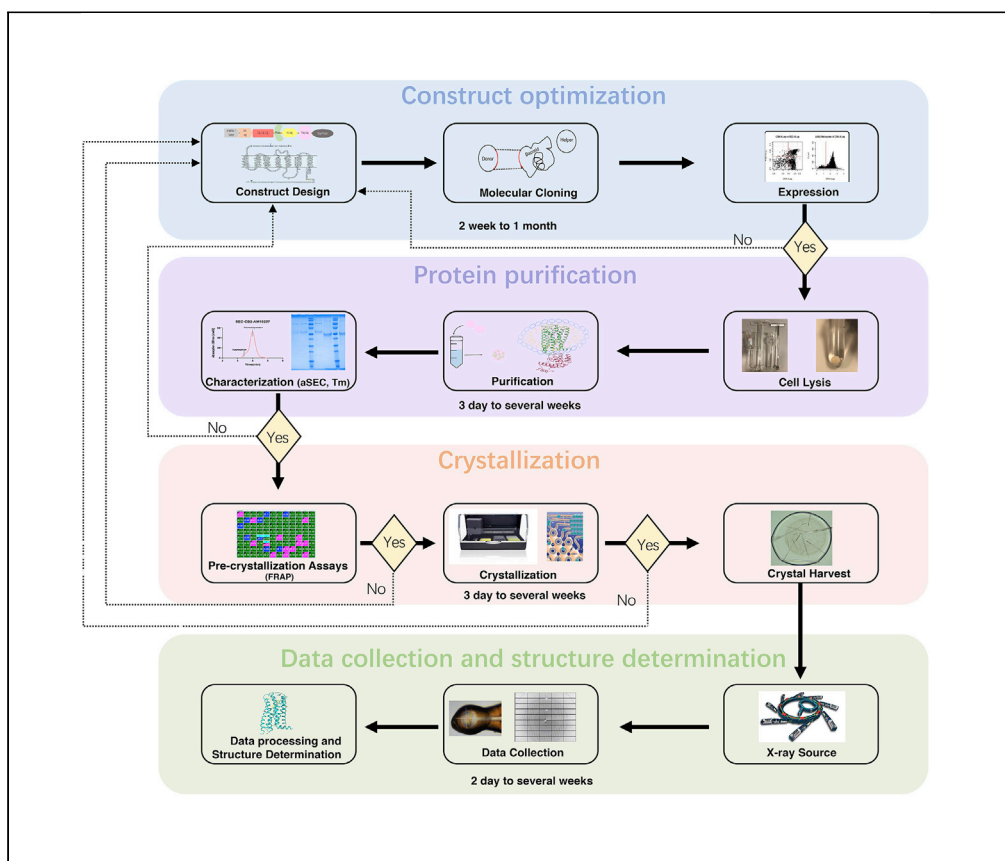


Protocol

Protocol for crystal structure determination of the antagonist-bound human cannabinoid receptor CB2



Human cannabinoid receptor CB2 plays an important role in the immune system and is an attractive therapeutic target for pain, inflammatory, and neurodegenerative diseases. However, the structural basis of CB2 agonist selectivity is still elusive. Here, we describe a detailed protocol for the determination of the crystal structure of antagonist AM10257-bound CB2. This methodology could be applied to the structural studies of CB2 with diverse antagonists and agonists or to other class A G-protein-coupled receptors.

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Highlights

Strategy to improve yield and stability of CB2 for crystallization in lipidic cubic phase

Optimized protocol to express and purify CB2 from insect cells

Crystallization and structural analysis of CB2 in the complex with antagonists

Protocol

Protocol for crystal structure determination of the antagonist-bound human cannabinoid receptor CB2

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SUMMARY

Human cannabinoid receptor CB2 plays an important role in the immune system and is an attractive therapeutic target for pain and for inflammatory and neurodegenerative diseases. However, the structural basis of CB2 agonist selectivity is still elusive. Here, we describe a detailed protocol for the determination of the crystal structure of antagonist AM10257-bound CB2. This methodology could be applied to the structural studies of CB2 with diverse antagonists and agonists or to other class A G-protein-coupled receptors.

For complete details on the use and execution of this protocol, please refer to Li et al. (2019).

BEFORE YOU BEGIN

This protocol contains complementary molecular biology, biochemical analysis and lipidic cubic phase (LCP) crystallization method, and a straightforward strategy for the crystal structure determination of CB2 in complex with different ligands (Li et al., 2019). The human cannabinoid receptor CB2 belongs to class A GPCRs, and it contains 360 amino acids with flexible N- and C- terminus and three extracellular and intracellular loops. The wild-type (WT) CB2 showed low surface expression and stability. In order to obtain a sufficient amount of stable CB2 protein for crystal structure determination experiments, about 400 expression constructs were designed and screened, which include purification tags, fusion proteins and its insertion sites, N-/C- terminal truncations as well as the thermostabilized mutations. The optimization process is mainly divided into three key steps, such as construct design and expression, purification and ligand screening, crystallization and diffraction characterization. The optimization of the whole processes is multidimensional and is iterated until the well-diffracting CB2 crystals are obtained for successful three-dimensional structure determination. Furthermore, each key step has its own criteria for the optimization process control. For example, the expression level and sample homogeneity are the quality control parameters for construct design and expression, sample's thermostability is used to judge the ligands suitability, the crystal diffraction quality is the guidance for crystallization and structure determination. It should be noted that the overall optimization strategy, result analysis and decision-making criteria are described in much more detailed fashion than that in the original publication (Li et al., 2019).

In this protocol, we first describe, in detail, the procedure for molecular cloning, expression, purification and characterization of the construct (CB2-T4L) for CB2 structure determination. Then we illustrate the methodology for analyzing the effects of antagonist AM10257 on CB2 protein's



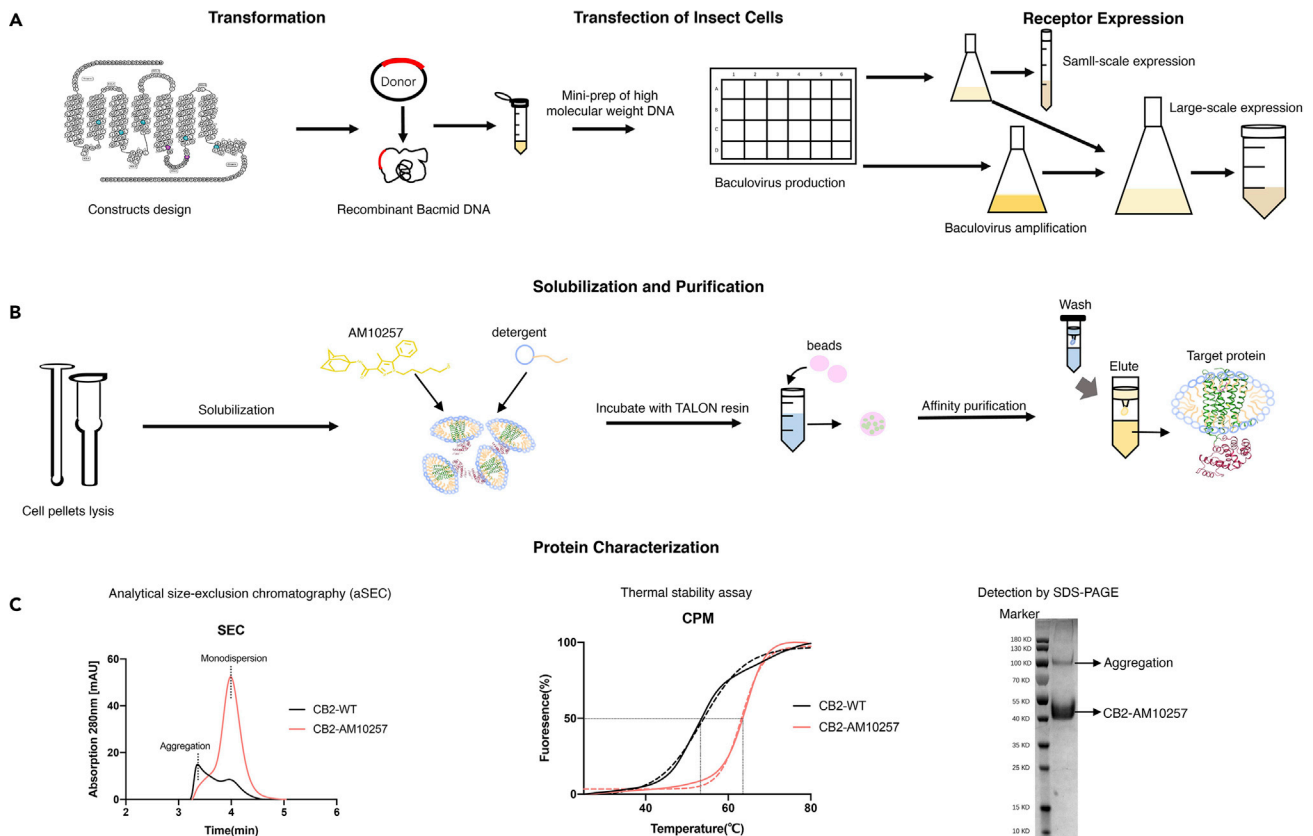


Figure 1. Diagram of the procedure for purification and verification of CB2-T4L with AM10257

(A) Construct design and preparation of expression in insect cells.

(B) Purification of the target protein by affinity chromatography.

(C) Characterization of the homogeneity, thermostability and purity of CB2.

homogeneity and thermostability. Finally, we demonstrate the crystallization process and structure determination methods.

Prepare the plasmids that encode CB2-T4L

⌚ Timing: 6–7 days (Figure 1A)

1. Obtain the sequence of human cannabinoid receptor 2 (CB2) (UniProt ID: P34972) and the bacteriophage T4 lysozyme (T4L) (UniProt ID: P00720) from the Uniprot database (<http://www.UniProt.org>). In addition, the codon-optimization for eukaryotic heterologous expression system is performed for CB2 gene and T4L gene, respectively (Genewiz).
2. Obtain the modified pFastBac 1 vector. The commercial pFastBac1 vector (Invitrogen) is modified with the hemagglutinin (HA) signal peptide (amino acid sequence: MKTIIALSIFYFLVFA) at the N terminus to enhance receptor expression and a PreScission protease site followed by a 10× histidine-tag (amino acid sequence: HHHHHHHHHH) as protein purification tag at the C terminus. In addition, a FLAG tag (amino acid sequence: DYKDDDK) is added following the 10× histidine-tag to improve the properties of CB2 protein.
3. Design the forward and reverse primers and amplify target gene by PCR. The main PCR strategies used in molecular cloning include: amplification of target gene fragments, site-directed mutations, and overlap extension PCR (Urban et al., 2007). Different from the traditional double

enzyme digestion method, the overlap extension PCR method used in this protocol significantly improves the success rate and efficiency of the molecular cloning experiments. The sequences of the primers are summarized in the [key resources table](#).

a. PCR reaction system for target gene fragment amplification.

Reagent	Final concentration	Amount
10× KOD buffer (TOYOBO™)	1×	5 μL
25 mM MgSO ₄	2 mM	4 μL
2 mM dNTPs	200 μM	5 μL
Primer forward (20 μM)	0.4 μM	1 μL
Primer reverse (20 μM)	0.4 μM	1 μL
Template DNA (200–300 ng/μL)	1 ng/μL	1 μL
KOD enzyme	1U	1 μL
ddH ₂ O	n/a	32 μL

b. PCR reaction program parameters to perform the thermocycler.

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min	
Denaturation	95	30 s	30 cycles
Annealing	56	1 min	
Extension	68	N min	
Final extension	68	20 min	
Hold	12	forever	

Note: “N” (1~2 min) depends on the efficiency of the enzyme and the size of the DNA fragment.

4. Run a 1% agarose gel to verify the fragment and purify the PCR product by using the E.Z.N.A.® Gel Extraction Kit (Omega) according to the manufacturer’s protocol (<http://www.omegabiotek.com.cn/template/productShow.aspx?m=129002&i=100000027416697>).

▮▮ **Pause point:** the amplified target gene fragment can be stored at 4°C for future use.

5. Fuse the target gene into the modified pFastBac1 vector by the overlap extension PCR method.
a. Overlap extension PCR reaction system

Reagent	Final concentration	Amount
5× Phusion HF buffer (NEB)	1×	4 μL
50 mM MgCl ₂	500 μM	0.2 μL
2 mM dNTPs	200 μM	2 μL
Gene DNA	200 ng	X μL
Vector DNA	200 ng	Y μL
Phusion HF enzyme	1 U	1 μL
ddH ₂ O	n/a	(12.8-X-Y) μL

Note: “X” and “Y” depend on the concentration of target gene and vector, usually 200 ng for each one.

b. Overlap extension PCR reaction program parameters to perform the thermocycler.

Steps	Temperature (°C)	Time	Note
Initial denaturation	98	5 min	
Denaturation	95	30 s	20~25 cycles
Annealing	58	1 min	
Extension	72	N min	
Final extension	72	10 min	
Hold	12	forever	

Note: "N" (4~6 min) depends on the efficiency of the enzyme and the size of the target gene and vector.

6. Introduce mutations into the target gene using the site-directed mutagenesis PCR.
 - a. Site-directed mutagenesis PCR reaction system

Reagent	Final concentration	Amount
5× Phusion HF buffer (NEB)	1×	5 µL
50 mM MgCl ₂	500 µM	0.2 µL
2 mM dNTPs	200 µM	2.5 µL
Primer forward (20 µM)	0.4 µM	0.5 µL
Primer reverse (20 µM)	0.4 µM	0.5 µL
Template DNA (200–300 ng/µl)	200 ng	0.5 µL
Phusion HF enzyme	1 U	0.5 µL
ddH ₂ O	n/a	40.3 µL

- b. Site-directed mutagenesis PCR reaction program parameters to perform the thermocycler.

Steps	Temperature (°C)	Time	Note
Initial denaturation	98	5 min	
Denaturation	95	30 s	20~25 cycles
Annealing	58	1 min	
Extension	72	N min	
Final extension	72	10 min	
Hold	12	forever	

7. Overlap extension PCR and site-directed mutagenesis PCR products need to be digested with Dpn I enzyme (NEB) at 37°C for 1 to 2 h, and then the digested products are transformed to DH5α competent cells (TIANGEN).
 - a. Thaw the competent cells on ice and pipette 50 µL to a pre-chilled 1.5 mL Axygen tube.
 - b. Add 10 µL digested PCR product into DH5α cells.
 - c. Keep the cells on ice for 10–20 min.
 - d. Heat shock at 42°C for 45 s.
 - e. Put the DH5α cells back on ice for 2 min.
 - f. Add 200 µL LB media to the tube.
 - g. Incubate the tube at 37°C in a Shaker with shaking at 225 rpm for 45–60 min.
 - h. Preparation agar plate containing 100 µg/mL ampicillin and incubate it to 37°C for 20 min.
 - i. Spread 30 µL of the cells and media on the ampicillin agar plate.
 - j. Incubate the plate at 37°C for 12–18 h.

△ CRITICAL: Avoid touching the bottom of the tube with DH5α competent cells because they are sensitive to heat. Do not mix the digested products with competent cells by pipetting up and down.

8. Pick a single clone for amplification in 5 mL LB media, and then incubate at 37°C with shaking at 225 rpm 12 h.
9. Extract the plasmids from the cells by a AxyPrep plasmid DNA mini kit (AXYGEN) using the manufacturer's protocol (<https://www.corning.com/catalog/cls/documents/protocols/RMI060002422.pdf>).
10. DNA sequencing. The verified plasmid is stored in the refrigerator at –20°C for the subsequent experiments.

△ **CRITICAL:** All plasmid DNA should be sequenced and verified that they are correct and within the open reading frame in the vectors. Additionally, make sure to add a stop codon after the target gene.

Generating the recombinant bacmid

⌚ **Timing:** 3 days

11. Follow the instructions below to prepare antibiotics and IPTG stock solutions for the recombinant bacmid generation experiment.

Antibiotics and IPTG stocks	Stock concentration	Solvent	Sterilization
IPTG	40 mg/mL	Milli-Q water	Filter sterilize
Ampicillin	100 mg/mL	Milli-Q water	Filter sterilize
Kanamycin	50 mg/mL	Milli-Q water	Filter sterilize
Gentamicin	7 mg/mL	Milli-Q water	Filter sterilize
Tetracycline	10 mg/mL	Ethanol	N/A
X-Gal	100 mg/mL	DMSO	N/A

12. Prepare the LB agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Blu-gal and 40 µg/mL IPTG. Weigh 20 g of LB agar (Sangon Biotech), dissolve it in 500 mL of single-distilled water, put it in a humid heat sterilization pot, and completely dissolve the LB agar. Wait for the agar to cool down to 55°C, then add 500 µL of kanamycin and gentamicin Tetracycline, IPTG and X-Gal stock prepared above, then pour 12 mL of LB agar into each 9 cm plate. The prepared plates can be stored at 4°C in dark place.
13. Transforming the purified plasmid DNA into DH10Bac competent cells.
 - a. Thaw the DH10Bac competent cells on ice and pipette 50 µl to a pre-chilled 1.5 mL AXYGEN tube.
 - b. Add 1 µL (~200 ng) extracted plasmid into DH10Bac cells.
 - c. Keep the cells on ice for 30 min.
 - d. Heat-shock the cells at 42°C for 45–90 s.
 - e. Immediately transfer the tubes back on ice for 2 min.
 - f. Add 200 µL LB media to the tube.
 - g. Incubate the tube at 37°C in a Shaker with shaking at 225 rpm for 3 h.
 - h. Spread 25 µL of the cells and media on a prepared LB agar plate from step 11.
 - i. Incubate the plate at 37°C for 36–48 h.

△ **CRITICAL:** DH10Bac competent cells are very sensitive to heat. Please pre-cool the tube on ice before transforming. Do not touch the bottom of competent cells during sample loading. The LB agar plates prepared in step 11 are sensitive to light. Please store the plates in dark place.

14. Use a sterile pipette tip to pick a single white clone (pFastBac™ Recombinant) from the agar plate and transfer it into 5 mL LB media containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline.
15. Incubate the culture at 37°C with shaking at 225 rpm 12 h.
16. Extract the recombinant bacmid DNA from the cells using a QIAGEN plasmid Maxi Kit (QIAGEN) according to the manufacturer's protocol (<https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiagen-plasmid-kits/?catno=12162>).
17. Analyze the recombinant bacmid DNA to verify successful transposition to the bacmid. PCR is used for verification according to the protocol from Bac-to Bac Baculovirus Expression System.

Expression of CB2-T4L using baculovirus expression system

Transfecting insect cells and isolating P0 viral stock

⌚ Timing: 2–3 days (Figure 1A)

18. Place the 24-deep well culture block and 96-well plate in the biosafety cabinet, and turn on UV light for 30 min.
19. Warm the transfection medium (Expression Systems™ ESF921) to 20°C~25°C.
20. Prepare the master mix:
 - a. Add 100 µL transfection medium into a sterile 1.5 mL Eppendorf tube.
 - b. In the same tube add 3 µL X-tremeGENE HP (Roche)
21. Add the following materials to each well of a sterile 96-well plate add the following:
 - a. Bacmid DNA sample: 5 µL (1 µg)
 - b. Master mix: 103 µL
22. Incubate the mixture at 20°C~25°C for 15 min.
23. Transfer 2.5 mL of Sf9 cells at 1×10^6 cells/mL into each well of a 24-deep well block (Thomson Instrument 931565-G, autoclaved)
24. Add the transfection mixture into each well of the 24-deep well block.
25. Cover with a BreathEasy (USA 9123-6100) plastic seal and incubate with shaking at 300 rpm for 96 h in Shel Lab incubator (Shellab).
26. After 96 h perform a GP-64 assay (Lv et al., 2016) for the P0 samples.
27. Remove cells by centrifugation at 2000 rpm for 15 min. Transfer the supernatant to a 15 mL tube and label it as the P0 virus and store at 4°C.

Preparing the P1 viral stock and 40 mL biomass production

⌚ Timing: 3 days

28. To 40 mL of cells at $2-3 \times 10^6$ cells/mL in 125 mL flask (Nest), add 400 µL of P0 virus.
29. Incubate the cells at 27°C with shaking 125 rpm for 48 h.
30. After 48 h perform a total and surface FITC expression assay (Lv et al., 2016).
31. Precipitate cells by centrifugation at 2000 rpm for 15 min in one of the 50 mL conical tubes (Corning).
32. Transfer the supernatant to another 50 mL conical tube labeled with P1.
33. Store the P1 virus at 4°C.
34. Store the 40 mL biomass pellet at –80°C for target protein purification.

Note: According to the results of the FITC expression assay, the surface expression level of different constructs can be judged.

Large-scale biomass (1L) production of CB2-T4L

⌚ Timing: 3 days

35. To the desired volume of cells at $2-3 \times 10^6$ cells/mL, add volume of virus that corresponds to desired MOI per the following equation: $(\text{Volume Cells} \times \text{Cell Count} \times \text{MOI})/\text{Titer} = \text{mL Virus Stock}$.
36. Incubate the cells at 27°C with shaking 125 rpm for 1 L (1.6 L Thoson flask).
37. After 48 h perform a Total and Surface FITC expression assay.
38. Precipitate cells by centrifugation at 2000 rpm for 20 min.
39. Store the biomass at -80°C for further use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HA Epitope Tag Antibody, Alexa Fluor® 488 conjugate (16B12)	Thermo Fisher Scientific Inc.	Cat# A-21287; RRID: AB_2535829
Bacterial and virus strains		
DH5a Competent Cells	TIANGEN	CB101-02
DH10Bac Competent Cells	Weidi Biotechnology	DL1071M
Chemicals, peptides, and recombinant proteins		
AM10257	This paper	N/A
Dimethyl sulfoxide	Sigma-Aldrich	Cat# D8418
EDTA-free complete protease inhibitor cocktail tablets	Roche	Cat# 5056489001
Iodoacetamide	Sigma	Cat# I1149
Lauryl Maltose Neopentyl Glycerol	Anatrace	Cat# 4216588
Cholesterol hemisuccinate (CHS)	Sigma	Cat# C6512
N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM)	Invitrogen	Cat# D10251
TALON IMAC resin	Clontech	Cat# 635507
1-Oleoyl-rac-glycerol (monoolein)	Sigma	Cat# M7765
Cholesterol	Sigma	Cat# C8667
X-tremeGENE™ HP	Roche	Cat# 06366236001
LB Broth Agar	Sangon Biotech	Cat# A507003
LB Broth	Sangon Biotech	Cat# A507002
Agarose	Abcone	Cat# A88490
Ampicillin	Solarbio	Cat# A8180
Kanamycin	Solarbio	Cat# K1030
Gentamycin sulfate	Solarbio	Cat# G8170
Tetracycline	Solarbio	Cat# T8180
X-GAL (5-Bromo-4-chloro-3-indolyl-β-galactopyranoside)	Biotium	Cat# 10011
IPTG	Solarbio	Cat# I8070
Transfection medium	Expression Systems	Cat# 95-020
Dpn I	NEB	Cat# R0176L
Imidazole	Sangon Biotech	Cat# A600277
4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich	Cat# H3375
Sodium chloride (NaCl)	Sigma-Aldrich	Cat# S3014
Potassium chloride (KCl)	Sigma-Aldrich	Cat# P9541
Magnesium chloride hexahydrate (MgCl ₂ ·6H ₂ O)	Sigma-Aldrich	Cat# M2670
2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris base)	Sigma-Aldrich	Cat# T1378

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Q5 Site-Directed Mutagenesis Kit	NEB	Cat# E0554S
KOD -Plus-	Toyobo	KOD-201
Phusion® High-Fidelity DNA Polymerase	NEB	Cat# M0530L
Deposited data		
CB2_AM10257 complex structure	This paper	PDB: 5ZTY
Experimental models: Cell lines		
<i>Spodoptera frugiperda</i> (Sf9)	A gift from Dr. Beili Wu (SIMM, CAS)	N/A
Oligonucleotides		
CB2_forward: GAAGACCATCAT CGCTCTGTCTACATC	This paper	N/A
CB2_reverse: TTACTIONATCGTC ATCGTCCTGTAGTCGT	This paper	N/A
N-terminal truncation_forward: TCGTGTTGCGCCGGCGCACC ACCTATGA	This paper	N/A
N-terminal truncation_reverse: TCATAGGTGGTGCGCCGGC GAACACGA	This paper	N/A
C-terminal truncation_forward: GAGGGGTCTGGGCGAATTC CTGGAGGTGCT	This paper	N/A
C-terminal truncation_reverse: AGCACCTCCAGGAATTCGC CCAGACCCCTC	This paper	N/A
ICL3-fusion(T4L) forward:AGCA TGTCGCGTCCAATATATTT GAAAT	This paper	N/A
ICL3-fusion(T4L) reverse:AGA CGCATGCGAGCATACGCG TCCCAAG	This paper	N/A
G78L_forward: TTGGCTCTGG CTGACTTC	This paper	N/A
G78L_reverse: GCTGCCAATGAAC AGGTATG	This paper	N/A
T127A_forward: GCTGGCCGCCATTGA	This paper	N/A
T127A_reverse: AGGAGGCTACCCACAGA	This paper	N/A
T153L_forward: GGTGCTCCTGGGCATC	This paper	N/A
T153L_reverse: AGTGCCCTCCACGG	This paper	N/A
R242E_forward: GATGTGGAG TTGGCCAAGAC	This paper	N/A
R242E_reverse: CAGCCTCATTGGGGCC	This paper	N/A
G304E_forward: GAGTGAAGAGATCCGCTCC	This paper	N/A
G304E_reverse: CGTAGAGCATAGA TGACAGGG	This paper	N/A
Software and algorithms		
XDS	Kabsch, 2010	Xds.mpimf-heidelberg.mpg.de
Scala	Collaborative Computational Project, 1994	www.ccp4.ac.uk/html/scala.html
Phaser	McCoy et al., 2007	www.phenix-online.org
Phenix	Adams et al., 2010	www.phenix-online.org
BUSTER	Smart et al., 2012	www.globalphasing.com/buster
Coot	Emsley et al., 2010	www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot
Prism v.7.0	GraphPad Software Inc.	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
100 kDa molecular weight cut-off Vivaspin 20 centrifuge concentrator	Sartorius	Cat# VS2042
100 kDa molecular weight cut-off Vivaspin 500 centrifuge concentrator	Sartorius	Cat# VS0142
PD Minitrap G-25 column	GE Healthcare	Cat# 28-9180-07
96-Well glass sandwich plates for LCP crystallization	NOVA	Cat# NOA90020
StockOptions™ Salt	Hampton	Cat# HR2-245
50 mL Conical tubes	Corning	Cat# 430290
125 mL Flask	Nest	Cat# 781011
Optimum Growth™ Flask Sterile 1.6 L	Thomson	Cat# 931113
Micro Bio-Spin Chromat Columns, pkg 100	Bio-Rad	Cat# 7326204
Poly-Prep Chromatography Columns, pkg 50	Bio-Rad	Cat# 7311550
Shaker	ZHICHENG Analytical Instrument Manufacturing Co., Ltd	N/A
SHEL LAB Incubators	SHEL LAB	N/A
0.22 µm Filter membrane	Sangon	Cat# F513134
Ultracentrifuge	Beckman Coulter	XPN-90
Ti45, Ti70 rotor	Beckman Coulter	N/A
Dounce tissue grinder complete 100 mL	Kimble	Cat#885300
15 mL Dounce tissue grinder	Wheaton	Cat#357544
2 mL Dounce tissue grinder	Wheaton	Cat#357422
Nanofilm SEC-250	Sepax Technologies	Cat#2012504625

MATERIALS AND EQUIPMENT

Hypotonic buffer		
Reagent	Final concentration	Amount
HEPES pH7.5 (1 M)	10 mM	10 mL
MgCl ₂ (1 M)	10 mM	10 mL
KCl (2 M)	20 mM	10 mL
Protease inhibitor (100×)	1×	10 mL
ddH ₂ O	N/A	960 mL
Total	N/A	1 L

Hypertonic buffer		
Reagent	Final concentration	Amount
HEPES pH7.5 (1 M)	10 mM	10 mL
MgCl ₂ (1 M)	10 mM	10 mL
KCl (2 M)	20 mM	10 mL
NaCl (5 M)	1 M	200 mL
Protease inhibitor (100×)	1×	10 mL
ddH ₂ O	N/A	760 mL
Total	N/A	1 L

Cell membrane cryopreservation solution

Reagent	Final concentration	Amount
HEPES pH7.5 (1 M)	10 mM	10 mL
MgCl ₂ (1 M)	10 mM	10 mL
KCl (2 M)	20 mM	10 mL
Glycerol (100%)	30%	300 mL
Protease inhibitor (100x)	1 x	10 mL
ddH ₂ O	N/A	660 mL
Total	N/A	1 L

2× Solubilization Buffer

Reagent	Final concentration	Amount
HEPES (1 M, pH7.5)	100 mM	5 mL
NaCl (5 M)	1 M	10 mL
LMNG/ CHS (6%/1.2%)	1.5%/0.3%	12.5 mL
ddH ₂ O	Up to 50 mL	22.5 mL

Wash buffer 1

Reagent	Final concentration	Amount
HEPES pH7.5 (1 M)	25 mM	1.25 mL
NaCl (5 M)	500 mM	5 mL
LMNG/CHS (6%/1.2%)	0.1%/0.02%	833 μL
Glycerol (100%)	10%	5 mL
Imidazole (2 M)	30 mM	750 μL
ddH ₂ O	N/A	37.17 mL
Total	N/A	50 mL

Wash buffer 2

Reagent	Final concentration	Amount
HEPES pH7.5 (1 M)	25 mM	1.25 mL
NaCl (5 M)	500 mM	5 mL
LMNG/CHS (6%/1.2%)	0.03%/0.006%	250 μL
Glycerol (100%)	10%	5 mL
Imidazole(2 M)	50 mM	1.25 mL
ddH ₂ O	N/A	37.25 mL
Total	N/A	50 mL

Elution buffer

Reagent	Final concentration	Amount
HEPES pH7.5 (1 M)	25 mM	1.25 mL
NaCl (5 M)	500 mM	5 mL
LMNG/CHS (6%/1.2%)	0.01%/0.002%	83.33 μL
Glycerol (100%)	10%	5 mL
Imidazole (2 M)	250 mM	6.25 mL
ddH ₂ O	N/A	32.42 mL
Total	N/A	50 mL

△ **CRITICAL:** All stocks need to be filtered by 0.22 µm filter membranes, and stored at 4°C for further use in 6 months.

STEP-BY-STEP METHOD DETAILS

Purification of CB2-T4L protein

GPCR as a membrane protein being purified, the whole process mainly includes cell pellets lysis, membrane washing, membrane solubilization, protein purification and property analysis (uniformity, thermal stability and purity).

Cell pellet lysis and membrane washing

⌚ **Timing:** 1 day

1. Preparation of cell membrane containing the CB2-T4L protein. Due to the relative instability of GPCRs, conventional cell disruption methods, such as ultrasonic disruption or high-pressure cell disruption, are not applicable. Thus, manual homogenization disruption method is used (Figure 1B).
 - a. 40 mL Biomass membrane washing steps
 - i. Thaw the cell pellets on ice.
 - ii. Lyse the cells and suspend the cell pellet evenly with 20 mL of hypotonic buffer containing protease inhibitor (EDTA-Free, DMSO, BIMAKE).
 - iii. Transfer the cell suspension to a 15 mL homogenizer, place it on ice and grind it up and down 40–50 times, pour the solution into a 26 mL ultracentrifuge tube (Beckman), wash the homogenizer with 5 mL hypotonic buffer and transfer the liquid to the same centrifuge Tube.
 - iv. After the tubes are balanced, place them in a Beckman Optima XPN-90 ultracentrifuge with a Ti70 rotor and centrifuge at 40000 rpm for 30 min.
 - v. After centrifugation, discard the supernatant, resuspend the cell membrane pellet with 20 mL hypertonic buffer, transfer to the same homogenizer, place it on ice and grind for 40–50 times, put it into a centrifuge tube, and wash with 5 mL hypertonic buffer.
 - vi. After centrifugation, discard the supernatant, resuspend the pellet with 2 mL cell membrane cryopreservation solution, grind it with a 2 mL homogenizer, and suspend the cell membrane evenly in the cryopreservation solution.
 - vii. Divide the cell membrane suspension evenly into two 2 mL Eppendorf tubes, quickly freeze with liquid nitrogen, and store at –80°C for subsequent purification experiments.
 - b. 1 L Biomass membrane washing steps
 - i. Take out the cell pellet frozen at –80°C and thaw it on ice.
 - ii. Cells are lysed with 100 mL of hypotonic buffer containing protease inhibitor (Roche).
 - iii. Transfer the cell suspension to a 100 mL homogenizer, place it on ice and grind it up and down 40–50 times, pour the solution into two 70 mL ultracentrifuge tubes, wash the homogenizer with 30 mL hypotonic buffer and transfer it to centrifuge tube.
 - iv. After the tubes are balanced, they are placed in a Beckman Optima XPN-90 ultracentrifuge with a Ti45 rotor and centrifuged at 45000 rpm for 35 min.
 - v. After centrifugation, discard the supernatant and repeat the above steps ii–iv.
 - vi. The supernatant is discarded in the end of centrifugation, hypertonic buffer with 100 mL membrane resuspended precipitate is transferred to the same homogenizer, and placed on ice after the same 40–50 triturated into a centrifuge tube, using a hypertonic buffer wash 30 mL Homogenizer.
 - vii. After the tubes are balanced, place them in a Beckman Optima XPN-90 ultracentrifuge using a Ti45 rotor, and centrifuge at 45000 rpm for 35 min.
 - viii. After centrifugation, discard the supernatant and repeat the above steps twice vi–vii.

- ix. The supernatant is discarded after centrifugation, the washed membrane is resuspended using 40 mL homogenizer milled uniformly suspended in cryopreservation solution membrane.
- x. The membrane suspension is evenly split two 50 mL tubes, frozen in liquid nitrogen and placed in -80°C for subsequent purification experiments.

▮▮ **Pause point:** In this step, put the membrane in the -80°C refrigerator and it can be paused.

Protein membrane solubilization

⌚ **Timing:** 1 day

2. The amphiphilic detergents are used to extract the CB2-T4L protein from the native membrane lipids (Figure 1B).
 - a. A small-scale biomass (40 mL) membrane solubilization steps
 - i. Take out the EP tube containing 1 mL of cell membrane suspension from the -80°C refrigerator and thaw it on ice.
 - ii. Add 1× protease inhibitor, 2 mg/mL iodoacetamide and 25 μM AM10257 (CB2 antagonist), incubate at 4°C for 1 h.
 - iii. Add 1 mL 2× Solubilization Buffer and incubate at 4°C for 1–2 h (The solubilization time can be optimized for different detergents and receptors).

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	100 mM	5 mL
NaCl (5 M)	1 M	10 mL
LMNG/CHS (6%, 1.2%)	1.5%, 0.3%	12.5 mL
ddH ₂ O	Up to 50 mL	22.5 mL

- iv. Centrifuge at 14000 rpm for 30 min after dissolving the membrane.
 - v. Transfer the supernatant into a 2 mL EP tube containing 50 μL TALON resin and 10 mM imidazole, and binding 12–16 h at 4°C (or ≥ 4 h).
 - b. A large-scale biomass (1 L) membrane solubilization steps
 - i. Take out the tube containing 25 mL of cell membrane suspension from the -80°C and thaw it on ice.
 - ii. Add 25 μM AM10257 and protease inhibitor (Roche), and binding at 4°C for 3 h.
 - iii. Add 2 mg/mL iodoacetamide and incubate at 4°C for 1 h.
 - iv. Add 25 mL of 2× Solubilization Buffer and incubate at 4°C for 2.5 h (The solubilization time can be optimized for different detergents and receptors).

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	100 mM	5 mL
NaCl (5 M)	1 M	10 mL
LMNG/CHS (6%, 1.2%)	1.5%, 0.3%	25 mL
ddH ₂ O	Up to 50 mL	10 mL

- v. Ultracentrifugation at 40,000 rpm for 30 min after dissolving the membrane.
 - vi. Transfer the supernatant into a 50 mL tube containing 0.6 mL TALON resin and 10 mM imidazole and binding 12–16 h at 4°C .

Purification of CB2-T4L protein

⌚ Timing: 1 day

3. Purification, using 10×His tag of the target protein for immobilized metal affinity chromatography (IMAC) to separate and purify the target protein (Figure 1B).
 - a. A small-scale (40 mL biomass) purification
 - i. Centrifuge the binding solution incubated 12–16 h at 4°C, at 800 g for 5 min. Discard 1 mL of supernatant, transfer TALON resin (Clontech) to the rinsed small purification column (Micro Bio-Spin Chromat Columns, pkg 100).
 - ii. Wash the protein with 20 column volume (CV) of Wash buffer (Wash buffer formula: 25 mM HEPES pH7.5, 500 mM NaCl, 10% Glycerol, 0.03% LMNG, 30 mM imidazole).

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	25 µL
NaCl (5 M)	500 mM	100 µL
LMNG/CHS (6%, 1.2%)	0.03%, 0.006%	5 µL
Glycerol (50%)	10%	200 µL
Imidazole (2 M, pH7.5)	30 mM	15 µL
AM10257(25 mM)	20 µM	0.8 µL
ddH ₂ O	Up to 1 mL	654.2 µL

- iii. Use 4 CV Elute buffer (Elute buffer formula: 25 mM HEPES pH7.5, 500 mM NaCl, 10% Glycerol, 0.03% LMNG, 250 mM imidazole) to elute the target protein.

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	5 µL
NaCl (5 M)	500 mM	20 µL
LMNG/CHS (6%, 1.2%)	0.03%, 0.006%	1 µL
Glycerol (50%)	10%	40 µL
Imidazole (2M, pH7.5)	250 mM	25 µL
AM10257(25 mM)	25 µM	0.2 µL
ddH ₂ O	Up to 200 µL	108.8 µL

- iv. Protein properties analysis.
 - b. A large-scale (1 L biomass) purification
 - i. Centrifuge the binding solution incubated 12–16 h at 4°C, at 800 g for 5 min. Discard 35 mL of the supernatant, transfer the TALON resin to the rinsed large purification column (Poly-Prep Chromatography Columns, pkg 50).
 - ii. Wash the protein with 15 CV of Wash buffer 1.

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	750 µL
NaCl (5 M)	500 mM	300 µL
LMNG/CHS (6%, 1.2%)	0.1%, 0.02%	50 µL
Glycerol (50%)	10%	600 µL
Imidazole (2M, pH7.5)	30 mM	45 µL
AM10257 (25 mM)	20 µM	2.4 µL
ddH ₂ O	Up to 3 mL	1252.6 µL

iii. Wash the protein with 15 CV of Wash buffer 2.

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	750 μ L
NaCl (5 M)	500 mM	300 μ L
LMNG/CHS (6%, 1.2%)	0.03%, 0.006%	15 μ L
Glycerol (50%)	10%	600 μ L
Imidazole (2M, pH7.5)	50 mM	75 μ L
AM10257(25 mM)	20 μ M	2.4 μ L
ddH ₂ O	Up to 3 mL	1257.6 μ L

iv. Elute the target protein with 3 CV Elute buffer.

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	15 μ L
NaCl (5 M)	500 mM	60 μ L
LMNG/CHS (6%, 1.2%)	0.01%, 0.002%	1 μ L
Glycerol (50%)	10%	120 μ L
Imidazole (2M, pH7.5)	250 mM	75 μ L
AM10257(25 mM)	25 μ M	0.6 μ L
ddH ₂ O	Up to 600 μ L	328.4 μ L

- v. The purified receptor is then concentrated to 18–25 mg/mL using a 100 kDa cut-off concentrator (Sartorius). The concentrated protein is transferred to a 1.5 mL Eppendorf tube, centrifuged at 14000 rpm for 30 min and used for crystallization experiments.
- vi. Save a small amount of protein samples for protein property analysis.

Protein property analysis

⌚ Timing: 1 day (Figure 1C)

Note: Protein property analysis, includes the purity, yield, uniformity and thermal stability of the protein sample. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot, analytical gel filtration chromatography and fluorescence thermal stability experiments are used.

4. Polyacrylamide gel electrophoresis, referred to as PAGE, is a common electrophoresis technique that uses polyacrylamide gel as a support medium. GenScripts ExpressPlus™ PAGE precast gels are used in this protocol.
5. Western blotting experiment. The FLAG tag and His tag at the C-terminus of CB2 are used for detection by using the anti-FLAG antibody and anti-His antibody, respectively.
6. Analytical size-exclusion chromatography (aSEC)
 - a. Use the ultrasonic degassed ddH₂O to flush the column at a flow rate of 0.2 mL/min for 1 h.
 - b. Use BSA buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 2% Glycerol, 0.03% LMNG, 0.01% CHS, 0.25 mg/mL BSA) to coat the column at a flow rate of 0.2 mL/min for \geq 4 h.

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	25 mL
NaCl (5 M)	150 mM	30 mL
LMNG/ CHS (6%, 1.2%)	0.03%, 0.006%	5 mL
Glycerol (50%)	2%	40 mL
BSA(10 mg/mL)	0.25 mg/mL	25 mL
ddH ₂ O	N/A	Up to 1 L

- c. Use HPLC buffer (25 mM HEPES pH 7.5, 500 mM NaCl, 2% Glycerol, 0.03% LMNG, 0.01% CHS) to equilibrate the column and the system (Agilent Technologies 1260 Infinity) at a flow rate of 0.5 mL/min for 2–3 h.

Reagent stock	Final concentration	Amount
HEPES (1 M, pH7.5)	25 mM	25 mL
NaCl (5 M)	500 mM	100 mL
LMNG/CHS (6%, 1.2%)	0.03%, 0.006%	5 mL
Glycerol (50%)	2%	40 mL
ddH ₂ O	N/A	Up to 1 L

- d. The protein (CB2-T4L) sample is centrifuged at 14000 rpm for 10 min and loaded into the HPLC for analysis. The peak position of GPCR monomer is about 4 min, and the oligomerization peak is about 3.3 min. The detection time of a sample is usually 11–15 min.

Note: According to the results of aSEC, the protein yield and homogeneity of different constructs can be judged.

7. Fluorescence thermal stability experiment

Note: Fluorescence thermal stability experiment uses chemical dye CPM (N-[4-(7-diethylamino-4-methyl-3-coumarin) phenyl] maleimide) for membrane protein. The principle and general procedure have been reported previously (Alexandrov et al., 2008).

- CPM dye powder (Invitrogen™) is dissolved in DMSO at the concentration of 4 mg/mL, and is stored at –80°C in the dark.
- Dilute 1:20 with elute buffer without imidazole before using the dye.
- 0.5 μL dye diluent and about 1 μg protein (CB2-T4L) solution are mixed in a final reaction volume of 50 μL in the Eppendorf tube, and incubated for 15 min in the dark at 20°C.
- The Eppendorf tube is placed in a real-time PCR instrument (QIAGEN Rotor-Gene Q), and the parameters are set (excitation light wavelength is 387 nm, emission light wavelength is 463 nm, and the temperature range for detecting fluorescence is usually 20°C~90°C).
- Use GraphPad Prism software for data analysis. According to the fluorescence thermal stability experiment, the constructs with relatively higher T_m values are selected for the following crystallization trials.

Note: The protein constructs or ligands showed higher T_m values were selected for the further crystallization trials. Based on the past experience, if the T_m value is higher than 60°C, it will have more chance to get the crystal hits (Popov et al., 2018).

Protein crystallization and crystal optimization

© Timing: 1 day

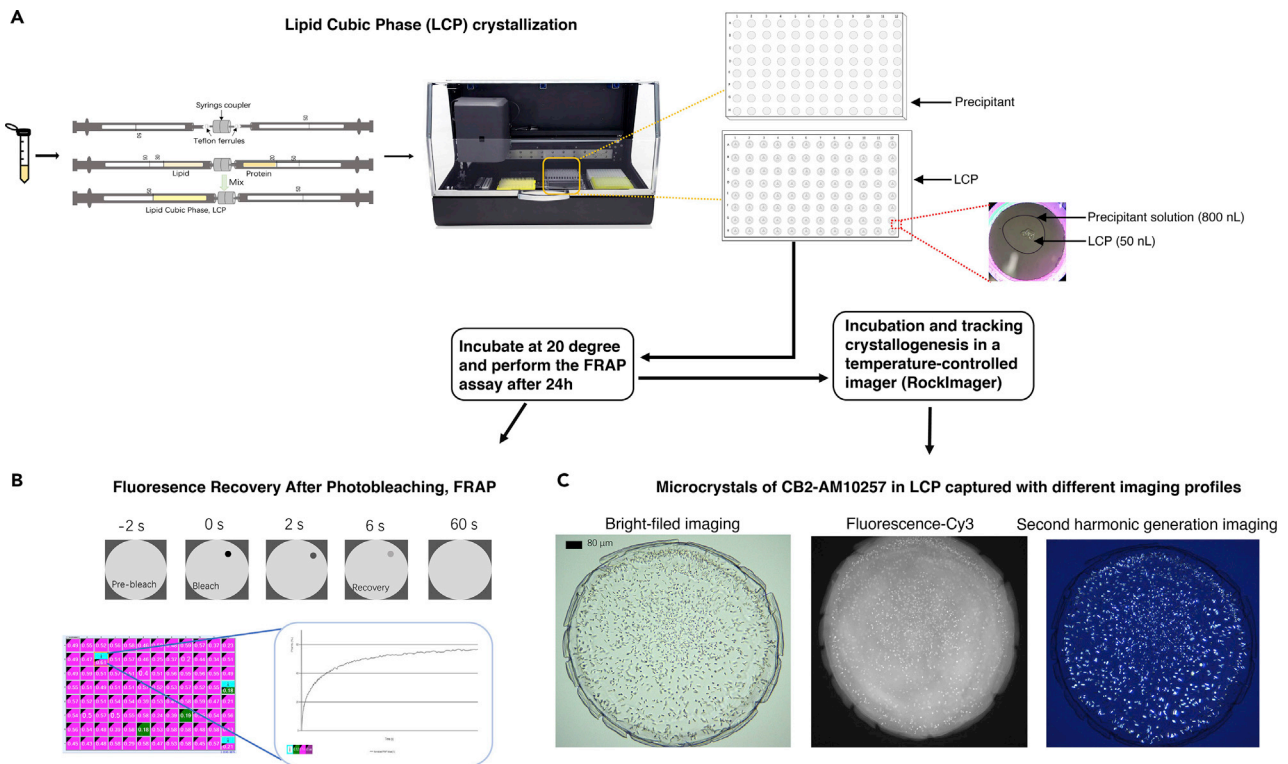


Figure 2. Crystallization experiment for CB2-AM10257

(A) The procedure for crystallization of CB2-AM10257 by LCP method.
 (B) The FRAP assay to test the diffusion activity of CB2-AM10257 in LCP.
 (C) Validation of crystals by visible light, fluoresce and SONICC.

Note: Most of the well diffracting crystals of GPCRs are successfully obtained using the lipid cubic phase (LCP) method. The principle is as described in the literature (Caffrey and Cherezov, 2009).

8. Preparation of Lipid-Cholesterol mixture. Because the grease is hygroscopic, take it out from -20°C and make it to 20°C before use (Figure 2).
 - a. Weigh monoolein: cholesterol in a 4 mL brown bottle (Agilent) according to the mass ratio of 9:1.
 - b. Add an appropriate amount of chloroform dropwise until the powder is completely dissolved and mixed.
 - c. Use nitrogen flow to volatilize chloroform until the solution becomes transparent and viscous.
 - d. Seal with aluminum foil, leave a vent with a syringe needle, and place it in a vacuum container (WELCH-ILMVAC).
 - e. Keep the container under vacuum for about 24 h to make the chloroform completely evaporate.
 - f. Take out the sample, tighten the cap and seal it with Parafilm, and store it at -20°C for future use.
9. Before sample preparation, two 100 μL Hamilton Syringes need to be washed with methanol and dried, the Lipid-Cholesterol mixture is taken out from -20°C , and the metal bath is heated at 42°C for dissolving the grease.
 - a. The protein sample (CB2-T4L) used for crystallization is centrifuged at 14,000 rpm, and put into one of the 100 μL Syringe with a relatively thin pipette tip (Avoid bubbles when transferring), and determine the protein solution weight.

- b. According to the weight ratio of 2 (Protein): 3 (Lipid), transfer the prepared lipid to another 100 μ L Syringe preheated at 42°C.
 - c. Use the coupler to connect two Syringes, put them at 20°C and then mix them to form a transparent LCP phase (Heat will be generated during the mixing process, so the pushing speed should not be too fast, just 1 s for 1 round of back and forth).
10. Crystallization experiment. The crystallization robot, Formulatrix NT8, is used for the high-throughput crystallization screening, which includes protein constructs, ligands, precipitants, and buffers.
- a. Follow the instructions of crystallization robot to perform the experiment.

Note: According to the past experience, the crystallization conditions for preliminary screening mainly include pH (5.0, 6.0, 7.0, 8.0), Hampton Salts (100 mM/400 mM 49 unique salts), PEG400 concentration (25%–40%) and commercial membrane protein crystallization kits.

- b. The initial small crystals were obtained in the condition of pH6.0, 30% PEG400 and 400 mM lithium sulfate monohydrate. The strategy for crystal optimization includes pH optimization with 0.2 intervals around pH6.0, adjusting the salt concentration and/or the PEG400 concentration.
 - c. Of note, when crystallizing CB2-AM12057 sample, there exist two different crystal forms in the same crystallization condition, which are in the bulk and long-rod shape, respectively. The ratio of those two crystal forms varies by changing the concentrations of PEG400, where the bulk crystals appear more in the lower PEG400 concentration while the long-rod crystals grow more in the higher concentration of PEG400. The diffraction resolution of bulk crystals is about 4 Å, while that of long rod crystals is about 3.0 Å.
 - d. Optimized crystals of CB2-AM12057 were obtained from precipitant conditions containing 100 mM sodium cacodylate trihydrate pH 6.2, 40% PEG 400, 400 mM lithium sulfate monohydrate, and reached a full size of 200 μ m after 2 weeks. Crystals were harvested from the LCP matrix using MiTeGen micromounts and immediately flash frozen in liquid nitrogen.
11. Fluorescence Recovery After Photobleaching, FRAP.

Note: The main principle is described in the previous literature (Cherezov et al., 2004). The LCP-FRAP experiments have been widely used in protein crystal screening for different optimized constructs and crystallization condition optimization (Cherezov et al., 2008). The key points of the good-diffracting crystal optimization are summarized as following:

- a. At first, the constructs with N-terminal fusion show good homogeneity and thermal stability, however, no crystals were obtained after extensive crystallization trials. This may be due to the flexibility of the N-terminal fusion. Then, the optimization of protein constructs was focused on the fusion proteins at ICL3.
- b. The aSEC and CPM results showed that inserting T4L (T4 Lysozyme) or Fla (Flavodoxin) fusion protein into ICL3 can improve the protein properties compared with other fusion proteins. Then, the FRAP assay was used to test the diffusion of CB2 constructs with T4L or Fla in the lipid cubic phase. The construct with T4L fusion protein shows better diffusion properties compared with that of Fla. Usually, the protein construct with good diffusion activity will facilitate the nucleation and crystal growth.
- c. Extensive optimization including crystallization conditions and junction sites of T4L were performed. However, the best diffraction resolution of the crystals is around 10 Å. In order to improve the resolution, a series of N-terminal truncation construct was further optimized and used for crystallization trials. The crystallization results showed that the N-terminal truncation played very important role in improving the diffraction of the crystals.
- d. Finally, the construct CB2-T4L describe in this protocol, which has T4L insertion at residues Ser222-Ala235, five mutations (Gly78^{2,48}Leu, Thr127^{3,46}Ala, Thr153^{3,45}Leu, Arg242^{6,32}Glu,

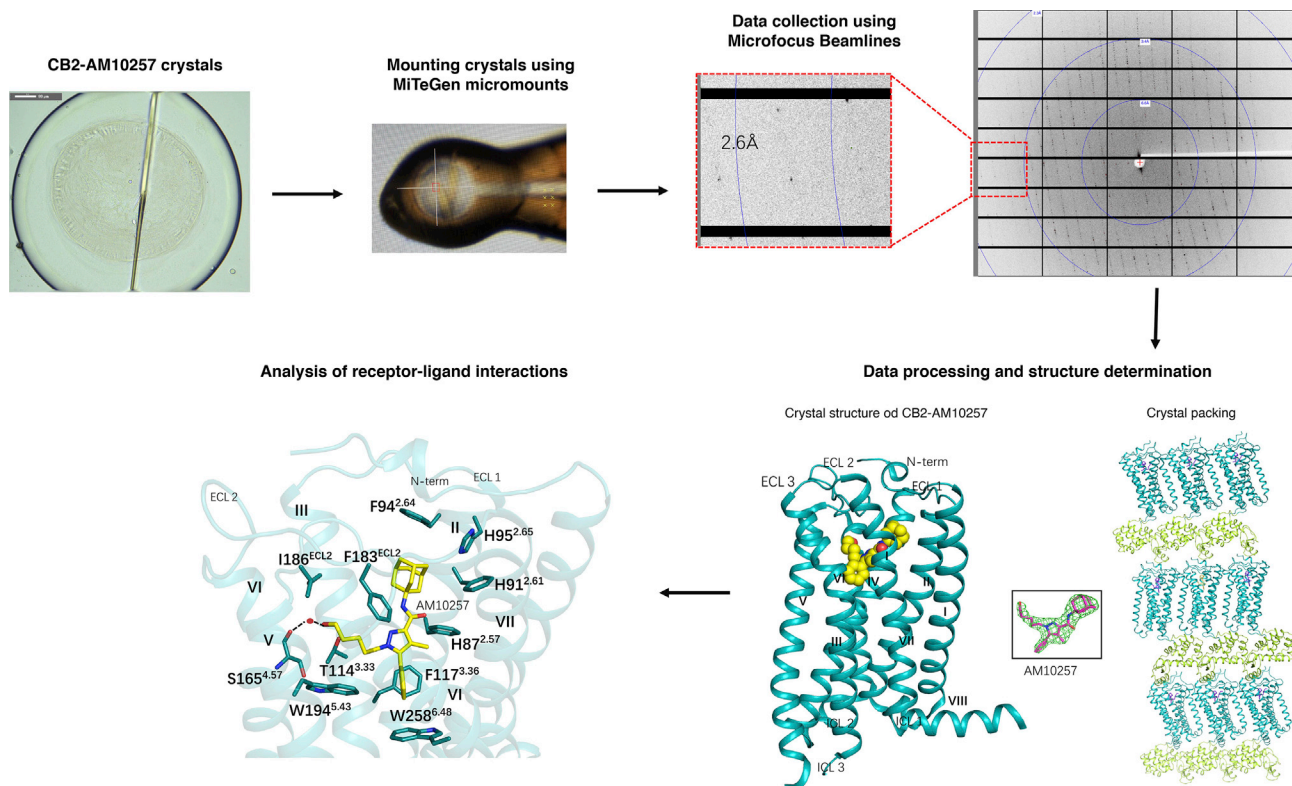


Figure 3. Elucidation of the 3D structure of CB2 and the binding mode of AM10257

Gly304^{8,48}Glu), N-terminal truncation of residues 1–20, as well as C-terminal truncation of residues 326–360, could significantly improve the crystal quality and used for the final structure determination of CB2 in complex with antagonist AM10257.

Crystal diffraction data collection, processing, and structure determination

© Timing: 7 days

12. GPCR crystals are relatively small, they are more suitable for Microfocus Beamlines for crystal diffraction data collection (Figure 3).
13. The frozen crystals in this project were mainly collected at the BL41XU beamline in the Spring-8 synchrotron radiation source in Japan.
14. The beamline station is equipped with an Eiger fast reading detector for collecting diffraction images, which greatly improved the data collection efficiency.
15. LCP coatings during the crystal fishing process, the diffraction scanning system (raster scan) is often used for automatic identification of crystal locations and diffraction quality screening.
16. Collecting a large amount of crystal diffraction data, XDS software was used for data processing.
17. The software CCP4, Phenix and Coot are generally used for structure determination and refinement (Emsley et al., 2010).

EXPECTED OUTCOMES

After the extensive constructs design process, expression and purification optimization of CB2, the samples with high yield, good uniformity and thermal stability are obtained. This optimized methodology should be suitable for many other membrane proteins, especially GPCRs. In our study, 1 L cell culture expressed in sf9 cells yields ~1 mg of CB2-T4L protein. The antagonist AM10257

can increase the homogeneity and thermostability of CB2 protein, indicating that the ligands are capable of stabilizing GPCRs to facilitate the structure determination. Finally, it is difficult to obtain well-diffracting GPCR crystals. The crystallization condition optimization strategy for CB2-T4L in this study may be useful for other GPCRs.

LIMITATIONS

Although the purification steps and biochemical methods we describe in this article may be applied to other GPCRs, however, the structure determination is a complicated try-and-error process and there is no unified protocol which works for every protein. Each protein has its own characteristics and we have to find the optimal conditions, such as construct design, cell lines, expression systems, ligands, detergents and lipids for individual proteins.

TROUBLESHOOTING

Problem 1

The cells are not expressing the receptor (steps 18–34)

Potential solution

Check if the cells are successfully infected. Titer the P0 virus as the infectivity could become reduced over time for some receptors. Reinfect with a MOI of 5. If the virus titer is too low, redo the transfection.

Problem 2

The cell survival is low (steps 30 and 37)

Potential solution

The basal signaling activity of some overexpressed receptor is toxic to the insect cells, which may lead to cell death during overexpression. Reduce the expression time or add a receptor ligand during the expression that could negatively modulate the receptor activity (such as antagonists or inverse agonists), which may be beneficial for cell survival. Optimize the expression constructs, such as introducing the inactive mutations, to reduce the basal activity of the receptor.

Problem 3

The purification yield is low with or without high aggregation (steps 3–7 in “Purification of CB2-T4L Protein”)

Potential solution

The purification conditions, including the detergent, buffer pH, the salt concentration, the incubation time and the ligands are not suitable for the receptor of interest. Test many different conditions on a new receptor construct and use the optimized conditions for the purification procedure. Screen different ligands of the receptor to choose the one that can stabilize the receptor. Add the appropriate ligand during cell culture, stabilization and purification buffers could help stabilize the receptor and increase the protein yield.

Problem 4

The LCP phase is not transparent (step 9 in “Protein crystallization and crystal optimization”)

Potential solution

The concentration of free detergent in the protein sample may be quite high. Try to reduce the concentration of detergent in the elution buffer or reduce the concentration times of the protein before crystallization.

Problem 5

No crystals or crystals are small (step 10 in “Protein crystallization and crystal optimization”)

Potential solution

Make sure the protein is relatively pure, conformationally homogeneous, and the protein concentration should be controlled within 20–30 mg/mL for crystallization experiment. The quality of the purified protein from each batch should be consistent, and pay attention to the cell viability and expression data for each batch of cell pellets.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tian Hua (huation@shanghaitech.edu.cn).

Materials availability

All reagents generated in this study will be made available on reasonable request.

Data and code availability

Coordinates and structure factors have been deposited in the Protein Data Bank with the PDB code 5ZTY.

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AUTHOR CONTRIBUTIONS

All authors contributed to writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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