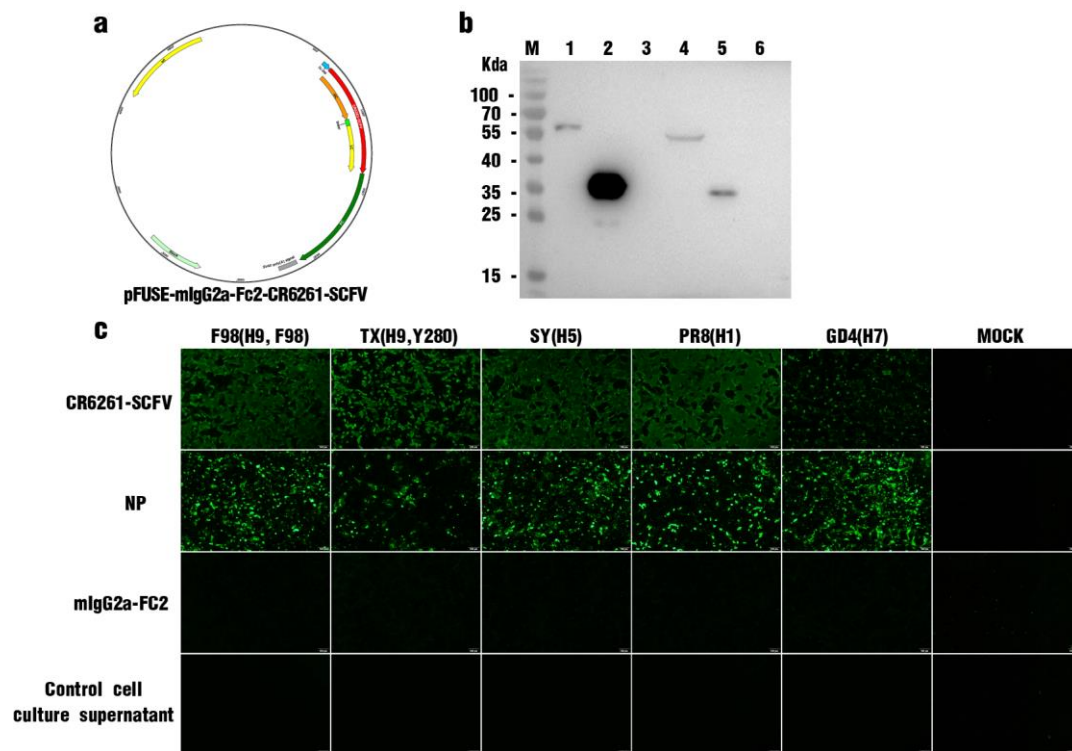
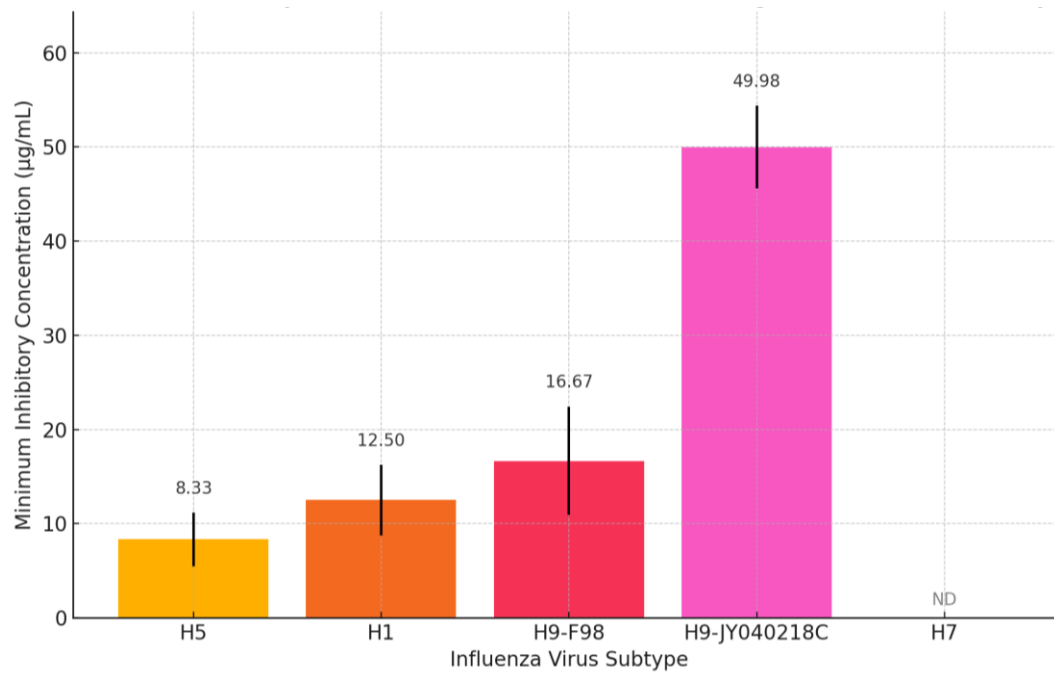


Supporting Information

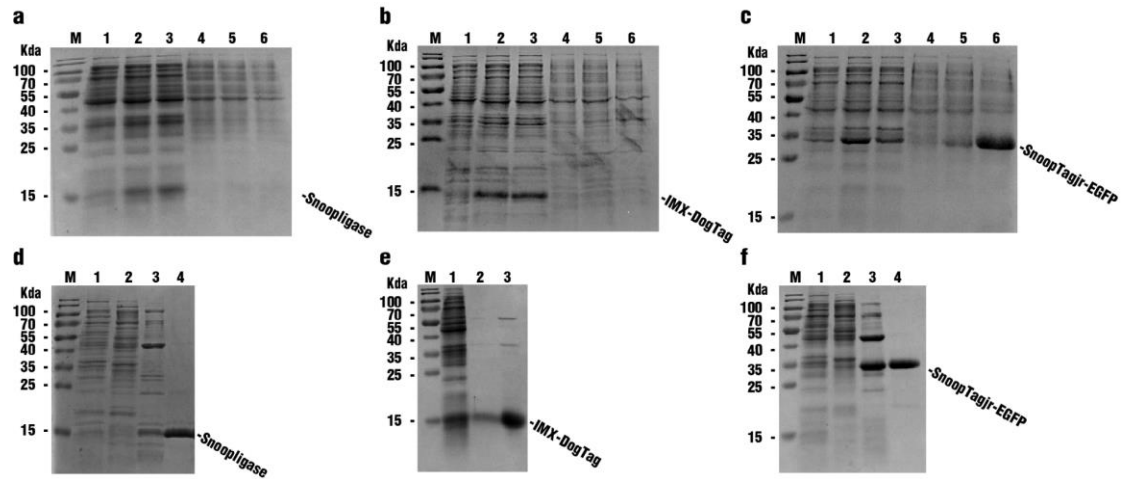
Development of a Broad-Spectrum Subunit Vaccine Against H9N2 Avian Influenza Using HA Stem Domain Scaffold and Snooplignase System



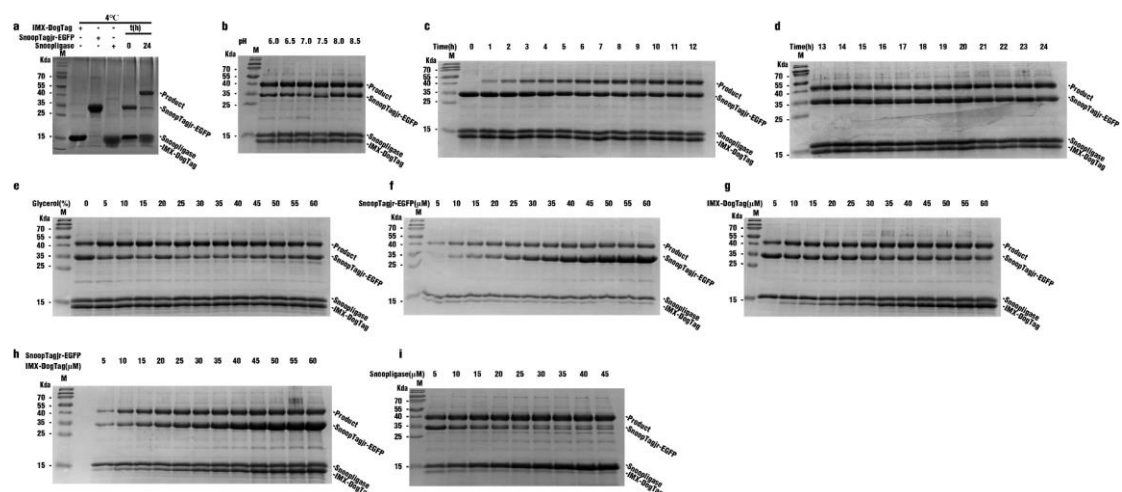
Supplementary Figure 1. Expression and characterization of CR6261-SCFV. a. Plasmid map of pFUSE-mIgG2a-Fc2-CR6261-SCFV. b. Expression verification of CR6261-SCFV. M Marker, 1 pFUSE-mIgG2A-Fc2-CR6261-SCFV transfected cell culture supernatant, 2 Empty vector-transfected cell culture supernatant, 3 Untransfected cell culture supernatant, 4 pFUSE-mIgG2A-Fc2-CR6261-SCFV transfected cell lysate supernatant, 5 Empty vector-transfected cell lysate supernatant, 6 Untransfected cell lysate supernatant. c. Reactivity of CR6261-SCFV with different subtypes of AIV.



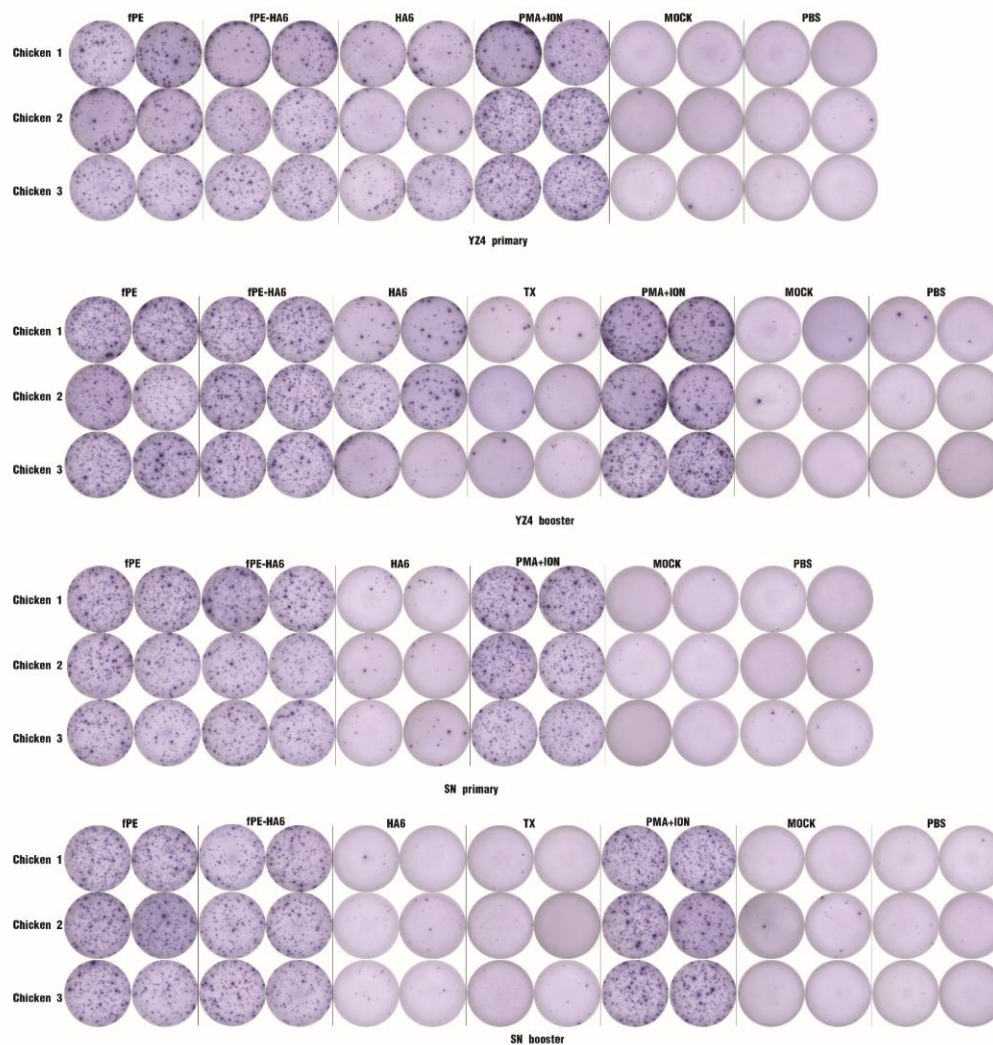
Supplementary Figure 2. Neutralization potency of CR6261-SCFV Against Influenza Subtypes. The minimum concentration of CR6261-SCFV required to inhibit detectable viral infection was measured for H5, H1, H7, and two H9 strains (F98 and JY040218C). Bars represent the mean $\pm$ standard deviation of at least three independent experiments. CR6261-SCFV demonstrated the highest potency against the H5 subtype, followed by H1, with strain-dependent neutralization observed within the H9 subtype group. H7 subtype was showed no measurable neutralizing activity



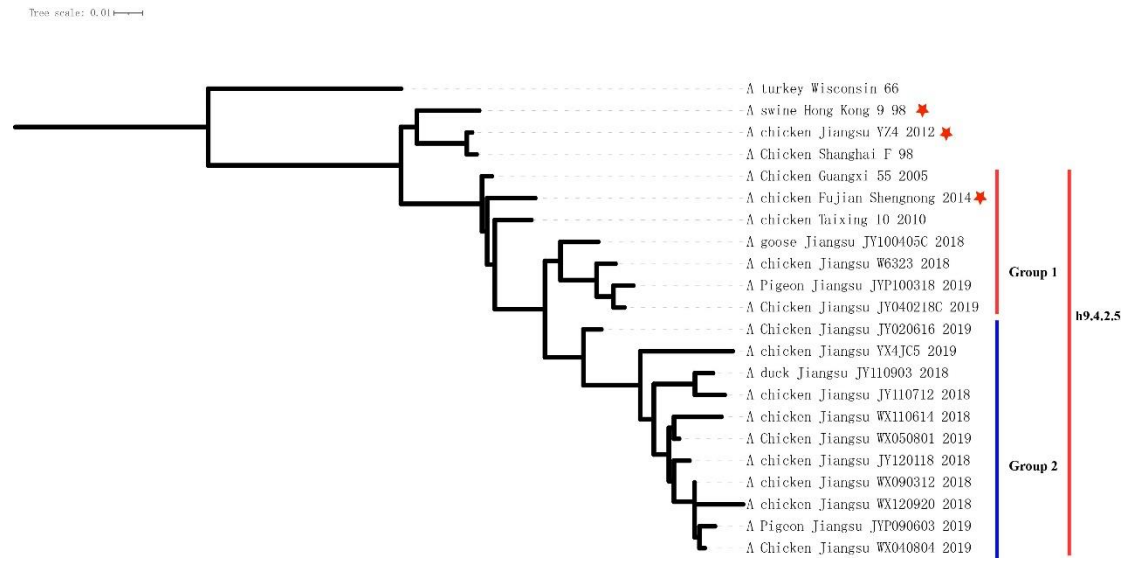
Supplementary Figure 3. Expression and purification of SnooPligase, SnooTagjr-EGFP, and IMX-DogTag. a. SnooPligase expression: 1-3 Supernatant after lysis at 16, 25, 37 °C, 4-6 Precipitate after lysis at 16, 25, 37 °C. b. IMX-DogTag expression: 1-3 Supernatant after lysis at 16, 25, 37 °C, 4-6 Precipitate after lysis at 16, 25, 37 °C. c. SnooTagjr-EGFP expression: 1-3 Supernatant after lysis at 16, 25, 37 °C, 4-6 Precipitate after lysis at 16, 25, 37 °C. d. SnooPligase purification: 1 load peak, 2 Elution with 10 mM imidazole, 3 Elution with 100 mM imidazole, 4 Elution with 250 mM imidazole. e. IMX-DogTag purification: 1 Supernatant after lysis, 2 Supernatant after incubation at 80 °C, 3 Precipitate after incubation at pH 2.0. f. SnooTagjr-EGFP purification: 1 load peak, 2 elution with 10 mM imidazole, 3 elution with 100 mM imidazole, 4 elution with 250 mM imidazole.



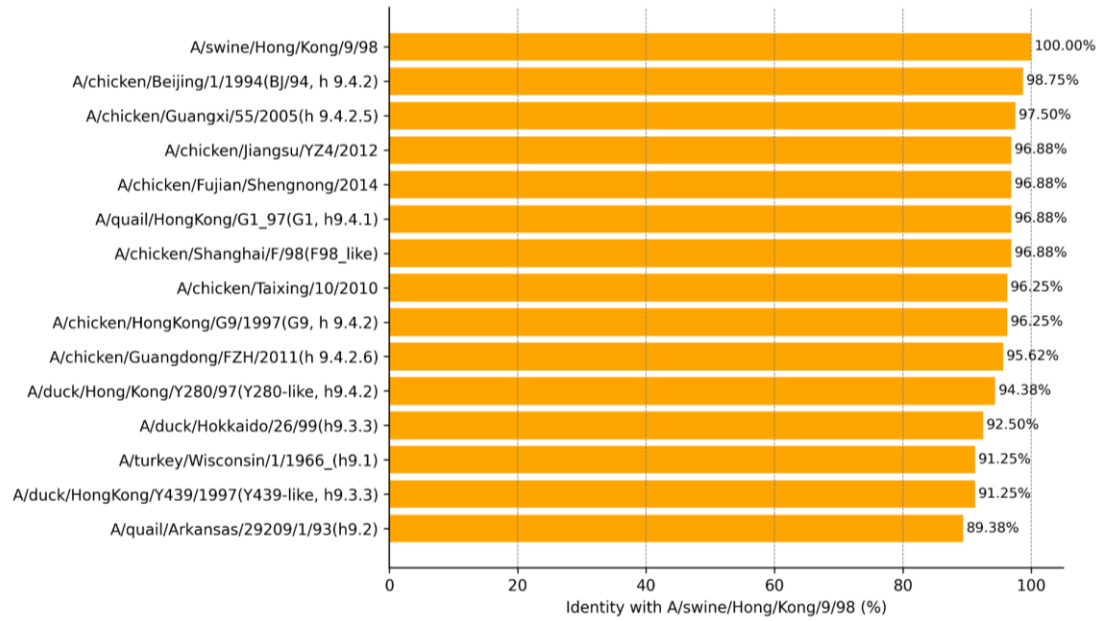
Supplementary Figure 4. Optimization of SnooPligase connection reaction conditions. a. Identification of SnooPligase coupling capability. b. pH optimization. c-d. Reaction time optimization. e. Glycine concentration optimization. f. SnooTagr substrate concentration optimization. g. DogTag substrate concentration optimization. h. SnooTagr and DogTag dual substrate concentration optimization. i. SnooPligase concentration optimization.



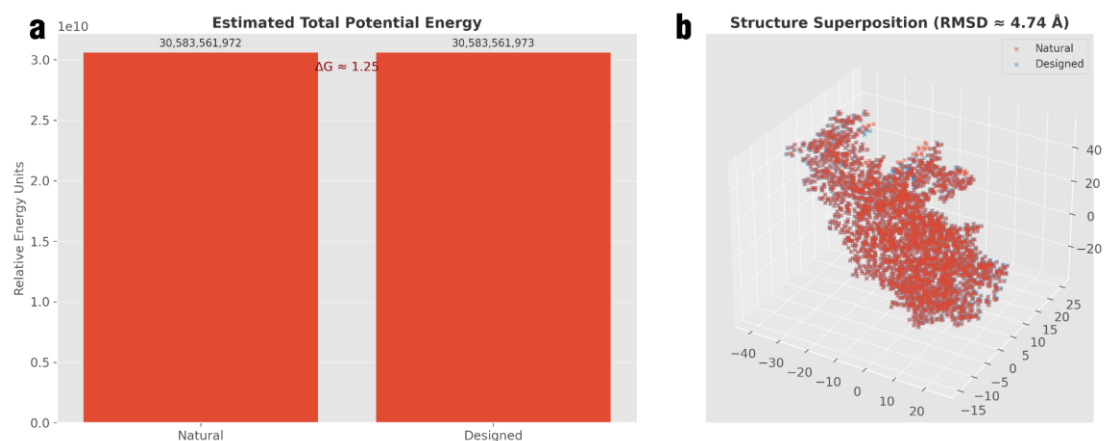
Supplementary Figure 5. ELISPOT determination of INF- γ results.



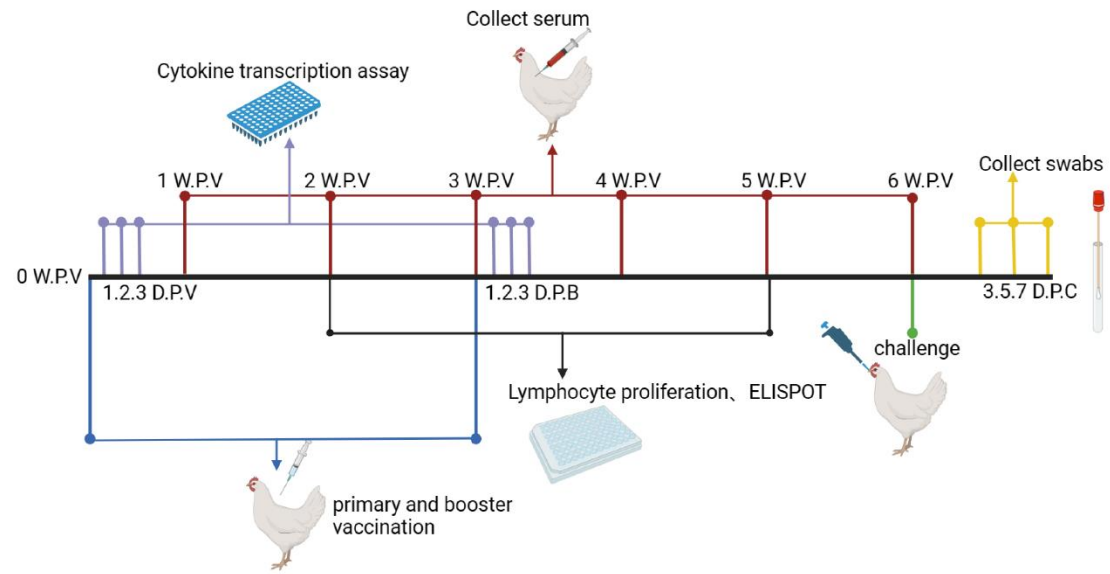
Supplementary Figure 6. Phylogenetic evolutionary assays of H9 HA amino acids.



Supplementary Figure 7. HA2 Amino Acid Sequence Identity with A/swine/Hong/Kong/9/98 The HA2 region of the HA6 immunogen shows high sequence identity with strains across multiple H9N2 sublineages, including the F98-like, Y280-like, and the G1-like lineage. HA6 shares 96.88% amino acid sequence identity with the G1-like strain. This high conservation supports the potential for cross-lineage protection by the fPE-HA6 vaccine.



Supplementary Figure 8. Structural and thermodynamic comparison of native and designed HA6. a. Estimated total potential energy indicates minimal Gibbs free energy difference between the wild-type and designed HA6 variants ($\Delta G \approx 1.25$). b. Structural superposition of all backbone atoms reveals a RMSD of ~ 4.74 Å, confirming high structural similarity post-design.



Supplementary Figure 9. Immunization process. Week post-vaccine, W. P. V. Day post-vaccine, D. P. V. Day post-booster, D. P. B. Day post-challenge, D.P.C.

Supplementary Table 1. Amino Acid Residues Involved in HA1 and HA2 Interaction

HA1		HA2	
Amino acid position	Amino acid motif	Amino acid position	Amino acid motif
1-9	DKICIGYQS	326-327	IA
16-20	VDTLT	330-335	IEGGWP
24	V	337-338	LV
26	V	340-348	GWYGFQHSN
28	H	368	I
30	K	371-372	KV
32	L	375-376	IV
99	E	379-391	MNKQYGIIDHEFS
102-103	RS	394	E
255-258	HGRI	405-406	DD
260	K	409	Q
282-285	TLPF	412-413	WT
290-302	KYAFGNCPKYVGV	416-418	AEL
305-312	LKLAVGLR	420-425	VLENQ
313-316	VPA	427-408	TL
		431	H
		435	V
		438-439	LY
		442	V
		446	L
		453	D
		456-460	GCFEL
		462-464	HKC
		469	M
		472-473	IR

Supplementary Table 2. Newly Exposed Amino Acid Positions in HA6

HA1	Amino acid	ΔASA ($\text{\AA}^2$)	ΔASA (%)	The hydrophobicity of amino acids
34	His	23.54	75.69	-3.2
35	Thr	5.23	7.61	-0.7
36	Ser	49.95	72.88	-0.8
281	Thr	69.35	200.67	-0.7
286	His	11.24	1628.99	-3.2
287	Asn	4.74	13.49	-3.5
288	Val	84.69	1036.60	4.2
289	Ser	29.91	347.39	-0.8
291	Tyr	25.12	40.54	-1.3
292	Ala	25.4	427.61	1.8
293	Phe	174.95	5831.67	2.8
294	Gly	28.07	106.73	-0.4
295	Asn	53.34	51.65	-3.5
296	Cys	12.52	108.40	2.5
297	Pro	11.16	117.47	-1.6
HA2	Amino acid	ΔASA ($\text{\AA}^2$)	ΔASA (%)	The hydrophobicity of amino acids
384	Gly	8.93	13.15	-0.4
385	Ile	6.15	211.34	4.5
386	Ile	57.2	103.96	4.5
387	Asp	7.62	7.87	-3.5
388	His	8.97	10.15	-3.2
389	Glu	121.49	231.06	-3.5
390	Phe	5.07	10.91	2.8
391	Ser	21.45	66.93	-0.8
392	Glu	6.8	10.94	-3.5

Supplementary Table 3. Average genetic distance of HK98, YZ4 and SN

Viruses name	Inter-group average genetic distance				
	HK98	YZ4	SN	Group 1	Group 2
HK98		0.042	0.067	0.084	0.110
YZ4			0.071	0.082	0.106
SN				0.055	0.072

Supplementary Table 4. CR6261 amplification primers

Primers	Sequence 5'-3'
CR6261-SCFV-F	tcttgca ^{ctt} gtcacga ^{attc} GGTACA ^{ACT} AGTCGAGTCAGGAGCT
CR6261-SCFV-R	ctctgggagatcta ^{acct} ggCTAAGACGGTCAATTTTCGTGCC

Annotation: Lowercase letters represent homologous arms.

Supplementary Table 5. HA2 and HA6 amplification primers

Primers	Sequence 5'-3'	Restriction endonucleases
HA6-F	CGC <u>GGATCC</u> GGTCTGTTTCGGCGCGAT	<i>Bam</i> HI
HA6-R	CCC <u>AAGCTT</u> ACGCGCCGGAACGTTAC	<i>Hind</i> III
HA2-F	CGC <u>GGATCC</u> GGTCTGTTTCGGCGCGAT	<i>Bam</i> HI
HA2-R	CCC <u>AAGCTT</u> TTTTCTGACGTTCCAGTTTGCTT	<i>Hind</i> III

Annotation: Underlined regions indicate enzyme cleavage sites.

Supplementary Table 6. Primers for constructing expression plasmids of Snoopligase, SnoopTagjr-EGFP, and IMX-DogTag

Primers	Sequence 5'-3'	Restriction endonuclease
BamHI-Snoopligase-F	CGCGGATCCAGCAGCGGCGGCCTGA	BamHI
Snoopligase-HindIII-R	CCCAAGCTTGGTAAATTCATATGTAGCCGGCACA	HindIII
EcoRI-IMX-F	CCGGAATTCATGGGCAGCAGCAAAAAACA	EcoRI
IMX-HindIII-R	CCCAAGCTTTTTTCGGCGGTATCGGTTCA	HindIII
Overlap-SnoopTagjr-EGFP-F	<i>aaactgggcagcatcgaattcatcaaagtgaacaaaggtagtggtagaagtgtgggtcaggg</i> ATGGTGAGCAAGGGCGAGG	
Overlap-SnoopTagjr-EGFP-R	CTTGACAGCTCGTCCATGCC	
TY-SnoopTagjr-EGFP-F	cagcaaatgggtcgcggatccAAACTGGGCAGCATCGAATTC	
TY-SnoopTagjr-EGFP-R	ctcgagtgcggccgcaagcttCTTGACAGCTCGTCCATGCC	

Annotation: 1 Underlined sequences represent enzyme recognition sites. 2 Italicized lowercase sequences denote SnoopTagjr-linker sequences. 3 Lowercase sequences represent homologous arms.

Supplementary Table 7. Primers for constructing expression plasmids for HA6, and fPE

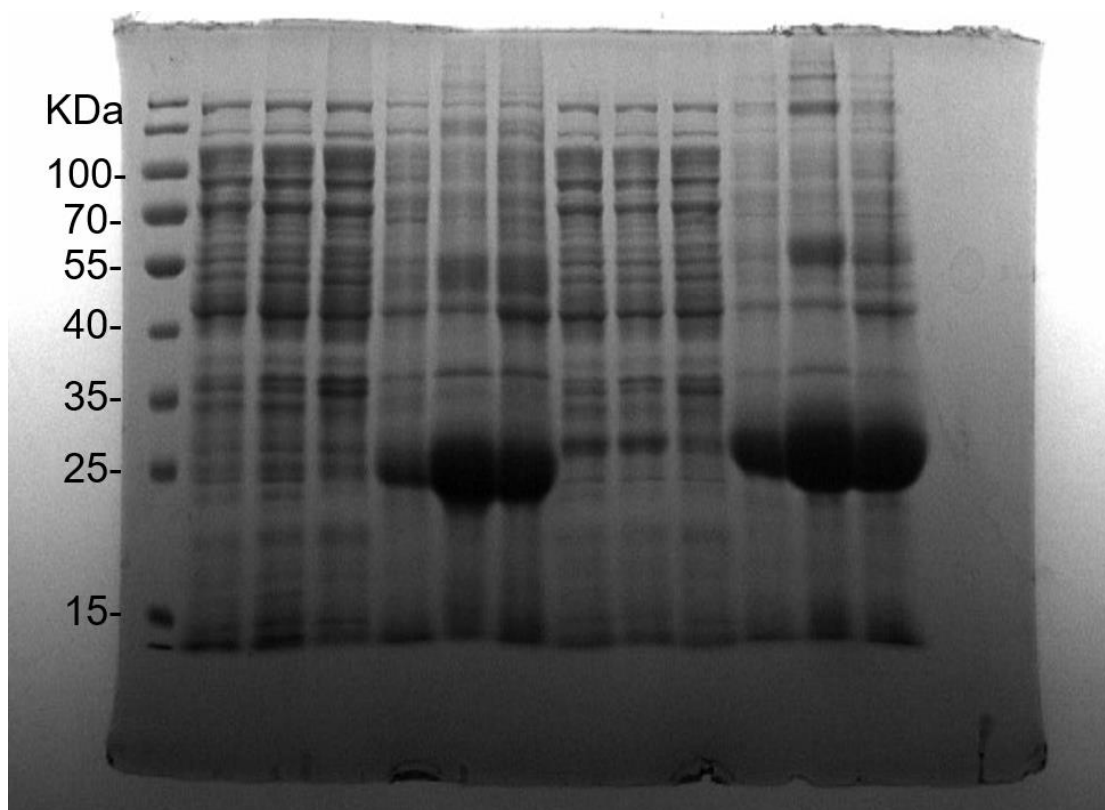
Primers	Sequence 5'-3'	Comments
DogTag-I-HA6-F	<u>ccaacgagccgattccaccgaaaggtagtggtgaaagtgggtgggtcag</u> ggGGTCTGTTCGGCGCGAT	Addition of DogTag to the N-terminus of HA6
DogTag-II-HA6-F	<u>acattccggcaacgtacgaatttaccgacggtaaacactacatta</u> CCA	
HA6-R	ACGAGCCGATTCCAC	
fPE-sTag-F	TTTTACCCAAGTTATCACTCAACAC	Addition of SnoopTagjr to the C-terminus of fPE
fPE-sTag-I-R	<u>gccggaaccctcaccggagccagagcc</u> GTTACCATCTGAC	
fPE-sTag-II-R	TTGATACGCAGT <u>tcatttggtcactttgatgaattcgatgctgccagttt</u> GCCGGAACCTCACCG	
TY-pET28-F	CACCACCACCACCACCACTG	Amplification procedures were conducted for the pET plasmid, intended for homologous recombination
TY-pET28-R	GGATCCGCGACCCATTTG	
TY-pET32-F	GATCCGGCTGCTAACAAAGC	
TY-pET32-R	GGATCCGATATCAGCCATGGC	
TY-DogTag-HA6-F	agcaaatgggtcgcggatccGACATTCCGGCAACGTAC GA	Amplification was conducted for HA6 and fPE fragments, each specifically designed for the incorporation of either DogTag or SnoopTagjr
TY-DogTag-HA6-R	cagtgggtggtggtggtgACGCGCCGGAACGTTACG	
TY-fPE-F	ccatggctgatcggatccTTTTACCCAAGTTATCACTCAACACC	
TY-fPE-R	gctttgttagcagccggatcTCATTTGTTCACTTTGATGAATTCG	

Supplementary Table 8. Immunization groups

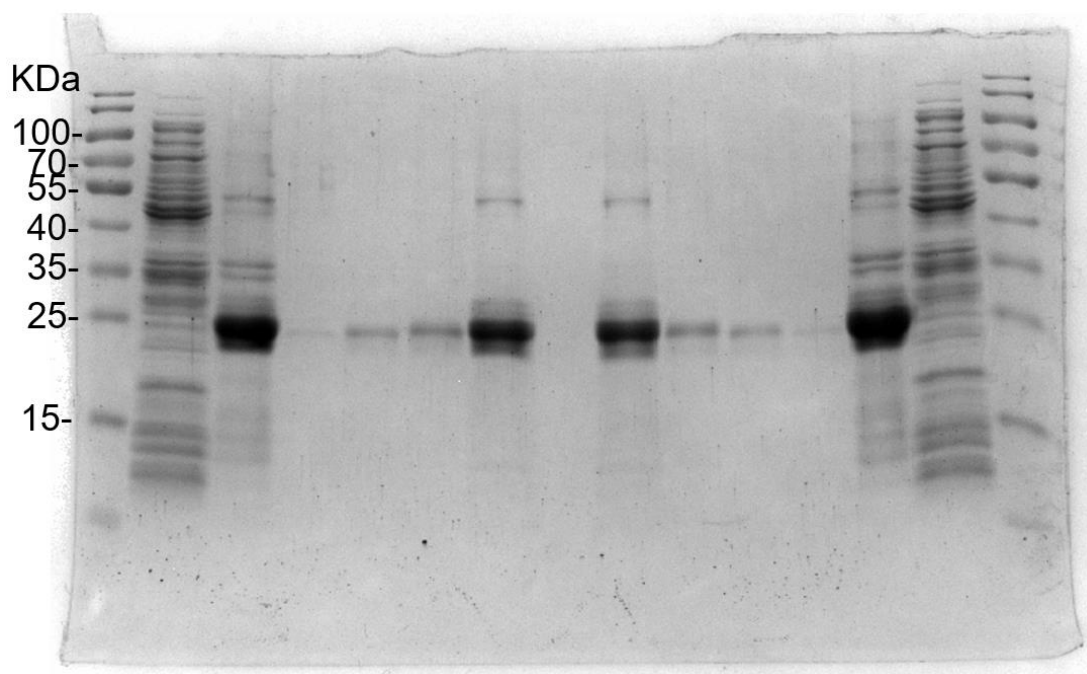
Immunization group	Challenge viruses	Challenge dose EID ₅₀ (Log ₁₀)
fPE-HA6 100 µg	YZ4	6
fPE-HA6 100 µg	SN	6
fPE 100 µg	YZ4	6
fPE 100 µg	SN	6
HA6 100 µg	YZ4	6
HA6 100 µg	SN	6
TX	YZ4	6
TX	SN	6
PBS	YZ4	6
PBS	SN	6

Supplementary Table 9. Real-Time Quantitative RT-PCR primers and probes

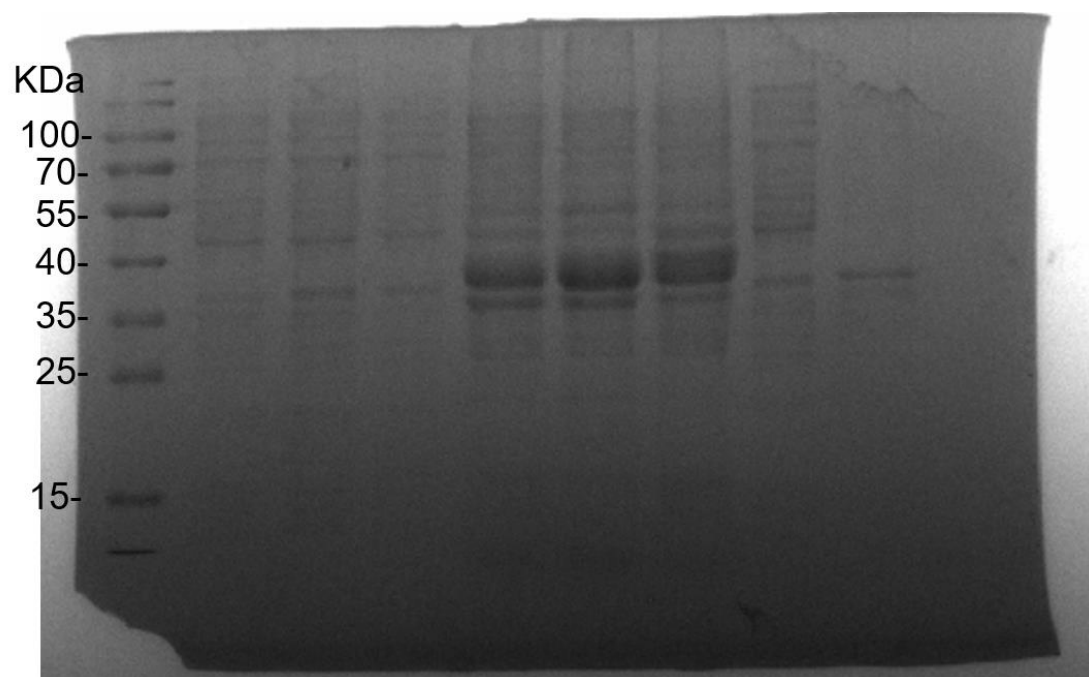
Primers and probes	Sequence 5'-3'
TaqMan M-R	CTTTAKCCAYTCCATGAGAGC
Taqman M-F	CTTCTAACCGAGGTCGAAACG
M-Probe	FAM-CCTCAAAGCCGAGATC-MGB
IL-2 F	TTCTGGGACCACTGTATGCTCTT
IL-2 R	TACCGACAAAGTGAGAATCAATCAG
IL-4 F	TGCACCCAGAAAAGCACAGA
IL-4 R	AGCTGACGCATGTTGAGGAA
IL-5 F	TGAGCACATCAGGACCATGAG
IL-5 R	AGGGATGGCCTCAGTTTGTC
IFN- γ F	GGGTGGGCCTCTTTTCTCAG
IFN- γ R	CTGCAGATCATCCACCGGAA
β -actin F	ATGAAGCCCAGAGCAAAAGA
β -actin R	GGGGTGTTGAAGGTCTCAA



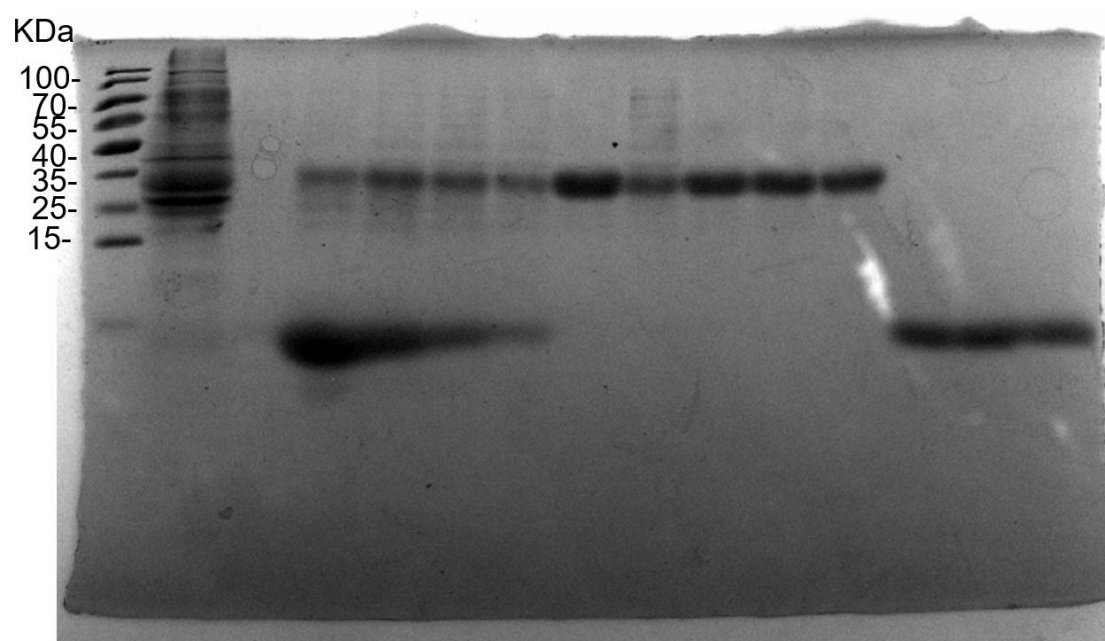
Raw SDS-PAGE for Figure 1d



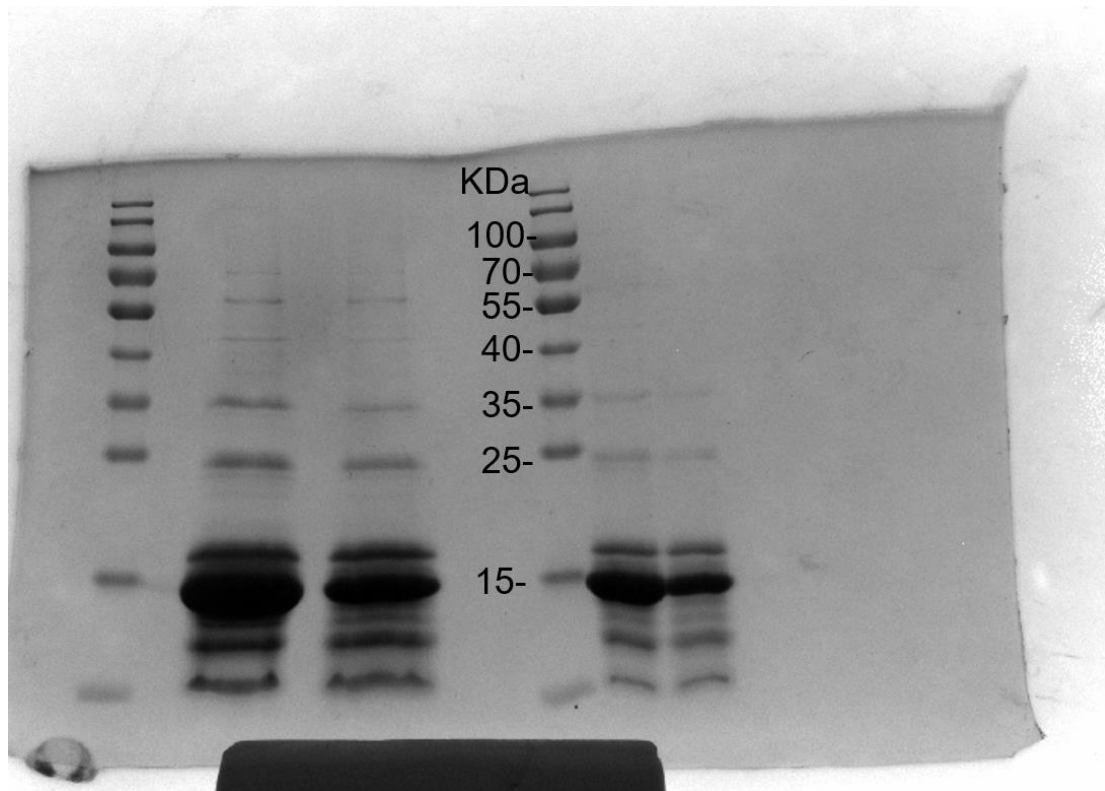
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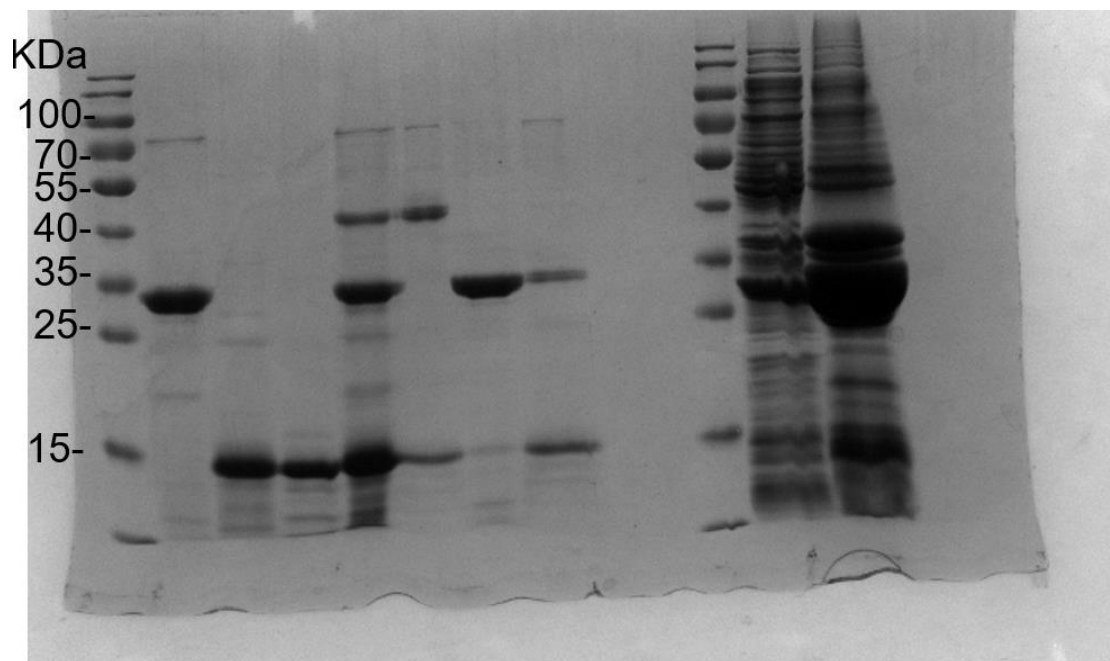
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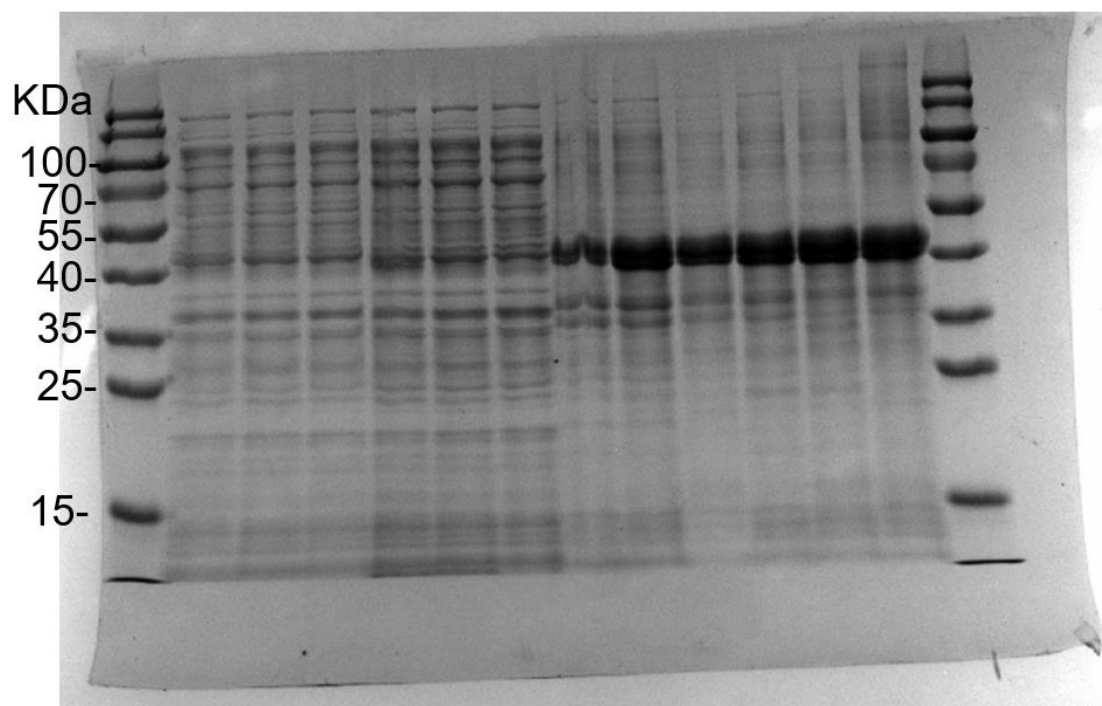
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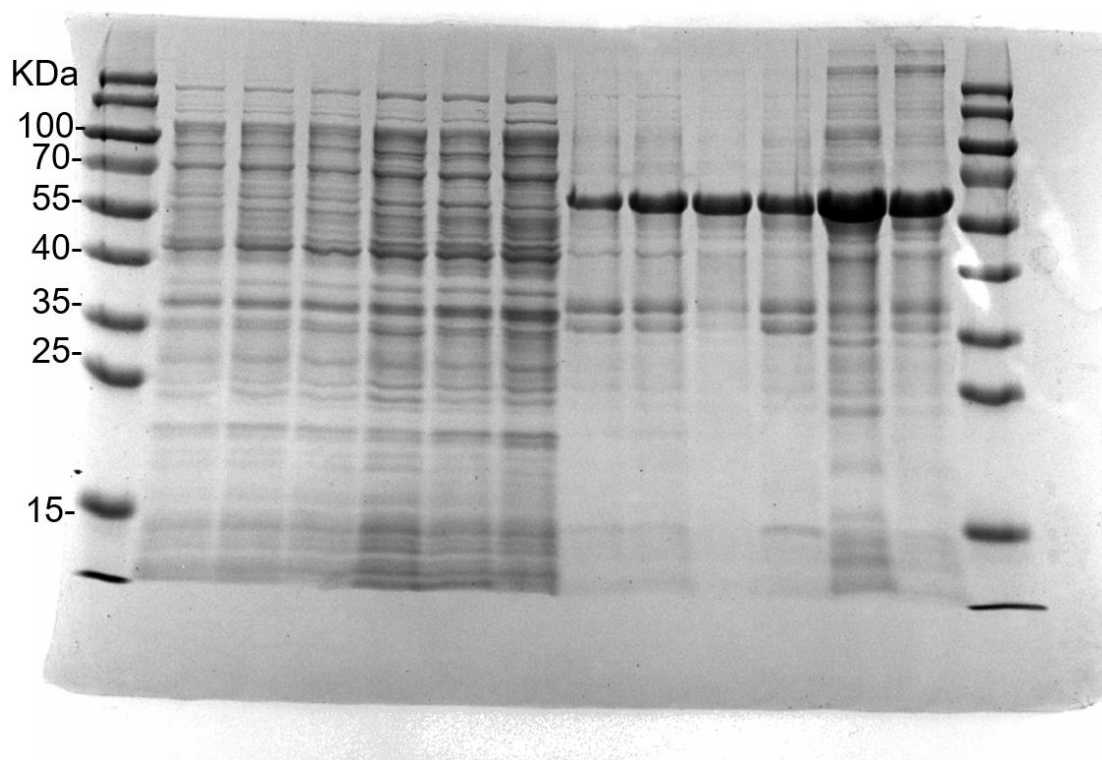
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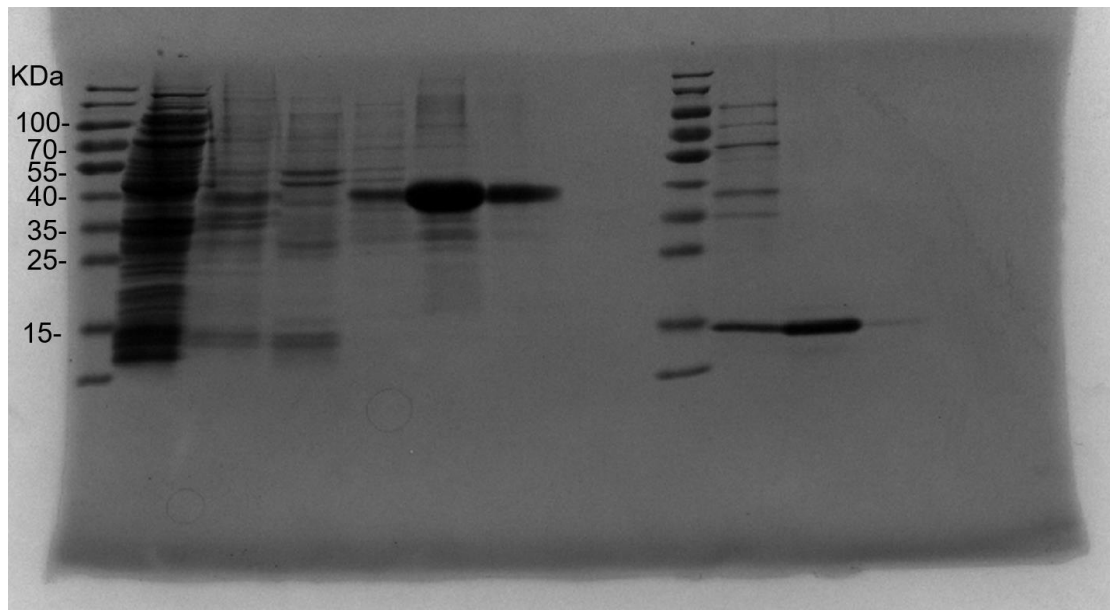
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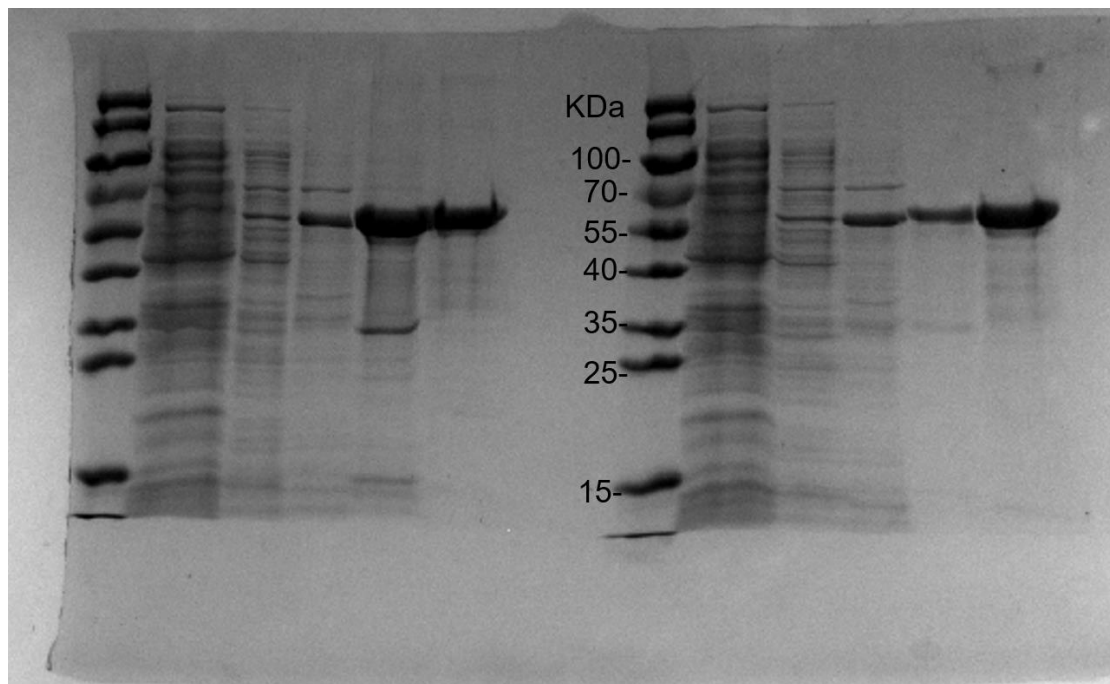
Raw SDS-PAGE for Figure 3a



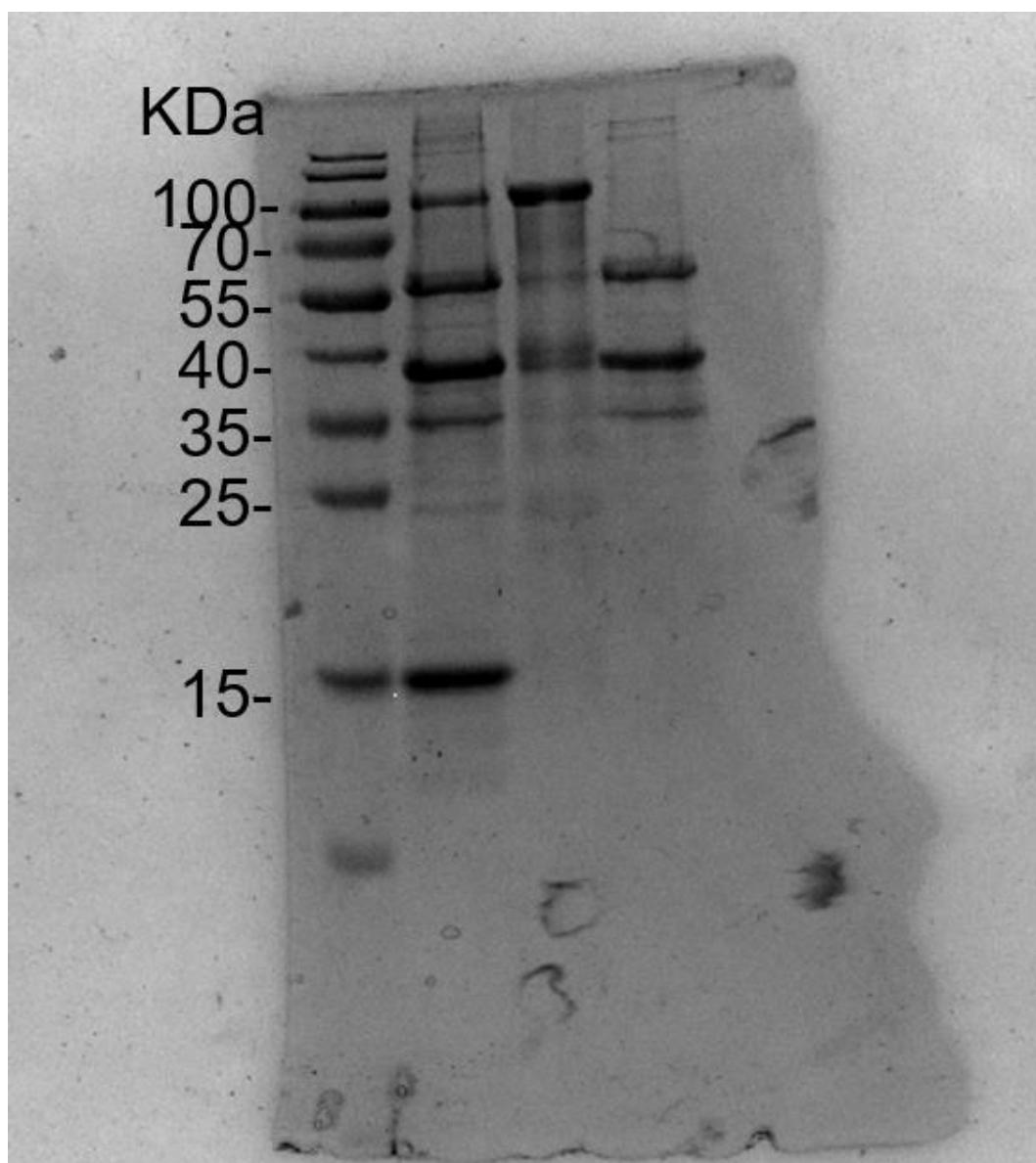
Raw SDS-PAGE for Figure 3b



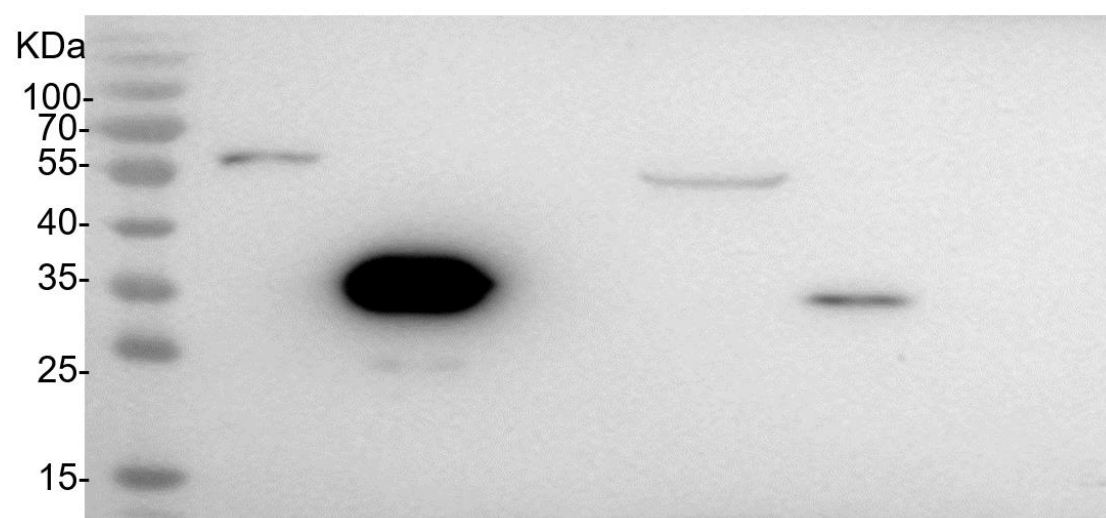
Raw SDS-PAGE for Figure 3c



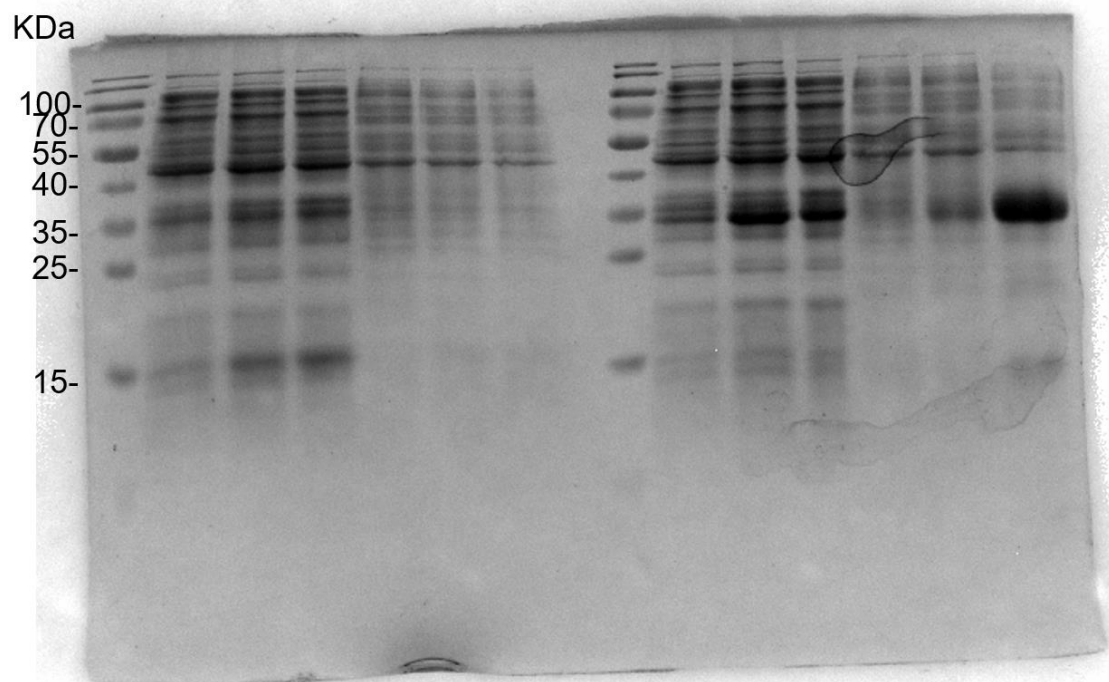
Raw SDS-PAGE for Figure 3d



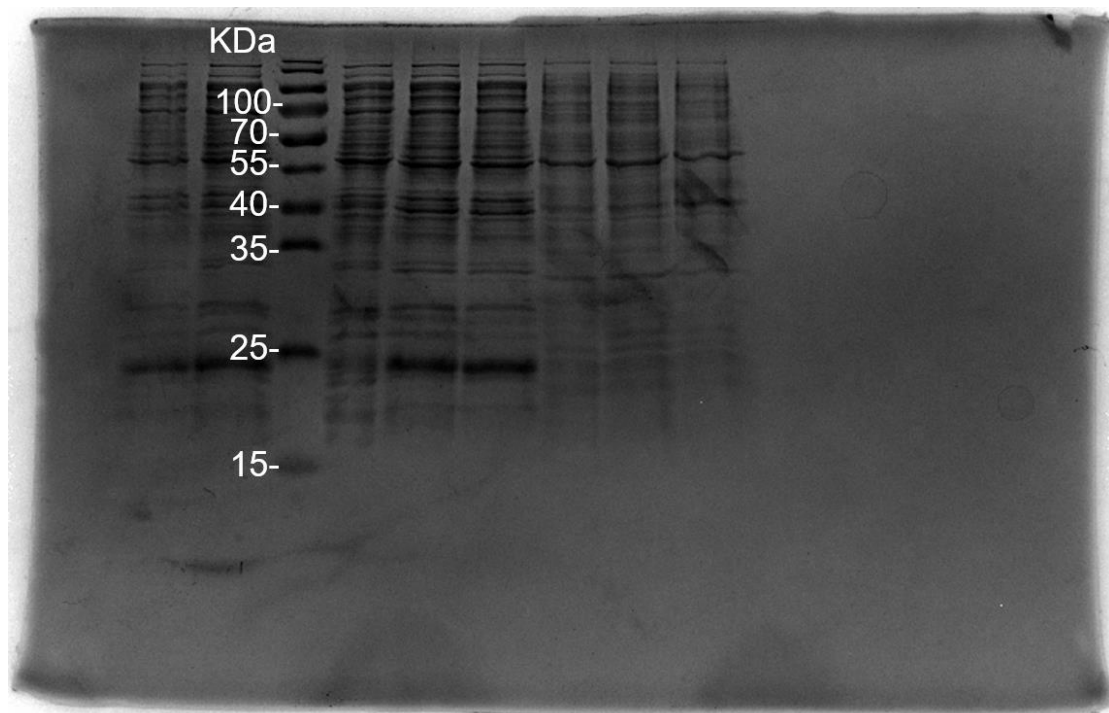
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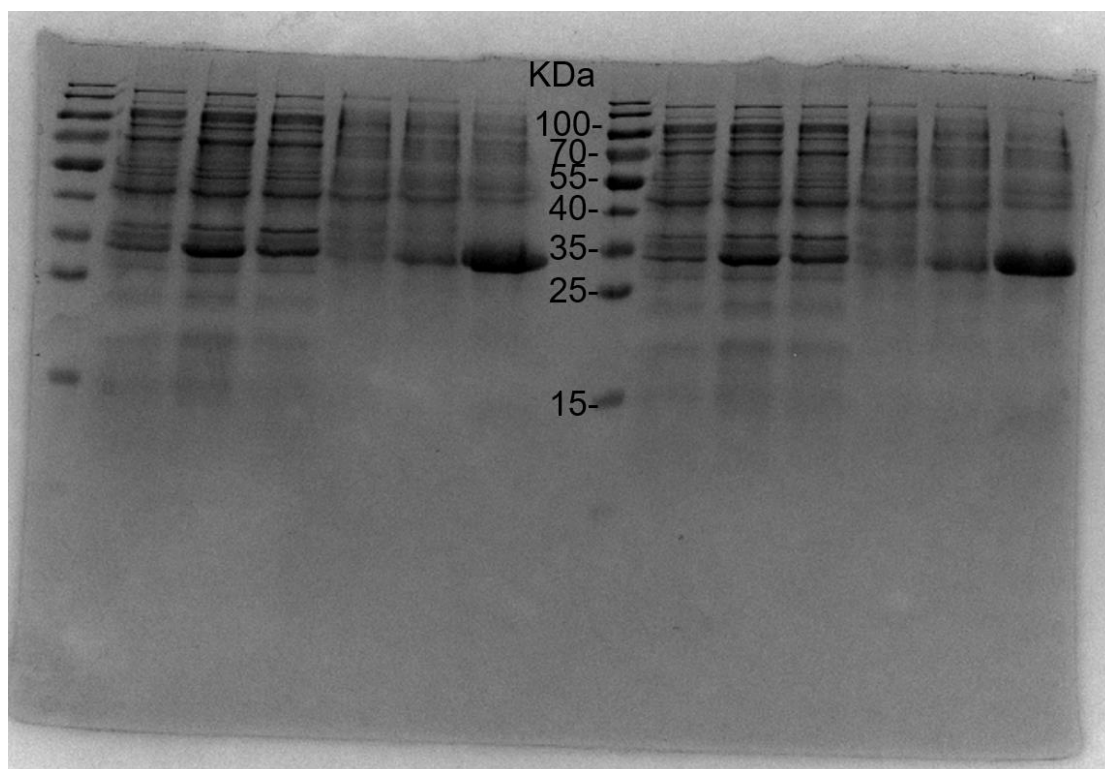
Raw blot for Supplementary Figure 1 b



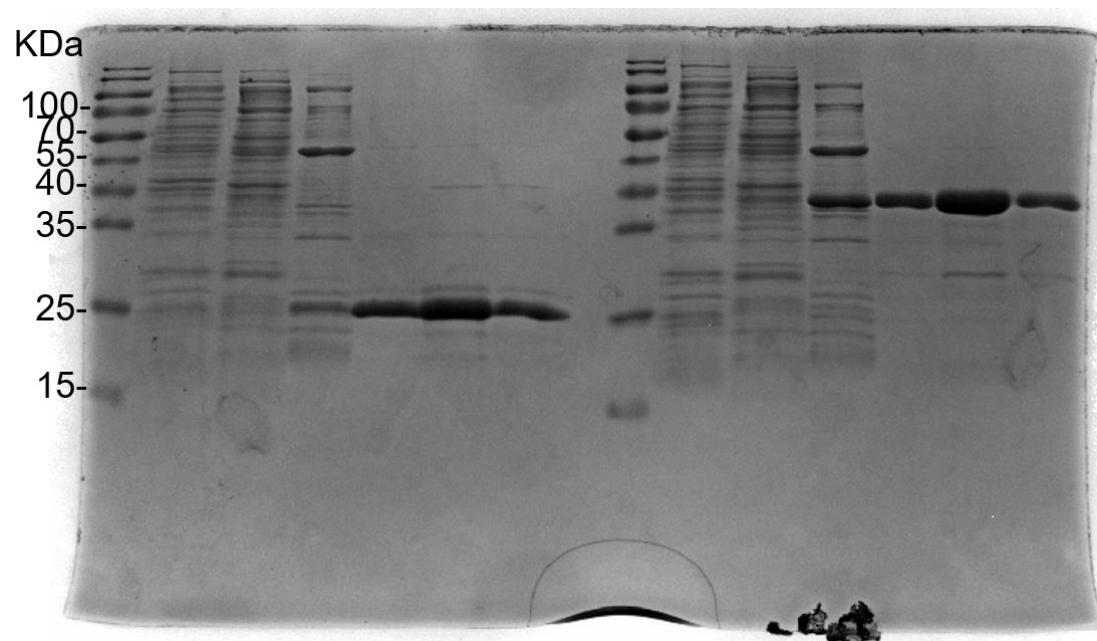
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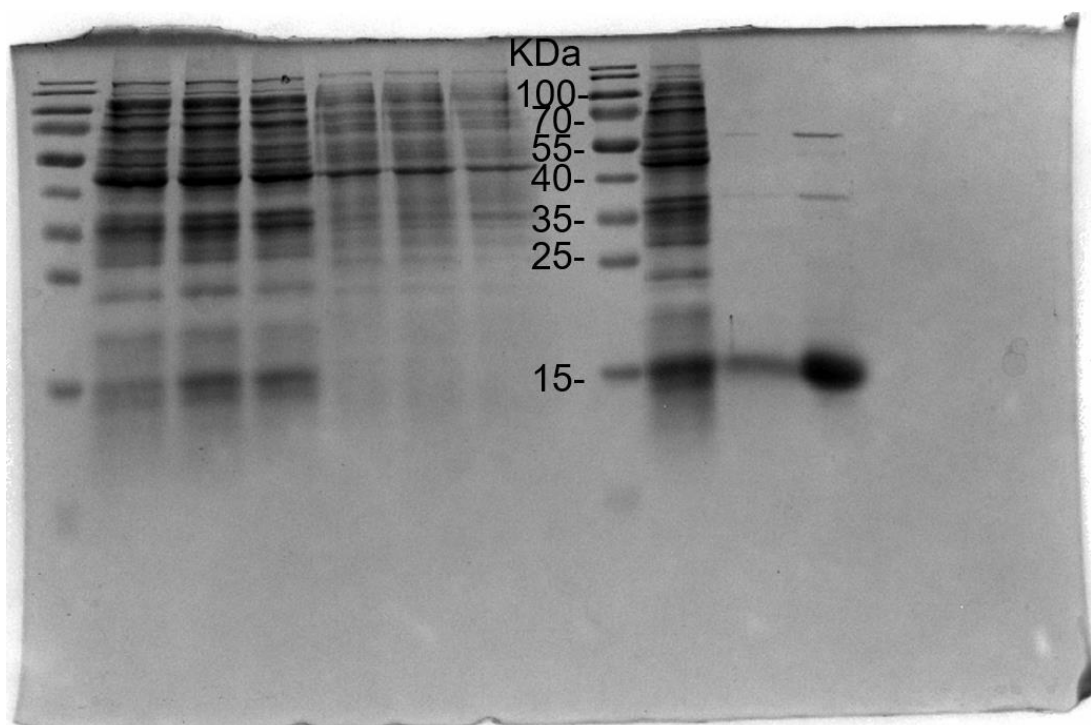
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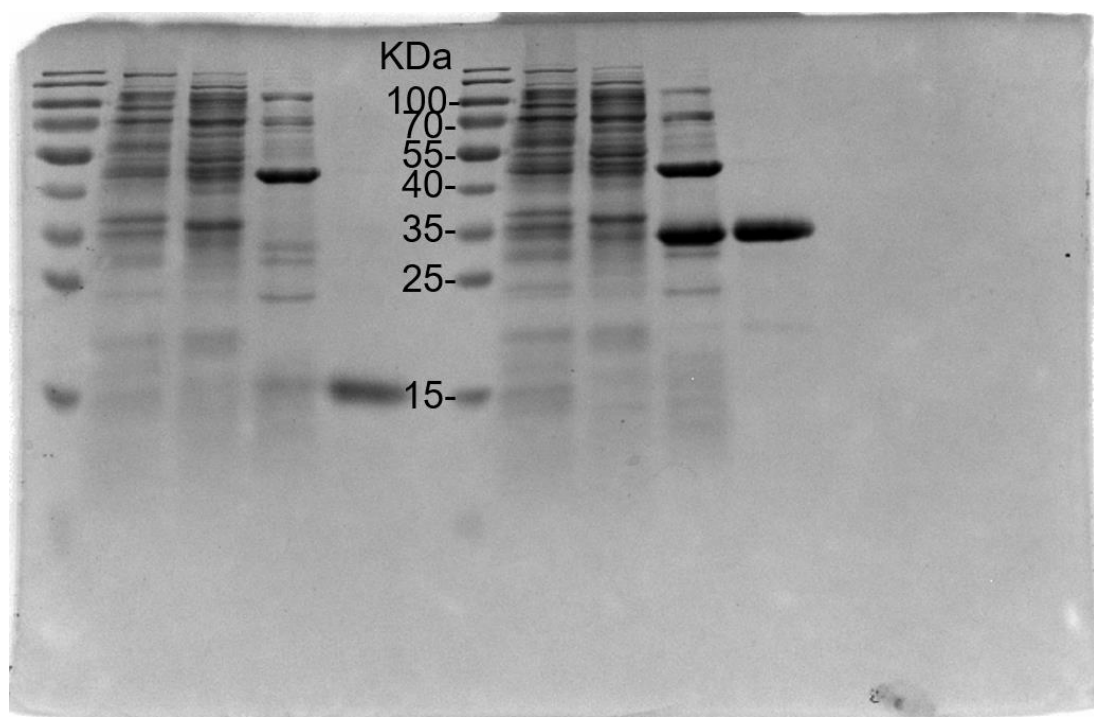
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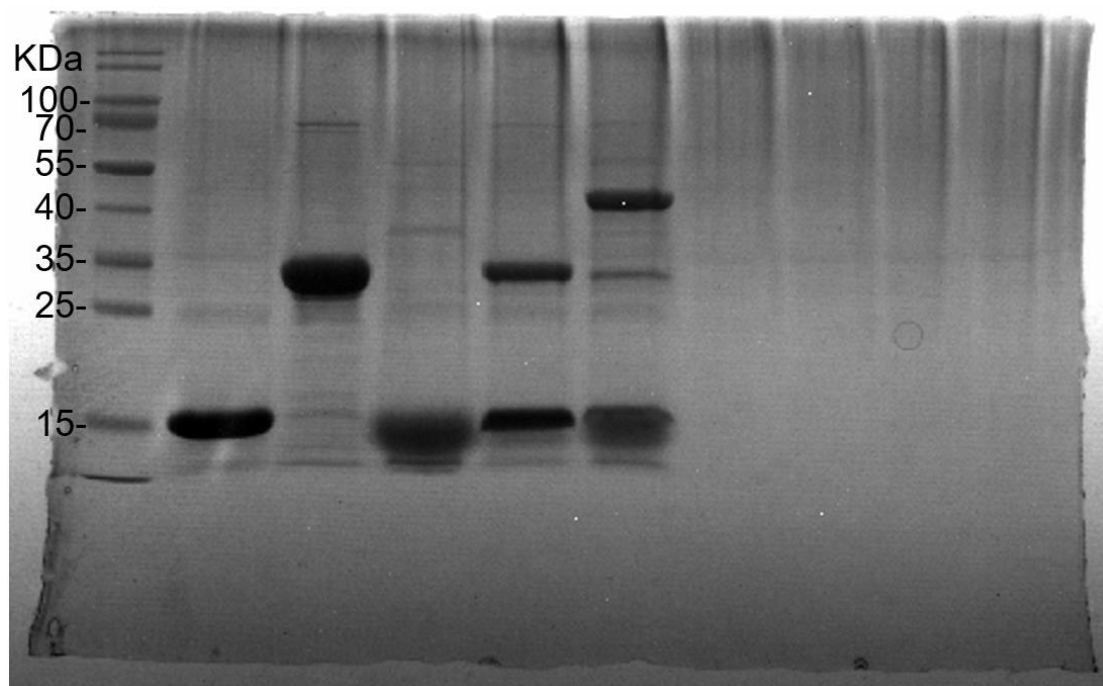
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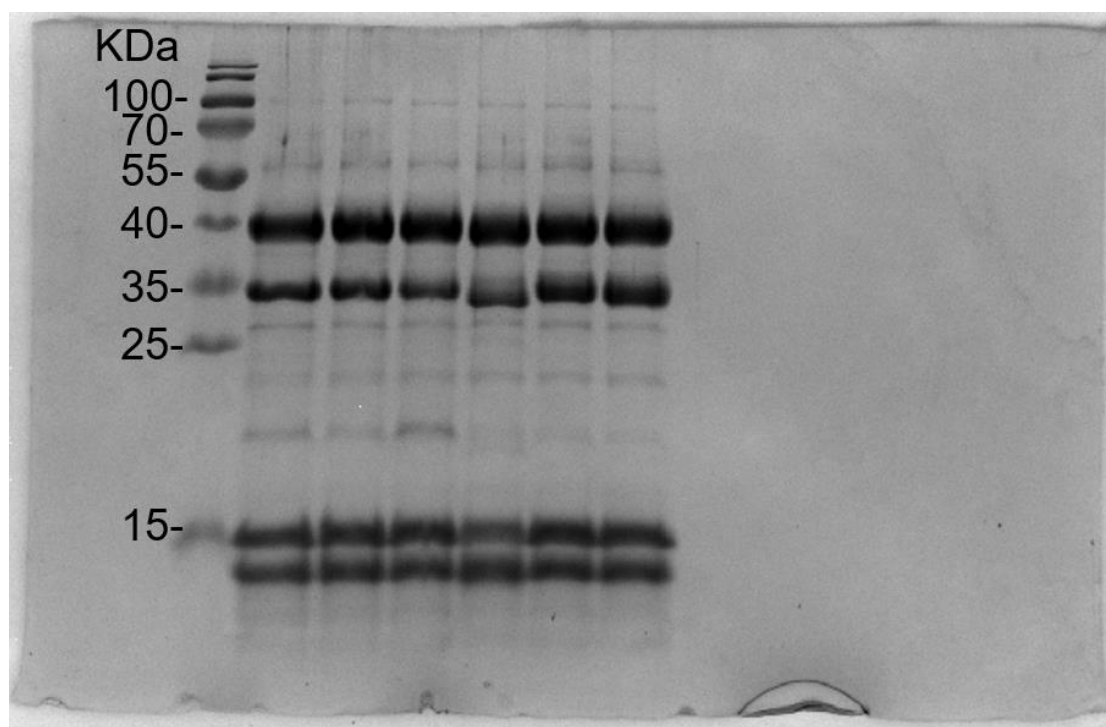
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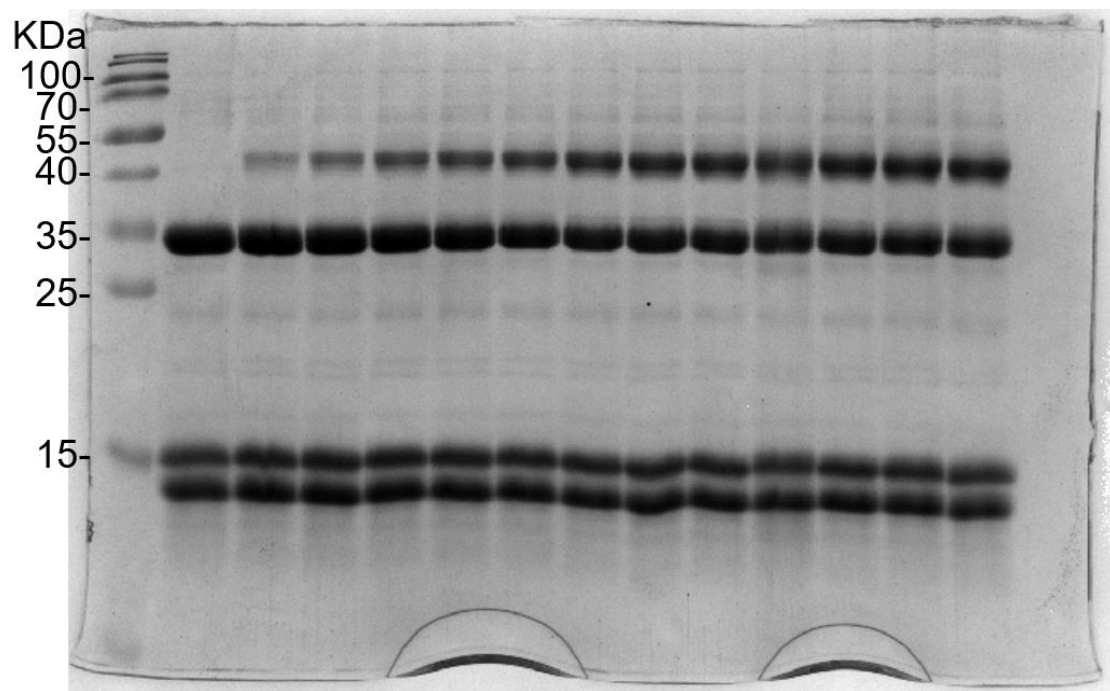
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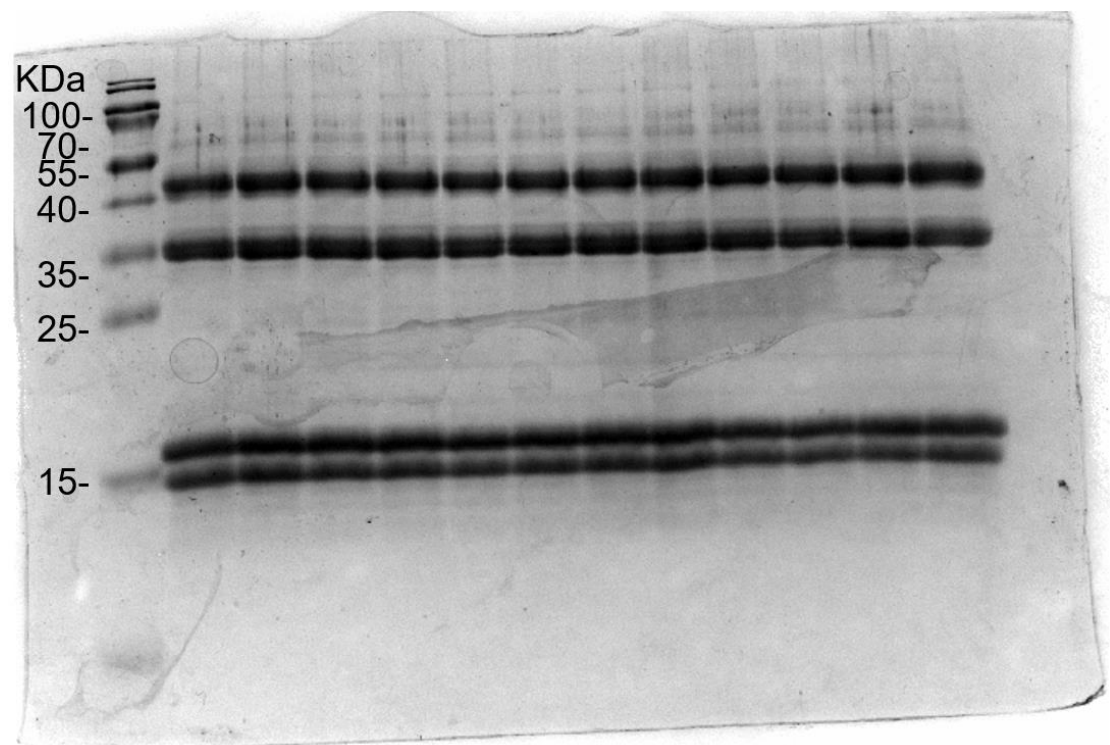
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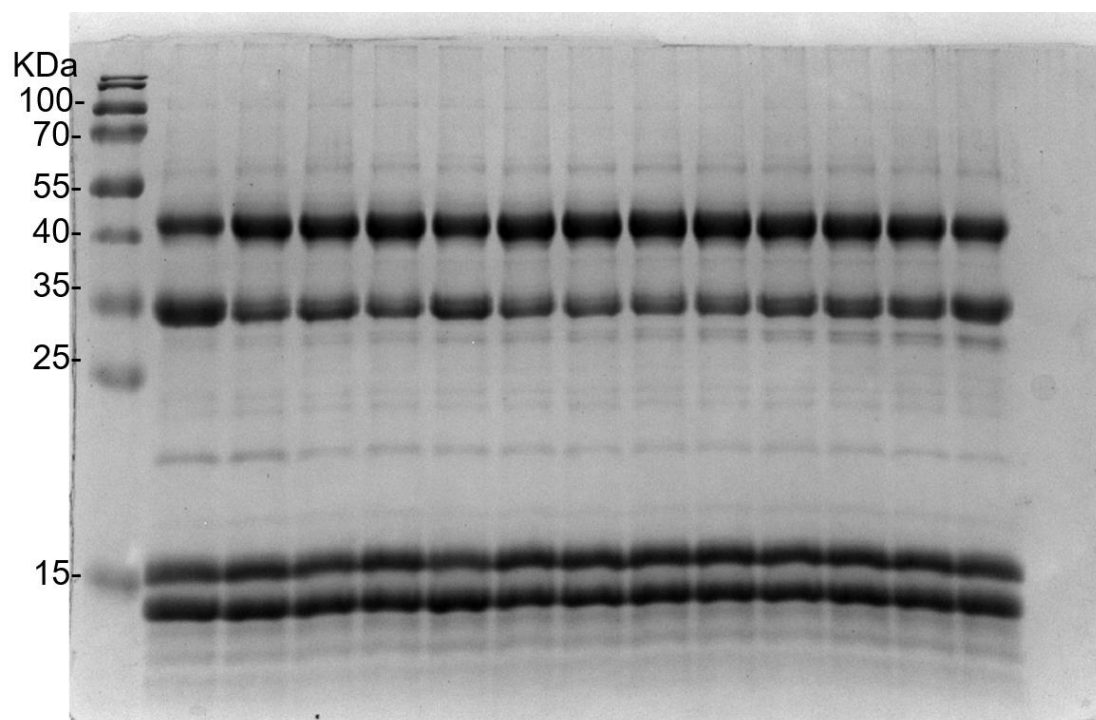
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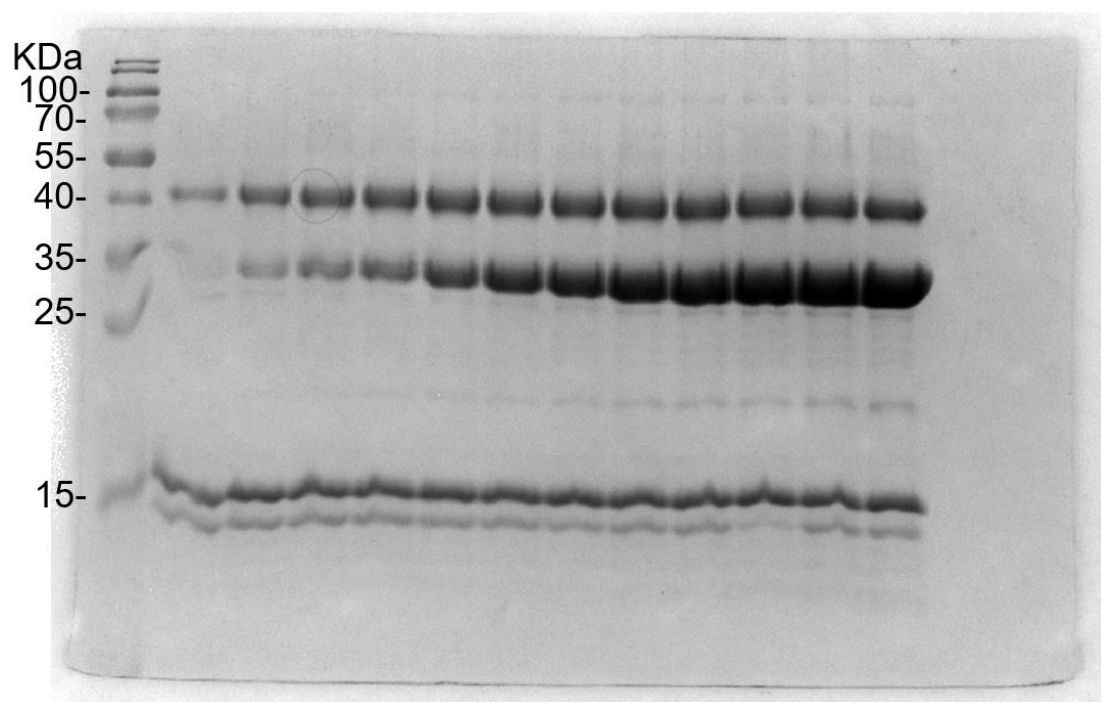
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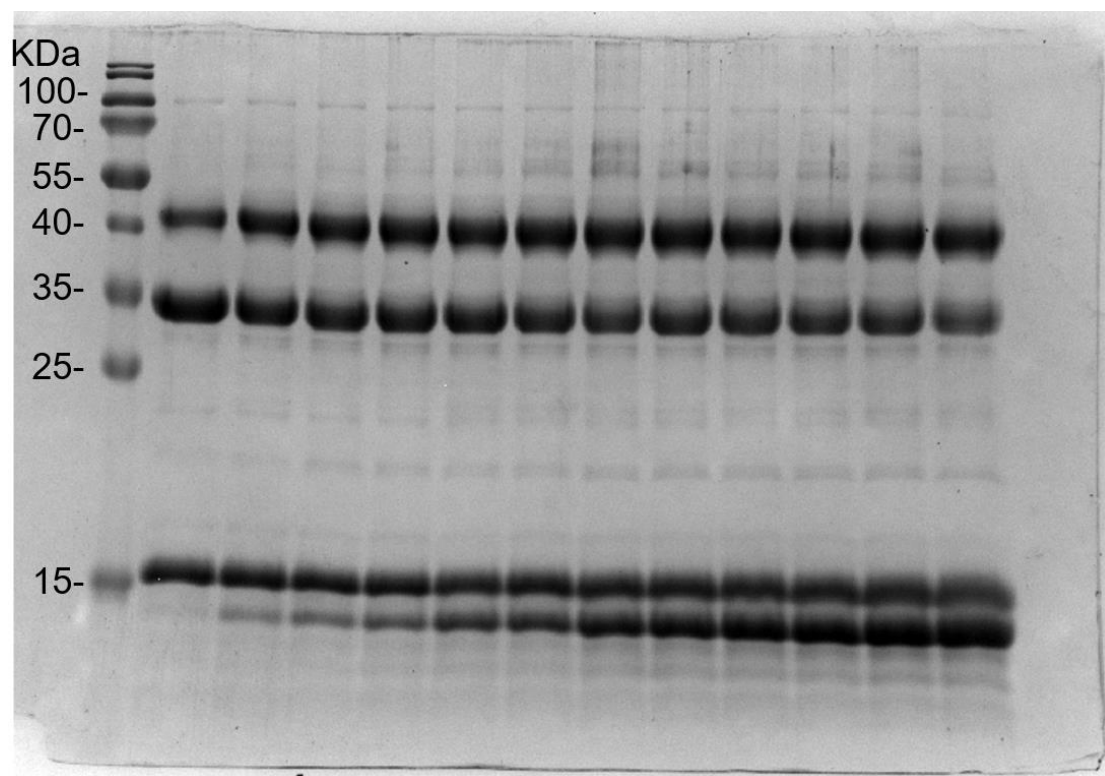
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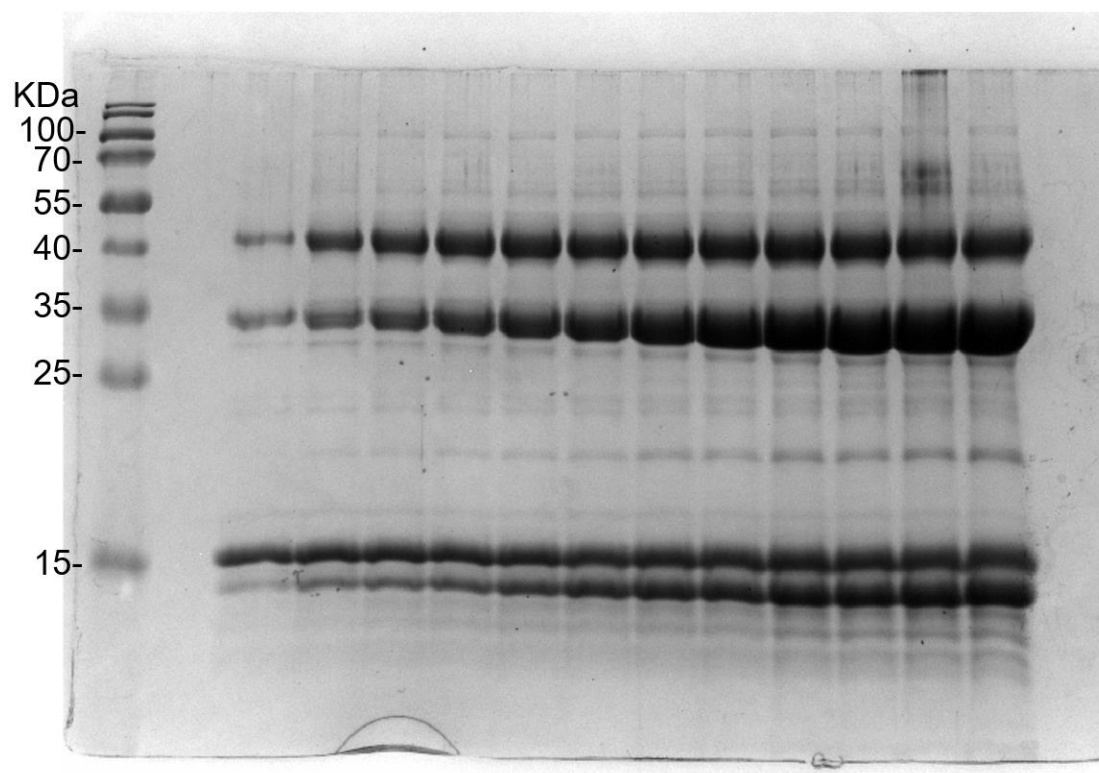
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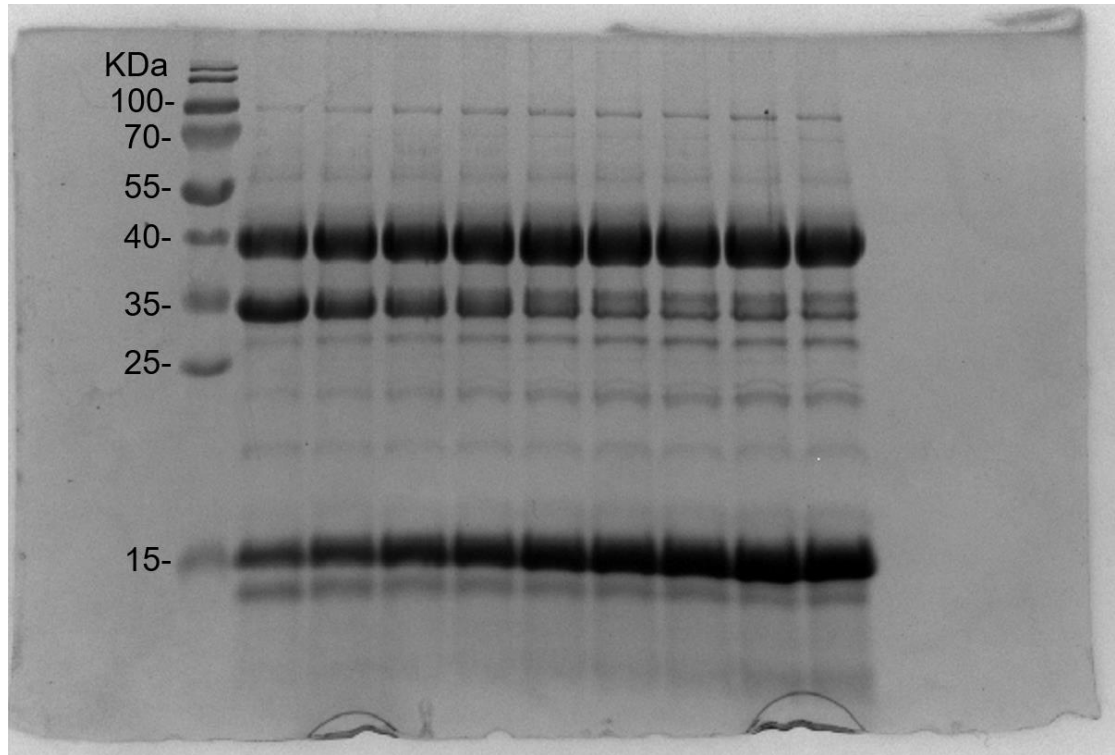
Raw SDS-PAGE for Supplementary Figure 4f



Raw SDS-PAGE for Supplementary Figure 4g



Raw SDS-PAGE for Supplementary Figure 4h



Raw SDS-PAGE for Supplementary Figure 4i