

Wild-type CA2

8.21

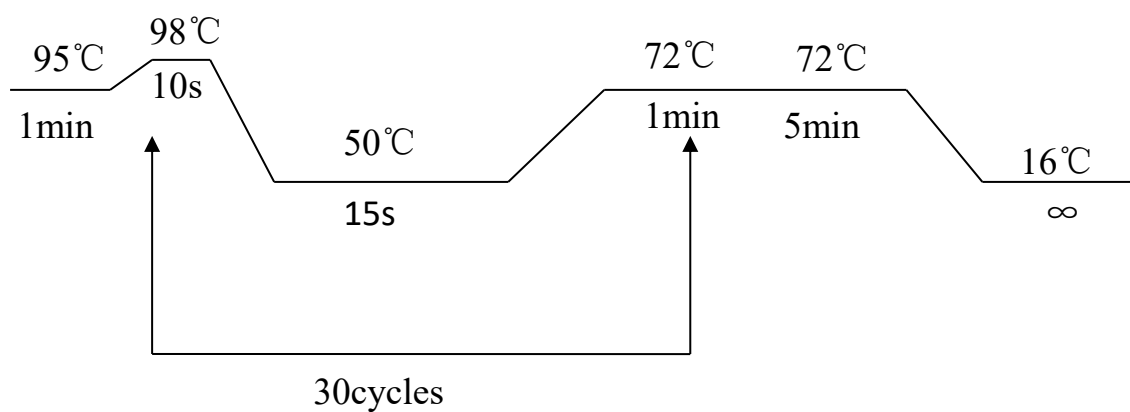
Diluting All Primers

CA2R	181.5 μ l ddH ₂ O
CA2F	207.9 μ l ddH ₂ O
MUF	251.4 μ l ddH ₂ O
MUR	281.9 μ l ddH ₂ O

8.22

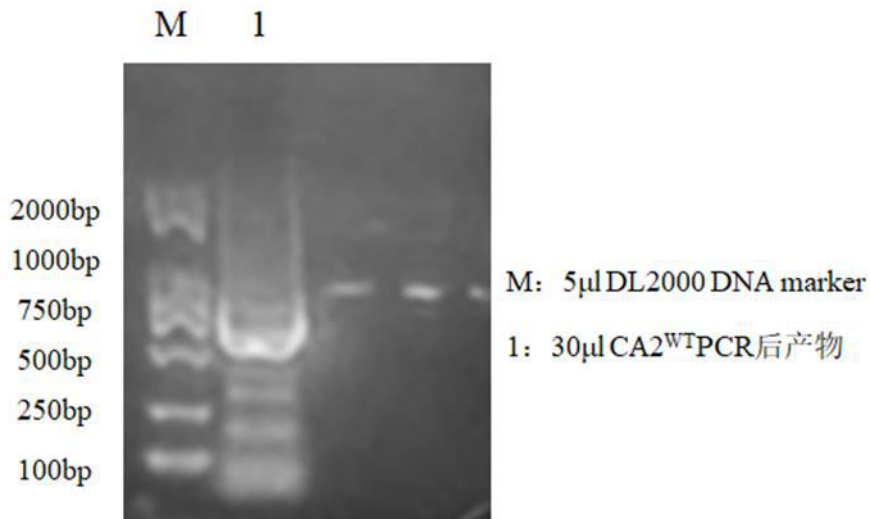
PCR

5 \times primer STAR Buffer (Mg ²⁺ plus)	10 μ l
dNTP Mixture(2.5mM each)	4 μ l
primer 1 (CA2F 10 μ M)	1 μ l
primer 2 (CA2R 10 μ M)	1 μ l
Template (CA2 ^{WT})	1 μ l
Primer STAR	0.5 μ l
ddH ₂ O	up to 50 μ l



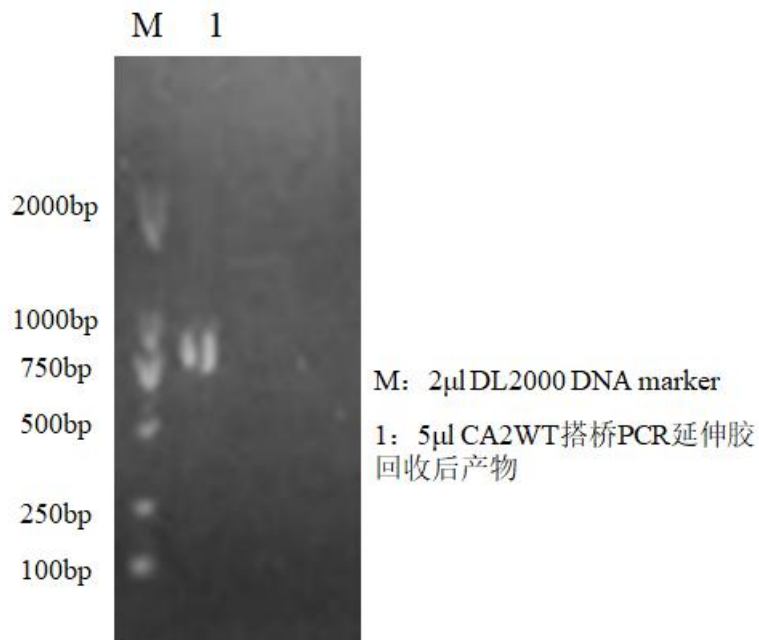
Recycling Gel

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



Gel Extraction
Identifying Gel

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



8.23

Enzyme Digestion at 37°C

restriction enzyme identification

reagent	volume
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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 ₂ O	up to 50μl

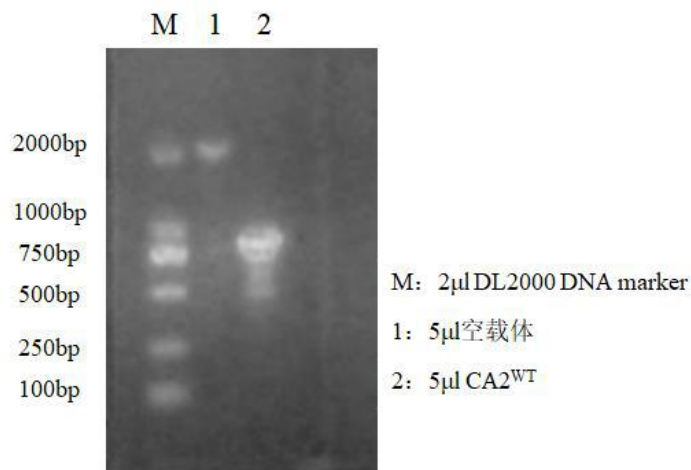
restriction enzyme identification

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 ₂ O	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°C 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 Monoclon

reagent	volume
LB (chl+)	3ml

8.26

Miniprep

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

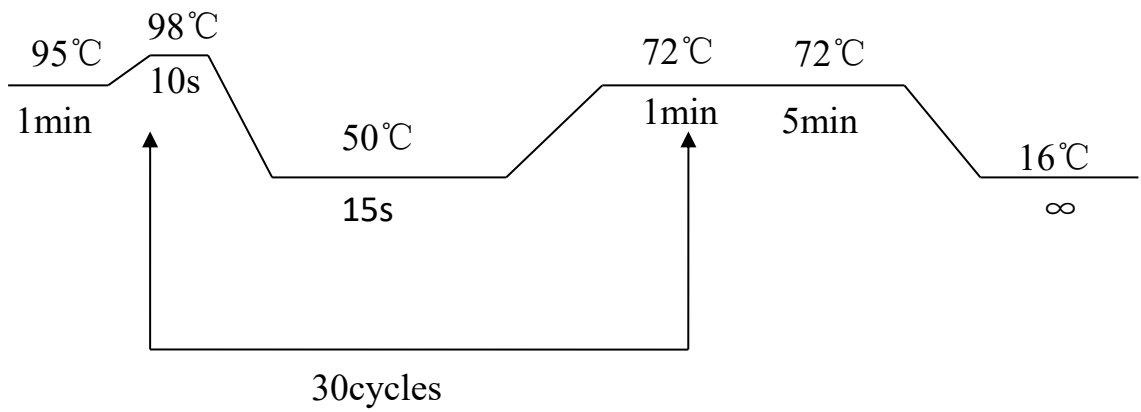
Mutant CA2^{L203k}

8.23

PCR

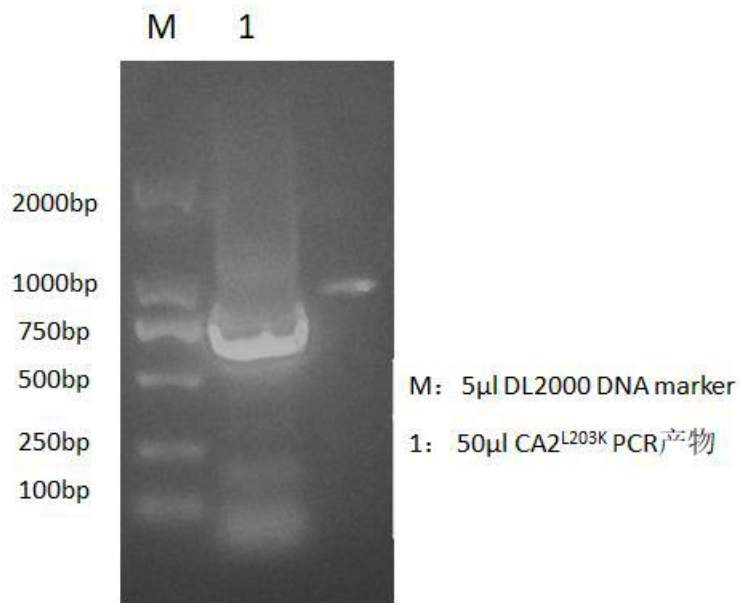
5×primer STAR Buffer (Mg ²⁺ plus)	10μl
dNTP Mixture(2.5mM each)	4μl
primer 1 (CA2F 10μM)	1μl
primer 2 (CA2R 10μM)	1μl

Template (CA2 ^{L203K})	1 μ l
Primer STAR	0.5 μ l
ddH ₂ O	up to 50 μ l



Recycling Gel

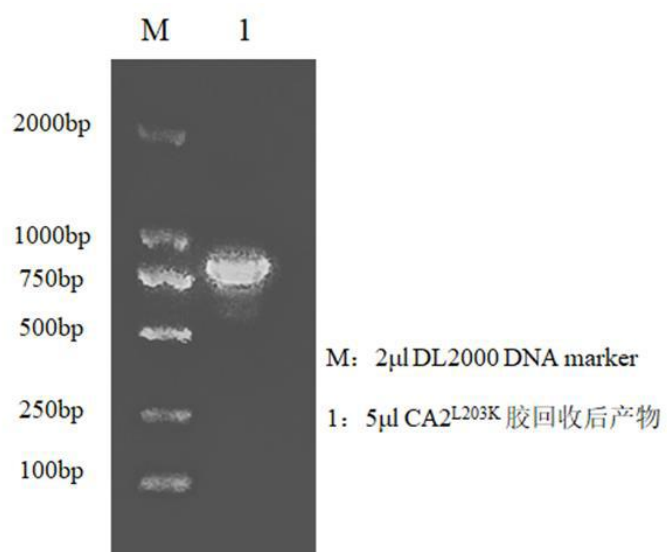
Lane1	Lane2
DL 2000DNA marker 2 μ l	PCR band of CA2 ^{L203K} 50 μ l



Gel Extraction

Identifying Gel

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



8.24

Enzyme Digestion at 37°C

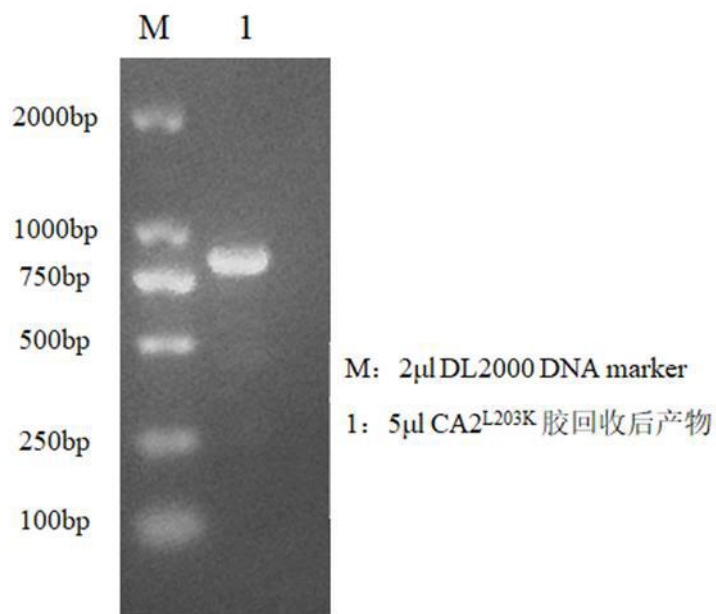
restriction enzyme identification

reagent	volume
CA ^{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 ₂ O	up to 50µl

Gel Extraction

Identifying Gel

Lane1	Lane2
DL 2000DNA marker 2µl	the product of gel extraction of CA ^{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 Monoclon

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°C

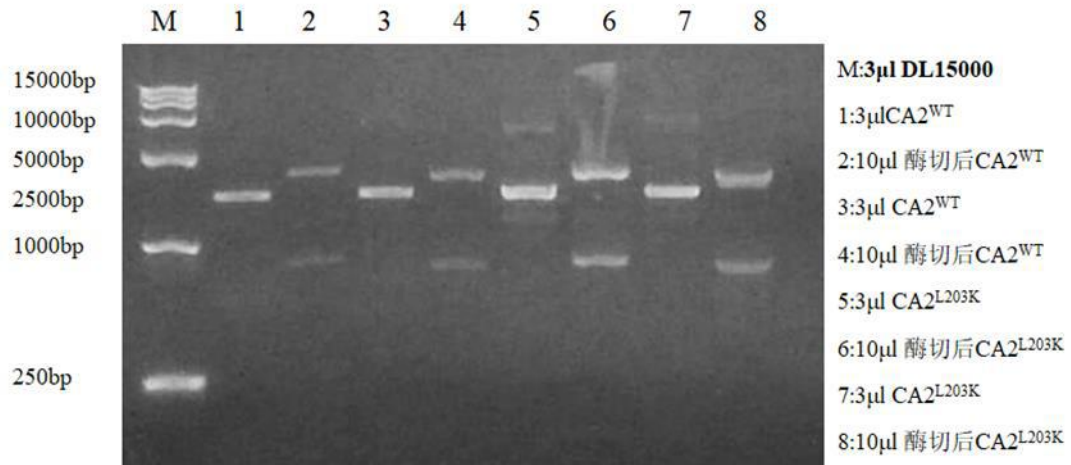
Miniprep

reagent	volume
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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 ₂ O	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+)	4ml*2
8.29 Fungal fluids cultivated	40 μ l*2

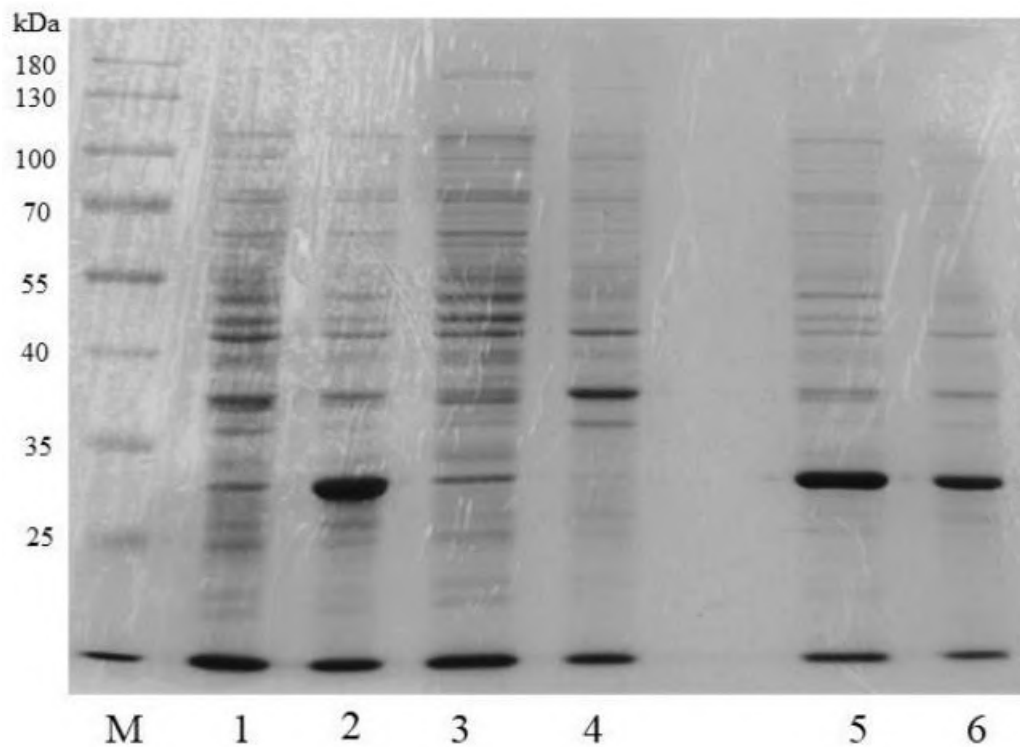
After the rocker culture is 4 H, add IPTG(final concentration is 0.5 mm) and then the rocker culture is 4 hours. Each of the two tubes absorbs 200 μ L of bacterial fluid, and in the frozen centrifuge, it is 4 °C and 12000 rpm centrifuge. 10 minutes, go to the clear, It is resuspended with 100 μ L 1x 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 2 μ l	IPTG-4h 30 μ l	IPTG+ 4h 30 μ l	IPTG- 4h exhaust 30 μ l	IPTG- 4hprecipitation 30 μ l
Pore canal 6	Pore canal 7			
IPTG- 4h exhaust 30 μ l	IPTG- 4h precipitation 30 μ l			

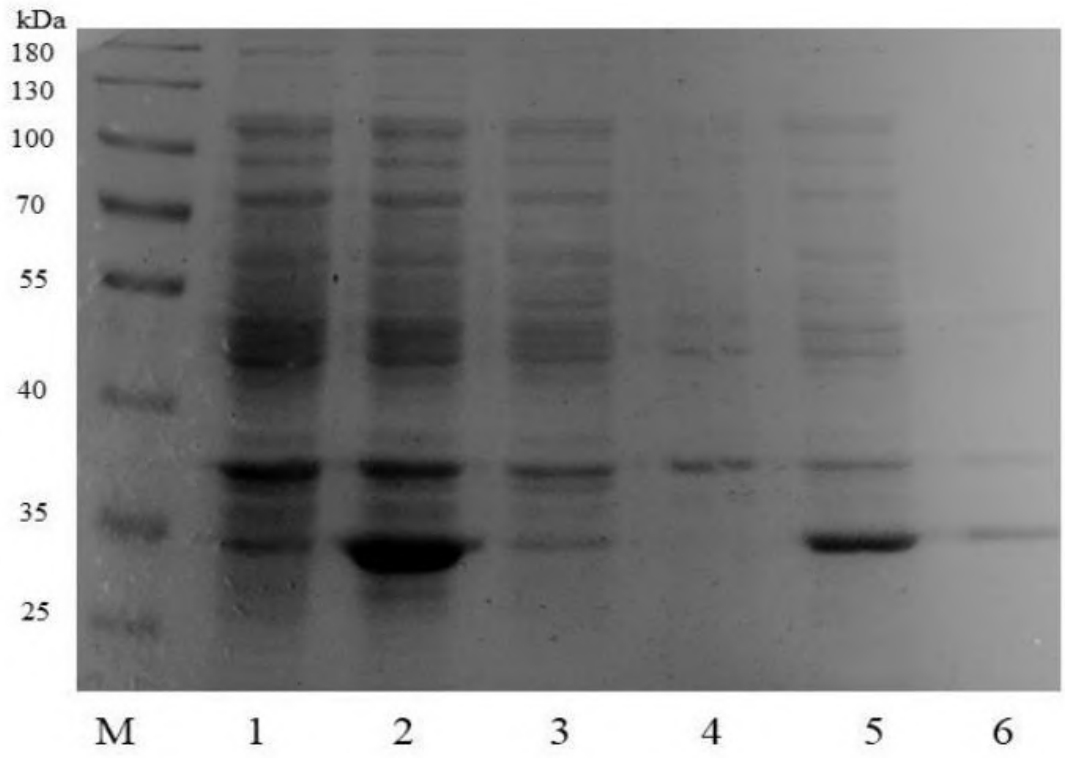


CA2^{WT}

CA2^{L203K}

Pore canal 1	Pore canal 2	Pore canal 3	Pore canal 4	Pore canal 5
Maker 2 μ l	IPTG-4h 30 μ l	IPTG+ 4h 30 μ l	IPTG- 4h exhaust 30 μ l	IPTG- 4h precipitation 30 μ l
Pore canal 6	Pore canal 7			

IPTG- 4h exhaust 30 μ l	IPTG- 4h precipitation 30 μ l
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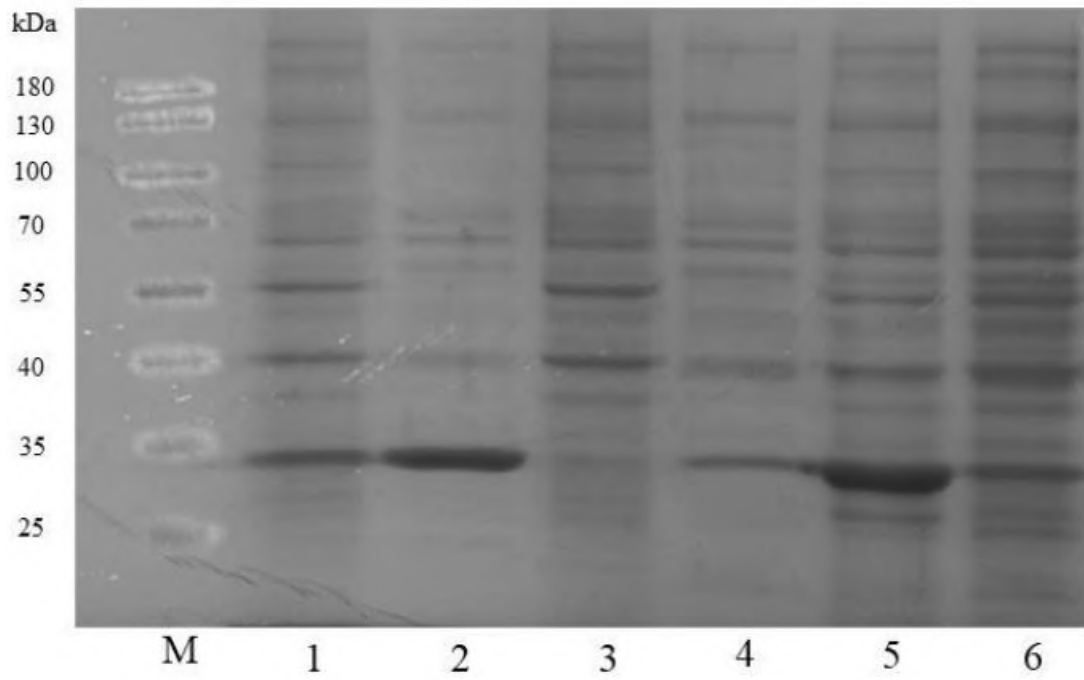


9.1

Purification of Protein

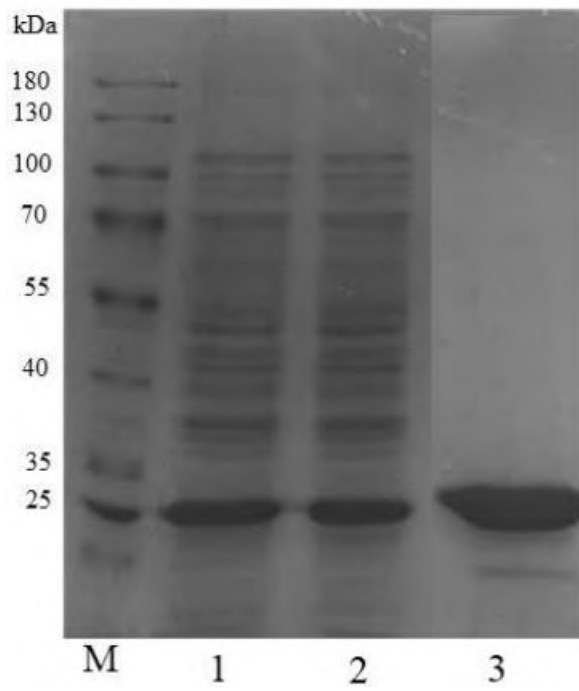
Prior to purification

Pore canal 1	Pore canal 2	Pore canal 3	Pore canal 4	Pore canal 5
Maker 2 μ l	IPTG+4h precipitation 30 μ l	IPTG+ 4h exhaust 30 μ l	IPTG- 4h precipitation 30 μ l	IPTG- 4h exhaust 30 μ l
Pore canal 6	Pore canal 7			
IPTG+4h 30 μ l	IPTG- 4h 30 μ l			



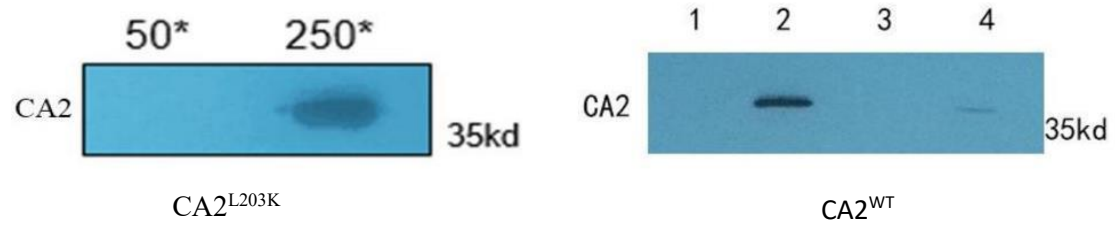
Purified

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

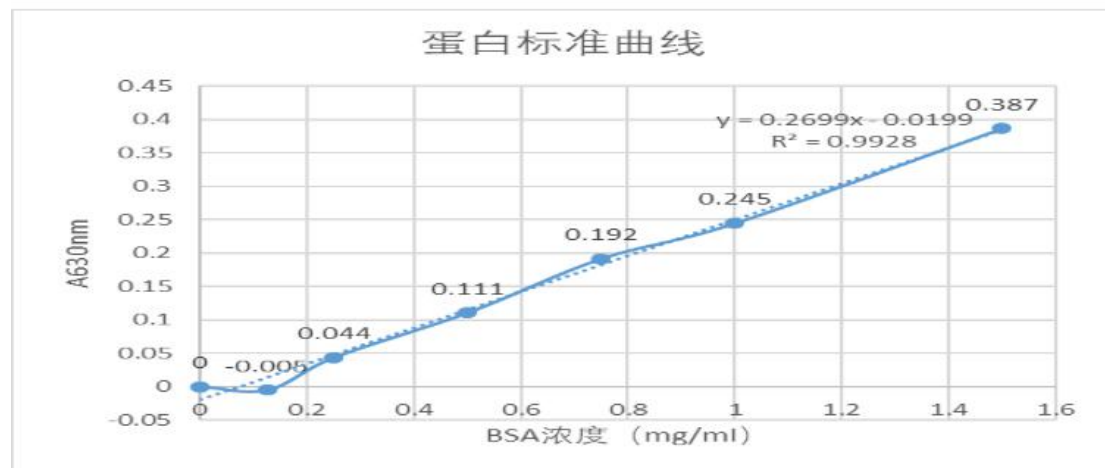
Western blot identification of proteins



9.4

Quantification of protein concentrations

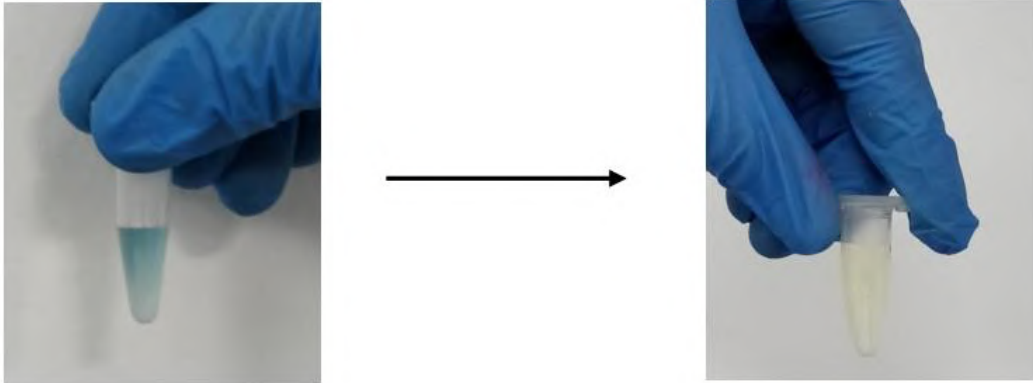
Standard	0	0.125	0.25	0.5	0.75	1	1.5
Protein							
Concentration							
n (mg/mL)							
OD value	1.405	1.400	1.449	1.516	1.597	1.650	1.792
A630 (nm)	0	-0.005	0.044	0.111	0.192	0.245	0.387



9.5

colorimetric enzyme activity

Add 250 μ l saturated CO₂ solution



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

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

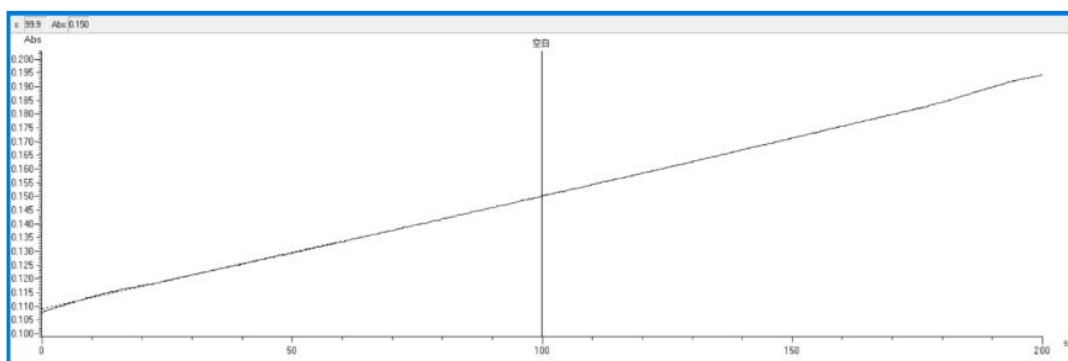
Unit of carbonic anhydrase activity (CA2^{WT})

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

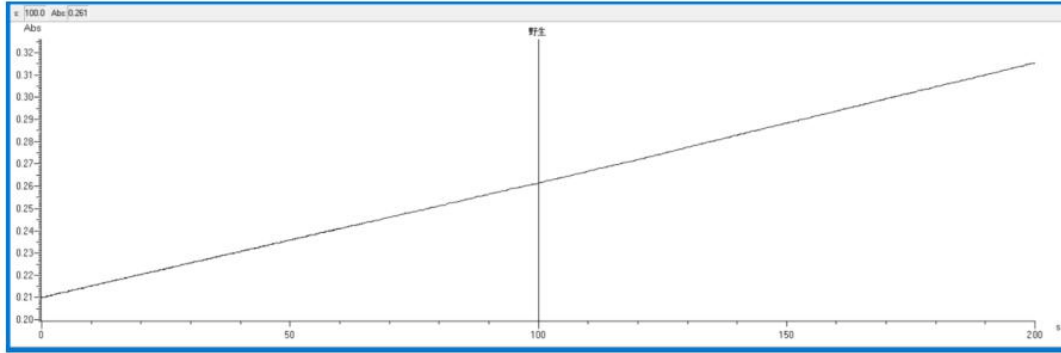
According to the average calculated in the table above, the unit enzyme activity of the mutated carbonic anhydrase enzyme is 0.3710(10⁵ WAU/ μ g), and the unit enzyme activity of wild carbonic anhydrase enzyme is 0.1755(10⁵ WAU/ μ g).

Test esterase activity

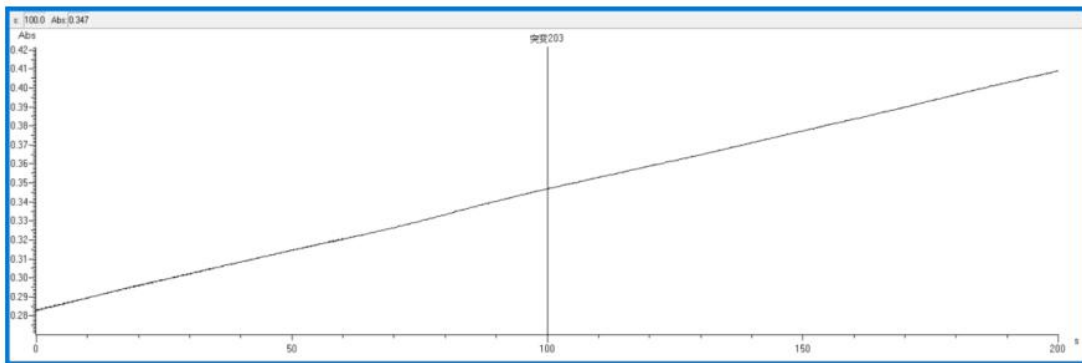
Determination curve without carbonated anhydrase:



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



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



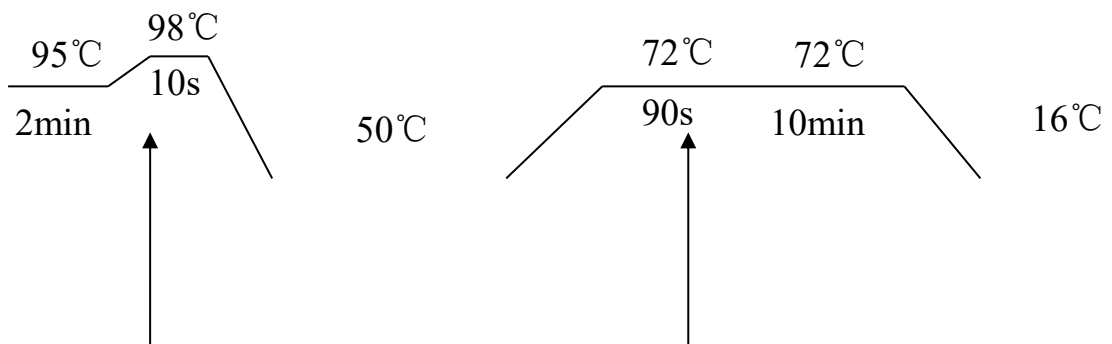
Wild carbonic anhydrase activity calculated as 0.5098 mol/g × min

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

9.13

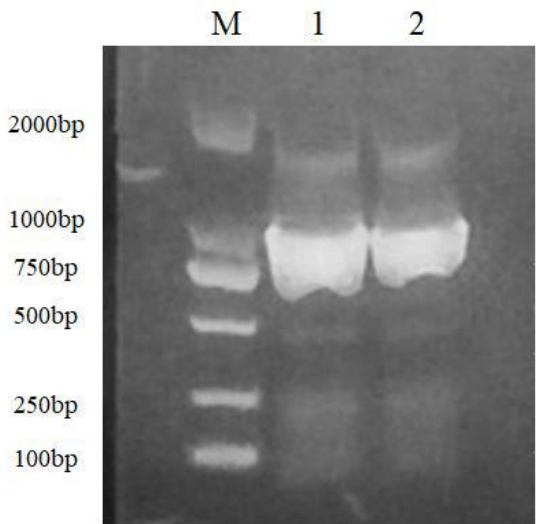
CA2-(csoS3) PCR

reagent	volume
5×primer STAR Buffer (Mg ²⁺ plus)	10 μ l
dNTP Mixture(2.5mM each)	4 μ l
primer 1 (CsoF 10 μ M)	1 μ l
primer 2 (CsoR 10 μ M)	1 μ l
Template	1 μ l
Primer STAR	0.5 μ l
ddH ₂ O	up to 50 μ l



15s

30cycles



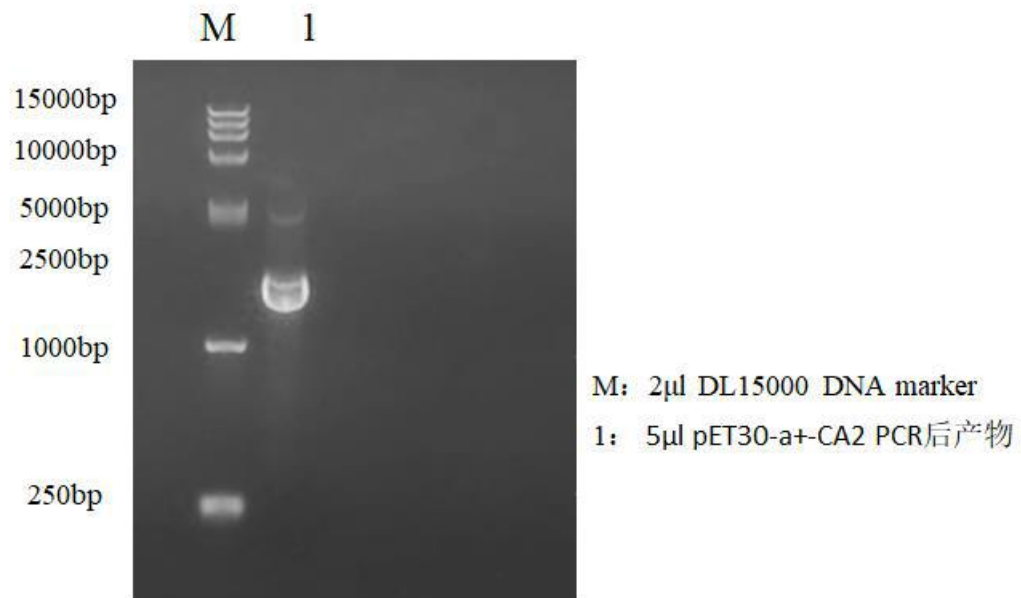
M: 5µl DL15000 DNA marker
1: 30µl PCR后产物
2: 20µl PCR后产物

3) Post-PCR product 20 µl

Rubber recovery
Configure 1 x recycled glue 30ml

1% Identification of gel 20ml

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



9.15

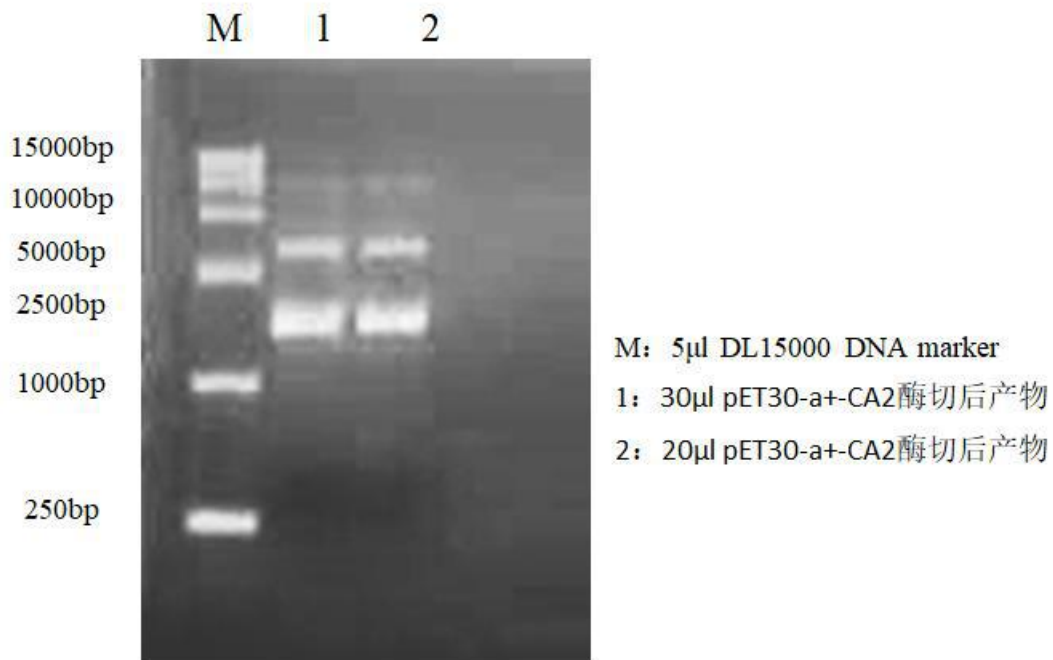
37°C Electrophoresis

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

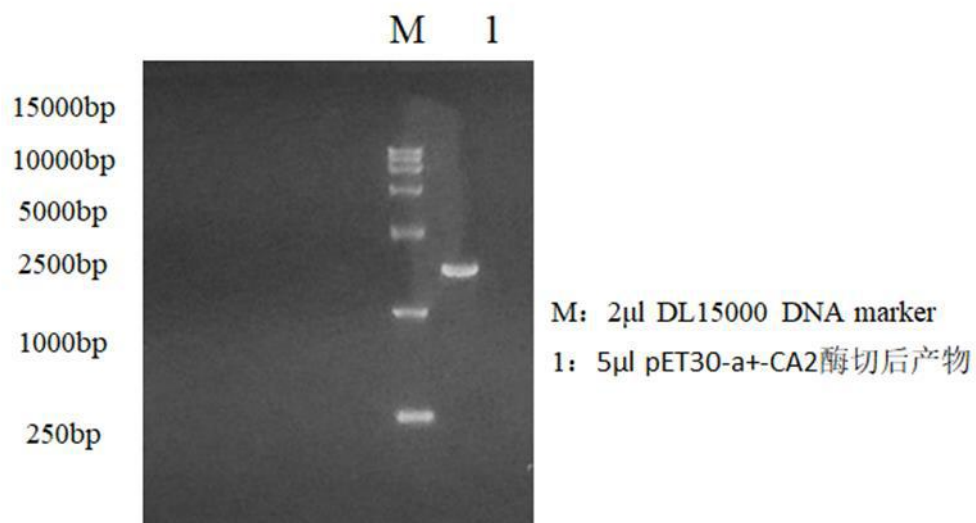
Lane 1	Lane 2	Lane 3
DL15000 DNA marker 5 μ l	CA2-(csoS3)Enzymatic product 30 μ l	CA2-(csoS3) Enzymatic product 20 μ l



Rubber recovery

1%Identification of gel 20ml

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



Connect PCR

reagent	volume
10×T4ConnectaseBuffer	1µl
T4Connectase	1.2µl
PSB1C3 snippet	0.5µl
after gel extraction CA2(csoS3)	0.6µl
ddH ₂ O	up to 10µl

9.19

Transformation

reagent	volume
CA2(csoS3) product after the connection	5µl
DH5asensory cells	100µl
LB (-)	200µl

Plate culture

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

9.20

Pick monoclonal (CA2(csoS3))

reagent	volume
LB (chl+) liquid	3ml

9.21

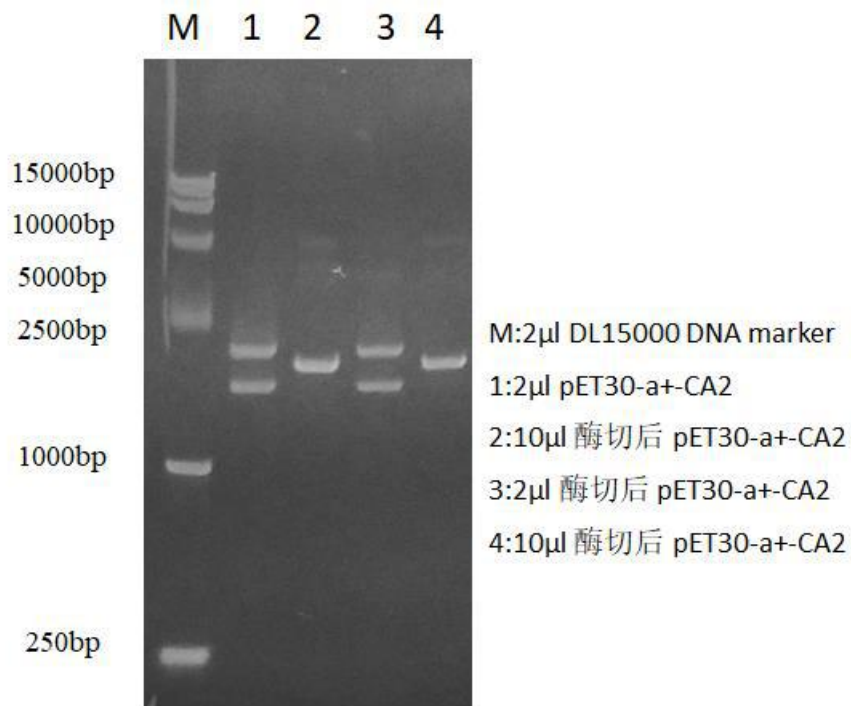
Miniprep

reagent	volume
9.20Cultivated CA2-(csoS3)	3ml

37°C 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 ₂ O	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
DH5asensory cells	100µl
LB (-)	200µl

reagent	volume
Original CA2-(csoS3)-Pet30-a (+)	5µl
DH5asensory cells	100µl
LB (-)	200µl

Plate culture

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

9.23

Pick monoclon

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-”

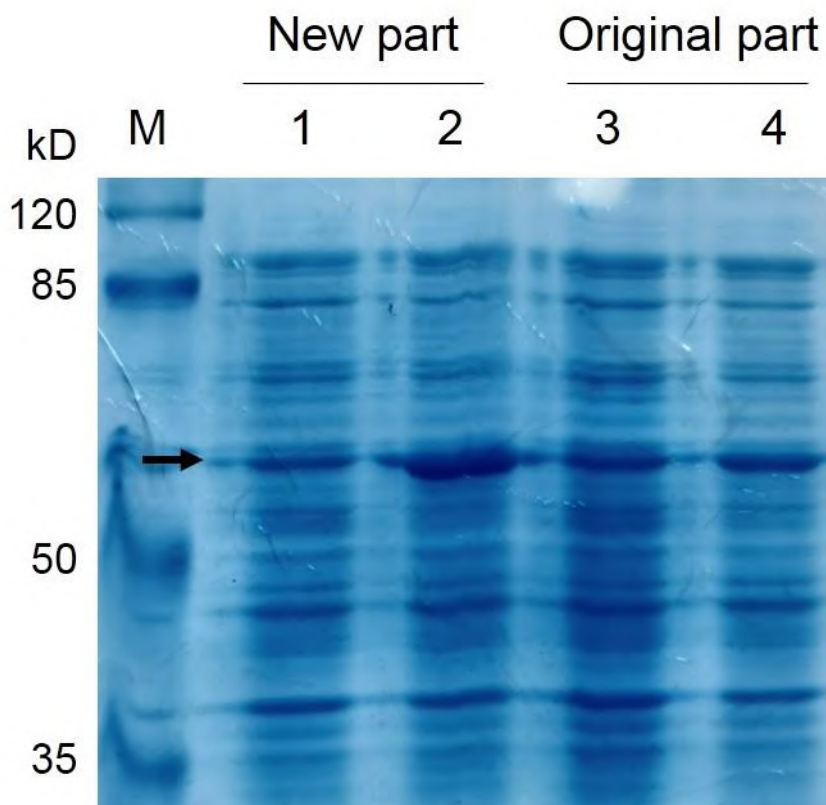
reagent	volume
Fungal fluids cultivated Original CA2-(csoS3)	80µl
LB (K+)	4ml

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.

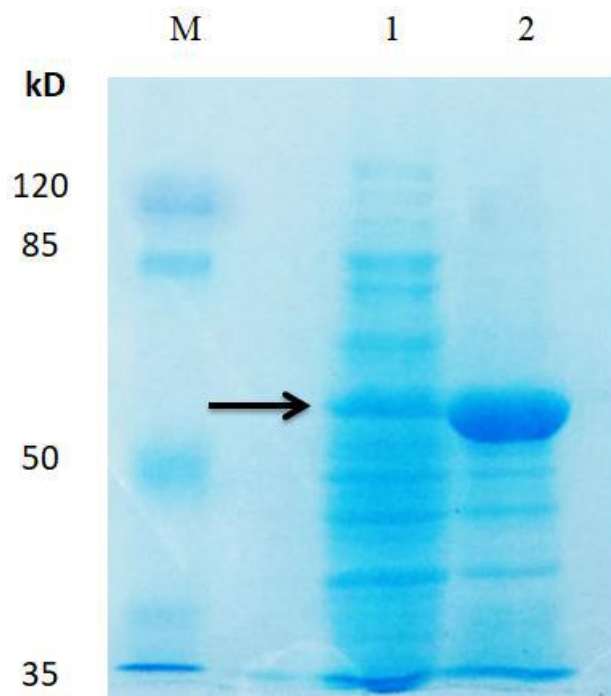
10.4

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)

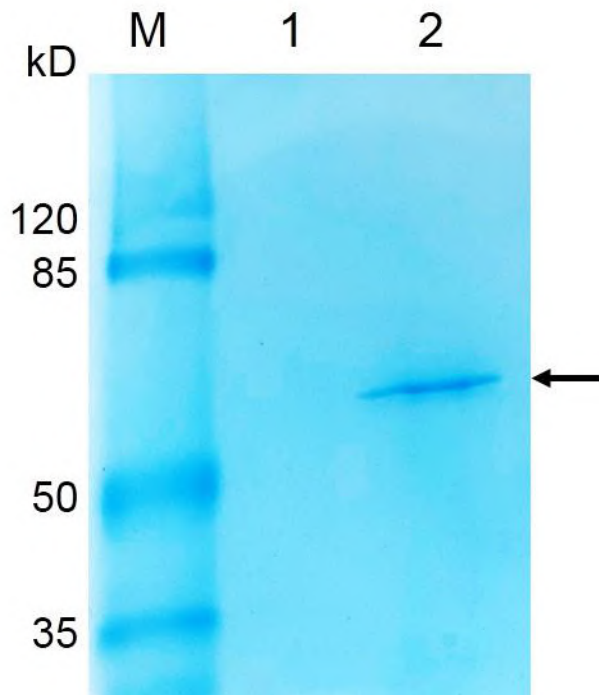
reagent	volume
LB(Chl+)	3ml

DDS-PAGE Identification of Protein

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

Lane 1	Lane 2	Lane 3

Marker 2μl	250× Imidazole elution 30μl	Purified protein 30μl
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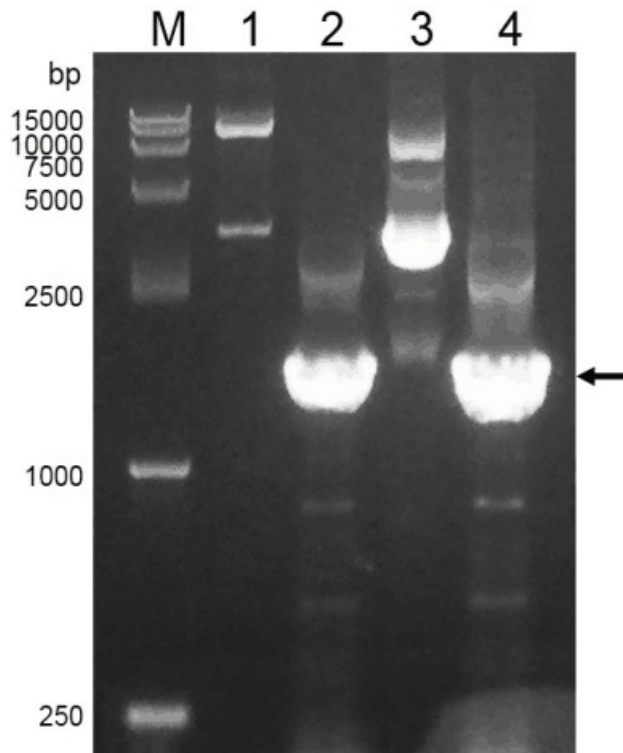
10.7

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

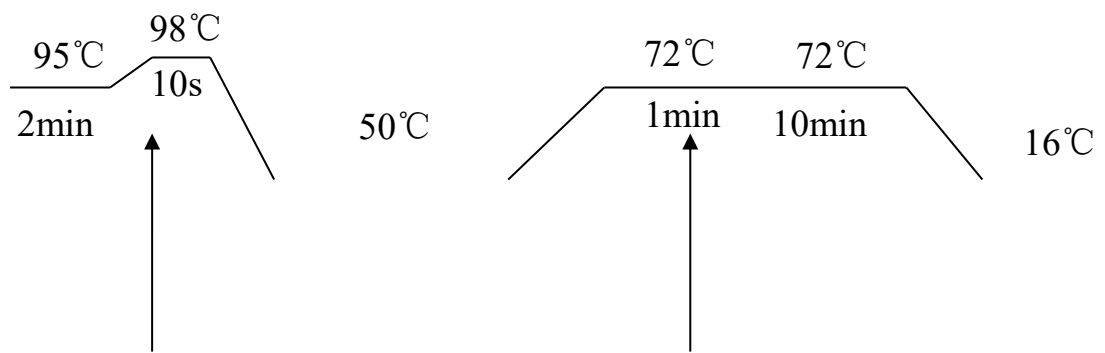
Determination of CSOS 3 Enzyme Activity by Esterase Method

Enzyme activity is about 22.84 U/ml

10.1

CA-(TSLV) PCR

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

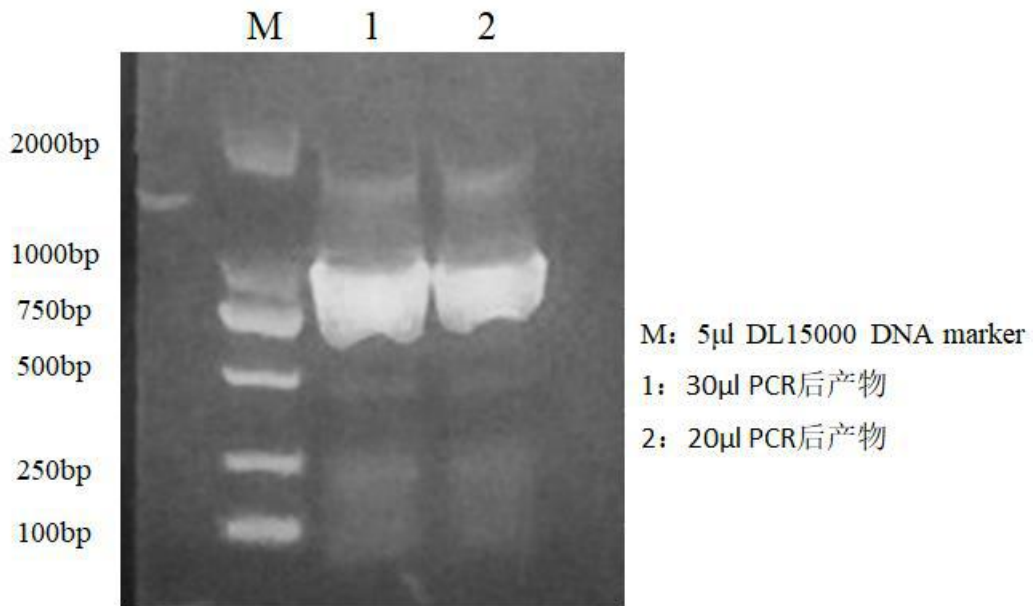


15s

30cycles

1%recycling used gel 30ml

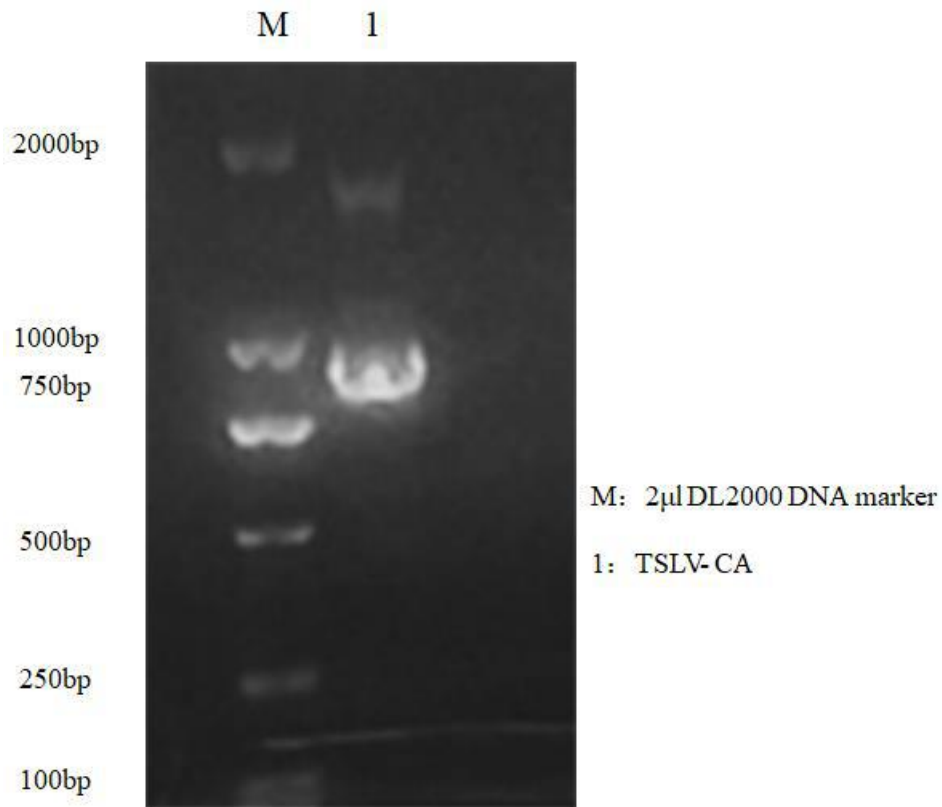
Lane 1	Lane 2
DL2000 5μl	Post-PCR product 50μl



gel extraction

Identification of gel

Lane 1	Lane 2
DL2000 5μl	TSLV- CA 5μl



37°C 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 ₂ O	up to 50µl

1% recycling used gel

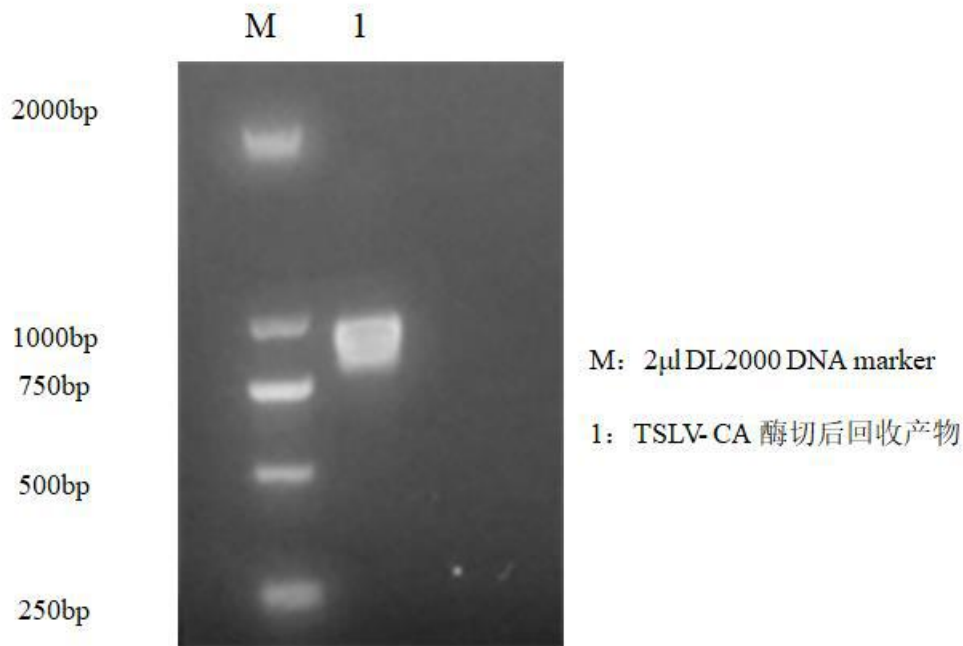
Lane 1	Lane 2
DL2000 5µl	TSLV-CA 50µl after enzyme digestion

gel extraction

1% Identification of gel

Lane 1	Lane 2

DL2000 2μl	The product of gel extraction 5μl
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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°Cconnection 10h

10.5

Transformation

reagent	volume
TSLV-CA2	5μl
DH5asensory cells	100μl
LB (-)	200μl

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

10.6

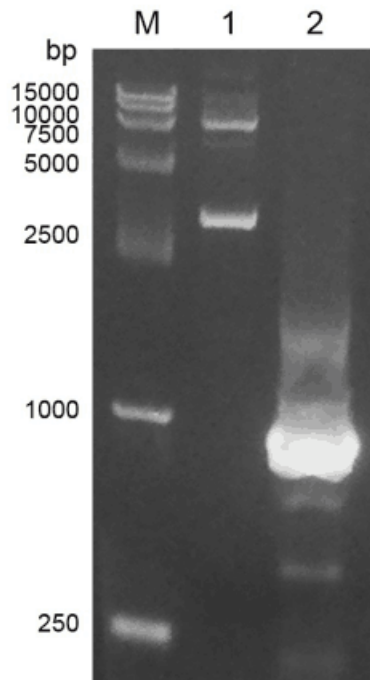
Select monoclonal

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”

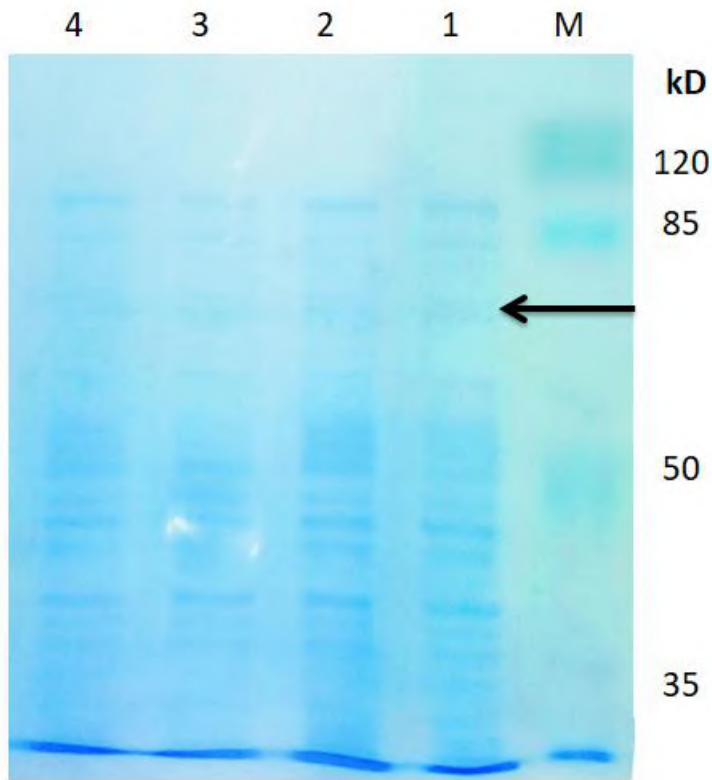
reagent	volume
Fungal fluids cultivated (CA2-(TSLV))	80μl
LB (K+)	4ml
IPTG (100mM)	20μl

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

