Standard curve preparation for experimental groups with cleaved reaction

8.1.1.1 Experimental groups containing target double-stranded DNA are referred to as groups where cleaved reaction has occurred. The fluorescence values at the plateau phase for single-stranded DNA fluorescent reporters of different concentrations, as well as blank controls, were selected, and the average fluorescence value of blank controls was calculated.

8.1.1.2 The average fluorescence value of the blank controls was subtracted by the fluorescence values of cleaved single-stranded DNA fluorescent reporters at different concentrations to obtain the net fluorescence values.

8.1.1.3 The average net fluorescence values of the cleaved single-stranded DNA fluorescent reporters at different concentrations represented by F_{cl} were then calculated.

8.1.1.4 A scatter plot was generated with the different concentrations (nmol/L) of the cleaved single-stranded DNA fluorescent reporters as the X-axis and the average net fluorescence values represented by F_{cl} as the Y-axis. Linear fitting was performed to create the standard curve, resulting in the standard curve diagram for the experimental groups with cleaved reaction, as shown in Figure 1, where $R^2 \geqslant 0.99$.

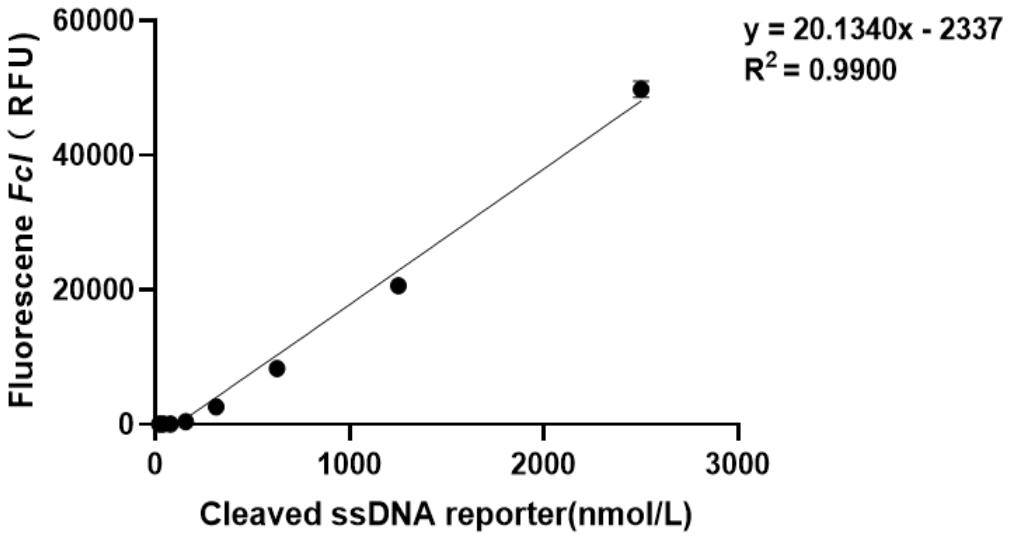

Figure 1

From Fig. 1, the slope of the fitted linear equation for the experimental groups with cleaved reaction represented by S_{cl} was 20.1340.

8.1.2 Standard curve preparation for experimental groups with uncleaved reaction

8.1.2.1 Experimental groups without target double-stranded DNA are defined as groups where cleaved reaction has not occurred. The fluorescence values at the plateau phase were selected for single-stranded DNA fluorescent reporters at different concentrations along with blank controls, and the average fluorescence value of the blank controls was calculated.

8.1.2.2 The average fluorescence value of the blank controls was subtracted by the fluorescence values of uncleaved single-stranded DNA fluorescent reporters at different concentrations to obtain the net fluorescence values.

8.1.2.3 The average net fluorescence values of uncleaved single-stranded DNA fluorescent reporters at different concentrations represented by F_{ucl} were then calculated.

8.1.2.4 A scatter plot was generated with the different concentrations (nmol/L) of the uncleaved single-stranded DNA fluorescent reporters as the X-axis and the average net fluorescence values represented by F_{ucl} as the Y-axis. Linear fitting was performed to create the standard curve, resulting in the standard curve diagram for the experimental groups with uncleaved reaction, as shown in Figure 2, where $R^2 \geqslant 0.99$.

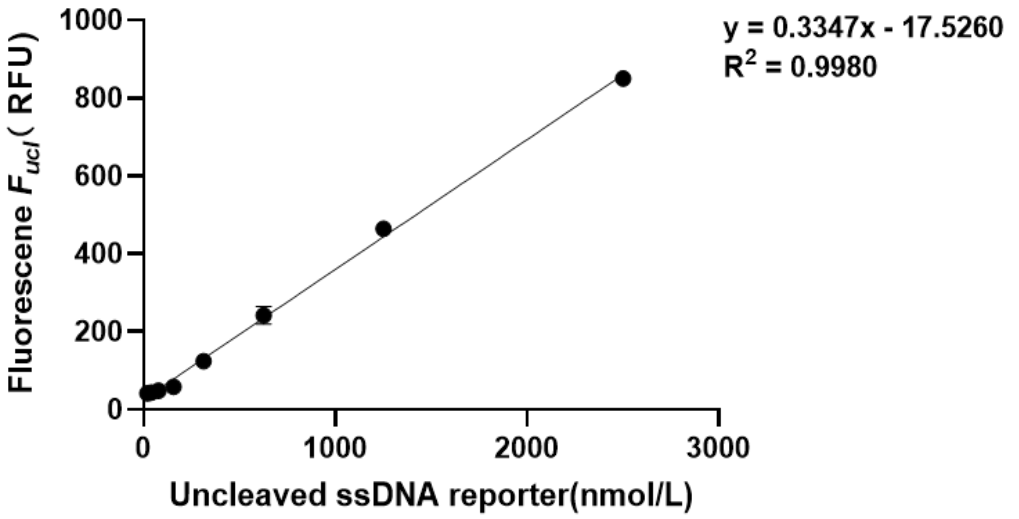

Figure 2

From Figure 2, the slope of the fitted linear equation for the experimental groups with uncleaved reaction represented by F_{ucl} was 0.3347.

8.2 Determination and calculation of the trans-cleavage activity of Cas12a protein.

8.2.1 Calculation of concentration of the cleaved single-stranded DNA fluorescent reporter probe

8.2.1.1 In trans-cleavage activity reactions with varying concentrations of Cas12a protein, using an initial single-stranded DNA fluorescent reporter concentration of 1000 nmol/L, the relationship between fluorescence signal value at time t and the corresponding concentration of trans-cleaved single-stranded DNA fluorescent reporter is given by Formula (1) as follows：

$$C_{cl}(t) = \frac{F(t) - C_0 S_{ucl}}{S_{cl} - S_{ucl}} = \frac{F(t) - 334.70}{19.7993} \quad \text{.....(1)}$$

In the Formula：

$C_{cl}(t)$ - The concentration of trans-cleaved single-stranded DNA fluorescent reporter probe at a time t, which is in units of nanomoles per liter (nmol/L).

$F(t)$ - The fluorescence signal value at a certain time t, which is in units of relative fluorescence unit (RFU).

C_0 - The initial concentration of the single-stranded DNA fluorescence reporter, which is 1000 nanomoles per liter (nmol/L).

S_{cl} -The slope of the fitted linear equation for the experimental groups with cleaved reaction, which is 20.1340.

S_{ucl} -The slope of the fitted linear equation for the experimental groups with uncleaved reaction, which is 0.3347.

8.2.2 Calculation of Cas12a protein trans-cleavage activity

8. 2. 2. 1 The raw fluorescence signal values were converted to substrate consumption (i.e., the concentration of single-stranded DNA fluorescent reporter consumed) by using Formula (1), and the maximum reaction rates (V_{max}) of the fluorescence curves for different concentrations of the single-stranded DNA reporter probes were calculated as the initial velocities (V_0) according to Formula (2).

$$V_0 = max\left|\frac{C_p(n+1)-C_p(n)}{\Delta T}\right| \quad \text{.....(2)}$$

In the Formula：

V_0- Initial velocity, here the maximum velocity of the fluorescence curve of different concentrations of single-stranded DNA fluorescent reporters is taken as the initial velocity, which is in units of picomoles per minute (pmol/min).

n - The number of samples, a total of 120 samples, but the 120th can't calculate the velocity, only reach n=119.

$C_p(n)$-The concentration of substrate consumption at the nth cycle(i.e.,the amount of consumption of the single-stranded DNA reporter at nth cycle), which is in units of picomoles (pmol).

ΔT- Sampling interval time, which is in units of minute (min).

8. 2. 2. 2 A scatter plot was generated with the concentrations of Cas12a protein [E] (pmol) as the X-axis and the initial velocities (pmol/min) of trans-cleavage reaction corresponding to the concentrations of Cas12a protein as the Y-axis. Linear fitting was performed using six data points with Cas12a concentrations of 0.0800 pmol, 0.0400 pmol, 0.0200 pmol, 0.0100 pmol, 0.0050 pmol, and 0.0025 pmol. The slope of this linear fit represents k_{cat}, namely $V_0 = k_{cat} \times [E]$. The results are shown in Figure 3, where $R^2 \geqslant 0.99$.

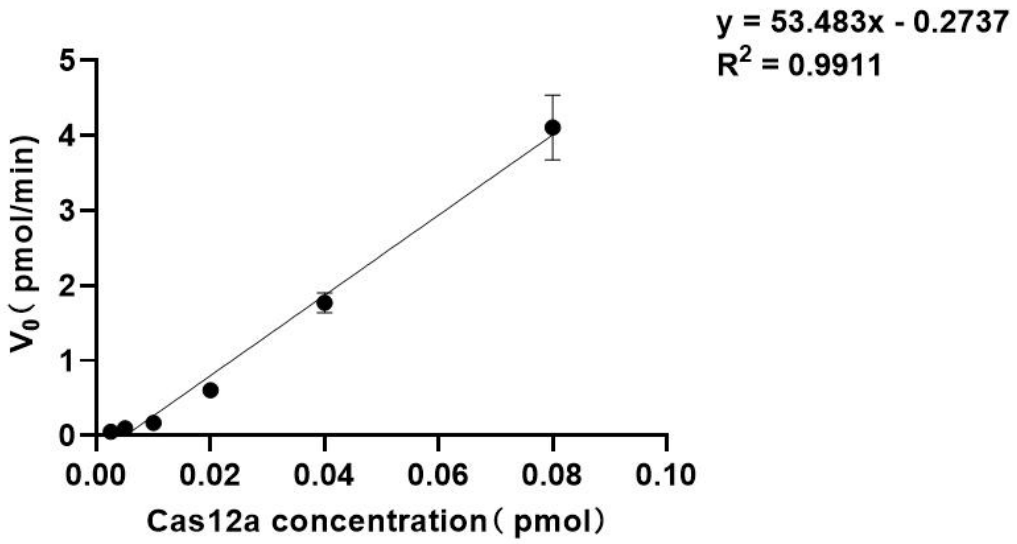

Figure 3

8. 2. 2. 3 According to the definition of trans-cleavage activity of Cas12a protein, the trans-cleavage activity of the sample is calculated by formula (3).

$$A(\ trans\text{-U}/pmol) = \frac{k_{cat}}{1\ pmol/min} \quad \text{.....(3)}$$

In the Formula：

$A(trans\text{-U}/pmol)$- trans-cleavage unit of Cas12a protein, which is defined as trans-cleavage units per picomole (*trans*-U/pmol).

k_{cat} - The catalytic constant of Cas12a protein, which is calculated to be 53.4830 from Figure 3.

$1\ pmol/min$-The velocity corresponding to the definition of Cas12a protein trans-cleavage activity, i.e.,when V_{max}= 1 pmol/min, the final Cas12a protein trans-cleavage activity of the sample measured in the example should be 53.4830 *trans*-U/pmol.

8.2.2.4 trans-cleavage unit of Cas12a protein(*trans*-U)：In a 20 μL reaction system, the amount of Cas12a protein required to cleave 1 pmol single-stranded DNA fluorescent reporters within 1 minute at 37 ℃ is 0.0187, which is in units of picomoles (pmol). Therefore, one trans-cleavage unit (*trans*-U) of the measured Cas12a protein sample corresponds to 0.0187 pmol Cas12a protein.

9 Precision

Under repeatability conditions, the absolute difference between two independent test results shall not exceed 10% of the arithmetic mean value.