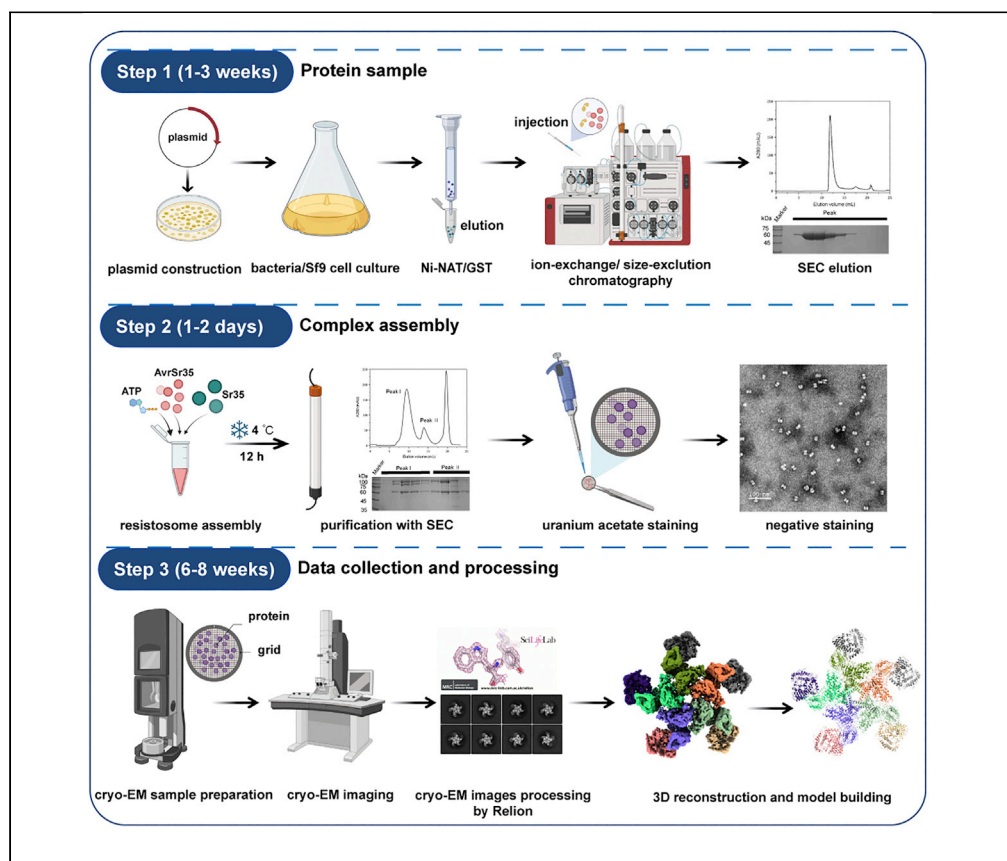


Protocol

Assembly and purification of AvrSr35-induced Sr35 resistosome and determination of its structure by cryo-EM



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Highlights

Expression and purification of recombinant plant NLR Sr35 proteins in mg quantities

Protocol for assembly of stable AvrSr35 induced Sr35 resistosome *in vitro*

Sr35 resistosome subjected to cryo-EM analysis produces high resolution

Sr35, a coiled-coil nucleotide-binding leucine-rich repeat receptor (CC-NLR) from the wheat species *Triticum monococcum* can directly recognize the pathogen avirulence factor AvrSr35 and confers immunity against wheat stem rust caused by *Puccinia graminis* f.sp. *tritici* race Ug99. Assembly of a stable Sr35 resistosome induced by AvrSr35 *in vitro* is usually limited by protein expression and low assembly efficiency. Here we describe the expression and purification of AvrSr35 and Sr35, *in vitro* assembly of Sr35 resistosome for structure determination by cryo-EM.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Assembly and purification of AvrSr35-induced Sr35 resistosome and determination of its structure by cryo-EM

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SUMMARY

Sr35, a coiled-coil nucleotide-binding leucine-rich repeat receptor (CC-NLR) from the wheat species *Triticum monococcum* can directly recognize the pathogen avirulence factor AvrSr35 and confers immunity against wheat stem rust caused by *Puccinia graminis* f.sp. *tritici* race Ug99. Assembly of a stable Sr35 resistosome induced by AvrSr35 *in vitro* is usually limited by protein expression and low assembly efficiency. Here, we describe the expression and purification of AvrSr35 and Sr35, *in vitro* assembly of Sr35 resistosome for structure determination by cryo-EM.

For complete details on the use and execution of this protocol, please refer to Zhao et al. (2022).

BEFORE YOU BEGIN

The Sf9 cells should be thawed and cultured suitably ahead of baculovirus-mediated expression of recombinant Sr35. In addition, all buffers used in the protocol should be prepared in advance and filtered using 0.22 µm filter (Merck-Millipore) and pre-cooled to 4°C before use unless stated otherwise.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
<i>Escherichia coli</i> Rosetta (DE3)	Weidi Biotechnology	CAT# EC1010
<i>Escherichia coli</i> DH5α	Weidi Biotechnology	CAT# DL1001
<i>Escherichia coli</i> DH10Bac	Weidi Biotechnology	CAT# DL1071
<i>Spodoptera frugiperda</i> Sf9 insect cells	Thermo Fisher Scientific	CAT# A35243
Chemicals, peptides, and recombinant proteins		
Cellfectin® Reagent	Thermo Fisher Scientific	CAT# A38915
KOD-plus-neo	Toyobo	CAT# KOD-401
T4 DNA ligase	Thermo Fisher Scientific	CAT# EL0014
T4 DNA polymerase	Thermo Fisher Scientific	CAT# 18005025

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BamHI	NEB	CAT# R0136S
XhoI	NEB	CAT# R0146S
SspI	NEB	CAT# R0132S
DpnI	NEB	CAT# R0176S
ATP	Sigma	CAT# 34369-07-8
MES	Sigma	CAT# 1266615-59-1
Glycerol	Sigma	CAT# 56-81-5
Tris base	Sigma	CAT# 77-86-1
Glycine	Sigma	CAT# 56-40-6
Sodium dodecyl sulfate (SDS)	Sigma	CAT# 151-21-3
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	BioLeaf	CAT# 367-93-1
Ampicillin	TransGen Biotech	CAT# GG101-01
Tris-HCl	Sinopharm Chemical Reagent	CAT# 77-86-1
NaCl	Sinopharm Chemical Reagent	CAT# 7647-14-5
Imidazole	Sinopharm Chemical Reagent	CAT# 288-32-4
Phenylmethylsulfonyl fluoride (PMSF)	Solarbio	CAT# 329-98-6
Dithiothreitol (DTT)	Sangon Biotech	CAT# 3483-12-3
Gentamicin sulfate	Solarbio	CAT# 1405-41-0
Tetracycline	Solarbio	CAT# 60-54-8
X-gal	TransGen Biotech	CAT# GF201-01
Sf-900 II medium	Gibco	CAT# 10902088
Uranyl acetate	Afine	CAT# 541-09-3
β-mercaptoethanol (BME)	Sigma	CAT# 77327-45-8
Gel Extraction Kit	Omega	CAT# D2500-02
Plasmid Mini Kit	Omega	CAT# D6943-02
Superose 6 Increase 10/300 GL column	GE Healthcare Life Sciences	CAT# 29091596
Superdex 200 Increase 10/300 GL column	GE Healthcare Life Sciences	CAT# 28990944
SP FF column	GE Healthcare Life Sciences	CAT# 17-5054-01
Deposited data		
Cryo-EM map for Sr35 resistosome	(Zhao et al., 2022)	EMDB: EMD-33153
Coordinates for Sr35 resistosome	(Zhao et al., 2022)	PDB: 7XE0
Recombinant DNA		
pMCSG7-AvrSr35 ^{ASP}	This paper	N/A
pMCSG7-AvrSr35 ^{ASP-R381A}	This paper	N/A
Sumo-pFastBac1-Sr35	This paper	N/A
Oligonucleotides		
AvrSr35 ^{ASP} amplification sense primer 5' cgcgatccatggtacaaatagatgaccgaaaggc 3'	This paper	N/A
AvrSr35 ^{ASP} amplification anti-sense primer 5' ccgctcgagtacaattgcttcatgaacattgg 3'	This paper	N/A
Sr35 amplification sense primer 5' cgcgatccatggagattgcatggggg 3'	This paper	N/A
Sr35 amplification anti-sense primer 5' ccgctcgagttatcaccatatatcgaggatgg 3'	This paper	N/A
Software and algorithms		
RELION 3.1	(Zivanov et al., 2018)	http://www2.mrc-lmb.cam.ac.uk/relion
CTFFIND	(Rohou and Grigorieff, 2015)	http://grigoriefflab.janelia.org/ctf
MotionCorr2	(Zheng et al., 2017)	https://msg.ucsf.edu/software
UCSF Chimera	(Pettersen et al., 2004)	http://www.cgl.ucsf.edu/chimera/
PHENIX	(Adams et al., 2010)	https://www.phenix-online.org/
Coot	(Emsley et al., 2010)	http://www2.mrc-lmb.cam.ac.uk/ Personal/pemsley/coot
SWISS-MODEL	(Waterhouse et al., 2018)	https://swissmodel.expasy.org/
Other		
Autogrid C-clip rings	FEI	CAT# 1036173
Autogrid C-clip	FEI	CAT# 1036171
Quantifoil R 1.2/1.3 Cu holey carbon grids	Electron Microscopy Sciences	CAT# Q51907



MATERIALS AND EQUIPMENT

Lysis buffer 1		
Reagent	Final concentration	Amount
NaCl (5 M)	150 mM	3 mL
Tris-HCl (1 M, pH 7.5)	25 mM	2.5 mL
BME (1 M)	5 mM	0.5 mL
PMSF (100 mM)	1 mM	1 mL
ddH ₂ O	N/A	93 mL
Total	N/A	100 mL

Note: β -mercaptoethanol (BME), a reducing agent used to prevent the formation of disulfide bonds, and the serine protease inhibitor phenyl methylsulfonyl fluoride (PMSF) should be added to the buffer immediately before use, after which the buffer can be stored at 4°C for up to 2 h.

△ CRITICAL: BME is toxic or even fatal if ingested, inhaled, or absorbed through the skin. It should also be kept away from open flames, hot surfaces and oxidizing agents. Store BME at 4°C avoiding exposure to light. BME should be handled inside a chemical fume hood while wearing appropriate protection, including gloves.

Lysis buffer 2		
Reagent	Final concentration	Amount
NaCl (5 M)	1 M	20 mL
Tris-HCl (1 M, pH 7.5)	25 mM	2.5 mL
BME (1 M)	5 mM	0.5 mL
PMSF (100 mM)	1 mM	1 mL
Glycerol 100% (v/v)	10% (v/v)	10 mL
ddH ₂ O	N/A	66 mL
Total	N/A	100 mL

Note: Add the PMSF before use, after buffer can stored at 4°C for up to 2 h.

△ CRITICAL: The addition of glycerol depends on the stability of the protein of interest. In this protocol, glycerol can improve solubility of AvrSr35 and Sr35 protein. The final concentration of glycerol should not exceed 10% to avoid high buffer viscosity.

Buffer A		
Reagent	Final concentration	Amount
NaCl (5 M)	100 mM	2 mL
MES (1 M, pH 6.0)	25 mM	2.5 mL
DTT (1 M)	2 mM	0.2 mL
ddH ₂ O	N/A	95.3 mL
Total	N/A	100 mL

Note: Dithiothreitol (DTT) should be added immediately before buffer use, after which the buffer can be stored at 4°C for up to 3–5 days.

Buffer B

Reagent	Final concentration	Amount
NaCl (5 M)	1 M	20 mL
MES (1 M, pH 6.0)	25 mM	2.5 mL
DTT (1 M)	2 mM	0.2 mL
ddH ₂ O	N/A	77.3 mL
Total	N/A	100 mL

Note: Dithiothreitol (DTT) should be added immediately before buffer use, after which the buffer can be stored at 4°C for up to 3–5 days.

SEC buffer 1

Reagent	Final concentration	Amount
NaCl (5 M)	150 mM	3 mL
Tris-HCl (1 M, pH 7.5)	25 mM	2.5 mL
DTT (1 M)	2 mM	0.2 mL
ddH ₂ O	N/A	94.3 mL
Total	N/A	100 mL

Note: Dithiothreitol (DTT) should be added immediately before buffer use, after which the buffer can be stored at 4°C for up to 3–5 days.

SEC buffer 2

Reagent	Final concentration	Amount
NaCl (5 M)	500 mM	10 mL
Tris-HCl (1 M, pH 7.5)	25 mM	2.5 mL
DTT (1 M)	2 mM	0.2 mL
ddH ₂ O	N/A	87.3 mL
Total	N/A	100 mL

Note: Dithiothreitol (DTT) should be added immediately before buffer use, after which the buffer can be stored at 4°C for up to 3–5 days.

SDS-PAGE running buffer

Reagent	Final concentration	Amount
Tris base	250 mM	30.2 g
Glycine	1.9 M	144 g
SDS	34.6 mM	10 g
ddH ₂ O	N/A	Fill to 1L
Total	N/A	1 L

Note: The buffer can be stored at 18°C–25°C for up to 2 months.

PCR reaction

Reagent	Final concentration	Amount
10×Buffer for KOD-Plus Neo	1×	5 μL
2 mM dNTPs	0.2 mM	5 μL
25 mM MgSO ₄	1.5 mM	3 μL

(Continued on next page)

Continued

Reagent	Final concentration	Amount
Forward primer (10 μ M)	0.3 μ M	1.5 μ L
Reverse primer (10 μ M)	0.3 μ M	1.5 μ L
Template DNA	10–200 ng	X μ L
KOD-Plus-Neo (1 U/ μ L)	1 U/50 μ L	1 μ L
Autoclaved distilled water	Add to 50 μ L	Y μ L
Total	N/A	50 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	98°C	30 s	25–35 cycles
Annealing	55°C	30 s	
Extension	72°C	1.5 min	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

Treatment with T4 DNA polymerase

Reagent	Final concentration	Amount
PCR-amplified insert/Vector	200 ng	X μ L
T4 polymerase 10 \times reaction buffer	1 \times	4 μ L
dCTP (100 mM)	2.5 mM	1 μ L
DTT (100 mM)	5 mM	2 μ L
T4 DNA polymerase (3 U/ μ L)	3 U/40 μ L	1 μ L
Autoclaved distilled water	Add to 50 μ L	Y μ L (X + Y = 32)
Total		40 μL

Vector linearization with SspI

Reagent	Final concentration	Amount
Vector	15 μ g	X μ L
SspI 10 \times reaction buffer	1 \times	6 μ L
SspI (5 U/ μ L)	10 U/60 μ L	2 μ L
Autoclaved distilled water	Add to 60 μ L	Y μ L (X + Y = 52)
Total		60 μL

Ligation-independent cloning (LIC) reaction

Reagent	Amount
Vector	15 ng
PCR-amplified insert	30–45 ng
Total	5–6 μL

BamHI/XhoI double digestion reaction

Reagent	Final concentration	Amount
PCR-amplified insert/plasmid (15 μ g)	15 μ g	X μ L
CutSmart® 10 \times reaction buffer	1 \times	5 μ L
BamHI (10 U/ μ L)	10 U/ 50 μ L	1 μ L
XhoI (10 U/ μ L)	10 U/ 50 μ L	1 μ L
Autoclaved distilled water	Add to 50 μ L	43-X μ L
Total		50 μL

Ligation reaction		
Reagent	Final concentration	Amount
PCR-amplified insert (1 kb)	0.076 pmol	50 ng (0.076 pmol)
Plasmid (3 kb)	0.025 pmol	50 ng (0.025 pmol)
10×T4 DNA ligase buffer	1×	2 μL
T4 DNA ligase (5 U/μL)	5 U/20 μL	1 μL
Nuclease-free water	Add to 20 μL	To 20 μL
Total		20 μL

Transfection Component	
Reagent	Amount
Sf9 cells Recombinant bacmid	8 × 10 ⁵ cells
Recombinant bacmid	1 μg (can be varied from 1 to 2 μg)
Cellfectin® Transfection Reagent	6 μL (can be varied from 1.5 to 9 μL)

STEP-BY-STEP METHOD DETAILS

We provide a detailed step-by-step protocol for purification of recombinant AvrSr35 and Sr35, assembly of the Sr35 resistosome, sample preparation for negative staining and cryo-EM, and cryo-EM structure determination.

Expression and purification of recombinant AvrSr35^{ASP}

⌚ **Timing:** 6–8 days total (4–5 days for step 1; 2–3 days for step 2)

The production of recombinant AvrSr35^{ASP} includes two main steps: plasmid construction and protein purification. The steps are described below.

1. Preparation of AvrSr35^{ASP} plasmid.

The process of recombinant AvrSr35^{ASP} plasmid construction are described as below steps.

- a. Obtain plasmid carrying the full-length coding sequence of wild-type AvrSr35.

Note: The full-length coding sequence of AvrSr35 was retrieved from the National Center for Biotechnology Information database (GenBank: MF474174.1) and cloned by company (Beijing Shengyuan Kemeng Gene Biotechnology Co., Ltd) into the pET28a vector between BamHI and XhoI sites.

- b. Design primers to remove sequence encoding 1–26 amino acids from the full-length coding sequence of AvrSr35 by PCR, thus generating the AvrSr35^{ASP} construct. Primer design is shown in Oligonucleotides of KRT.

Note: Amino acid residues 1–26 of AvrSr35 have been predicted to encode a signal peptide that is not required for this study.

- c. Perform PCR with the pET28a-AvrSr35 vector as a template to generate the AvrSr35^{ASP} construct. For a detailed description of the PCR reaction system and cycling conditions, please refer to PCR reaction and PCR cycling conditions tables.
- d. Run a 1% agarose gel at 120 V to verify the size and purity of PCR products. The size of the target PCR product is 1,653 bp. Excise the target PCR product (i.e., PCR-amplified insert) from the gel and recover it using the OMEGA Gel Extraction Kit or another similar kit. The recovered product can be quantified by Nanodrop.
- e. Treat the recovered PCR-amplified AvrSr35^{ASP} insert with T4 DNA polymerase.

- i. Prepare the reaction system as detailed in the Treatment with T4 DNA polymerase table, adding 1 μL of T4 DNA polymerase into 200 ng of the PCR-amplified AvrSr35^{ASP} insert.
- ii. Incubate the reaction at 20°C–25°C for 30 min.
- iii. Afterward, incubate the reaction system at 75°C for 20 min to eliminate T4 DNA polymerase activity.
- iv. Keep at 4°C or –20°C until the ligation-independent cloning (LIC) step.
- f. Linearize the pMCSG7 vector using the SspI restriction endonuclease. Prepare the reaction system according to the Vector linearization with SspI table and incubate the reaction at 37°C for 2 h.
- g. Treat the linearized pMCSG7 vector with T4 DNA polymerase as described in step e.

Note: Following treatment with T4 DNA polymerase, vectors and inserts can be stored long-term at –80°C after flash freezing via liquid nitrogen.

- h. Ligation-independent cloning. Mix the PCR-amplified AvrSr35^{ASP} insert and the pMCSG7 vector as described in Ligation-independent cloning (LIC) reaction table and incubate on ice for 30 min.
- i. Transform the ligation product pMCSG7-AvrSr35^{ASP} into DH5 α competent cells and plate the transformants onto LB agar plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin.
 - i. Incubate the plates at 37°C for 12–15 h.
 - ii. Use a single large bacterial colony to inoculate 5 mL of LB medium supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin. Culture at 37°C and 220 rpm for 12 h.
 - iii. Extract and purify plasmid DNA using the Omega Plasmid Mini Kit or similar plasmid extraction kit.
 - iv. Sequence the recombinant plasmid to confirm that the sequence of the pMCSG7-AvrSr35^{ASP} is correct before proceeding to the next step.

Pause point: The bacterial cultures can be stored short-term at 4°C as suspensions or long-term at –20°C – –80°C as pellets. Extracted plasmid samples can be stored at –20°C until further use.

Note: The crystal structure of AvrSr35^{ASP} reported by Zhao et al. (Zhao et al., 2022), shows that the asymmetric unit contains two AvrSr35 molecules forming a homodimer. The residue R381 located at the dimeric interface plays a key role in maintaining the homodimer conformation. Replacing R381 with alanine almost completely abolished the AvrSr35-AvrSr35 interaction and significantly weakened the interaction between AvrSr35 and Sr35. To explore the relationship between AvrSr35 dimerization and the activation of Sr35, the AvrSr35^{ASP_R381A} mutant was also cloned into the pMCSG7 vector to analyze its capacity for inducing the Sr35 resistosome.

2. Purification of recombinant AvrSr35^{ASP}.

This section describes the purification of AvrSr35^{ASP} by Ni-NTA affinity chromatography, ion-exchange chromatography and size-exclusion chromatography.

- a. Transform the pMCSG7-AvrSr35^{ASP} plasmid into the *E. coli* Rosetta (DE3) protein expression strain. Plate the transformants onto LB agar plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 50 $\mu\text{g}/\text{mL}$ chloramphenicol.
- b. Pick a single, large colony with a sterile pipette tip and use it to inoculate 100 mL of liquid LB medium supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin and 50 $\mu\text{g}/\text{mL}$ chloramphenicol. Culture at 37°C and 220 rpm for 12 h.
- c. Expand the bacterial culture. Dilute the 100 mL starter culture at a 1:100 ratio in 1 L of LB medium supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin and 50 $\mu\text{g}/\text{mL}$ chloramphenicol. Culture at 37°C and 220 rpm until the OD₆₀₀ value of the culture reaches 0.6–0.8.

- d. Induce overexpression of AvrSr35^{ASP}. Add 300 μ L of 1 M IPTG to each flask with bacterial culture (final concentration of IPTG is 0.3 mM). Express the protein at 18°C and 220 rpm for 12–15 h.
- e. Pellet the bacteria by centrifuging the culture at 3,100 \times g and 4°C for 15 min. Discard the supernatant and collect the pelleted bacteria.

Note: Unless proceeding immediately to the next step, pelleted bacteria should be stored at –20°C – –80°C.

- f. Resuspend the bacterial pellet in pre-chilled Lysis buffer 1 by vortexing or rocking on a platform until completely resuspended.

Note: BME and PMSF should be added immediately before using Lysis buffers.

Note: The volume of Lysis buffer used for resuspending the bacterial pellets depends on the size of bacterial pellets. In this study, we used 20 mL of Lysis buffer per 1 L of pelleted bacterial culture.

- g. Disrupt the bacteria using a French press at 800 bar.

Note: Due to the poor thermal stability of the protein, the French press must be pre-cooled to 4°C before lysis.

Alternatives: Cells can be disrupted by sonication instead of French press.

- h. Centrifuge the lysate at 10,540 \times g for 30 min at 4°C to separate supernatant from cell debris and unbroken cells.
- i. Load the supernatant onto a Ni-NTA affinity column. Allow the supernatant to pass through the column at a flow rate of \sim 0.8 mL/min to ensure efficient binding of the target protein binds column matrix.

Note: Before loading the supernatant, Ni-NTA affinity column should be equilibrated with 5 column volumes of Lysis buffer 1.

- j. Wash the Ni-NTA beads with 5 column volumes of Lysis buffer 1.
- k. Carry out elution with Lysis buffer 1 containing 20 mM imidazole, followed by Lysis buffer 1 containing 200 mM imidazole. Collect the eluate in separate tubes.

Note: The Bradford reagent can be used to detect the presence of proteins in the eluate. Once no protein can be detected with the Bradford reagent, stop washing the column with the same buffer and then continue the elution process with a buffer containing a higher concentration of imidazole.

Alternatives: In this protocol, the process of Ni-NTA purification is carried out on the bench. The high Performance-liquid chromatography (HPLC) can also be used for the purification. Protein elution process can be monitored by the absorbance at 280 nm.

- l. Collect the eluate from each tube and mix it with protein loading buffer. Perform SDS-PAGE gel analysis (Figure 1A). Select the eluates containing AvrSr35^{ASP} with good purity. Monitor the absorbance at 280 nm and the ratio of A260/A280 via Nanodrop. The A260/A280 ratio should be 0.5–0.7.

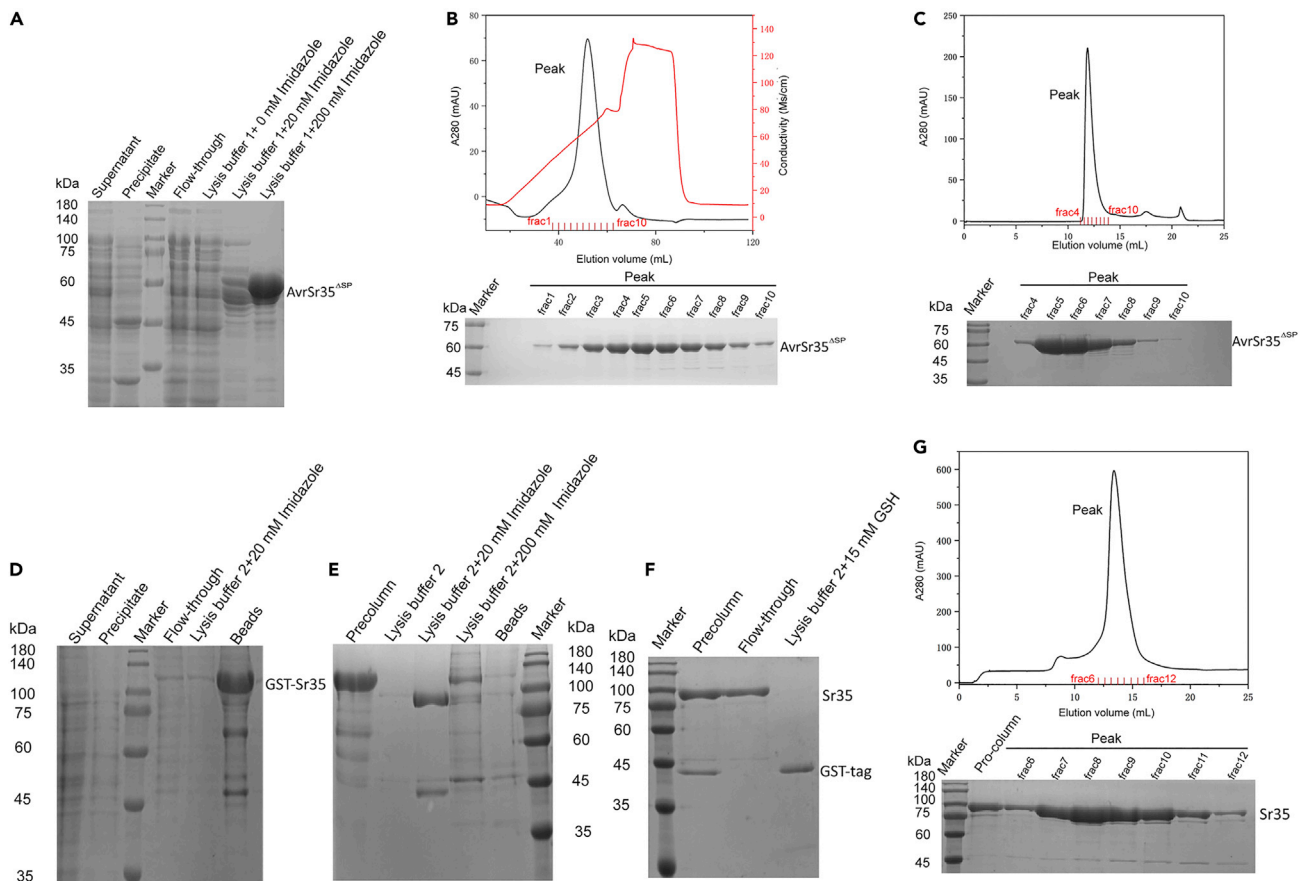


Figure 1. Purification of AvrSr35^{ASP} and Sr35

- (A) SDS-PAGE gel showing the results of AvrSr35^{ASP} purification with Ni-NTA affinity chromatography.
 (B) Chromatogram and SDS-PAGE gel showing the results of AvrSr35^{ASP} purification with ion-exchange chromatography (SP FF column).
 (C) Chromatogram and SDS-PAGE gel showing the results of AvrSr35^{ASP} purification with size-exclusion chromatography (Superdex 200 (10/300 GL) column). Each gel lane corresponds to the fraction of peak.
 (D) SDS-PAGE gel showing the results of Sr35 purification with Ni-NTA affinity chromatography.
 (E) SDS-PAGE gel showing results of SUMO tag digestion reaction by PreScission Protease.
 (F) SDS-PAGE gel analysis of removal of PP enzyme with GST column.
 (G) Chromatogram and SDS-PAGE gel demonstrating the results of Sr35 purification with size-exclusion chromatography. Each gel lane corresponds to the fraction of peak.

- m. Pool the selected protein samples using a centrifugal filter with molecular weight cut-off (MWCO) value of 50 kDa and replace the buffer system into Buffer A (25 mM MES (pH 6.0), 100 mM NaCl, 2 mM DTT). Concentrate and adjust the sample volume to 15 mL.
- n. Load the protein sample onto an SP FF cation exchange chromatography ion column at 4°C and at a flow rate of 1 mL/min using a peristaltic pump.

Note: Before sample loading, the SP FF column should be washed using at least five column volumes of Buffer B (25 mM MES (pH 6.0), 1 M NaCl, 2 mM DTT), followed by equilibration with five column volumes of Buffer A. Washing and equilibration can be performed at a flow rate of 2 mL/min. Moreover, the flow rate of sample loading can also be increased but should not exceed 2 mL/min to allow efficient binding of the protein sample onto column matrix.

Alternatives: This step can also be completed using an HPLC.

- o. Purification with ion-exchange chromatography (Figure 1B).

- i. Mount the SP FF column onto a chromatography system, such as the ÄKTA pure, at 4°C. Elute the target protein using a NaCl gradient between Buffer A and Buffer B at a flow rate of 1 mL/min and steady buffer B increase from 0% to 60% over 20 min. An elution peak can be observed between a 45%–60% NaCl gradient.
- ii. Then, resume the buffer B increase from 60% to 100% after the peak is fully eluted. Collect fractions containing protein peaks and run SDS-PAGE analysis.
- iii. Select fractions corresponding to purified AvrSr35^{ASP} for further purification.
- p. Using centrifugal filter (MWCO = 50 kDa), pool the purified AvrSr35^{ASP}, replace the buffer system into SEC buffer 1 (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM DTT), and then concentrate the sample.
- q. Quantify the concentration (30–40 absorption units/mL when concentrated to 0.5 mL) using a spectrophotometer and aliquot it appropriately in a sterile 1.5 mL microcentrifuge tubes so that the absorbance at 280 nm (A280) does not exceed 1000 absorption units. Adjust the volume of each sample to the required volume for SEC.

Note: Before injecting the concentrated AvrSr35^{ASP} sample into a size-exclusion chromatography (SEC) column centrifuge it at 7,440 × *g* at 4°C for 15 min. Some denatured protein may precipitate out of solution. In such cases, the sample should be injected carefully to avoid resuspending the sediment and clogging the pores within column matrix.

- r. Purification with size-exclusion chromatography (Figure 1C).
 - i. Inject the prepared AvrSr35^{ASP} sample via the sample loop into a Superdex 200 (10/300 GL) SEC column of 24 mL volume which is equilibrated with at least one column volume of SEC buffer 1.
 - ii. Perform the purification at the flow rate of 0.5–0.8 mL/min.
 - iii. Collect fractions containing protein peaks and run SDS-PAGE analysis.

Note: SEC buffer 1 should be degassed by sonication or negative pressure chamber and cooled to 4°C before use.

- s. Using centrifugal filter (MWCO = 50 kDa), pool and concentrate the fractions containing pure and homogeneous AvrSr35^{ASP} to 15–30 mg/mL. The purified AvrSr35^{ASP} can be stored at –80°C until further use.

Note: The protein samples were flash-frozen in liquid nitrogen and then stored at –80°C.

Expression and purification of recombinant Sr35

⌚ **Timing:** about 20 days total: 4–5 days for step 3; ~1 week for step 4; 8 hr for step 5; ~7–8 days for step 6; ~2 days for step 7

Since Sr35 was difficult to express in *E. coli*, SF9 insect expression system was used instead. Detailed protocols for preparation of Sr35 plasmid and expression and purification of recombinant Sr35 are provided below.

3. Preparation Sumo-pFastBac1-Sr35 plasmid.

The process of recombinant plasmid construction is described as below.

- a. Retrieve full-length coding sequence of the Sr35 from NCBI database (GenBank: MT165900.1). The sequence should be inserted between BamHI and XhoI sites of the pFastBac1 transformation vector containing a 6×His-tag and SUMO tag upstream of the multiple cloning site. Primer design is shown in KRT.

- b. Use the primers containing the BamHI and XhoI recognition sites for PCR amplification of full-length Sr35 gene. The PCR reaction system and reaction conditions are described in the PCR reaction and PCR cycling conditions tables.
- c. Run a 1% agarose gel electrophoresis with the PCR products. Excise the PCR-amplified insert band from the gel (2,757 bp) and recover it using OMEGA Gel Extraction Kit or another similar kit. Quantify the concentration of recovered PCR-amplified insert.
- d. Double-digest the PCR-amplified insert and SUMO-pFastBac1 vector separately using BamHI and XhoI restriction enzymes by incubating at 37°C for 2 h. Detailed reaction system is described in the BamHI/XhoI double digestion reaction table.
- e. Run a 1% agarose gel electrophoresis. Excise the digested PCR-amplified insert (919 bp) and SUMO-pFastBac1 (~5,000 bp) vector bands and recover them using the OMEGA Gel Extraction Kit or another similar kit. Quantify the concentration of recovered digestion products.
- f. Mix the recovered PCR-amplified insert and SUMO-pFastBac1 vector at a molar concentration ratio of 3:1 and incubate with T4 DNA ligase at 37°C for 4 h. Detailed reaction system is described in the Ligation reaction table.
- g. Transform the ligation product into DH5 α competent cells and plate the transformants onto LB agar plates containing 100 μ g/mL ampicillin.
 - i. Incubate the plates at 37°C for 12–15 h.
 - ii. Use a single large bacterial colony to inoculate 10 mL of LB medium supplemented with 100 μ g/mL ampicillin. Culture at 37°C and 220 rpm for 12 h.
 - iii. Extract and purify plasmid DNA using Omega Plasmid Mini Kit or similar plasmid extraction kit.
- h. Sequence the recombinant plasmid to confirm that the sequence of the pFastBac1-Sr35 is correct before proceeding to the next step.

▮▮▮ Pause point: The bacterial cultures can be stored short-term at 4°C as suspensions or long-term at –20°C – –80°C as pellets. Extracted plasmid samples can be stored at –20°C until further use.

4. Preparation and isolation of bacmid.

In the Bac-to-Bac baculovirus expression system, DH10Bac competent cells are commonly used to produce recombinant bacmids. DH10Bac competent cells contain a tetracycline resistance gene and can improve the transposition efficiency of Sumo-pFastBac1. The isolation of Sumo-pFastBac1-Sr35 bacmids is carried out as follows:

- a. Place vials with 100 μ L of DH10Bac competent cells on ice until the cells are thawed.
- b. Mix 100 ng of the Sumo-pFastBac1-Sr35 with thawed DH10Bac competent cells using a sterile pipette tip. Incubate on ice for 30 min, heat shock cells in a 42°C water bath for 45 s, and then return to the ice for 2–3 min.
- c. Add 900 μ L of SOC media to the DH10Bac competent cells. Incubate at 37°C and 220 rpm for 4–5 h.
- d. Plate the cells onto SOC agar containing 50 mg/mL kanamycin, 7 mg/mL gentamicin, 10 mg/mL tetracycline, 200 mg/mL Bluo-Gal, and 40 mg/mL IPTG. Incubate at 37°C for 48–72 h.

Note: During incubation, the plates need to be completely protected from light. The common method is to wrap the plates in aluminum foil.

- e. After 48–72 h of incubation, blue and white colonies will appear on the plates. Use a single, large white colony to inoculate 20 mL of SOC medium supplemented with 50 μ g/mL kanamycin, 7 μ g/mL gentamicin, and 10 μ g/mL tetracycline. Culture at 37°C and 220 rpm for 24 h.
- f. Centrifuge the culture at 3,100 \times g for 30 min. Discard the supernatant and keep the cell pellet. Collect cell pellets in a 1.5 mL-microcentrifuge tube.
- g. Perform bacmid extraction with a QIAGEN Plasmid Mini Kit.

Note: The following steps of bacmid extraction need to be performed under sterile conditions in a laminar flow hood.

Alternatives: We use reagents from the QIAGEN mini prep kit. Reagents from other mini prep kits can also be used.

- h. Add 300 μL of Buffer P1 to the tube and resuspend the cells thoroughly.

Note: Ensure that RNase A has been added to Buffer P1.

- i. Add 300 μL of Buffer P2 and gently invert the microcentrifuge tube 4–6 times to allow thorough mixing. Leave at room temperature until the color of the solution fully changes to blue (approximately 5 min). The color change is due to the addition of LyseBlue reagent to Buffer P1.
- j. Add 300 μL of ice-cold Buffer P3. Mix by gently inverting the microcentrifuge tube 4–6 times, or until the blue color disappears completely. Afterward, incubate the tube for 5 min on ice.
- k. Centrifuge at $7,440 \times g$ and 4°C for 10 min.
- l. In the meantime, equilibrate the QIAGEN-tip by adding 5 volumes of Buffer QBT. Allow the column to empty by gravity flow.
- m. Transfer supernatant containing DNA onto an equilibrated QIAGEN-tip and allow it to flow through the column.
- n. Wash the QIAGEN-tip twice with 2 mL of Buffer QC.
- o. Elute bacmid DNA by adding 800 μL of Buffer QN to the QIAGEN-tip and allowing it to flow through the column. Collect the eluate into a sterile 1.5 mL microcentrifuge tube.
- p. Add 560 μL of sterile isopropanol into the tube containing eluted DNA. Centrifuge at $7,440 \times g$ and 4°C for 30 min.
- q. Precipitated DNA should appear on the bottom of the tube in form of a white pellet. Carefully decant the supernatant and add 1 mL of ice-cold 70% ethanol. Invert the tube gently several times to wash the DNA pellet.
- r. Centrifuge at $7,440 \times g$ and 4°C for 10 min. Discard the supernatant carefully.
- s. Air-dry the pellet for 15–20 min at room temperature, and then dissolve the DNA in 30 μL of TE buffer.
- t. Store the purified recombinant bacmid at -20°C .

Note: The recombinant bacmid can be stored long-term at -80°C .

This next section describes the expression of recombinant Sr35 in SF9 insect expression system and the process of purification.

5. Transfection of recombinant bacmid
- Culture Sf9 insect cells in Sf-900 II medium containing 50 units/mL penicillin and 50 $\mu\text{g}/\text{mL}$ streptomycin. The cells should look healthy, and no cell clumping should be observed.
 - Pipette Sf9 cells into a six-well plate so that each well contains up to 8×10^5 cells. Add fresh antibiotic-free Sf-900 II medium to increase the total volume of the cell suspension to 2.5 mL per well.
 - Allow the cells to adhere by incubating them at 27°C in the dark for 30 min to 1 h.
 - Once the cells adhere to the bottom of the 6-well plate, change the medium to Grace's Un-supplemented Insect Medium using a sterile pipette tip.
 - Take two sterile 1.5 mL microcentrifuge tubes and add 100 μL of Sf-900 II SFM medium to each tube. Add 6 μL of transfection reagent to Tube 1 and 1 μg bacmid DNA to Tube 2. Mix the contents within each tube and leave them at room temperature for 30 min.
 - Transfer the contents of Tube 1 to Tube 2 and mix well by pipetting up and down. Leave the mixture at room temperature for 30 min.

- g. Add the bacmid: transfection reagent mixture to the cell in the well. Mix gently by shaking the plate. Incubate at 27°C for 5 h.
- h. Replace the transfection mixture with fresh Sf-900 II medium and culture at 27°C for 4–5 days.

Note: DNA concentration, Cellfectin® II transfection reagent concentration, and cell density are three key factors determining the efficiency of bacmid transfection. Transfection conditions can thus be optimized by varying these three factors. Based on our experience, we recommend the concentrations of each component as Transfection Component table.

6. P1-P3 virus production

- a. After transfection, observe the cells under a microscope every other day. Visible signs of infection usually begin to appear 48 h after transfection.

Note: Compared to control cells, successfully transfected cells should display the following phenotypic characteristics: 1) the cell number does not increase since transfection or is significantly lower than the number of control cells; 2) cell diameter is notably increased, black spots (granules) can be seen inside the cells and some cells are ruptured; 3) some cells are no longer attached to surface of the well and are instead suspended in medium.

- b. Four days after transfection, the phenotypic characteristics of infected cells should be obvious.

Note: During incubation, if the incubator does not have a humidification system, the sides of the six-well plate need to be sealed with tape to prevent evaporation.

- c. Aspirate the cell supernatant with a pipette, transfer it to two 1.5 mL microcentrifuge tubes or a 15 mL centrifuge tube. Centrifuge at 100 × g for 10 min to remove cell debris and collect supernatant containing clarified first-generation baculovirus (P1 virus) stock.
- d. Since P1 virus titers are too low for expression of recombinant protein, it should be amplified in two further rounds of infection that would yield P2 and P3 viral stocks, respectively.

Note: The preparation of P2 and P3 viral stocks requires T75 cultures flasks to enable infection of larger cell volumes.

- e. Prepare healthy Sf9 cells. Add 8×10^6 cells to a T75 cell culture flask and then add the Sf-900 II medium to the final volume of 15 mL.

Note: The medium should be pre-warmed to 28°C in a thermostatic water bath.

- f. Lay the culture flasks horizontally and place them at 28°C in the dark for more than 20 min.
- g. After the cells are attached to the surface of a T75 culture flask, add 0.4 mL of P1 virus stock to the flask. Lay the flask horizontally and shake it gently to mix evenly.
- h. Incubate at 28°C in the dark for 72 h and monitor cell behavior under a microscope every other day.
- i. After 72 h of infection, cells appear lysed, and the viral particles are released into the medium. Decant the medium into a 50 mL centrifuge tube and centrifuge at 100 × g and 4°C for 10 min.
- j. Transfer the clarified supernatant containing the high-titer P2 viral stock to a new centrifuge tube. Store at 4°C in the dark.

Note: P1 and P2 viral stocks can be stored at 4°C for a long time (more than half a year). If stored at –80°C, repeated freezing and thawing should be avoided. Otherwise, viral titers will be greatly reduced. Long-term storage is generally recommended at –80°C degrees.

- k. Refer to the operating steps for P2 virus production to prepare P3 virus for large-scale protein expression.

Note: Protein expression levels were tested by inoculating 100 mL of Sf-900 II medium with P2 viral stock at a volume ratio of 30:1 and incubating at 28°C in the dark for 72 h. Quantity and purity of expressed Sr35 were considered to determine whether to culture the P3 virus.

- l. Prepare 750 mL healthy Sf9 cells at $1.0\text{--}1.2 \times 10^6$ concentration in a cell culture flask and infect them with P3 virus stock at a volume ratio of 1:30. Incubate at 27°C and 120 rpm for 72 h.
- m. Harvest infected cells by centrifuging at $3,100 \times g$ and 4°C for 15 min. Discard the supernatant and collect the pelleted cells.

▮▮▮ **Pause point:** The cell pellets can be stored at –20°C or –80°C for long-term storage.

7. Purification of recombinant Sr35

- a. Resuspend the cell pellet in ice-cold Lysis buffer 2 by rocking on a platform or by vortexing until the cell pellet is fully dissolved.
- b. Disrupt the cells by sonication under the following conditions: two cycles, cycle time 18 min (36 min total), 150 W, 3 s pulse on, 8 s pulse off.

Note: During the entire sonication process, the cell suspension should be placed into water-ice mixture to prevent overheating and protein denaturation.

- c. Centrifuge the cell lysate at $10,540 \times g$ and at 4°C for 30 min to remove cell debris and unbroken cells. Collect the clarified supernatant.
- d. Incubate the clarified supernatant with Ni-NTA resin at 4°C for 1 h. Before incubation with supernatant, the Ni-NTA resin should be equilibrated with five column volumes of Lysis buffer 2.

Note: The supernatant with Ni-NTA mixture should rotate slowly and uniformly to prevent resin deposition.

- e. Transfer the mixture of supernatant and Ni-NTA resin onto a column. Allow the supernatant to flow through the column. Wash the column extensively with Lysis buffer 2 and then with Lysis buffer 2 containing 20 mM imidazole. Each wash step should be carried out until no protein can be detected in the eluate when using Bradford reagent.
- f. Equilibrate the column with 5 column volumes of Lysis buffer 2. Leave 5 mL of the buffer in the column and close the column to prevent the buffer from flowing out. Add 100 µL of PreScission Protease (PP) at a 1:100 concentration (w/w). Mix PP well with beads and incubate about 12 h at 4°C.
- g. Allow the enzyme digestion system to flow through the Ni-NTA column and wash the column extensively with Lysis buffer 2.
- h. Elute the Sr35 protein (sumo tag-free) with Lysis buffer 2 that contained 20 mM imidazole.

Note: After washing away protein impurities, the recombinant SUMO-Sr35 remains bound to the Ni-NTA resin (Figure 1D).

Optional: In this protocol, PP protease was directly added to the Ni-NTA column bed to remove the SUMO tag. This procedure was used not only to avoid repetitive protein concentration steps but also to shorten overall purification time, which also reduces the probability of protein aggregation and denaturation. However, when necessary, the SUMO tag can also be removed after eluting the target protein from the Ni-NTA column.

- i. After SDS-PAGE gel analysis (Figure 1E), select the eluate containing Sr35 with better protein purity at higher quantity for further purification.
- j. To remove the remaining PP protease, the selected Sr35 sample should undergo a round of purification with GST affinity chromatography. Pass the Sr35 sample through a GST column and collect the eluate containing purified tagless Sr35 (Figure 1F).

Note: The PP protease carries a GST-tag, so purification with GST affinity chromatography results in PP protease bound to glutathione resin, whereas the target protein flows through the column.

- k. Using centrifugal filter (MWCO = 50 kDa), pool the purified Sr35, replace the buffer system into SEC buffer 2 (25 mM Tris-HCl (pH 7.5), 500 mM NaCl, 2 mM DTT). Concentrate the purified Sr35 to the required volume for SEC.

Note: According to our experience, Sr35 precipitates at concentrations above 5 mg/mL. Therefore, it is important to regularly measure protein concentration during the concentration process to prevent precipitation. Reducing the time of the concentration process can also reduce precipitation.

- l. Aliquot and dilute the Sr35 sample in a sterile 1.5 mL microcentrifuge tubes to achieve concentration below 5 mg/mL in each tube. Centrifuge at $7,440 \times g$ and 4°C for 15 min.
- m. Purification with size-exclusion chromatography (Figure 1G). Inject the prepared Sr35 sample via sample loop into a Superdex 200 (10/300 GL) SEC column mounted. Be careful not to disturb precipitated protein during sample injection. Perform the purification at the flow rate of 0.6 mL/min. Collect fractions containing protein peaks and run SDS-PAGE analysis.

Note: The Superdex 200 (10/300 GL) SEC column should be equilibrated with at least one column volume of SEC buffer 2 previously degassed by sonication.

- n. Pool and concentrate the fractions corresponding to pure and homogeneous Sr35 using a centrifugal filter (MWCO = 50 kDa). The concentrated and purified Sr35 can be stored at -80°C until further use.

Assembly and purification of Sr35 resistosome

⌚ **Timing:** 1 day

Mix the purified AvrSr35^{ASP} and Sr35 proteins to assemble the Sr35 resistosome *in vitro* and purify the complex with size-exclusion chromatography according to the following steps.

8. Assembly and purification of AvrSr35^{ASP}-induced Sr35 resistosome
 - a. Mix the purified Sr35 and AvrSr35^{ASP} at a $\sim 1:2$ molar ratio and add ATP to the final concentration of 1 mM. Incubate at 4°C for 2 h.

Note: Before ATP can be added, the sodium chloride concentration of the Sr35:AvrSr35^{ASP} mixture needs to be adjusted to 150 mM. This same protocol can be used for the assembly of AvrSr35^{ASP_R381A}-induced Sr35 resistosome.

- b. Centrifuge the Sr35: AvrSr35^{ASP} mixture at $7,440 \times g$ and 4°C for 15 min. Inject the mixture and purify with size-exclusion chromatography (Superose 6 Increase 10/300GL, GE Healthcare) (Figures 2A and 2B). Perform the purification at the flow rate of 0.6 mL/min. Before sample injection, the column should be equilibrated with SEC buffer 1.

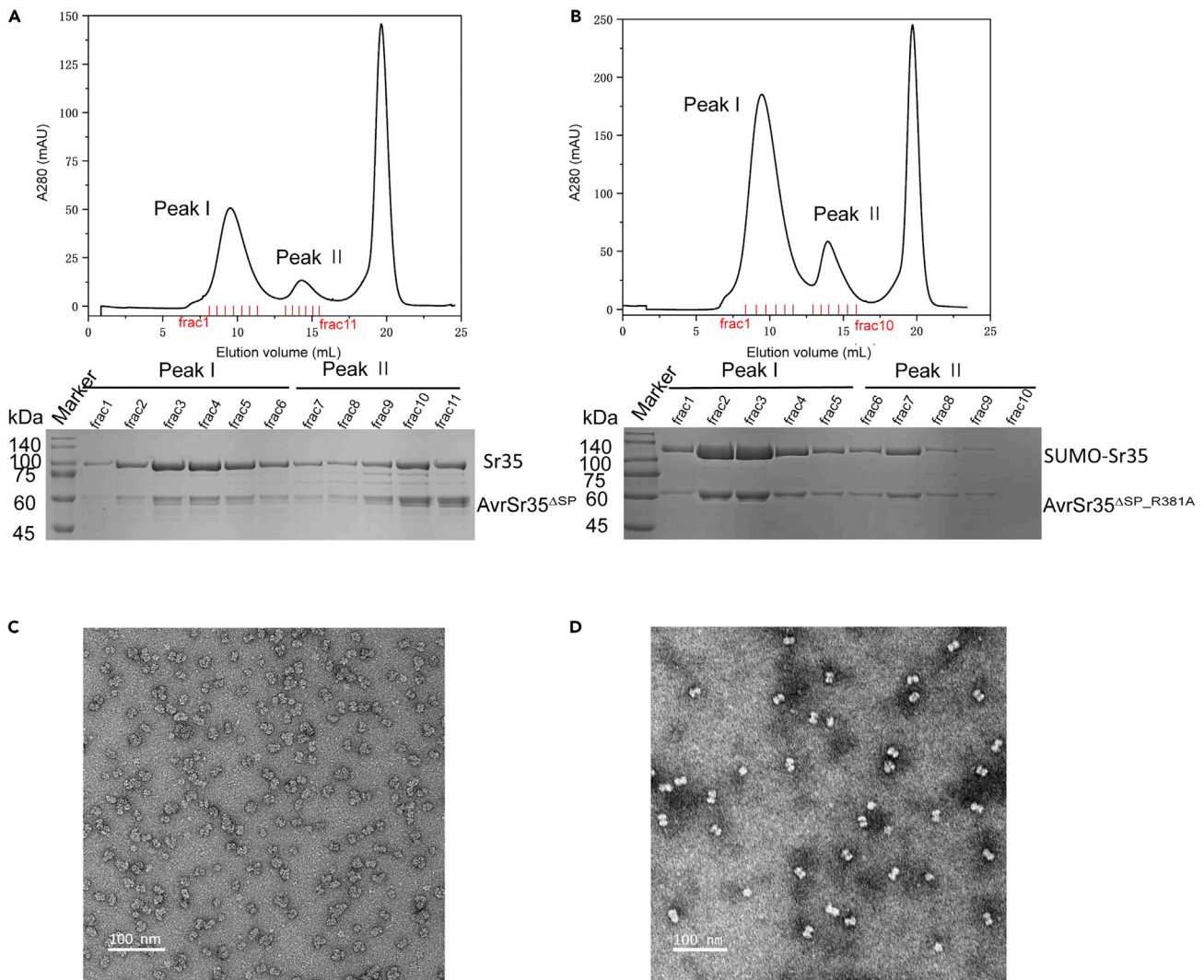


Figure 2. In vitro assembly and purification of Sr35 resistosome

(A) Chromatograms and SDS-PAGE results for Sr35 resistosome induced by AvrSr35^{ASP}.

(B) Chromatograms and SDS-PAGE results for SUMO-Sr35 resistosome induced by AvrSr35^{ASP_R381A}.

(C) Representative negative staining micrograph depicting the Sr35 resistosome induced by AvrSr35^{ASP}. The protein sample corresponds to peak I as shown in (A). Scale bar, 100 nm.

(D) Representative negative staining micrograph depicting the SUMO-Sr35 resistosome induced by AvrSr35^{ASP_R381A}. The protein sample corresponds to peak I as shown in (B). Scale bar, 100 nm.

- c. Collect fractions containing protein peaks and run SDS-PAGE analysis (Figures 2A and 2B). Select fractions corresponding to Sr35 resistosome for cryo-EM experiments.
- d. Pool and concentrate the fractions containing purified Sr35 resistosome to 0.5–3 mg/mL in SEC buffer 1 using centrifugal filter (MWCO = 100 kDa).

Negative staining electron microscopy

© Timing: about 3 h

To check the sample homogeneity, the Sr35 resistosome is stained by uranyl acetate or tungsten phosphate staining solution and observed by electron microscopy (Talos L120C G2). The detailed process of the sample preparation is described below.

9. Sample preparation of negative staining

- a. Dilute the Sr35 resistosome sample to 0.01–0.05 mg/mL and centrifuge it at 4°C for 15 min.

Note: Before sample loading, the grid should undergo a glow discharge treatment (30–45 s at 15 mA).

- b. Transfer 5 μ L of sample supernatant onto a 2–4 nm carbon-coated 300-mesh copper grid and let it stand for 1 min.
- c. Absorb excess liquid with filter paper.

Note: The liquid should not be completely blotted to avoid removal of protein from the grid.

- d. Drop 4–6 μ L of uranyl acetate onto the grid and quickly absorb excess liquid. Repeat this step three times.
- e. Drop 4–6 μ L of uranyl acetate onto the grid and let it stand for 1 min.
- f. Absorb excess uranyl acetate with filter paper. After natural drying, observe the grid by transmission electron microscope (Figures 2C and 2D).

Note: The parameters of protein concentration and staining time can be adjusted according to protein properties.

Cryo-EM structure determination

⌚ **Timing:** about 2 weeks total: ~1 day for step 10; ~1–2 weeks for step 11

The successfully assembled of Sr35 resistosome is prepared for the structure determination by cryo-EM. The following steps describe the process in detail, including frozen-hydrated specimen preparation, screening, data collection and processing.

10. Grid sample preparation and screening.

Frozen-hydrated specimens of Sr35 resistosome were prepared using a Vitrobot Mark IV plunger (Thermo Fisher Scientific) and screened by cryo-EM. The flow chart is shown as Figure 3. Detailed steps are described below.

- a. Ultracentrifuge the Sr35 resistosome sample at 7,440 \times g and 4°C for 10 min in a Micro-Ultracentrifuge (Thermo Fisher Scientific). Transfer the supernatant into sterile 1.5 mL microcentrifuge tubes.
- b. Dilute the Sr35 resistosome sample to five gradient concentrations, including 3, 2.5, 2, 1.5, 1, and 0.5 mg/mL.
- c. Pipette 5 μ L of each protein solution onto a separate freshly glow-discharged grid (QuantiFoil-1.2/1.3-Cu 300 mesh holey carbon grid). Absorb excess solution from the grid with 3–6 s of blotting at 100% humidity and 4°C before plunging the grids into liquid ethane.

Optional: The grid needs to undergo a glow discharge treatment (30 s at 15 mA) before the specimen is loaded. Glow discharge parameters are based on sample hydrophilicity and the existence of a supporting film. When the hydrophilicity of the sample is low, the time of glow discharge can be extended appropriately, or the power can be increased. Moreover, the user can optimize various grid types and vitrification parameters including blot time, blot force and wait time for specimens as required.

- d. Transfer the grid into a pre-cooled sample box. Keep in liquid nitrogen until further experiments.

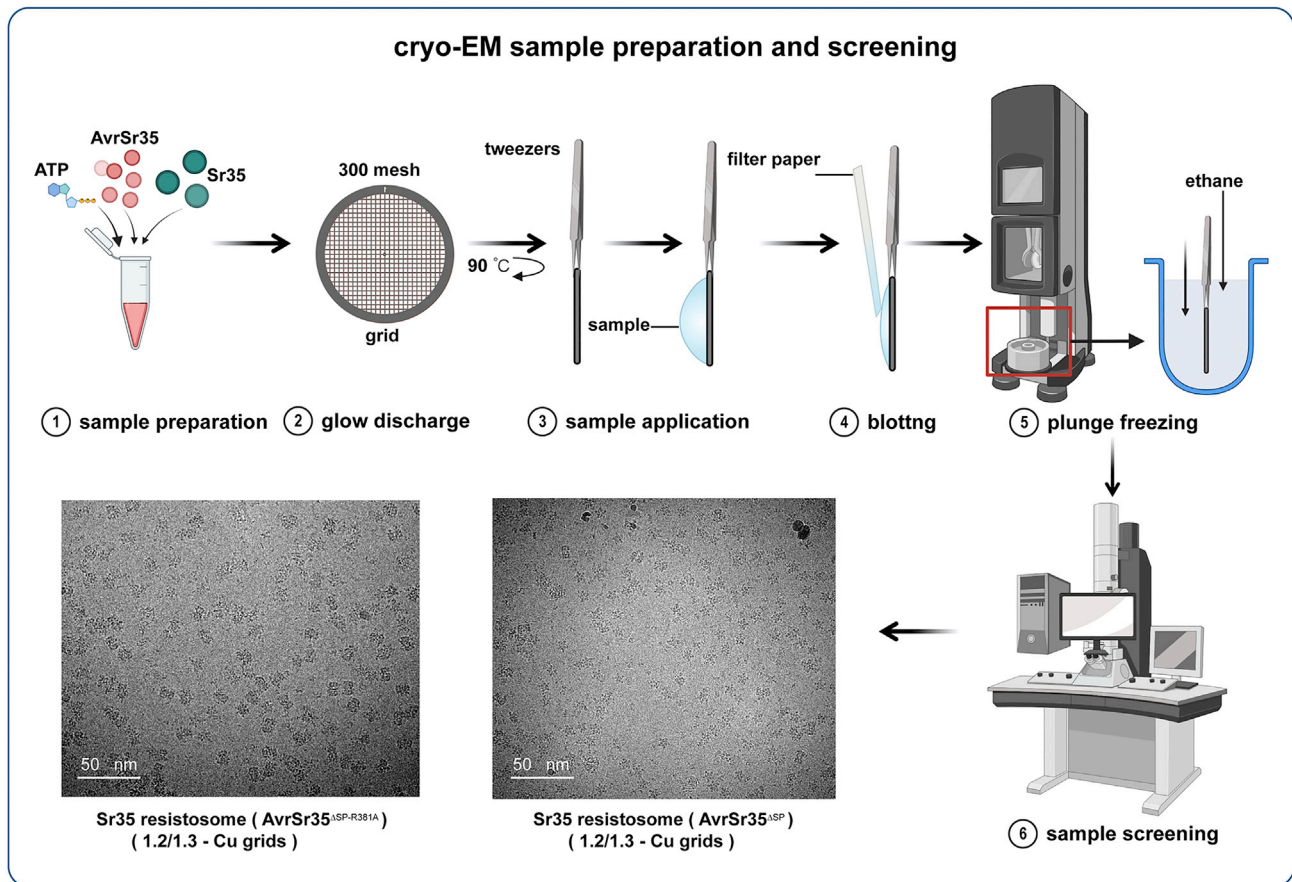


Figure 3. Cryo-EM sample preparation and screening flow chart

Note: Some image in this figure elements such as instruments and materials were exported from BioRender. This figure is edited in Adobe Illustrator software. Scale bar, 50 nm.

Note: After grids are plunge-frozen, the grid must be transferred into liquid nitrogen and remain at cryogenic temperatures. Frozen grids can be stored in liquid nitrogen before loading onto the microscope.

To assess particle distribution and orientation as well as ice quality, sample grids are tested using a transmission electron microscope (TEM). Screening results indicated that the Sr35 resistosomes induced by *AvrSr35^{ΔSP}* and *AvrSr35^{ΔSP-R381A}* form intact particles and have a better contrast in holey carbon grid (Figure 3).

Optional: To obtain protein particles with better contrast on the grid, ice thickness and distribution can be adjusted by varying parameters including blot time, blot force, and waiting time. The adjustment of parameters depends on ice quality.

11. Cryo-EM data collection and processing.

FEI Titan Krios transmission electron microscope equipped with a Gatan K3 Summit direct detector and Gatan BioQuantum energy filter was used for data collection. The following are detailed steps for collection and processing of cryo-EM data, which are displayed in Figure 4.

- a. Acquire data using EPU software to control the 300 kV transmission electron microscope operated at a nominal magnification of 105,000 \times with a pixel size of 1.095 Å. Record

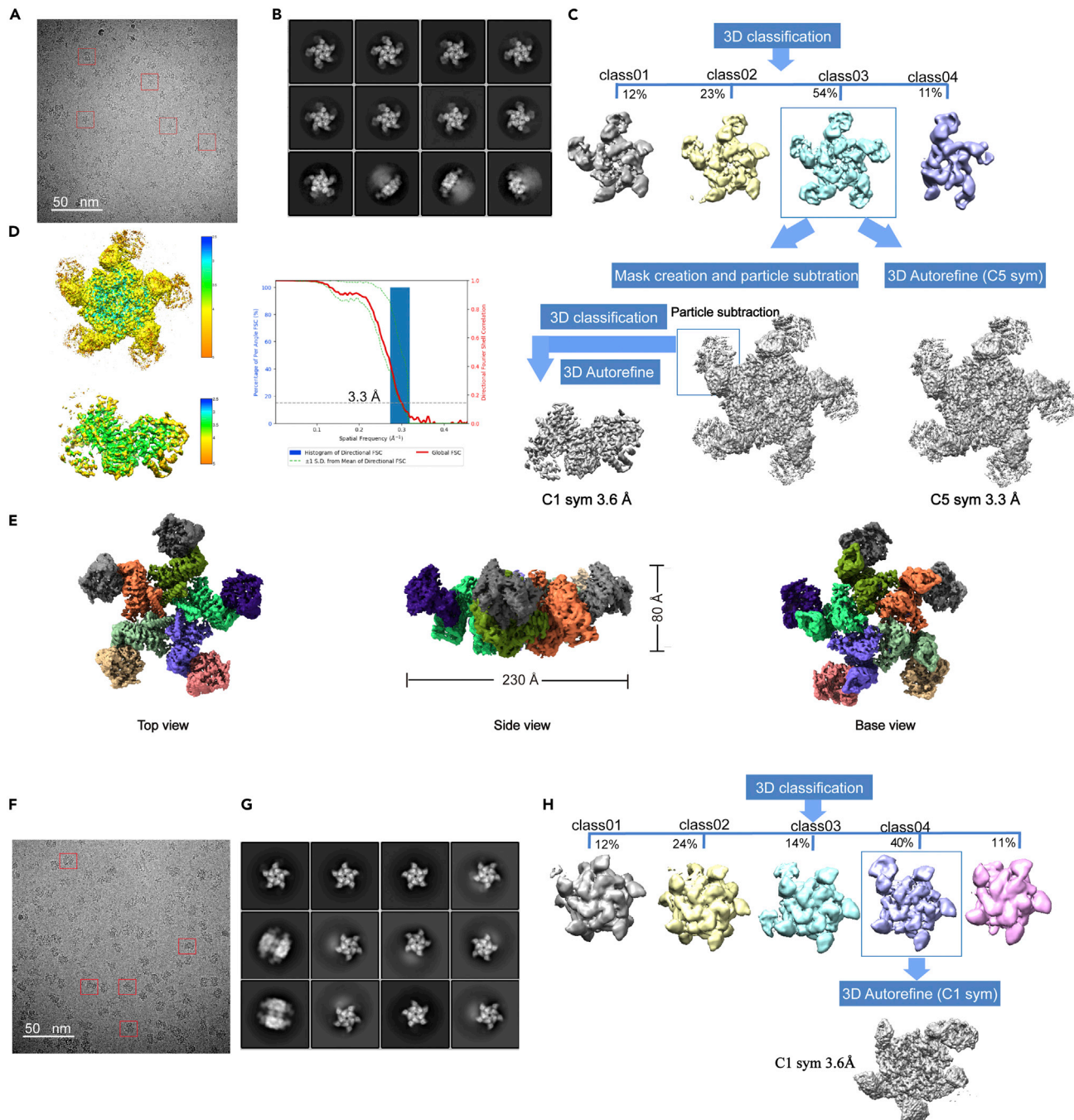


Figure 4. 3D reconstruction of the Sr35 resistosome induced by AvrSr35^{ASP} and SUMO-Sr35 resistosome induced by AvrSr35^{ASP}_{R381A}

(A) Representative electron micrograph of the Sr35 resistosome embedded in vitreous ice. Scale bar, 50 nm.

(B) Representative views of 2D class averages.

(C) Flowchart of Cryo-EM data processing and 3D reconstruction of the Sr35 resistosome induced by AvrSr35^{ASP}.

(D) Local resolution map calculated using Relion for the Sr35 resistosome induced by AvrSr35^{ASP} (top) and subtraction part (bottom). Right panel: FSC curves at 0.143 of the final reconstruction of the Sr35 resistosome induced by AvrSr35^{ASP}.

(E) Composite density map of the Sr35 resistosome from three cryo-EM reconstructions.

(F) Representative electron micrograph of AvrSr35^{ASP}_{R381A}-induced SUMO-Sr35 resistosome embedded in vitreous ice. Scale bar, 50 nm.

(G) Representative views of 2D class averages.

(H) Flowchart of Cryo-EM data processing and 3D reconstruction of the AvrSr35^{ASP}_{R381A}-induced SUMO-Sr35 resistosome.

- pre-screening specimen images with a defocus range of -1.2 to -1.8 μm in super-resolution mode with 32 frames per movie and a total dose of 50 electrons per \AA^2 .
- Align, dose-weight, and sum the 32 frames of each movie in a super-resolution model using MotionCor2, resulting in summed micrographs in a pixel size of 1.095 \AA per pixel.
 - Except for the motion correction of raw movies, processing of other data are completed in Relion/3.1.2.

Optional: All the processing steps can also be performed in new/updated CryoEM software, such as cryoSPARC.

- Import the micrographs with dose-weighting in Relion software and estimate contrast transfer function (CTF) parameters by running CTFFIND-4.1.
- 3,851 micrographs with visible thon rings at better than 4 \AA resolution are selected and performed in particles auto-picking by Laplacian-of-Gaussian filter with diameter for LoG filter to 200–250 \AA . $\sim 20,000$ particles from the first autopick are extracted with “Particle box size” set to 400, “Rescaled size” set to 100. Two-dimensional (2D) class averaging is performed with “Number of classes” set to 200, “mask diameter” set to 340 \AA to generate templates for further template-based auto picking.
- Select any orientation particle images from 2D classification to generate template images and perform for a template-based auto picking. Then, extract the particles as step e for further 2D classification.
- After two rounds of reference-2D classification, select $\sim 150,000$ particles from well-defined particle images and use them for generation of the initial 3D model.
- Subject particles to 3D classification into 4 classes according to C1 symmetry. Select the best classes, which should correspond to the Sr35 resistosome decamer.
- Extract particles for the best 3D classes without binning to the original pixel size (1.095 \AA /pixel).
- Select the optimal classification with 35,510 particles for the final 3D auto-refinement with an imposing symmetry C5. At this step, the post-processed map had a resolution of 3.3 \AA (FSC = 0.143).
- To enhance the resolution of the C terminal domain of Sr35 that interacted with Avr35, create five local masks corresponding to different coordinates in local maps for particle subtraction.
- Carry out particle subtraction in 3D classification without performing image alignment. After two rounds of 3D classification, carry out 3D auto-refinement of an optimal local electron density map. After processing, electron density map in the Zhao et al. study had a resolution of 3.6 \AA (FSC = 0.143).

Note: Parameters for cryo-EM data collection for Sr35 resistosome induced by AvrSr35^{ASP_R381A} and data processing are the same as for the Sr35 resistosome induced by AvrSr35^{ASP}.

- EM density map of Sr35 resistosome induced by AvrSr35^{ASP} at a 3.3 \AA resolution was used for model building.

Note: Data analysis indicated that the residue R381 of AvrSr35 does not only mediate AvrSr35 homodimerization, but also plays role in recognition of AvrSr35 by Sr35. The Sr35 resistosome induced by AvrSr35^{ASP_R381A} exhibits lower rates of ligand binding. Moreover, no intact ligand-binding particles were observed in 3D classification.

Model building and refinement

⌚ Timing: about 3–4 weeks

12. Model building and refinement of Sr35 resistosome
 - a. Perform initial model building in the SWISS-MODEL server using the structure of ZAR1 resistosome (PDB ID: 6J5T) as a template. Dock the structure of the ZAR1 resistosome into the EM density of Sr35 resistosome in Chimera, followed by docking of five ATP molecules into the density using COOT.
 - b. Refine the model of five ATP-bound Sr35-AvrSr35 molecules in PHENIX based on the EM map in real space with secondary structure and geometric constraints.
 - c. Verify the structural model was verified using MolProbity included in the PHENIX package.

EXPECTED OUTCOMES

Approximately ~ 10 mg of purified Sr35 can be obtained from 1.5 L insect cells and approximately ~ 12 mg of purified AvrSr35^{ASP} can be obtained from E. coli. Finally, approximately 0.5 mg of Sr35 resistosome can be obtained when mixing the purified Sr35 (1.2 mg) and AvrSr35^{ASP} (2.4 mg) at a ~1:2 molar ratio and adding ATP to the final concentration of 1 mM. High-resolution complex structure of Sr35 resistosome induced by AvrSr35 is determined by single-particle cryo-EM.

LIMITATIONS

The Sr35 protein has poor stability and is prone to degradation and precipitation, which increased the difficulty of complex preparation. Moreover, Sr35 resistosome assembled *in vitro* exhibited a low rate of binding to ligands and the intact ligand binding particles were observed at a low percentage in cryo-EM movies.

TROUBLESHOOTING

Problem 1

AvrSr35 is significantly degraded during purification.

Potential solution

Shorten the protein purification time of Ni-NTA affinity chromatography and ensure that the process of purification is carried out at a low temperature (4°C) at steps (steps 2j, 2k, and step 2p) that can effectively reduce the rate of protein degradation.

Problem 2

Sr35 degrades and precipitates during purification.

Potential solution

Lyse the cells in a high salt (1 M NaCl) buffer containing 5 mM BME and maintain buffer NaCl concentration at 500 mM throughout purification (steps b–h of [purification of recombinant Sr35](#)). When preparing the Sr35-AvrSr35^{ASP} complex, decrease the buffer NaCl concentration gradually to 150 mM using the method of concentrated liquid exchange after mixing the two proteins (step a of [assembly and purification of Sr35 resistosome](#)).

Problem 3

Sr35 and Avrsr35 proteins precipitate or aggregate easily.

Potential solution

Reducing the time and frequency of the concentration process can efficiently reduce protein precipitation and aggregation (step p and s of [Purification of recombinant AvrSr35^{ASP}](#); step k and n of [purification of recombinant Sr35](#)).

Problem 4

The yield of Sr35 resistosome is low.

Potential solution

Prolonged incubation time of the Sr35:AvrSr35^{ASP} mixture with ATP (step a of [assembly and purification of Sr35 resistosome](#)).

Problem 5

Too high or too low particle density on the grid.

Potential solution

- Adjust the protein concentration accordingly (step b of [Grid sample preparation and screening](#)).
- Varying the blot time and blot force in Vitrobot can adjust the ice thickness (step c of [Grid sample preparation and screening](#)).
- Extend waiting time when using Vitrobot to improve the protein adsorption rate (step c of [Grid sample preparation and screening](#)).

Problem 6

Protein samples were easily damaged during preparation of frozen samples.

Potential solution

This is a common situation in the preparation of frozen samples. In this protocol, we have tried different grid materials and preparation conditions, including blot time and blot force until we identified the most suitable conditions (step c of [Grid sample preparation and screening](#)). Depending on protein characteristics, different detergents may also affect particle distribution and orientation, as well as sample quality.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Songying Ouyang (ouyangsy@fjnu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original/source data for figures in the paper is available. The EM density maps of the Sr35 resistosome generated in this study have been deposited in the EMDB under the accession code EMD-33153. Atomic coordinates have been deposited in the PDB under the accession code 7XE0.

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AUTHOR CONTRIBUTIONS

Design and supervision: S.O.; Protein expression and purification: Y.B.Z., T.T.C.; Data collection and refinement statistics of Sr35 resistosome cryo-EM structure: M.X.L., Z.K.L.; M.X.L. wrote the manuscript and Y.B.Z. revised and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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