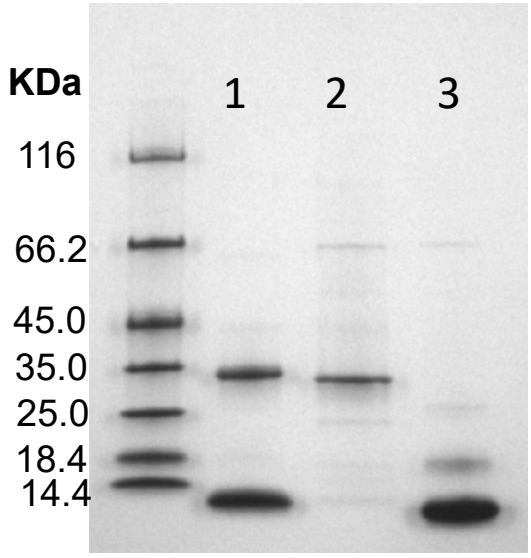
Supplementary Information



1. Stx2a holotoxin
2. Stx2a-A1 subunit
3. Stx2a B subunit

Amino acid sequences:

Stx2a holotoxin:

MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLR
LIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDA
SRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNC
HHQGARSVRAVNEESQPECQITGDRPVIKINNTLWESNTAAAFLNRKSQFLYTTGK

taaaggagttaagc

MKKMFMAVLFALASVNAMAADCAKGKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQSAQLTGMTVTIKSSTCESGSGFAE
VQFNNDLEHHHHHH

DNA sequence sandwiched between the A and B subunit
Signal peptide

Stx2a-A1:
MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLR
LIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDA
SRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNC
HHQGARSVRAVNEESQPEGGSLEHHHHHH

Stx2a-B:

MKKMFMAVLFALASVNAMAADCAKGKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQSAQLTGMTVTIKSSTCESGSGFAE
VQFNNDHHHHHH

Figure S1. An SDS-PAGE (4-15%) image of the purified proteins under reducing conditions and their protein sequences.

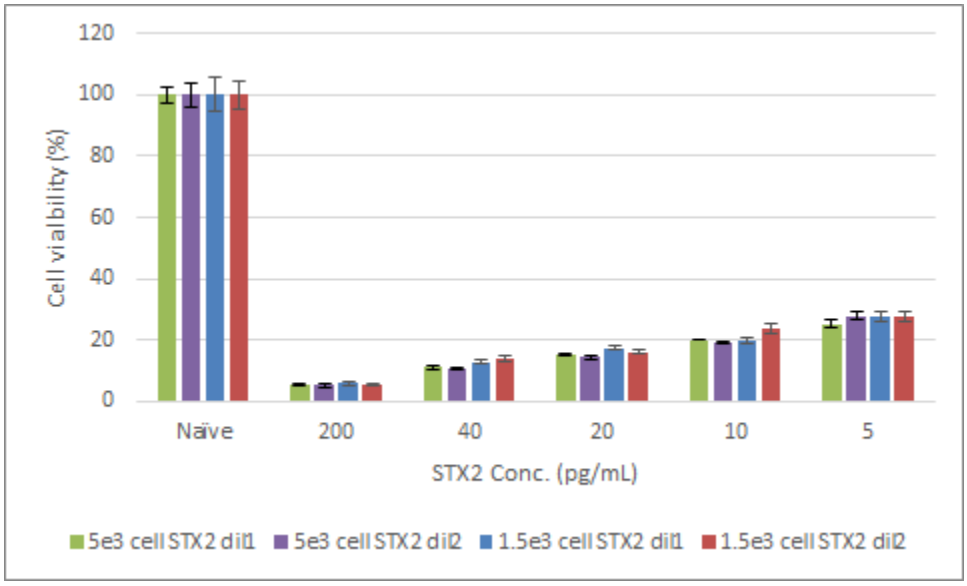


Figure S2. Toxicity of holotoxin Stx2a. Vero E6 cells ($1.5x10^3$ cells/well) were mixed with the appropriately diluted Stx2a and the mixture was added to 96-well plate. The cell viability was quantified 3 days later using the CellTiterGlo assay.

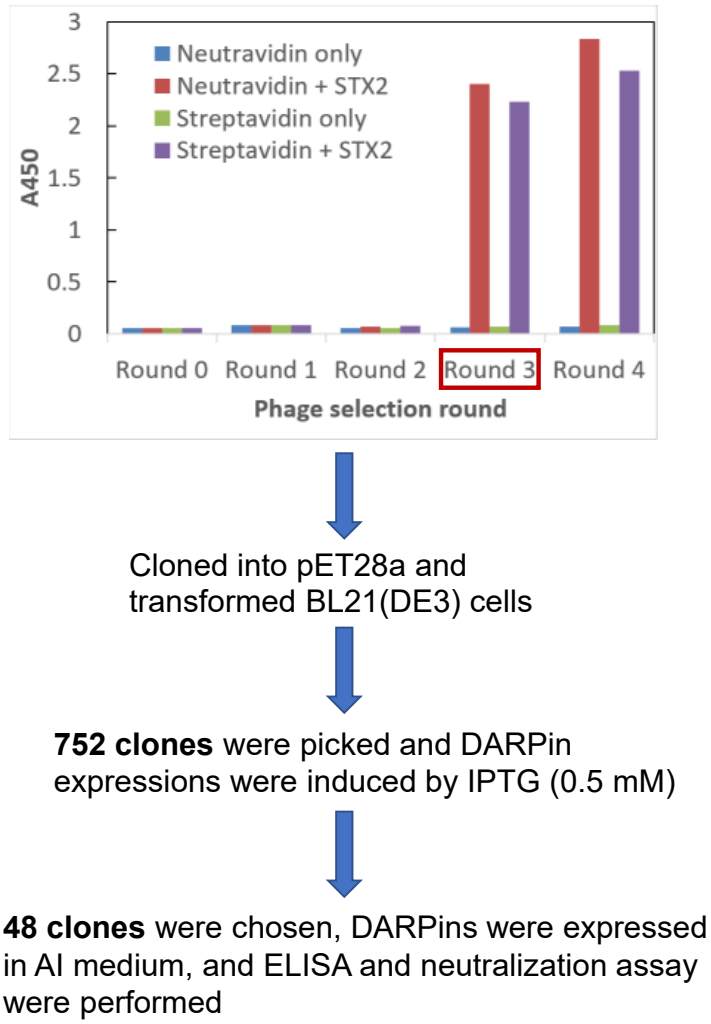


Figure S3. Enrichment of STX2-binding DARPins after phage panning. Successive rounds of a DARPin-phage library were panned against STX2 using streptavidin-coated magnetic beads coated with 100 nM biotinylated-STX2 and MaxiSorp 96-well plates coated with neutravidin (66 nM) and 100 nM biotin-STX2 in alternative rounds. The binding of phage recovered from successive rounds of panning to STX2 was quantified using ELISA. Significant binding was observed after three rounds of panning.

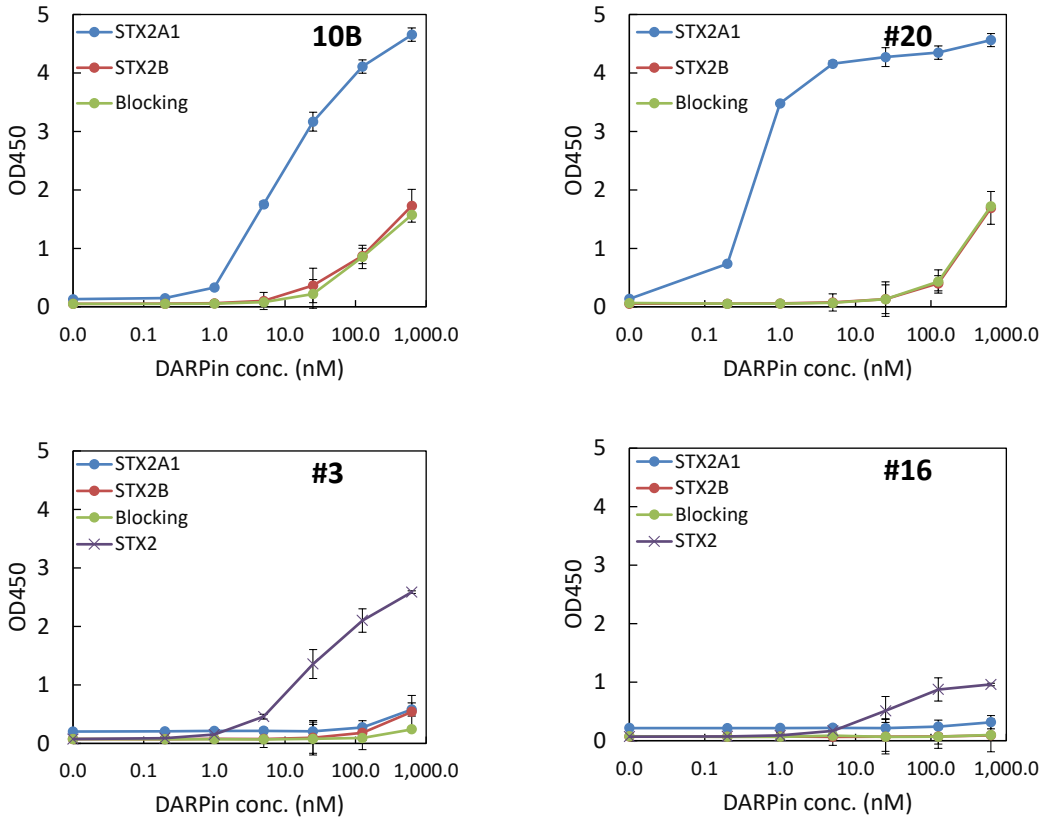
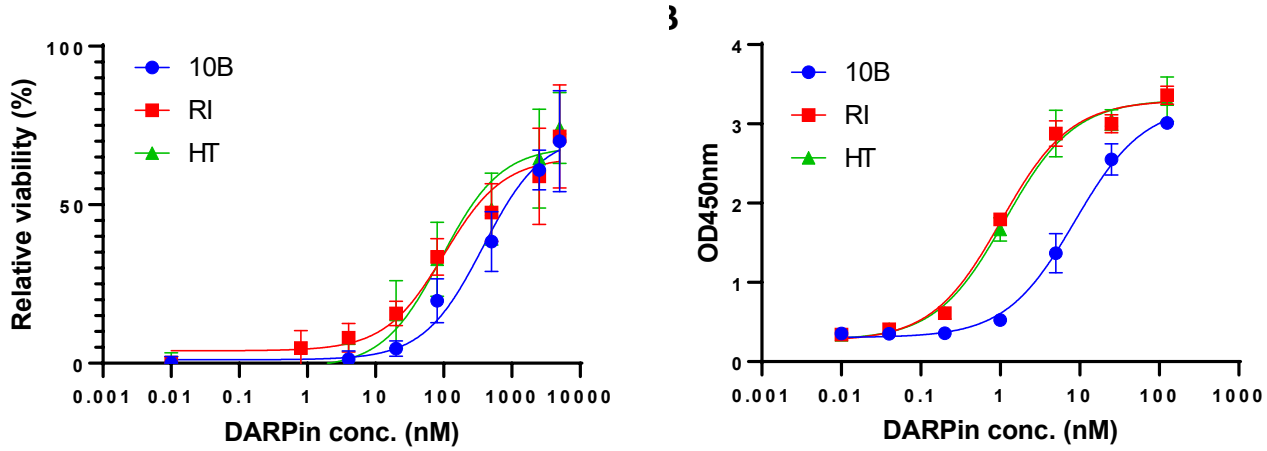


Figure S4. ELISA-based assay to evaluate the ability of monomeric DARPins to bind the A- and B-subunits of Stx2a. The error bars represent the mean deviation of duplicate samples from one representative experiment.



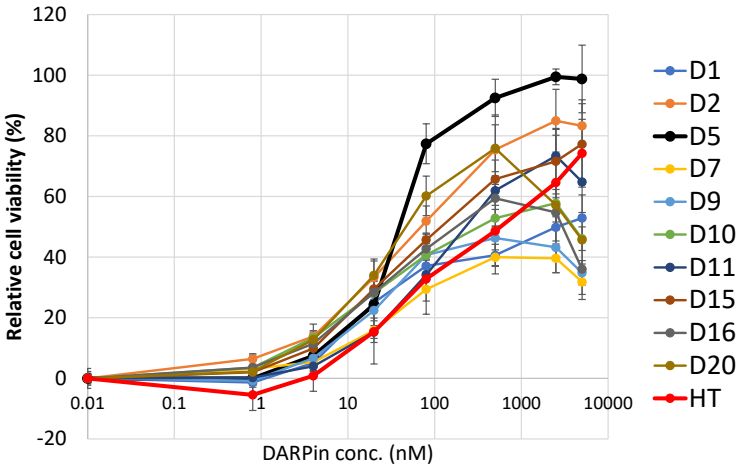
HT:

GKKLLEAARAGQDDEVRILMANGADVNA/GDPFGFTPLHLAAMFGHLEIVEVLLKNGADVNA/HDEYGFTPLHLAA
VVGHLEIVEVLLKNGADVNA/CDNQGGTPLHLAAHTGHLEIVEVLLKHGADVNA/QDKFGKTAFDISIDNGNEDLA
EILQ

RI:

GKKLLEAARAGQDDEVRILMANGADVNA/GDPFGFTPLHLAAMFGHLEIVEVLLKNGADVNA/HDEYGFTPLHLAA
VVGHLEIVEVLLKNGADVNA/CDNQGGTPLHLAARIGHLEIVEVLLKHGADVNA/QDKFGKTAFDISIDNGNEDLA
EILQ

Figure S5. Relative viability **(A)** and binding ability **(B)** of DARPin HT, RI and 10B. **(C)** Amino acid sequences of DARPin HT and RI. The ELISA plates were coated with the full-length Stx2a.



DARPin Dimer	N-DARPin	C-DARPin	Rank @ 80 nM
D5	#3	HT	1st
D20	#16	RI*	2nd
D2	#1	RI	3rd
D15	#16	HT	4th
D16	#12	#16	5th
D10	#9	#16	6th
D9	#20	#1	7th
D1	#20	#16	8th
D11	10B	#1	9th
D7	10B	#9	10th

* Variant contains additional point mutations

Figure S6. Activity comparison of DARPin dimers.

Figure S7. Sequence alignment of off-rate mutants.

ID	Motif	NCAP / AR1 / AR2 / AR3 / CCAP
		1--------10--------20--------30--------40--------50--------60--------70--------80--------90--------100-------110-------120--------130-------140-------150
		GKKLLEAARAGQDDEVRILMANGADVNA/XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA/XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA/XDXXGXTPLHLAAXXGHLEIVEVLLKXGADVNA/QDKFGKTAFDISIDNGNEDLAEILQ
	RI	GKKLLEAARAGQDDEVRILMANGADVNA/GDPFGFTPLHLAAMFGHLEIVEVLLKNGADVNA/HDEYGFTPLHLAAVVGHLEIVEVLLKNGADVNA/CDNQGGTPLHLAARIGHLEIVEVLLKHGADVNA/QDKFGKTAFDISIDNGNEDLAEILQ
	HT	GKKLLEAARAGQDDEVRILMANGADVNA/GDPFGFTPLHLAAMFGHLEIVEVLLKNGADVNA/HDEYGFTPLHLAAVVGHLEIVEVLLKNGADVNA/CDNQGGTPLHLAAHTGHLEIVEVLLKHGADVNA/QDKFGKTAFDISIDNGNEDLAEILQ
#1	#1-A4	----------/----------/-E----------N-------/----------HT----------/----------I----------
#2	#1-A10	-----V----------/----------/-E----------/----------HT----------/----------
#3	#2-A9	----------/----------/-E----------S-M-------/----------HT----------/----------
#4	#2-G5	----------/----------/-E----------/----------RM----M----------/----------
#5	#2-H7	----------/----------/-E----------/----------RI----------/----------
#6	#2-H8	----------V--------/----------/-E----------R-------/----------HT----------/----------
#7	#3-A6	----------V--------/----------/-E----------R-------/----------HT----------/----------
#8	#3-A7	----------/----------/-E----------/----------HT----------/----------
#9	#3-A8	----------/----------/-E----------/----------HT----------/----------
#10	#3-A10	----------/----------/-E----------L---------/----------HT----------/----------GY----------
#11	#3-B2	----------V/----------/-E----------/----------HT----------/----------
#12	#3-C4	----------/----------LY----------/-E----------/----------HT----------/----------
#13	#3-D10	----------/----------Y----------/-E----------/----------HT----------/--------Y----------
#14	#3-D12	----------V----------/----------A---S--------/-E----------G---------/----------RI----------/----------
#15	#3-E9	----------/----------/-E----------/----------HT----------/----------
#16	#3-E10	-----D---T----------/----------L----------/-E----------/----------RI----------Q------/----------
#17	#3-E11	----------/----------M-------/-E----------G----------/S----------RI----------/----------
#18	#3-F8	----------/----------G----------/-E----------/----------RI----------/----------I----------
#19	#3-F12	----------/----------F----------/-E----------/----------HT----------/----------I----------
#20	#4-A2	----------T----------/----------Y----------/-E----------/----------HT----------/----------
#21	#4-A10	----------/----------/-E----------/----------HT----------/----------
#22	#4-B5	----------/----------G---/-E----------L---------/R-----S------HT----------R------/----------
#23	#4-E1	----------/C----------/-E----------/----------HT----------/----------
#24	#4-F2	----------/----------/-E----------N-------/----------HT----------/----------
#25	#4-F9	----------/----------M-----A--/-E----------/----------HT----------/----------
#26	#4-G7	----------/----------/-E----------/----------SHT----------/----------T----------
#27	#4-H5	----------F----------/----------E--/-E----------/----------HT----------/----------
#28	#4-H7	----------/----------Y----------/-E----------/----------RI----------/----------
Syn	SHT	----------V--------/----------LY----------/-E----------S-M-------N-------/R-----S-----SHT----------Q------/--------Y-----I----------

A

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Stx2a-A     MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSV    60
Stx2c-A     MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSV    60
            ************************************************************

Stx2a-A     SVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI    120
Stx2c-A     SVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI    120
            ************************************************************

Stx2a-A     SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAV    180
Stx2c-A     SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAV    180
            ************************************************************

Stx2a-A     LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGV    240
Stx2c-A     LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGV    240
            ************************************************************

Stx2a-A     RVGRISFNNISAILGTVAVILNCHHQGARSVRAVNEESQPECQITGDRPVIKINNTLWES    300
Stx2c-A     RVGRISFNNISAILGTVAVILNCHHQGARSVRAVNEDSQPECQITGDRPVIKINNTLWES    300
            ************************************:***********************

Stx2a-A     NTAAAFLNRKSQFLYTTGK   319
Stx2c-A     NTAAAFLNRKSQFLYTTGK   319
            ************ *****:


Stx2a-B     MKKMFMAVLFALASVNAMAADCAKGKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQS    60
Stx2c-B     MKKMFMAVLFALVSVNAMAADCAKGKIEFSKYNENDTFTVKVAGKEYWTSRWNLQPLLQS    60
            ************.********************:******* *****************

Stx2a-B     AQLTGMTVTIKSSTCESGSGFAEVQFNND  89
Stx2c-B     AQLTGMTVTIKSSTCESGSGFAEVQFNND  89
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B

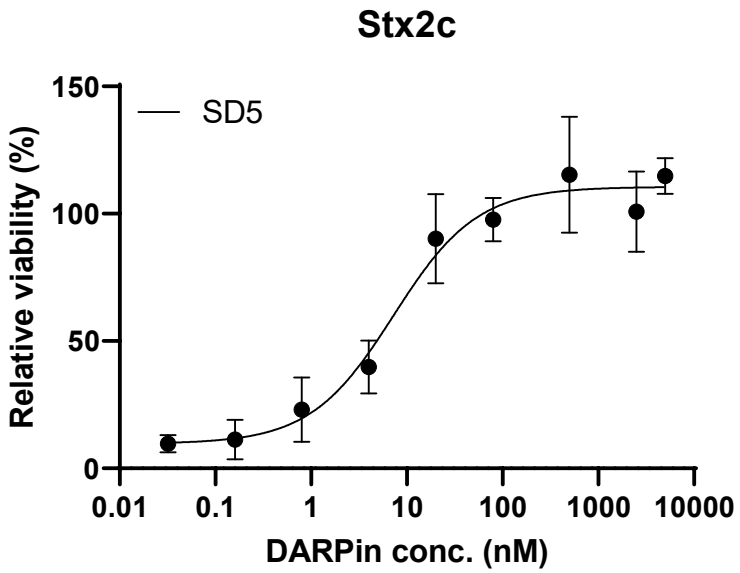

Figure S8. (A) Sequence alignment of Stx2a and Stx2c. (B) Protection of Vero cells by SD5. Vero E6 cells ($1.5x10^3$ cells/well) were incubated with 60 pg/mL Stx2c and the appropriately diluted DARPin for 3 days. Error bars represent the standard deviation of two independent experiments performed in duplicate.

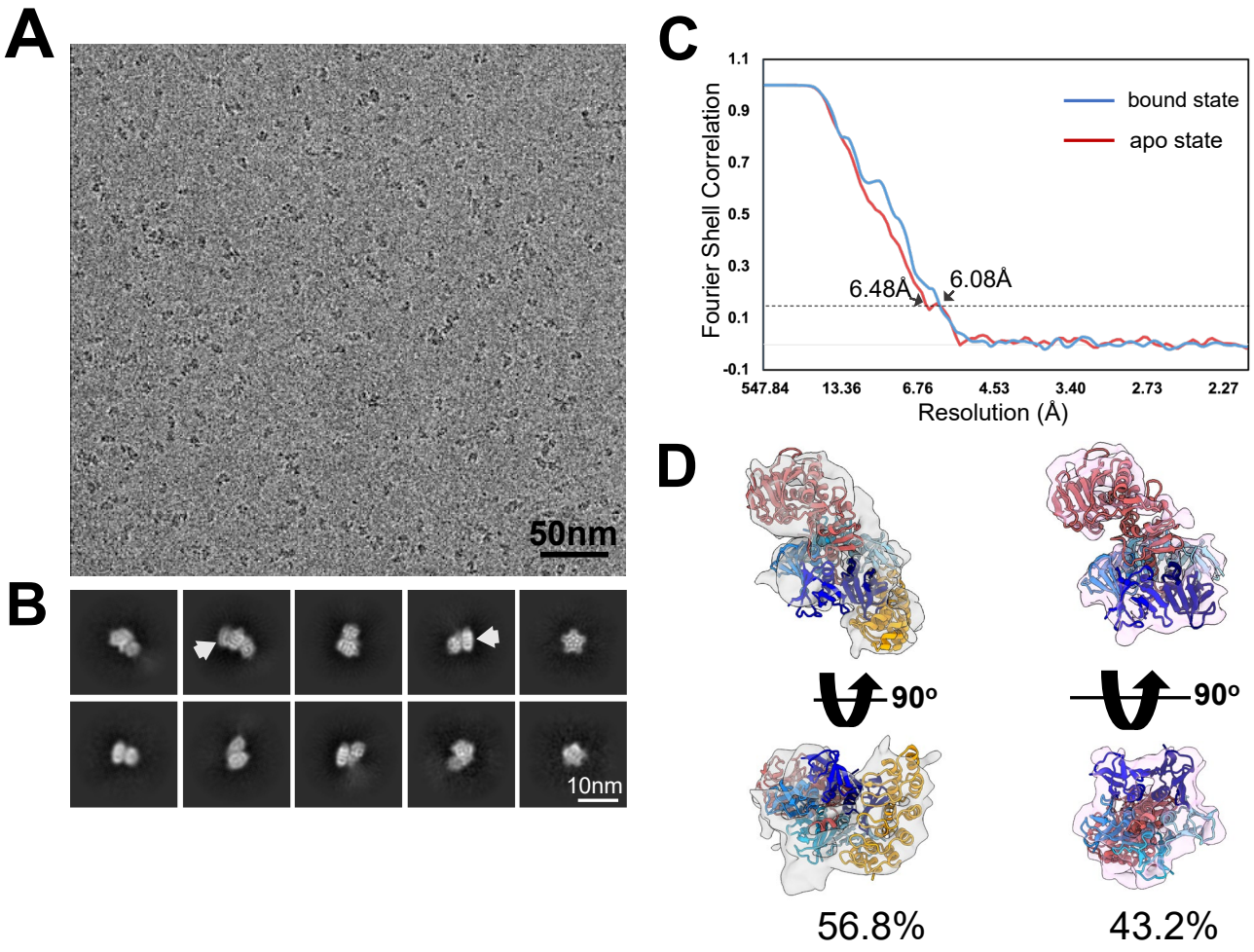

Figure S9. Cryo-EM analysis on Stx2a and DARPin #3 complex. **(A)** Representative micrograph of the cryo-EM data. **(B)** Representative 2D classification of the complex. The white arrows highlight the extra density of DARPin. **(C)** FSC of the bound-state map and apo-state map. **(D)** The density maps of the bound state (left) and the apo state (right) with the refined models docked in. The A subunit is colored red. The B subunit is colored blue. The DARPin is colored orange.

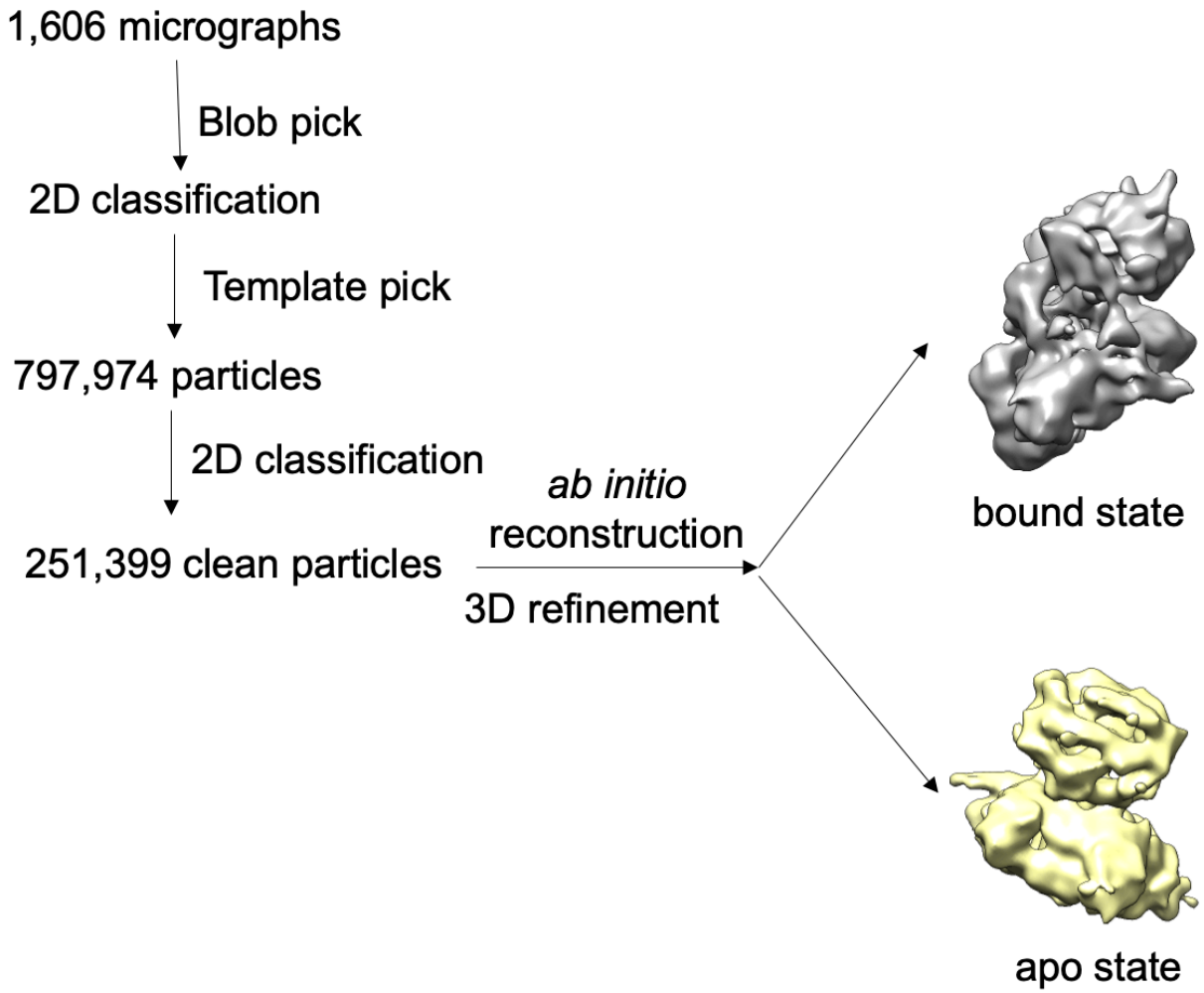

Figure S10. Data processing for the complex between Stx2a and DARPin #3.