Aug 13, 2019

15 Determination of Enzyme Activity

DOI
dx.doi.org/10.17504/protocols.io.6gthbwn

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Protocol Citation: Tjusls China 2019. 15 Determination of Enzyme Activity. **protocols.io** https://dx.doi.org/10.17504/protocols.io.6gthbwn

Protocol status: Working
We use this protocol and it's working

Created: August 13, 2019

Last Modified: August 13, 2019

Protocol Integer ID: 26867

Materials

MATERIALS

- Ultrasonic Processor **Cole-Parmer** **Catalog #**UX-04714-52
- DMSO **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D8418
- General 96-well plates (Black) **Catalog #/**
- Infinite M1000 Pro Automatic Microplate Reader **Catalog #/**
- Multi-channel adjustable pipette **Catalog #/**
- Fluorescent Probe(CDC-1) **Catalog #/**
- Target Enzyme(beta-lactamase) **Catalog #/**

System setup – protein concentration

1 Soak the 96-well plates in 75% ethanol and put the container in ultrasonic cleaner for 30min to 1 hour, then use ddH_2O to wash these plates several times. Put these clean plates in drying oven at 55°C.
00:30:00 ~ 01:00:00
55 °C

2 Dilute the enzyme using its buffer. There we pipet 1 µL protein stock solution in 1mL buffer and mix gently. Then pipet 100 µL protein solution and mix with 400µL buffer each time, in order to dilute it as a 5-time gradient.

3 Pipet 94µL protein solution into 9 wells in plate, usually choosing B2-D4 area, to set 3 parallel controls. Pipet 94 µL buffer without protein into 3 wells as negative controls. Then add 6µL fluorescent substrate into wells.

4 Set up the program in Infinite M1000 Pro Automatic Microplate Reader.
Shake for 10 sec at 654 rpm.
00:00:10 654 rpm
Kinetic Cycle (to read fluorescent intensity each cycle)
Fluorescent measure, 75 cycle, 10sec for each cycle.

5 Put the plate in Microplate reader, and click Start button.

6 When the facility ends testing, save data and import it into GraphPad Prism Software. Use “nonlinear fit” – “straight line” and compare R^2 of lines under different concentrations to pick up the best linear fit one, whose R^2 is most close to 1 .

7 Take this concentration as standard value, then set up parallel gradient of its 2x, 0.5x, 0.25x, etc. Repeat step 3-5.

8 Calculate the ratio of emission(rE = Q_0/Q_m, Q_0 means the maximum fluorescent intensity of each reaction under different protein concentrations, Q_m means the maximum fluorescent intensity of all reactions under different protein concentrations). Use GraphPad Prism Software to calculate EC80 value. Set log(concentration of protein) as X, the rate of emissionas Y. Use “nonlinear fit” – “log(agonist) vs. response—Find ECanything”, input 80 as the value of F parameter.

9 Usually we use the EC80 value as suitable protein concentration, and it can be adjusted according to the actual situation.

System setup – buffer

10 Design experimental groups with the “N+(N-1) principle”.
Since we use PBS as our protein buffer, and class B beta-lactamases are depend on Zn^{2+}, so we choose the concentration of NaCl, the concentration of $ZnCl_2$, and pH, as variables.

11 Repeat step 3-5 to measure.

12 When the facility ends testing, save data and import it into GraphPad Prism Software. Use “nonlinear fit” – “straight line” to calculate the initial velocity of each reaction a.k.a. its slope value. Choose the condition with higher initial velocity.

Kinetic Constant Measurement

13 Dilute protein again with the ensured most suitable solution into proper concentration.

14 Dilute the fluorescent substrate as 2-time gradient for 8 groups.

15 Repeat step 3-5 to measure.

16 When the facility ends testing, save data and import it into GraphPad Prism Software. Use “nonlinear fit” – “straight line” to calculate the initial velocity of each reaction a.k.a. its slope value.

17 Use “nonlinear fit” – “Michaelis-Menten” to fit Michaelis plot of this beta-lactamase. At the same time the software will calculate kinetic constants Km, Vmax automatically.

18 Dilute protein as 2-time gradient for several groups. Repeat step 3-5 to measure. Take FI as Y, [S] as X, then use “nonlinear fit” – “straight line” to calculate fluorescent calibration value.

19 Calculate kcat value. Kcat = Vmax/[E].