

# *Cloning and Protein Purification of 2201LBD Mutants*

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**Abstract:** In this study, we employed molecular cloning techniques involving PCR, DNA agarose gel electrophoresis, and restriction enzyme digestion to successfully clone 2201LBD mutants with point mutations at residues 172 and 142. Following enzymatic digestion of DNA and plasmid vectors using restriction enzymes, the T4 DNA ligase was used to ligate the modified DNA fragments into the vectors. Subsequently, the resulting constructs were transformed into *Escherichia coli* BL21 (DE3) strains for protein expression and purification. These engineered mutants, 2201LBD-Y172A and 2201LBD-R142A, were purified to facilitate subsequent experimental investigations.

## **1. Gene cloning construction**

For now, PCR serves as the foundational technique upon which various other experimental procedures rely [1]. It is a rapid method utilized for the amplification and replication of short DNA segments. This process, necessitates the use of DNA polymerase (such as Taq polymerase), which enables the addition and extension of nucleotides to form phosphodiester bonds. Successful execution of a PCR experiment also requires primers for both the sense and antisense DNA strands to initiate the reaction. Additionally, a suitable reaction buffer with moderate Mg<sup>2+</sup> concentration and containing all four types of deoxyribonucleotide triphosphates (dNTPs) are essential components.

The designated template for this PCR experiment is ctCNB1\_2201, a genetic sequence present within the bacterial strain CNB1 [2].

After the addition of these reagents to small PCR tubes, centrifugation is employed to ensure thoroughly mixing the reaction solution at the bottom of the tubes. The reaction protocol involves cycling through three distinct temperatures: denaturation at 94 °C for 45-60 seconds, annealing (duration determined based on primer and template melting temperatures), for 1 minute, and extension at 72 °C for a duration dictated by the size of the targeted DNA fragment. The initial denaturation step lasts for 5-10 minutes, followed by a series of 25-30 amplification cycles, and concludes with a final extension step lasting 10 minutes. PCR reaction system is: 45ul mix, 1/1 forward and reverse primers, template (ctCNB1\_2201) 1ul, ddH<sub>2</sub>O:2ul, total:50ul.

2201LBD-R142A F AACCTATATTGCCAGCGCGGACAAGGTCAGTCA

2201LBD-R142A R CGCGCTGGCAATATAGGTTTTGCGCACCTG

2201LBD-Y172A F CGGCCGCCAGGGCGCGCTGAAGCTGCT

2201LBD-Y172A R CGCGCCCTGGGCGGCCGGCACATAGCTGTT

## 2. Amplification of 2201LBD-Y172A and R142I DNA Sequences via PCR

For the amplification of the DNA sequences 2201LBD-Y172A and 2201LBD-R142A, the following protocol was employed: A DNA template was introduced into individual PCR tubes, along with either the 2201LBD-Y172F/R or 2201LBD-R142F/R primers [3]. This mixture was combined with the PCRr-mix. The reaction included an initial denaturation step at 98 °C for 5 minutes, followed by 30 cycles of denaturation at 98 °C for 20 seconds, annealing at 78 °C for 20 seconds, and extension at 72 °C for 15 seconds. A final extension was performed at 72 °C for 5 minutes.

Subsequently, the amplified PCR products underwent nucleic acid electrophoresis to determine their respective sizes. The outcomes of the experiments are visually represented in the provided figure 1. In the gel electrophoresis results, lane 1 corresponds to the 2201LBD-Y172A sample, while lane 2 corresponds to the 2201LBD-R142A sample. Notably, the analysis revealed that both lane 1 and lane 2 exhibited a band at 436 base pairs (bp), which concurs with the expected size of the target clones in the experimental design.

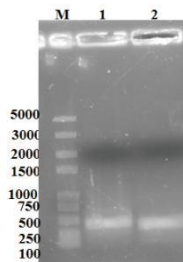


Figure 1: DNA Agarose Gel Electrophoresis for Size Analysis of 2201LBD-Y172A and 2201LBD-R142A Mutants.

## 3. Agarose Gel Electrophoresis for Nucleic Acids

Agarose gel electrophoresis stands as a widely employed method for the separation and identification of mixed DNA and RNA molecules [4]. This approach employs an agarose gel matrix, capitalizing on the electrostatic and sieving effects that guide the migration of DNA molecules, thereby achieving their separation within the mixture.

### 3.1 Fundamental Principles

DNA molecules bear a negative charge above their isoelectric point in a solution, prompting migration towards the anode in an electric field. The velocity of DNA molecule migration is influenced mainly by the molecular sieving effect, wherein molecular size and configuration serve as primary determining factors. The migration rate of DNA molecules is inversely proportional to their relative molecular weight.

### 3.2 Factors Influencing Electrophoretic Migration

Migration is influenced by the nucleic acid molecule's size (inversely proportional to the logarithm of relative molecular weight), gel concentration (inversely proportional to concentration, with 1% gel being common for DNA separation), DNA conformation (supercoiled DNA migrates fastest, linear

DNA next, and open loop DNA slowest under general conditions), voltage (typically 5V/cm, mobility directly proportional to voltage within a suitable range), and base composition (with minor impact, carried out within a temperature range of 4~30 °C, generally at room temperature; excessive current can lead to gel temperature rise and solution evaporation causing aberrant electrophoresis).

### 3.3 DNA Staining

Post-electrophoresis, the gel is stained in an aqueous solution of fluorescent dye ethidium bromide. The basic principle hinges on the fact that ethidium bromide-DNA complexes emit visible orange fluorescence upon ultraviolet light exposure. Rough estimation of DNA sample concentration is feasible based on fluorescence intensity.

**DNA Gel Recovery Procedure:** Dilute the concentrated SPW buffer with an appropriate volume of ethanol. Post-electrophoresis, meticulously excise the desired DNA fragments under a UV lamp, ensuring removal of excess DNA as much as possible. Place the targeted gel slice into a 1.5ml centrifuge tube, add the Binding Buffer in an equal volume to the gel, incubate the mixture at approximately 65 °C for 7 minutes until complete gel dissolution, gently mixing every 2-3 minutes. Load 700 µl of DNA agarose solution onto a HiBind™ DNA column, positioned within a clean 2ml collection tube, and centrifuge at 10,000 × g for 1 minute. Discard the liquid. Note: If the DNA agarose mixture volume exceeds 700 µl, transfer 700 µl first, followed by the remaining solution post-centrifugation. Repeat step four for at least three cycles. Wash the DNA with SPW buffer twice. Centrifuge at 13,000g for 3 minutes to eliminate residual ethanol from the column. Place the purification column in a new centrifuge tube and elute with 30-50 µl preheated Elution Buffer or ddH<sub>2</sub>O at 60 °C. Allow it to stand for 1-2 minutes. Centrifuge at 3,000g for 1 minute to obtain high-purity DNA in the resulting eluent.

Subsequent to amplification of the target DNA fragment and DNA recovery, a double enzyme digestion was conducted on the pET28a vector and the recovered DNA using EcoR1 and Xho1 enzymes at 37 °C for 2.5 hours. Following enzyme digestion, the pET28a vector underwent nucleic acid gel electrophoresis. Results, depicted in the figure2, demonstrated a clear and appropriately sized pET28a band post-enzyme digestion. Gel extraction was conducted to prepare for ensuing experiments.

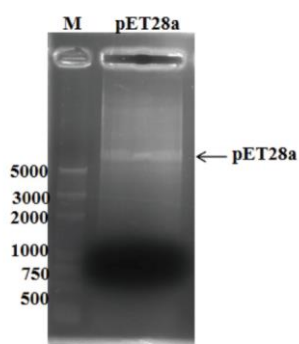


Figure 2: DNA Agarose Gel Electrophoresis of Restriction Enzyme Double Digestion of pET28a

### 4. Gene Fragment and Vector Ligation

**Method:** Utilization of T4 DNA Ligase [5]

**Reaction System:** The reaction system comprises DNA fragments, vectors (carriers), T4 DNA Ligase enzyme, and the appropriate reaction buffer.

**Reaction Temperature:** The ligation reaction is carried out at 25 °C for a duration of 1 hour.

The procedure entails the mixing of the previously digested pET28a vector with the digested DNA fragments in a molar ratio of 1:5. Subsequently, 1  $\mu\text{L}$  of T4 DNA Ligase and 1  $\mu\text{L}$  of T4 DNA Ligase buffer are added to the reaction mixture. The reaction is then conducted at a temperature of 25  $^{\circ}\text{C}$  for a duration of 1 hour within a PCR tube.

## 5. Plasmid Transformation

The ligation product, resulting from the connection between DNA and the vector, is introduced into TG1 host cells [6].

Method: Initially, TG1 host cells are chilled on ice for a duration of 5 minutes. Subsequently, the ligation products are added to the cells and co-incubated for 30 minutes. A heat shock step is executed at 42  $^{\circ}\text{C}$  for 90 seconds, followed by a return to ice for 5 minutes. The cells are then revitalized by the addition of 800  $\mu\text{L}$  of LB medium and cultured at 37  $^{\circ}\text{C}$  for 1 hour. Post-revitalization, the cells are plated onto solid LB agar.

Following overnight incubation, single colonies are cultivated on an LB agar plate containing the appropriate resistance marker. Validation through PCR is carried out on these colonies, using 2201LBD-Y172F/R or 2201LBD-R142F/R primers along with PCR-mix, mixed with the template extracted from each individual colony. Subsequent PCR amplification is performed, and the experimental outcomes are depicted in the subsequent figure3. Lane 1 corresponds to the 2201LBD-Y172A product, while lane 2 corresponds to the 2201LBD-R142A product. It is evident that both lanes 1 and 2 exhibit bands at 436 base pairs, aligning precisely with the anticipated size of the target clones. This confirms the successful connection of 2201LBD-Y172A and 2201LBD-R142A with the pET28a vector.

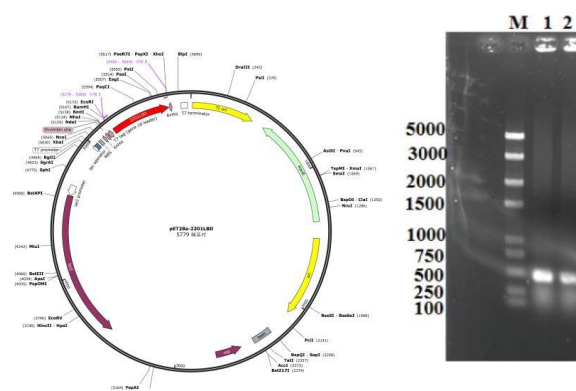


Figure 3: DNA agarose gel electrophoresis of 2201LBD-R142A/2201LBD-Y172A ligase with pET28a

## 6. Plasmid Extraction

Collect bacterial precipitates by centrifuging 1.5-5 ml of bacterial solution at room temperature for 1 minute. Carefully discard the supernatant and add 250  $\mu\text{L}$  of Solution I/RNase. Resuspend bacteria by vortexing or pipetting. Introduce 250  $\mu\text{L}$  of Solution II, inverting the mixture 4-6 times to achieve a clear lysate. Incorporate 125  $\mu\text{L}$  Buffer N3, gently mixing until white flocculent precipitates appear. Allow complete reaction at room temperature for 2-3 minutes. Centrifuge at 12,000  $\times g$  (room temperature or 4  $^{\circ}\text{C}$ ) for 10 minutes, gather supernatant, and transfer it to a new centrifuge tube. Add the same volume of ETR Binding Buffer as the supernatant, invert 7-10 times, and let it sit at room temperature for 5 minutes. Bind Plasmid - Place the binding column into a 2 ml collection tube, transfer 700  $\mu\text{L}$  of mixed solution to the column each time, and centrifuge at 8,000  $\times g$  for 1 minute.

Discard filtrate. Repeat step 7 until all supernatants have been filtered and discard the filtrate. Add 500  $\mu$ l ETR Wash Buffer to the column, centrifuge at  $8,000 \times g$  for 1 minute, discarding the filtrate. Introduce 500  $\mu$ l Buffer EHB to the column, centrifuge at  $8,000 \times g$  for 1 minute, and discard the filtrate. Add 700  $\mu$ l of DNA Wash Buffer to the column, centrifuge at  $8,000 \times g$  for 1 minute, and discard the filtrate. Note: Concentrated DNA Wash Buffer must be diluted with anhydrous ethanol as per label instructions before usage. We need to centrifuge at a maximum speed of  $13,000 \times g$  for 2 minutes to dry the column. We need to place the binding column in a new 1.5 ml centrifuge tube, add 30-50  $\mu$ l Endotoxin-Free Elution Buffer, incubate at room temperature for 2-5 minutes, and centrifuge at maximum speed for 1 minute to elute plasmid DNA.

The extracted DNA clone plasmid was transformed into the *Escherichia coli* BL21 host cells to initiate subsequent steps for protein expression and purification.

## 7. Protein Purification

The expressed protein was purified following the previously described method [7].

Procedure:

(1) Suspension and Lysis: Bacterial pellets obtained previously are resuspended in a 1:10 ratio using protein lysis buffer. The mixture is thoroughly shaken. (2) Ultrasonic Disruption: The resuspended bacterial suspension undergoes ultrasonic disruption for 4-5 cycles until achieving a clear and transparent solution. (3) Centrifugation: The disrupted bacterial lysate is subjected to high-speed, low-temperature centrifugation at  $4^\circ\text{C}$  and 12,000 rpm for 40 minutes. The resulting sediment is discarded, and the supernatant is collected. (4) Affinity Chromatography: The collected supernatant is carefully loaded onto a pre-equilibrated Ni<sup>2+</sup>-Bind affinity chromatography column. All of the supernatant is allowed to flow through the column. (5) Washing: The washing buffer is employed to wash the column, typically requiring 4-5 column volumes. During the final column volume wash, 5  $\mu$ l of the eluate is mixed with 100  $\mu$ l of Coomassie Brilliant Blue to verify the presence of any foreign proteins on the column surface. Note: A blue color change indicates the presence of foreign proteins, while no color change indicates the absence of foreign proteins. (6) Elution: The Elution Buffer is applied to elute the target Tce1 protein. Coomassie Brilliant Blue is used to monitor the elution process, ensuring complete elution of the target protein. (7) SDS-PAGE and Dialysis: The purified protein is analysed through SDS-PAGE to verify its purity and size accuracy. Upon confirmation of the correct band size, the purified protein is subjected to dialysis and divided for further utilization.

Experimental result:

As depicted in the Figure 4, the expected molecule weight of the purified protein is 17 kD. The experimental analysis yielded a protein with molecule weight of 17 kD, confirming the successful purification process.

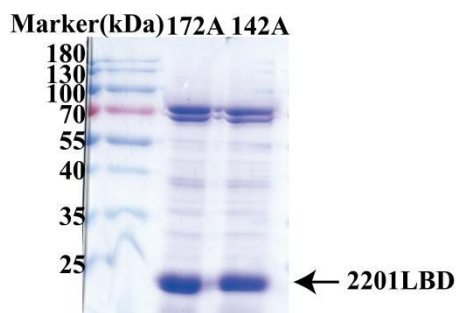


Figure 4: SDS-PAGE to determine the molecular weight of the mutated protein at position 2201BD.

## 8. Reagents and Instruments

DNA Gel Recovery Kit (Beijing TianGen Biotech Company), Plasmid Miniprep Kit (Beijing TianGen Biotech Company), T4 DNA Ligase (NEB), DNA Polymerase (Beijing TianGen Biotech Company), Restriction Endonucleases (Takara), DNA Marker (TransGen), Protein Marker (TransGen), Bacterial Genomic DNA Extraction Kit (Beijing TianGen Biotech Company), etc.

Agarose, Peptone, Agar, Yeast Extract, NaCl, Kanamycin (50 µg/mL), Tris, TBE Buffer (Tris Borate EDTA Buffer), Concentrated Hydrochloric Acid, Ethylenediaminetetraacetic Acid Sodium Salt (EDTA Na), Acrylamide, N,N,N',N'-Tetramethylethylenediamine (TEMED), Ammonium Persulfate (APS), Sodium Dodecyl Sulfate (SDS), Coomassie Brilliant Blue R-250, Coomassie Brilliant Blue G-250, Triton-X100, etc.

LB Medium: 5 g/L Yeast Extract, 10 g/L Peptone, 10 g/L Sodium Chloride.

1×PBS Buffer: 8 g/L NaCl, 0.2 g/L KCl, 1.42 g/L Na<sub>2</sub>HPO<sub>4</sub>, 0.27 g/L KH<sub>2</sub>PO<sub>4</sub>, pH 7.4.

5×SDS-PAGE Protein Electrophoresis Buffer: 15.1 g/L Tris, 94 g/L Glycine, 5.0 g/L SDS.

50×TAE Buffer: 242 g/L Tris, 37.2 g/L Na<sub>2</sub>EDTA 2H<sub>2</sub>O, 57.1 mL/L Acetic Acid, pH 8.5.

Protein Lysis Buffer: 50 mM Tris, 150 mM NaCl, pH 7.5.

Protein Purification Washing Buffer: 50 mM Tris, 150 mM NaCl, 30 mM Imidazole, pH 7.5.

Protein Purification Elution Buffer: 50 mM Tris, 150 mM NaCl, 300 mM Imidazole, pH 7.5.

Laboratory Instruments: TC-XP Gene Amplification PCR System (Xi'an ZhongTuan Biotech Company), Nucleic Acid Electrophoresis Apparatus, Protein Electrophoresis Apparatus and Related Equipment (Tanon), Water Bath, Incubator (Shanghai YiHeng Co., Ltd.), Constant Temperature Oscillating Incubator ZQZY-85BN (Shanghai ZhiChu Instrument Co., Ltd.), Gel Imaging System (Bio-Link Instruments, USA), pH Meter (Beijing 61 Instrument Factory), P70D20TL-D4 Galanz Microwave Oven (Guangdong Galanz Microwave Appliance Manufacturing Co., Ltd.), Protein Gel Scanner, Ultrasonic Cell Disruptor (Ningbo Xinzhi Biotech Corporation), High-Capacity High-Speed Freezing Centrifuge, High-Speed Freezing Centrifuge (Anhui Zhongke Zhongjia Scientific Instruments Co., Ltd.), High-Pressure Steam Sterilization Pot (Japanese Sanyo Corporation), Protein De-staining Shaker (Haimen Qilin Beier Instrument Manufacturing Co., Ltd.), Micropipette (Jinan Qiansi Biotech Co., Ltd.), Multi-Function Microplate Reader (Bio-Techne Instruments, USA), SW-CJ-1FD Ultra-Clean Workbench (Suzhou Purification Equipment Co., Ltd.), Haier Medical Low-Temperature Storage Refrigerator (Qingdao Haier Special Electrical Appliances Co., Ltd.), Electronic Precision Balance (Sartorius Scientific Instruments, Beijing), XMTD-8222 Constant Temperature Water Bath, Mcra Pulser Electroporation System (Bio-Rad Laboratories, USA)

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