TEV-protease FRET activity assay kit

Description:

Product number: 2808

Number of reaction: 10 protease samples + 2 calibration curves

Application: Assay for precise TEV protease activity measurement using protein substrate based on FRET. For comparison of different lots and types (mutants) of TEV protease with our standard TEV protease.

Introduction:

The TEV-protease fluorescent activity assay uses protein substrate based on fluorescent energy transfer (FRET). The principle of FRET relies on energy transfer of excitation energy of a donor fluorophore to a nearby acceptor fluorophore. The 54 kDa FRET substrate protein is composed by two fluorescent proteins green 26 kDa and red 28 kDa linked with TEV protease recognition sequence ENLYFQ|SG. The substrate is specifically cleaved to fluorescent monomers, which results in quantitative decrease of fluorescent intensity at 580-650 nm (emission range, the excitation range is 490-515 nm). The TEV-protease fluorescent activity assay is suitable for precise protease activity measurements, monitoring or high-throughput screening of TEV protease variants, substrate specificity measurements, etc. All components are manufactured in certified laboratory environment and could be used in GMP certified downstream processes. Recombinant proteins (TEV-protease and FRET TEV protease substrate) are purified by affinity chromatography, size exclusion chromatography and desalting.

Precautions and Disclaimer:

This kit is manufactured under ISO 9001 and ISO 13485. It is not intended to use for a direct clinical diagnostic use. Country of origin: Czech Republic. The kit does not contain animal products.

Components:

Supplied:

- FRET TEV protease substrate (0,5 mg/ml; **1,5 ml)**
- reference TEV protease (10 kU/ml, **50 µl)**
- Protease reaction buffer, PRB (**1 ml**)
- DTT (1M DTT; volume **100 µl**)

NOTE: Reference TEV protease serves as positive control.

Supplied by user:

- ultra pure water

Storage:

-20˚C (for several days 4˚C)

Example Protease Assay Procedure:

IN SEPARATE TUBES:

1. Determine the desired number of reactions (NOTE: always add blank reaction – without protease).
2. Let the reagents thaw on ice properly. **Leave on ice during all preparation.**
3. Prepare mixture for reactions together to eliminate pipetting errors.
   For 1 reaction (final volume 375 µl):
   - 238 µl ultra pure H_2O
   - 100 µl FRET TEV protease substrate (0,5 mg/ml)
   - 37,5 µl Protease reaction buffer
   - 0,375 µl 1M DTT

   *(For example for **2 reactions with protease** and **1 blank reaction** you will need 714 µl ultrapure H2O; 300 µl FRET TEV protease substrate; 112,5 µl Protease reaction buffer and optionally 1,125 µl DTT)*

4. Prepare calibration curve.
   It is suitable to choose your highest point of calibration as a mixture for 1 reaction (final amount of FRET in reaction is 50 µg). We recommend to prepare minimally 6 points calibration plus zero.

Table 1: Example of calibration curve and its preparation:

m_{FRET} (µg)	V_{FRET} (µl)	V_{H2O} (µl)	V_{PRB} (µl)	V_{DTT} (µl)
0	0	388,000	37,5	*0,375*
1,5	3,125	334,875	37,5	*0,375*
3,0	6,250	331,750	37,5	*0,375*
6,0	12,500	325,500	37,5	*0,375*
12,5	25,000	313,000	37,5	*0,375*
25,0	50,000	288,000	37,5	*0,375*
50,0	100,000	238,000	37,5	*0,375*

5. We recommend to dilute proteases 10x to slower the digest and to gain precise results. To 375 µl reaction mixture add 5 µl of protease (for blank reaction use water). Measure fluorescence immediately at excitation wave length 490-515 nm. Incubate at 30˚C or 6 ˚C according to your needs. Measure repeatedly every hour or as desired.
   Activity of proteases should decrease after 6 hours approximately, due to fully digested substrate.

6. *Optional: After ending measurements run SDS-Page to see the result of digest. Prepare 15% separating polyacrylamide gel. To 30 µl of reaction mixture add 6 µl Protein Loading Buffer (not supplied), boil for 5 minutes. After SDS-PAGE not digested FRET is visible as 54 kDa band and digested fragments as 26 kDa and 28 kDa bands.*

Data evaluation:

1. Emission wave length range is 580-650 nm. In case your fluorometer has more channels with different wave length range, make sure you are using the correct one. (**Excitation and emission wave length ranges differ!**)

2. Get rid of background. Subtract blank sample for each hour from protease sample of corresponding hour. (This step serves as correction of raising fluorescence in time. Amount of FRET is decreasing with time, but fluorescence of the not digested FRET is increasing – this effect is visible at data of blank sample)

3. Plot a graph from gained numbers.

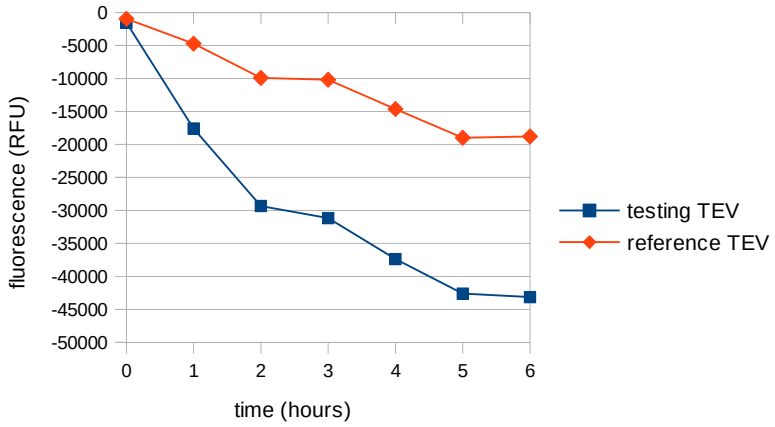

Image 1: Example graph of decreasing fluorescence (RFU) against time (hours). Fluorescence is decreasing into negative numbers after subtracting blank sample. The start (0 hours) correspond to 0 fluorescence after subtraction of background.

4. Calibration curve. Subtract measured fluorescence of sample with highest concentration (blank sample for calibration) from all points of calibration. Plot a graph against amounts of FRET. Add a linear trend line and show the equation of trend line **(y=A·x+B)**. You will get constants A and B, in this equation **y=measured fluorescence** and **x=amount of FRET**. Apply this equation on your measured data and you will get precise amounts of FRET for your sample for each hour.

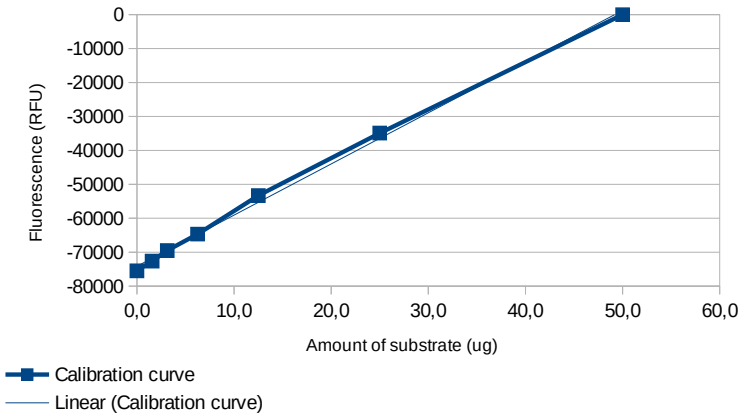

Image 2: Example graph of modified calibration curve. Fluorescence is decreasing into negative numbers after subtracting blank sample (highest point of calibration). Highest point of calibration corresponds to 0, which is equal to 0 hours in proteases samples, where this means that no substrate is digested yet.

5. Calculation of enzyme activity. Subtract the amount of FRET substrate gained from last measurement from starting amount of FRET substrate, to get amount of digested FRET substrate. Your results will be in µg, to convert them to µmol use the molar mass of substrate (54 kDa = 54 g/mol). Then subtract the result with time of reaction and volume of reaction.

$$enzyme\,activity = \frac{n_{t1} - n_{t2}}{(t_2) - (t_1) * V} = U/ml = \mu mol/min/ml$$

Where:

n_{t1}, n_{t2} are amount of non digested FRET substrate (µmol)

t_1 is time of first reading (min) *NOTE: preferred to be 0*

t_2 is time of second (last) reading (min)

V is sample volume (ml)