Wild-type CA2

8.21

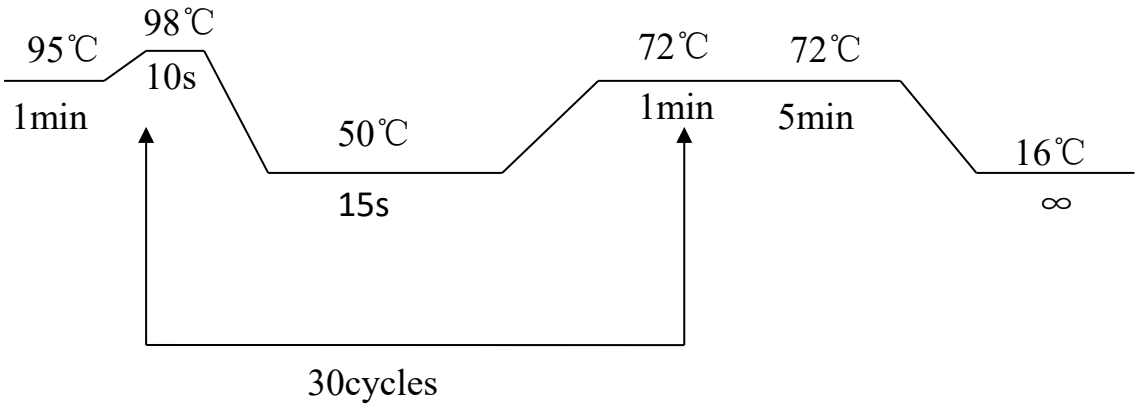
Diluting All Primers

CA2R	181.5μl ddH_20
CA2F	207.9μl ddH_20
MUF	251.4μl ddH_20
MUR	281.9μl ddH_20

8.22

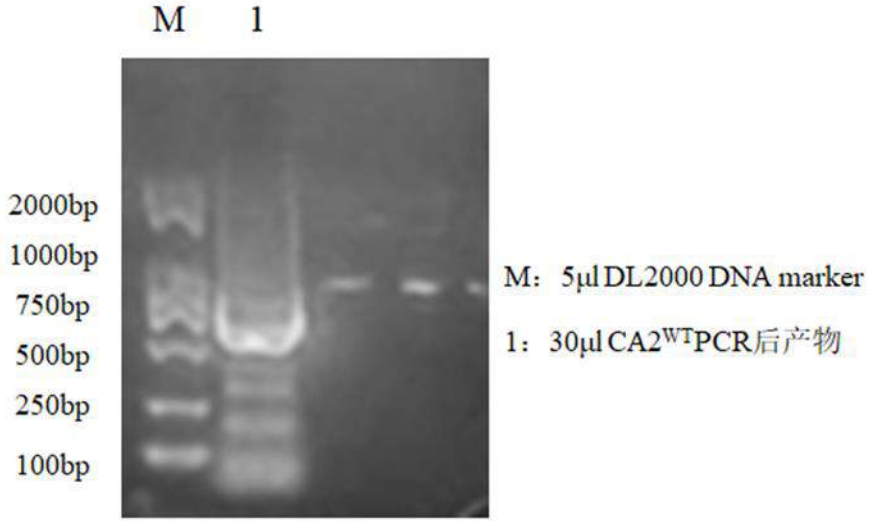
PCR

5×primer STAR Buffer (Mg2+ plus)	10μl
dNTP Mixture(2.5mM each)	4μl
primer 1 (CA2F 10μM)	1μl
primer 2 (CA2R 10μM)	1μl
Template ($CA2^{WT}$)	1μl
Primer STAR	0.5μl
ddH_2O	up to 50μl



Recycling Gel

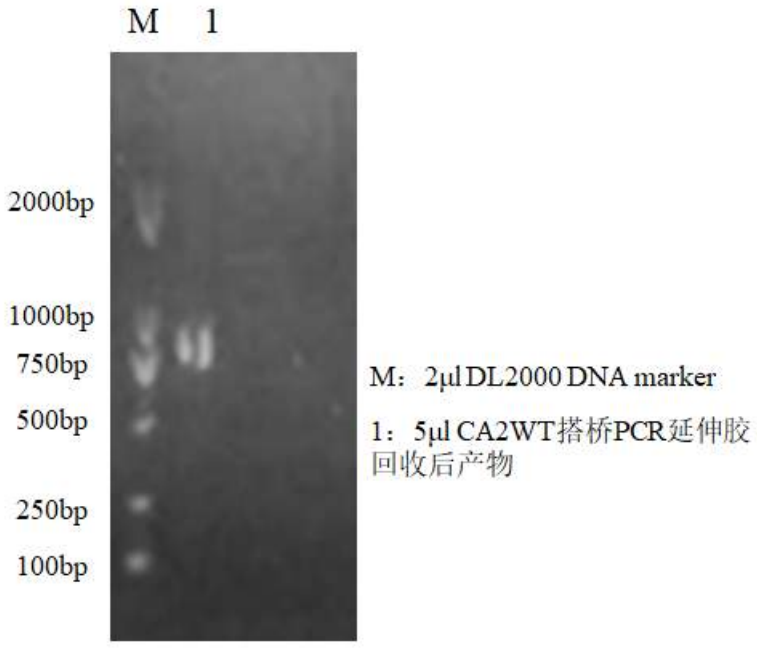
Lane 1	Lane2
DL 2000DNA marker 2μl	RCR band of $CA2^{WT}$ 30μl



Gel Extraction

Identifying Gel

Lane1	Lane2
DL 2000DNA marker 2μl	RCR band of $CA2^{WT}$ 5μl



8.23

Enzyme Digestion at 37℃

restriction enzyme identification

reagent	volume

$CA2^{WT}$	15μl
10×NEB buffer 2	5μl
BSA(0.1%)	0.5μl
enzyme 1(Pst I)	1.2μl
enzyme 2 (EcoRI-HF)	1.2μl
ddH_2O	up to 50μl

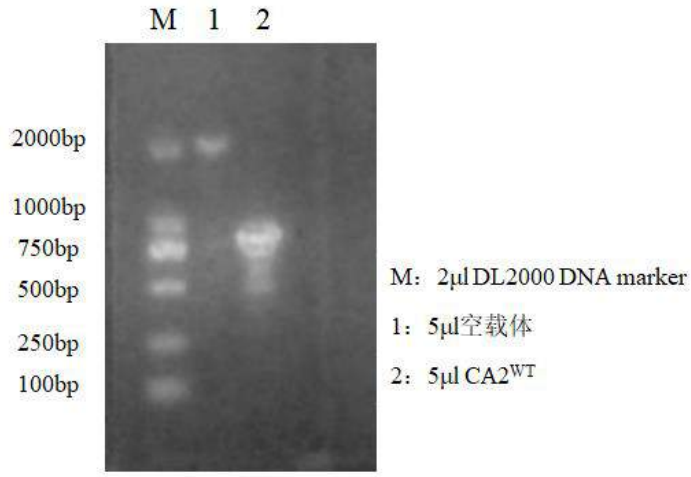
restriction enzyme identification

reagent	volume
PSB1C3	15μl
10×NEB buffer 2	5μl
BSA(0.1%)	0.5μl
enzyme 1(Pst I)	1.2μl
enzyme 2 (EcoRI-HF)	1.2μl
ddH_2O	up to 50μl

Gel Extraction

Identifying Gel

Lane1	Lane2	Lane3
DL 2000DNA marker 2μl	PSB1C3 5μl	$CA2^{WT}$ 5μl



reagent	volume
10×T4 DNA Ligase Buffer	1μl

T4 DNA ligase	1.2μl
PSB1C3	1.5μl
$CA2^{WT}$	1.5μl
ddH2O	up to 10μl

PCR：16℃connection 10h

8.24

Transformation

reagent	volume
$CA2^{WT}$	5μl
competent cell	100μl
LB（-）	400μl

Plate culture

reagent	medium
$CA2^{WT}$	LB(chl＋)

8.25

Pick Monoclone

reagent	volume
LB（chl+）	3ml

8.26

Miniprep

试剂（reagent)	体积（volume)
Colony ($CA2^{WT}$)	3ml

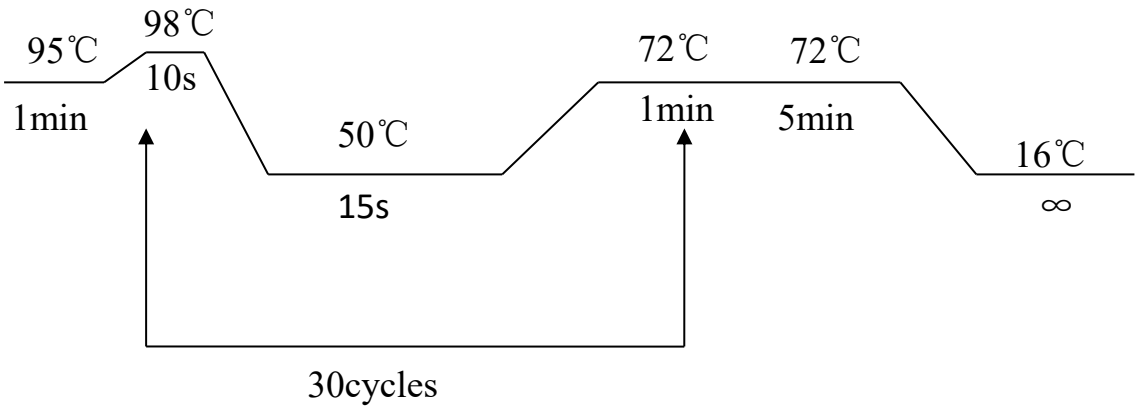
Mutant $CA2^{L203k}$

8.23

PCR

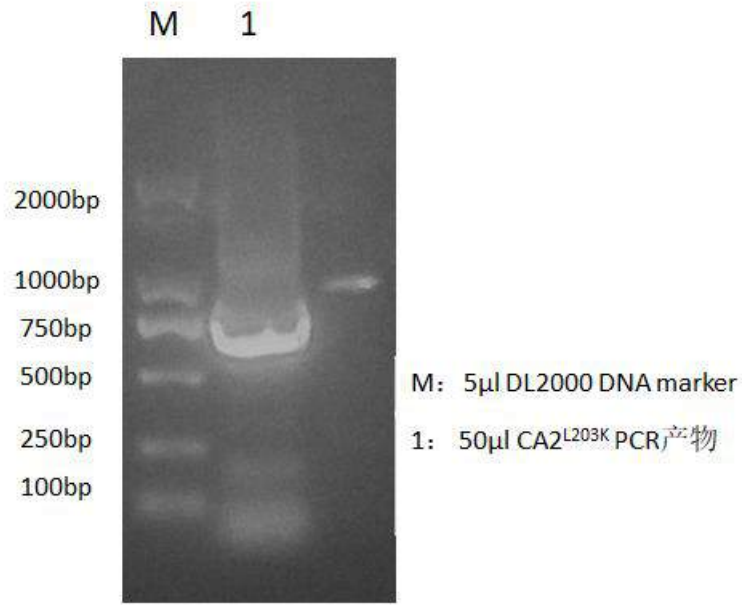
5×primer STAR Buffer (Mg2+ plus)	10μl
dNTP Mixture(2.5mM each)	4μl
primer 1 (CA2F 10μM)	1μl
primer 2 (CA2R 10μM)	1μl

Template (CA2 ^{L203K})	1μl
Primer STAR	0.5μl
ddH_2O	up to 50μl



Recycling Gel

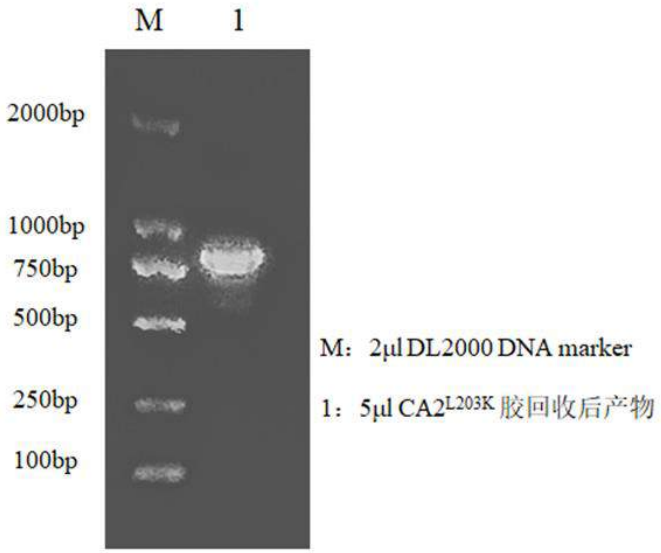
Lane1	Lane2
DL 2000DNA marker 2μl	PCR band of CA2^{L203K} 50μl



Gel Extraction

Identifying Gel

Lane1	Lane2
DL 2000DNA marker 2μl	the product of gel extraction of CA2^{L203K} 5μl



8.24

Enzyme Digestion at 37℃

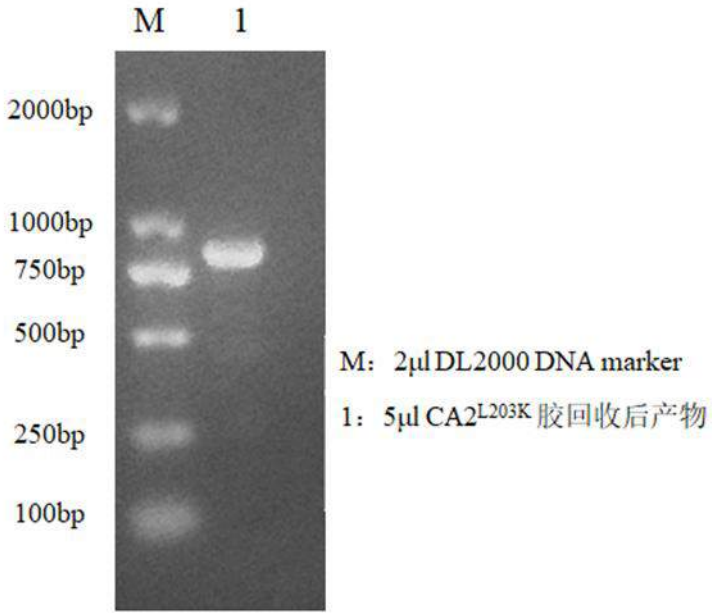
restriction enzyme identification

reagent	volume
$CA2^{L203K}$	15μl
10×NEB buffer 2	5μl
BSA(0.1％)	0.5μl
enzyme 1(Pst I)	1.2μl
enzyme 2 (EcoRI-HF)	1.2μl
ddH_2O	up to 50μl

Gel Extraction

Identifying Gel

Lane1	Lane2
DL 2000DNA marker 2μl	the product of gel extraction of $CA2^{L203K}$ 5μl



8.25

Transformation

reagent	volume
$CA2^{L203K}$	5μl
competent cell DH5α	100μl
LB（-）	200μl

Plate culture

reagent	medium
$CA2^{WT}$	LB(chl＋)

8.26

Pick Monoclone

reagent	volume
Liquid LB（chl+）	3ml

8.27

Miniprep

reagent	volume
cultivated $CA2^{WT}$ on 26, August	1.5ml

8.28

Enzyme Digestion at 37℃

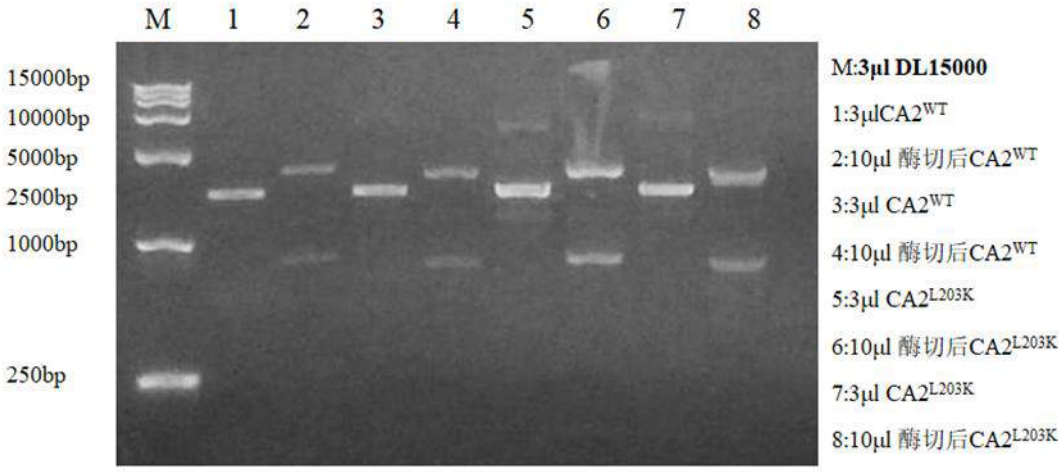
Miniprep

reagent	volume

$CA2^{L203K}$	5μl（recycled）
10×NEB buffer 2	5μl
BSA(0.1％)	0.5μl
enzyme 1(Pst I)	1.2μl
enzyme 2 (EcoRI-HF)	1.2μl
ddH_2O	up to 50μl

Identifying Gel

Lane1	Lane2	Lane3	Lane4	Lane5
DL 2000DNA marker 3μl	$CA2^{WT}$ 3μl	$CA2^{WT}$ 10μl after enzyme digestion	$CA2^{WT}$ 3μl	$CA2^{WT}$ 10μl after enzyme dig
Lane7	Lane8	Lane9		
$CA2^{L203K}$ 10μl after enzyme digestion	$CA2^{L203K}$ 3μl	($CA2^{L203K}$ 10μl after enzyme digestion		



8.29

Pick Clone

reagent	volume
LB（K+）liquid	4ml

8.30

Pilot Expression of Protein

“IPTG+ 4h” “IPTG- 4h”

reagent	volume
LB（K+）	4㎖*2
8.29 Fungal fluids cultivated	40μl*2

After the rocker culture is 4 H, add IPTG(final concentration is 0.5 mm) and then the rocker culture is 4 hours. Each of the two tubes absorbs 200 μL of bacterial fluid, and in the frozen centrifuge, it is 4 °C and 12000 rpm centrifuge. 10 minutes, go to the clear, It is resuspended with 100 μL 1x SDS Loading buffer, then heated in a water bath pot at 100 °C for 10 minutes and stored in a -20 °C refrigerator.

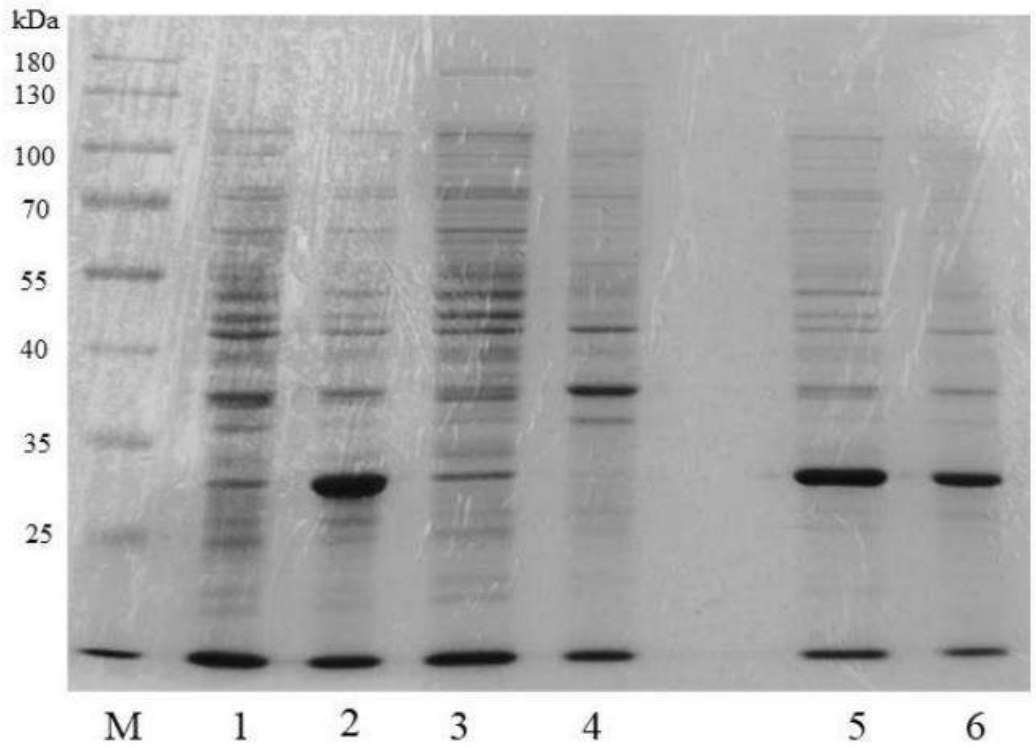
8.31

Protein extraction

$CA2^{L203K}$

Pore canal1	Pore canal 2	Pore canal 3	Pore canal 4	Pore canal 5
Maker 2μl	IPTG-4h 30μl	IPTG+ 4h 30μl	IPTG- 4h exhaust 30μl	IPTG- 4hprecipitation 30μl

Pore canal 6	Pore canal 7
IPTG- 4h exhaust 30μl	IPTG- 4h precipitation 30μl



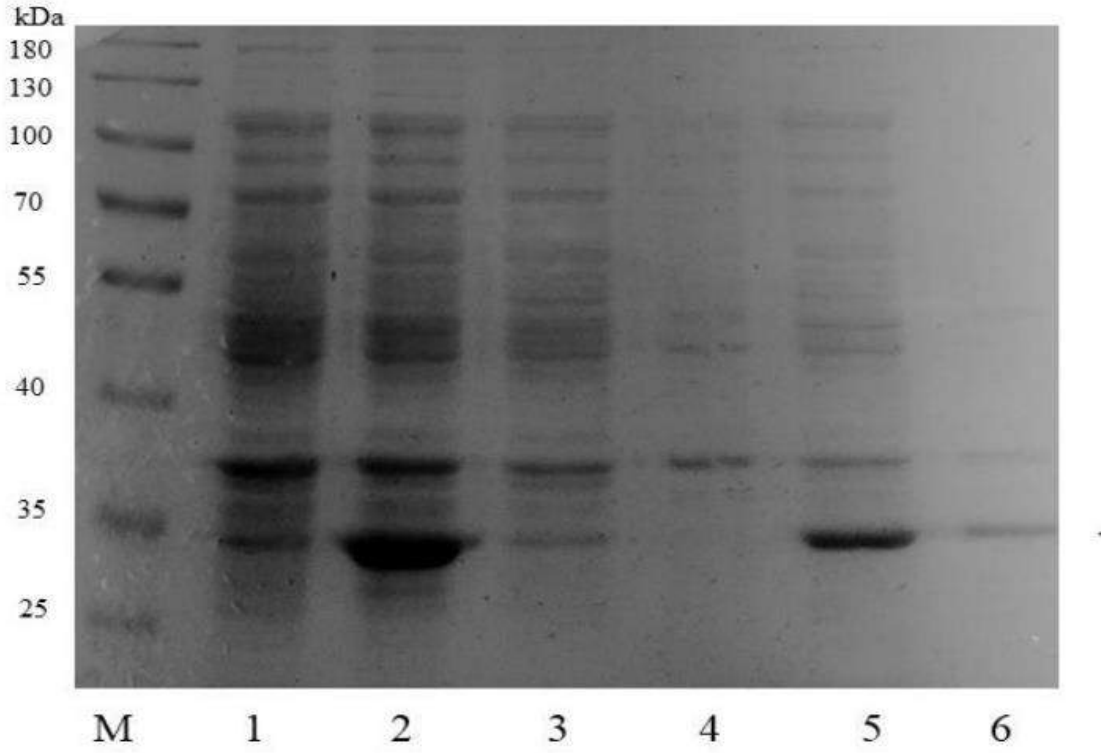
$CA2^{WT}$

$CA2^{L203K}$

Pore canal 1	Pore canal 2	Pore canal 3	Pore canal 4	Pore canal 5
Maker 2μl	IPTG-4h 30μl	IPTG+ 4h 30μl	IPTG- 4h exhaust 30μl	IPTG- 4h precipitation 30μl

Pore canal 6	Pore canal 7

IPTG- 4h exhaust 30 μl	IPTG- 4h precipitation 30 μl



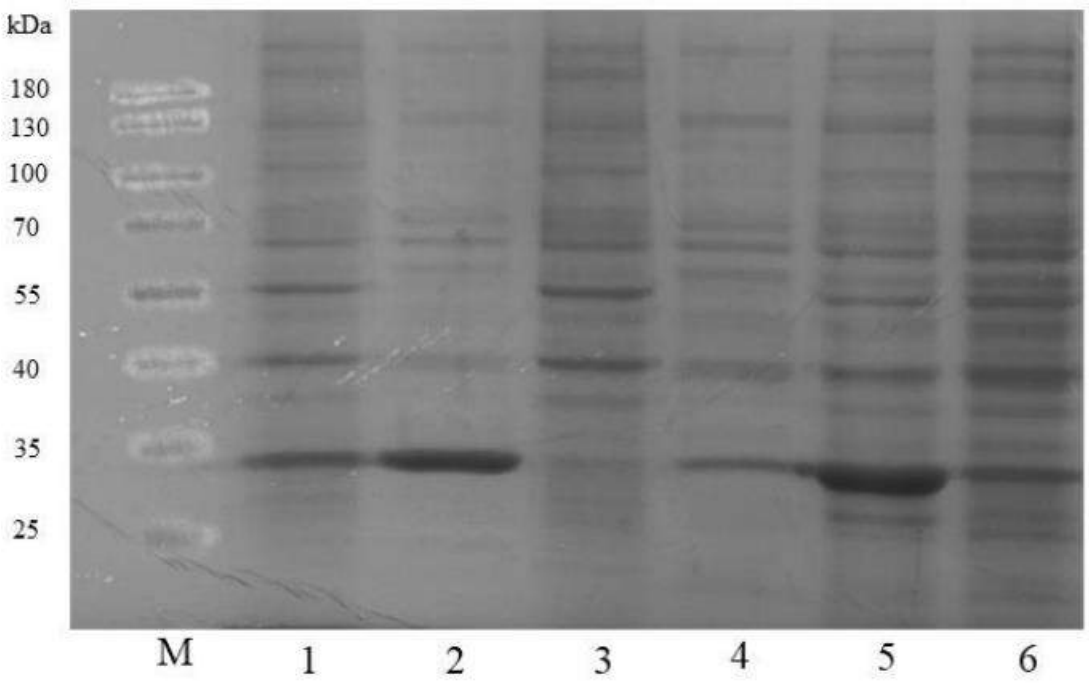
9.1

Purification of Protein

Prior to purification

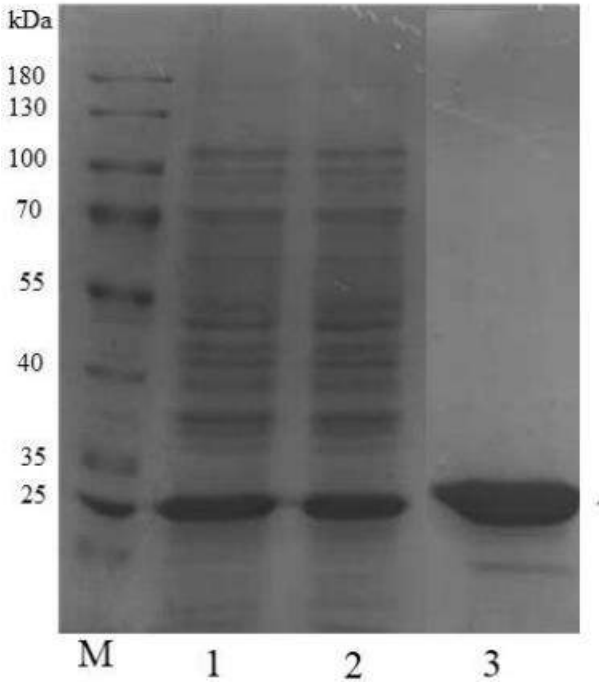
Pore canal 1	Pore canal 2	Pore canal 3	Pore canal 4	Pore canal 5
Maker 2 μl	IPTG+4h precipitation 30 μl	IPTG+ 4h exhaust 30 μl	IPTG- 4h precipitation 30 μl	IPTG- 4h exhaust 30 μl

Pore canal 6	Pore canal 7
IPTG+4h 30 μl	IPTG- 4h 30 μl



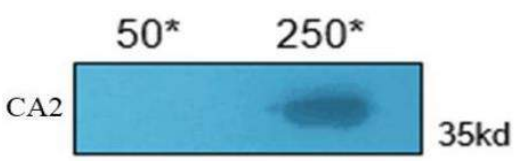
Purified

Pore canal 1	Pore canal 2	Pore canal 3	Pore canal 4
Maker 2 μ l	IPTG+4h exhaust 30 μ l	IPTG+ 4h precipitation 30 μ l	IPTG- 4h 30 μ l

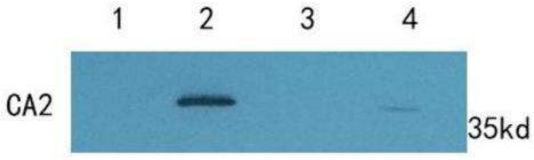


9.3

Western blot identification of proteins



$CA2^{L203K}$

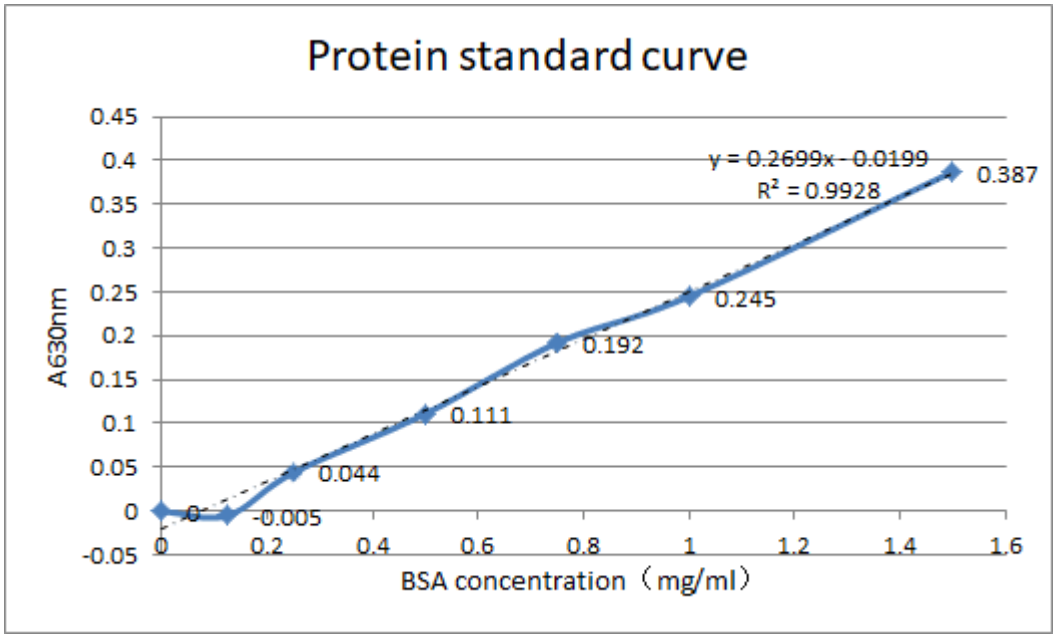


$CA2^{WT}$

9.4

Quantification of protein concentrations

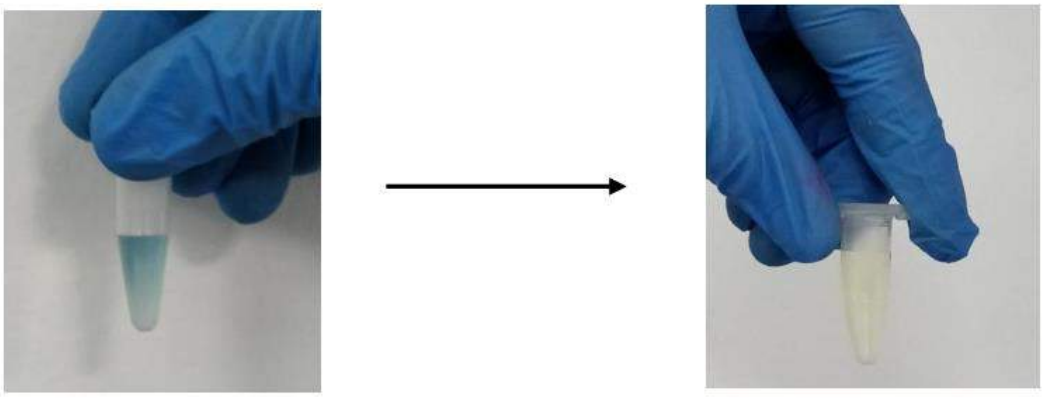
Standard Protein Concentration（mg/mL）	0	0.125	0.25	0.5	0.75	1	1.5
OD value	1.405	1.400	1.449	1.516	1.597	1.650	1.792
A630（nm）	0	-0.005	0.044	0.111	0.192	0.245	0.387



9.5

colorimetric enzyme activity

Add 250 μl saturated CO2 solution



Unit of carbonic anhydrase activity（$CA2^{L203K}$）

sequence number	Unit Enzyme（10^5 WAU/ μg）
2	0.3900
3	0.4110
4	0.3640
5	0.3192

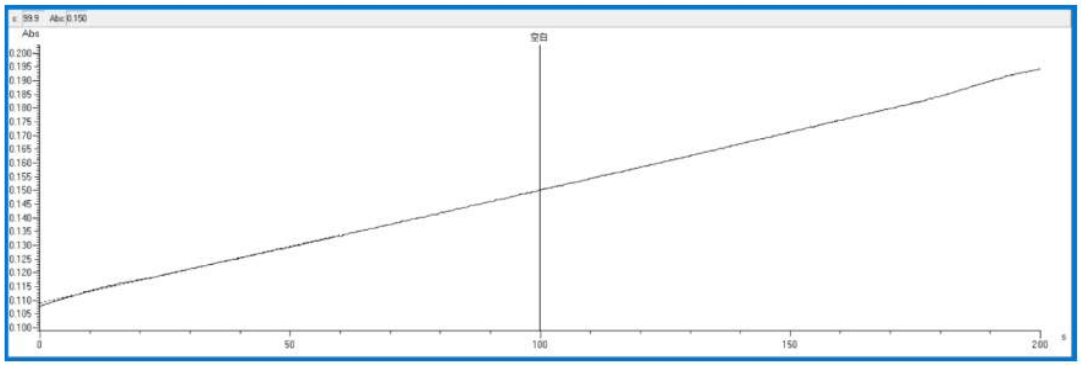
Unit of carbonic anhydrase activity（$CA2^{WT}$）

sequence number	Unit Enzyme（10^5 WAU/ μg）
2	0.1568
3	0.1885
4	0.1651
5	0.1917

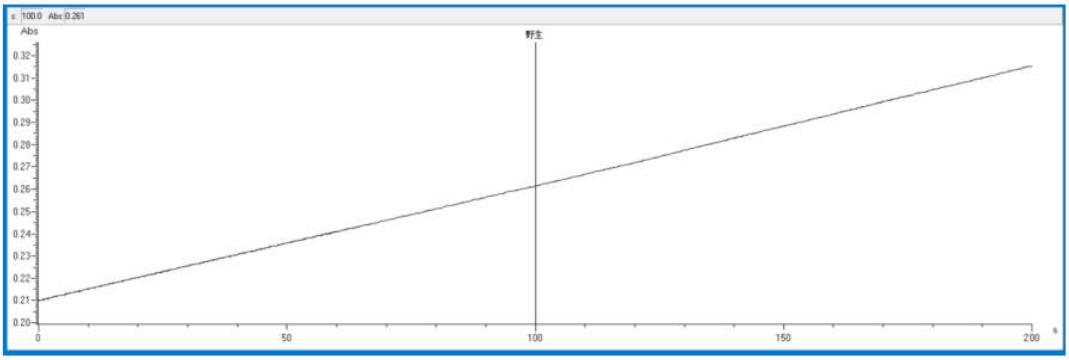
According to the average calculated in the table above, the unit enzyme activity of the mutated carbonic anhydride enzyme is 0.3710(10 5 WAU/μg), and the unit enzyme activity of wild carbonic anhydride enzyme is 0.1755(10 5 WAU/μg).

Test esterase activity

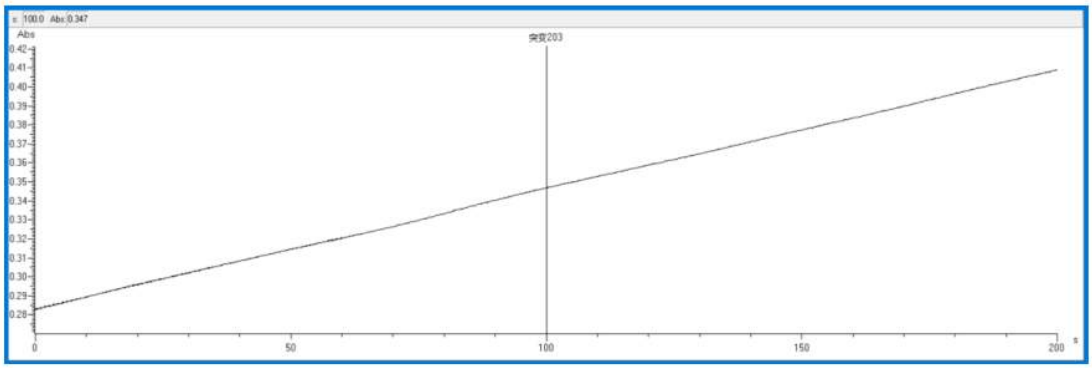
Determination curve without carbonated anhydrase:



Determination curve with carbonated anhydrase（$CA2^{WT}$）



Determination curve with carbonated anhydrase（$CA2^{L203K}$）



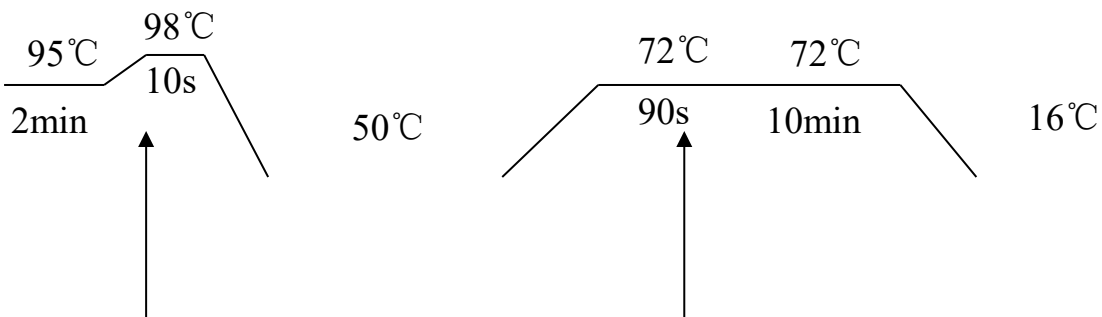
Wild carbonic anhydrase activity calculated as 0.5098 mol/g × min

The mutated carbonic anhydrase enzyme activity was calculated to be 1.0392 mol/g * min.

9.13

CA2-(csoS3) PCR

reagent	volume
5×primer STAR Buffer (Mg2+ plus)	10μl
dNTP Mixture(2.5mM each)	4μl
primer 1 (CsoF 10μM)	1μl
primer 2 (CsoR 10μM)	1μl
Template	1μl
Primer STAR	0.5μl
ddH_2O	up to 50μl



∞

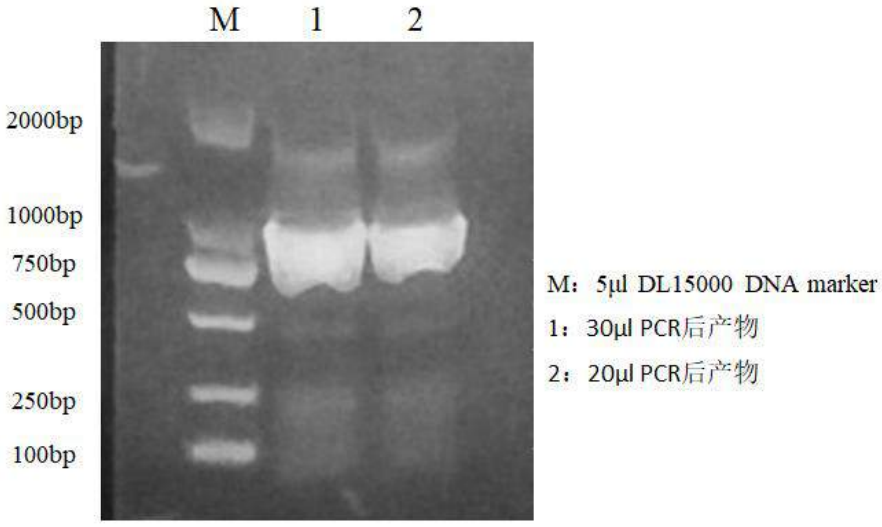
15s

30cycles

Rubber recovery

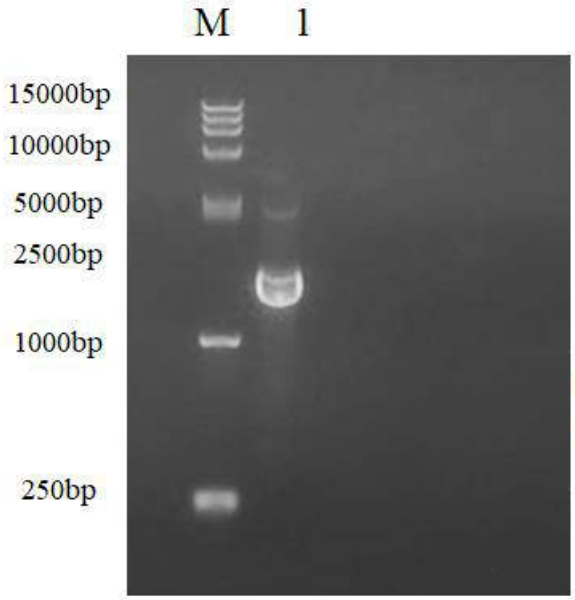
Configure 1 x recycled glue 30ml

Lane1	Lane 2	Lane 3
DL15000 DNA marker 5μl	CA2-(csoS3)Post-PCR product 30 μl	CA2-(csoS3) Post-PCR product 20 μl



1%Identification of gel 20ml

Lane 1	Lane 2
DL15000 DNA marker 2μl	CA2-(csoS3))Post-PCR product 30 μl



M：2μl DL15000 DNA marker

1： 5μl pET30-a+-CA2 PCR后产物

9.15

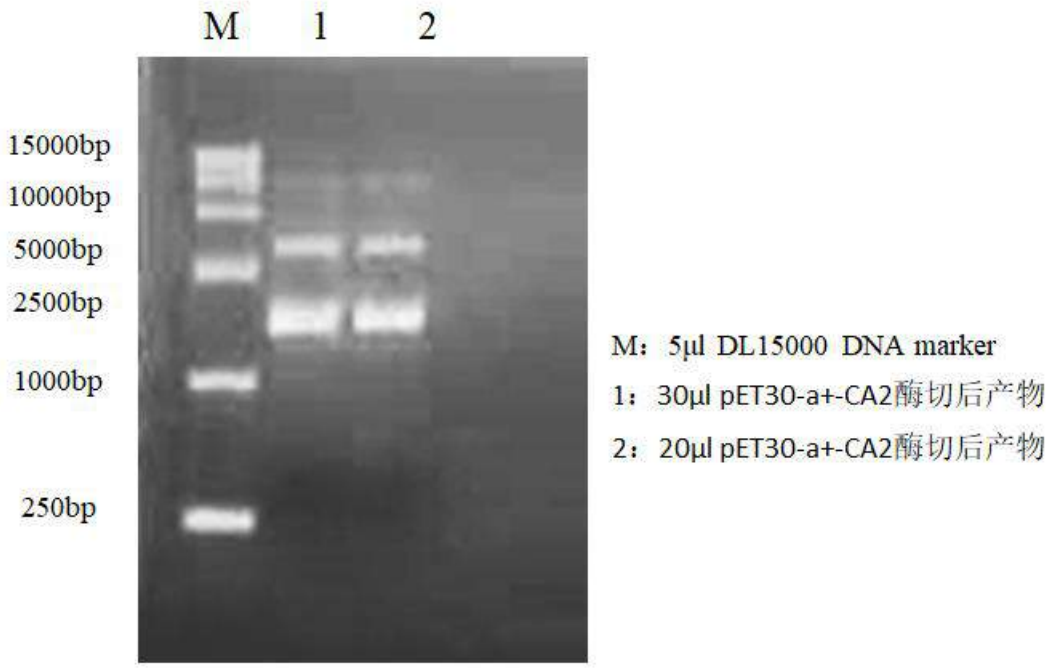
37℃ Electrophoresis

CA2(csoS3) double enzyme digestion

reagent	volume
CA2-(csoS3) Products after rubber recovery	15μl
10×NEB buffer 2	5μl
BSA(0.1%)	0.5μl
enzyme 1 (Pst I)	1.2μl
enzyme 2 (EcoRI-HF)	1.2μl
ddH2O	up to 50μl

1%recycling used gel 30ml

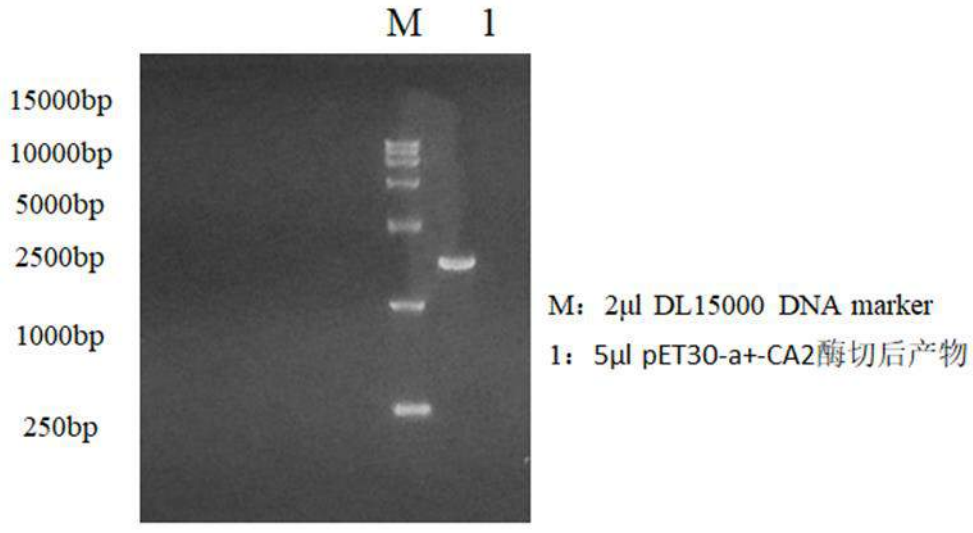
Lane 1	Lane 2	Lane 3
DL15000 DNA marker 5μl	CA2-(csoS3)Enzymatic product 30μl	CA2-(csoS3) Enzymatic product 20μl



Rubber recovery

1%Identification of gel 20ml

Lane 1	Lane 2
DL15000 DNA marker 2μl	CA2 (csoS3) 5μl after enzyme digestion



Connect PCR

reagent	volume
10×T4ConnectaseBuffer	1μl
T4Connectase	1.2μl
PSB1C3 snippet	0.5μl
after gel extraction CA2(csoS3)	0.6μl
ddH2O	up to 10μl

9.19

Transformation

reagent	volume
CA2(csoS3) product after the connection	5μl
DH5αsensory cells	100μl
LB（-）	200μl

Plate culture

reagent	medium
CA2-(csoS3) product after the connection	LB(chl＋)

9.20

Pick monoclone（CA2(csoS3)）

reagent	volume
LB（chl+）liquid	3ml

9.21

Miniprep

reagent	volume
9.20Cultivated CA2-(csoS3)	3ml

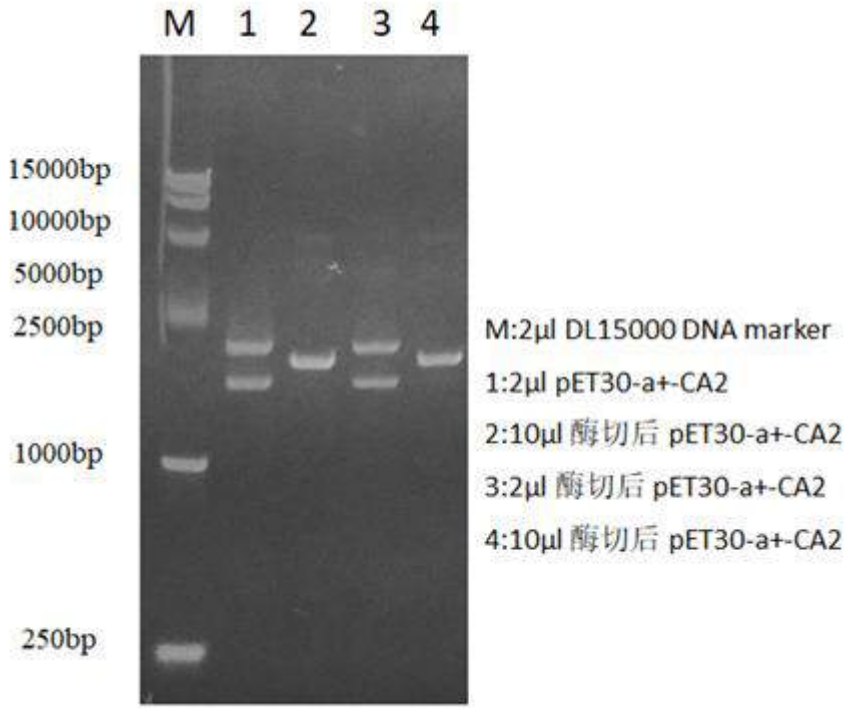
37℃ Electrophoresis

CA2(csoS3)Double Electrophoresis System

reagent	volume
Recombinant plasmid	5μl
10×NEB buffer 2	2μl
enzyme 1 (Pst I)	0.6μl
enzyme 2 (EcoRI-HF)	0.6μl
ddH_2O	up to 20μl

1% Identification of gel

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
DL15000 DNA marker 2μl	① CA2-(csoS3) 2μl	① CA2-(csoS3) 10μl after enzyme digestion	② CA2-(csoS3) 2μl	③ CA2-(csoS3) 10μl after enzyme digestion



9.22

Transformation

reagent	volume
CA2(csoS3)-Pet30-a(+)	5μl
DH5αsensory cells	100μl
LB（-）	200μl

reagent	volume
Original CA2-(csoS3)-Pet30-a（+）	5μl
DH5αsensory cells	100μl
LB（-）	200μl

Plate culture

reagent	medium
CA2-(csoS3)	LB(K＋)
Original CA2-(csoS3)	LB(K＋)

9.23

Pick monoclone

reagent	volume
LB（K+）liquid *2	4ml

9.24

Induction and extraction of protein

“①IPTG+ ”“①IPTG-”

reagent	volume
Fungal fluids cultivated CA2-(csoS3)	80μl
LB（K+）	4ml

“②IPTG+ ”“②IPTG-”

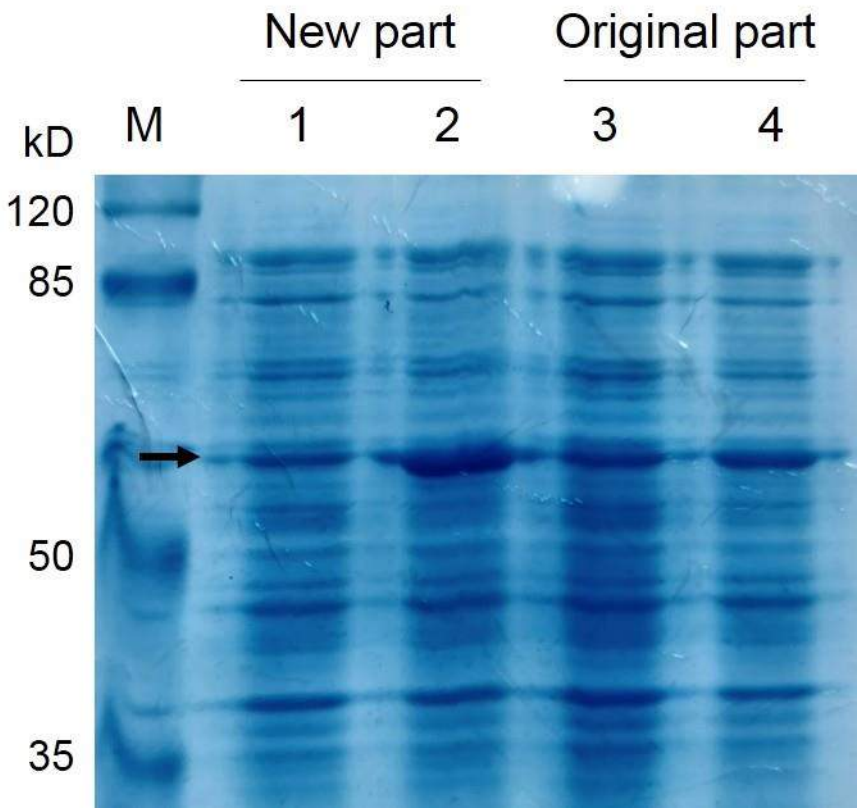
reagent	volume
Fungal fluids cultivated Original CA2-(csoS3)	80μl
LB（K+）	4ml

The shaker is cultivated for 4 hours and 20 μl IPTG is added to "①1 IPTG +" and "②2 IPTG +" to cultivate 6h. The 300 μl bacteria solution is separated and stored using a frozen centrifuge of 10,000 RPM centrifuge 15 min -20 °C.

9.25

Extraction of Protein

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Marker 2μl	①IPTG- 6h 20μl	①IPTG+ 6h 20μl	②IPTG+ 6h 20μl	②IPTG- 6h 20μl



10.2

Protein extraction and identification(Exhaust, precipitation)

reagent	9.24 Saved modified CSOS 3 solution ： IPTG+ 6h、IPTG- 6h			
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Maker 2μl	IPTG-6hExhaust 30μl	IPTG+ 6 Exhaust 30μl	IPTG- 6h precipitation 30μl	IPTG+ 6h precipitation 30μl

Select monoclonal（csoS3-CA2）

reagent	volume
LB(kana+)	4ml

10.3

Exploration of Protein Induction Conditions

“IPTG+ 4h”“IPTG- 4h” “IPTG+ 6h” “IPTG -6h”

reagent	volume
Fungal fluids cultivated （CA2-(csoS3)）	80μl
LB（K+）	150ml
IPTG	End Concentration 0.05mM

After adding IPTG, the shaker culture 4, 6 H, frozen centrifuge 4500 rpm centrifuge 15 min, -20 °C preserved.

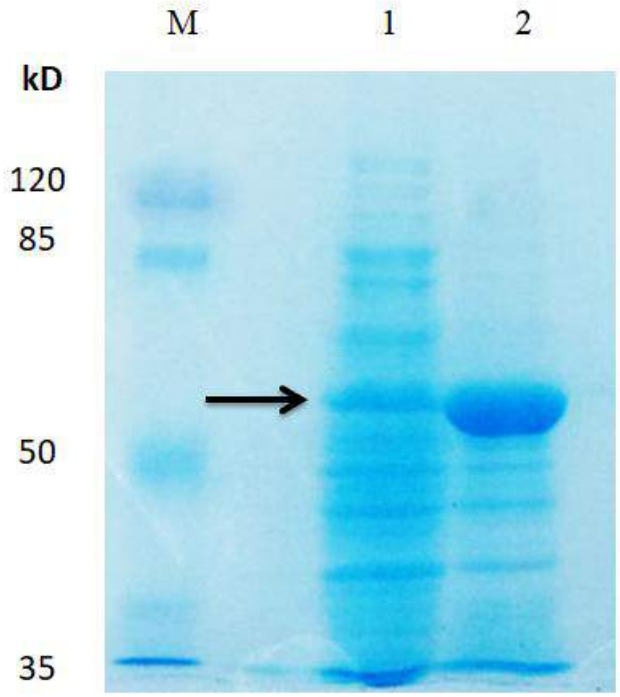
10.4

Expression and Identification of Protein

10.3 Preserved bacteria, protein extraction(exhaust, precipitation)

reagent	volume
5*loading（exhaust）	70μl
1*loading（precipitation）	300μl

Lane 1	Lane 2	Lane 3
Maker 2μl	IPTG+ 6h exhaust 30μl	IPTG+ 6h precipitation 30μl



10.5

Protein purification

10.6

Select monoclonal（①CSOS3、②CSOS3）

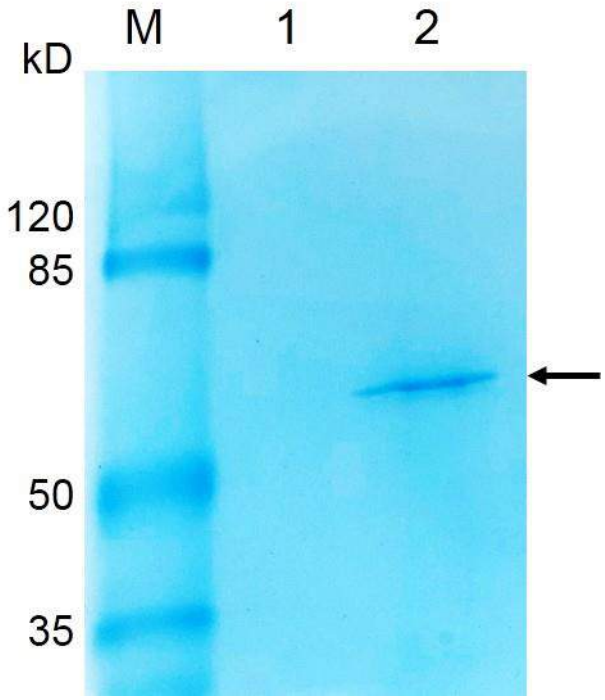
reagent	volume
LB(Chl+)	3ml

DDS-PAGE Identification of Protein

reagent	volume
10.5 Purified protein	500μl
5*loading	80μl

Lane 1	Lane 2	Lane 3

Maker 2μl	250× Imidazole elution 30μl	Purified protein 30μl



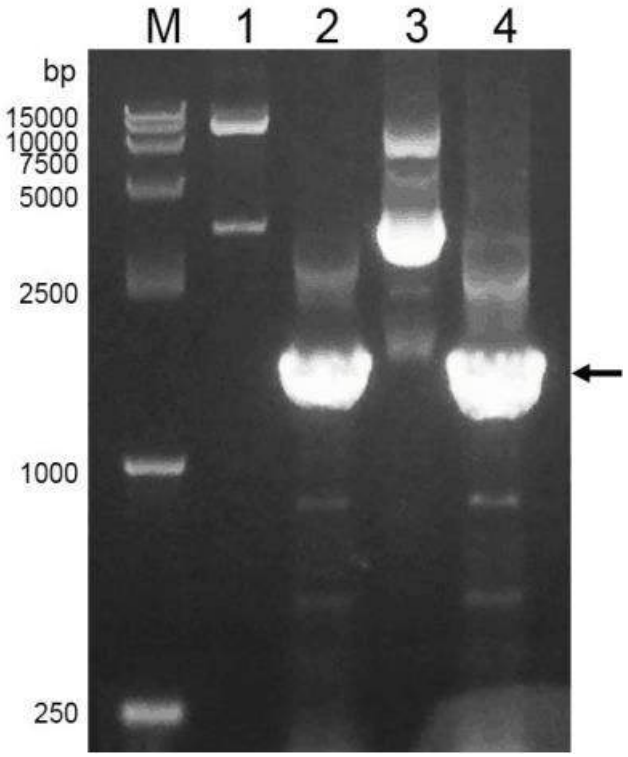
10.7

Miniprep

CsoS3 Plasmid①	50μl
CsoS3 Plasmid②	50μl

1% Identification of gel

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
DL 15000 DNA marker 2μl	Cso S3 Plasmid① 5μl	Cso S3 PCR product 5μl	Cso S3 Plasmid② 5μl	Cso S3 PCR product 5μl



10.8

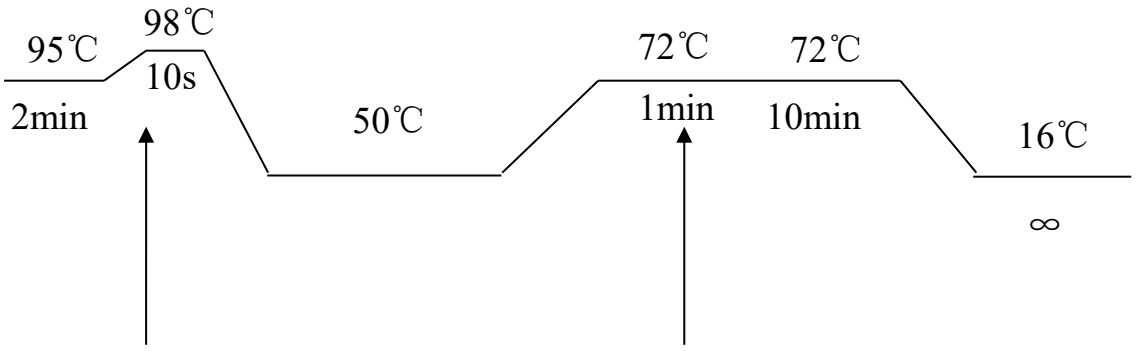
Determination of CSOS 3 Enzyme Activity by Esterase Method

Enzyme activity is about 22.84 U/ml

10.1

CA-(TSLV) PCR

reagent	volume
5×primer STAR Buffer (Mg2+ plus)	10μl
dNTP Mixture(2.5mM each)	4μl
primer 1 (TSLVF 10μM)	1μl
primer 2 (TSLVR 10μM)	1μl
Template	1μl
Primer STAR	0.5μl

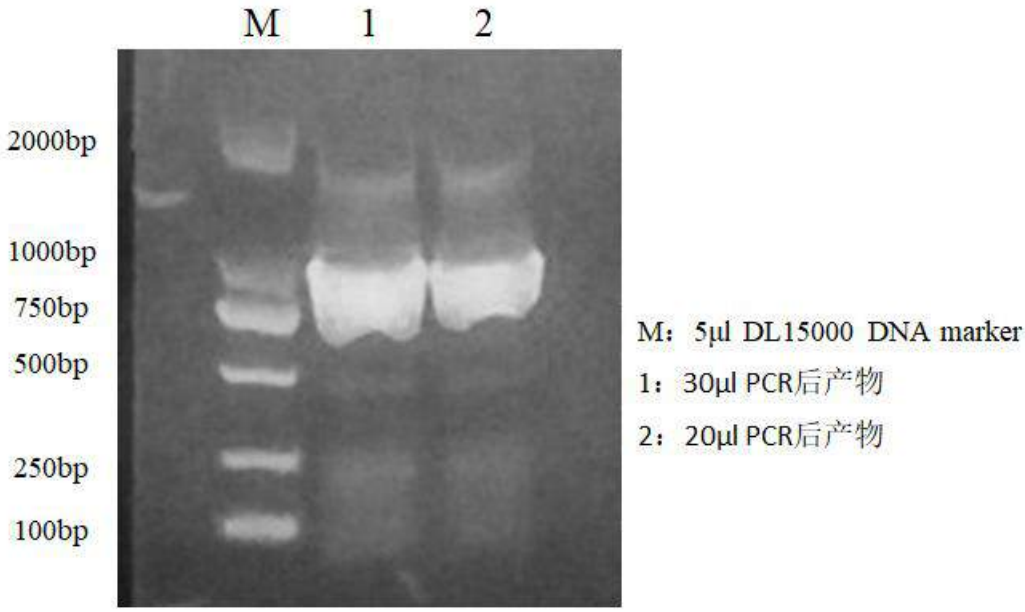


15s

30cycles

1％recycling used gel 30ml

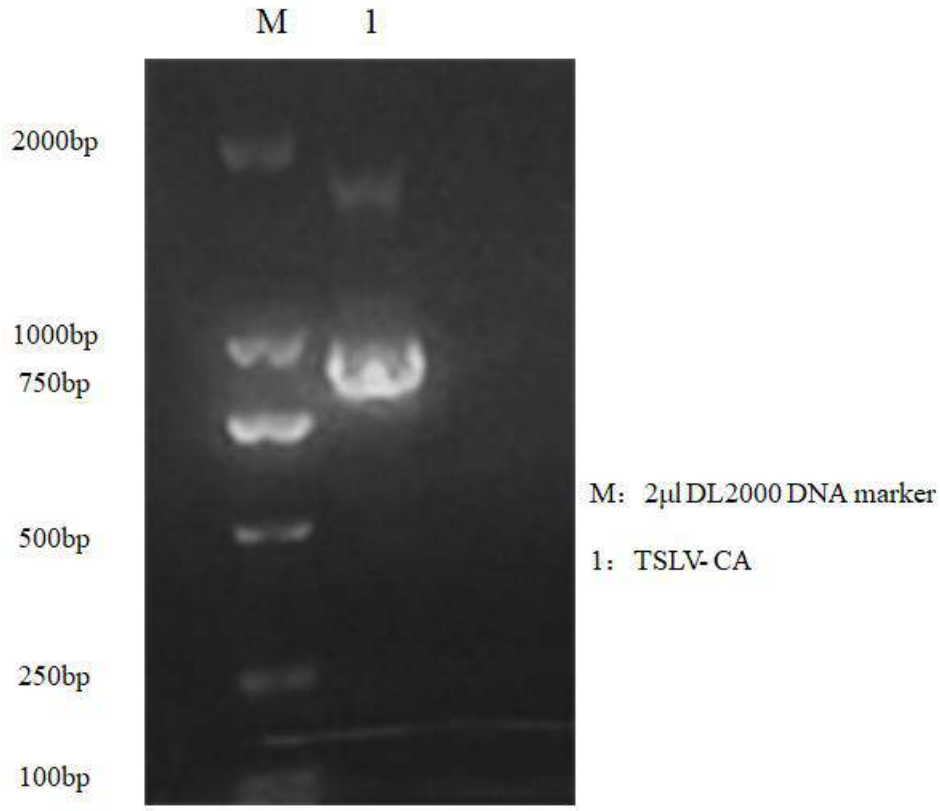
Lane 1	Lane 2
DL2000 5μl	Post-PCR product 50μl



gel extraction

Identification of gel

Lane 1	Lane 2
DL2000 5μl	TSLV- CA 5μl



37℃Electrophoresis

reagent	volume
TSLV-CA2 Products after rubber recovery	20μl
10×NEB buffer 2	5μl
BSA(0.1%)	0.5μl
enzyme 1 (Pst I)	1.2μl
enzyme 2 (EcoRI-HF)	1.2μl
ddH_2O	up to 50μl

1% recycling used gel

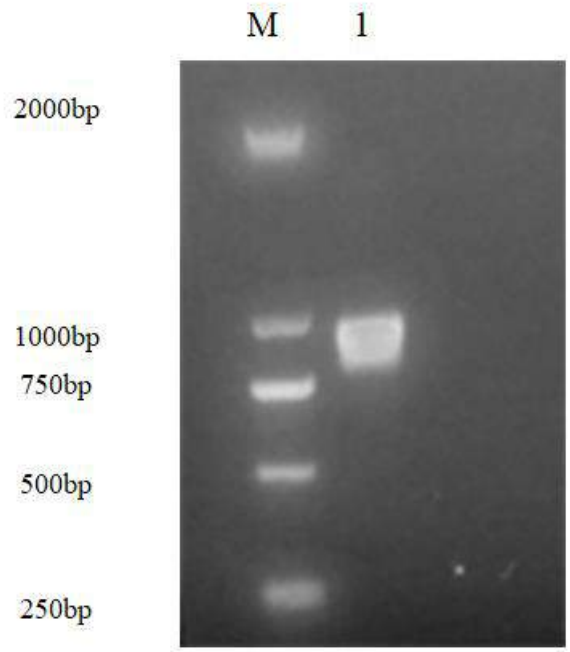
Lane 1	Lane 2
DL2000 5μl	TSLV-CA 50μl after enzyme digestion

gel extraction

1% Identification of gel

Lane 1	Lane 2

DL2000 2μl	The product of gel extraction 5μl



M：2μl DL2000 DNA marker

1：TSLV-CA 酶切后回收产物

10.2

Connect PCR

reagent	volume
10×T4ConnectaseBuffer	1μl
T4Connectase	1.2μl
PSB1C3 snippet	1μl
The product of gel extraction TSLV-CA2	0.8μl
ddH2O	up to 10μl

PCR：16℃connection 10h

10.5

Transformation

reagent	volume
TSLV-CA2	5μl
DH5αsensory cells	100μl
LB（-）	200μl

The Transformation failed many times, so the company used to provide the bacteria.

10.6

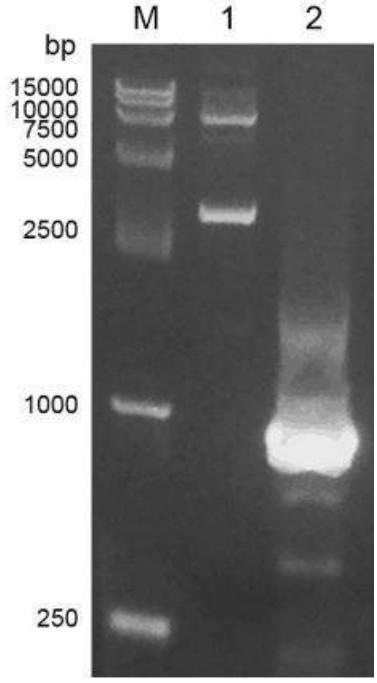
Select monoclonal

reagent	volume
LB(kana+)	4ml
BL21 Bacterial fluid （TSLV）	10μl

Miniprep

reagent	volume
TSLV Plasmid	50μl

Lane 1	Lane 2	Lane 3
DL 15000 DNA marker 2μl	TSLV Plasmid 5μl	TSLV PCR product 5μl



Protein induction

“IPTG+ 4h”“IPTG- 4h” “IPTG+ 6h” “IPTG -6h”

reagent	volume
10.6 Fungal fluids cultivated （CA2-(TSLV)）	80μl
LB（K+）	4ml
IPTG （100mM）	20μl

After adding IPTG, the shaker culture is 6h, and the 300 μl bacteria solution is packed. All of them use a frozen centrifuge 10000 rpm centrifuge 15min, and 1 * SDS loading 200μl is added to the separated bacteria solution, and the protein is cooked. 10min, intermittent shock, -20 °C.

10.8

DDS-PAGE Identification of Protein

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Marker 2μl	IPTG- 4h 20μl	IPTG+ 4h 20μl	IPTG- 6h 20μl	IPTG+ 6h 20μl

Failed, no picture

Select monoclonal colony

reagent	volume
LB（K+）liquid	4ml
BL21 Bacterial fluid （TSLV）	10μl

Delimit plate

reagent
LB（K+）solid
BL21 Bacterial fluid （TSLV）

10.9

Protein induction

“IPTG+ 4h”“IPTG- 4h” “IPTG+ 6h” “IPTG -6h”

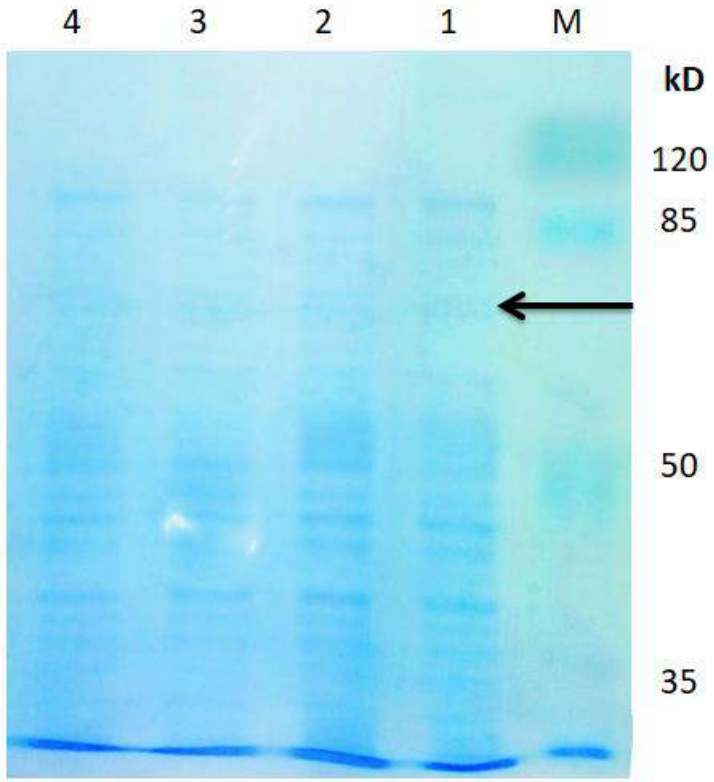
reagent	volume
Fungal fluids cultivated （CA2-(TSLV)）	80μl
LB（K+）	4ml
IPTG （100mM）	20μl

After adding IPTG, the shaker culture is 6h, and the 300 μl bacteria solution is packed. All of them use a frozen centrifuge 10000 rpm centrifuge 15min, and 1 * SDS loading 200μl is added to the separated bacteria solution, and the protein is cooked. 10min, intermittent shock, -20 °C.

10.10

DDS-PAGE Identification of Protein

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
Marker 2μl	IPTG- 4h 20μl	IPTG+ 4h 20μl	IPTG- 6h 20μl	IPTG+ 6h 20μl



DDS-PAGE Identification of Protein

Lane 1	Lane 2	Lane 3	Lane 4
1*SDS loading buffer 10μl	IPTG- 4h（10.9） 20μl	IPTG+ 4h（10.9） 20μl	IPTG- 6h（10.9） 20μl
Lane 5	Lane 6	Lane 7	Lane 8
IPTG+ 6h（10.9） 20μl	IPTG+ 6h（10.8） 20μl	IPTG- 6h（10.8） 20μl	IPTG+ 4h（10.8） 20μl
Lane 9			
IPTG- 4h（10.8） 20μl			

10.11

Western blot identification of proteins