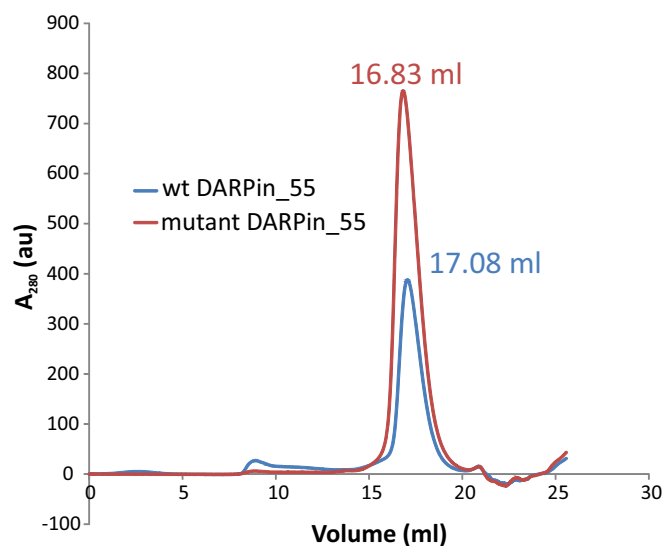
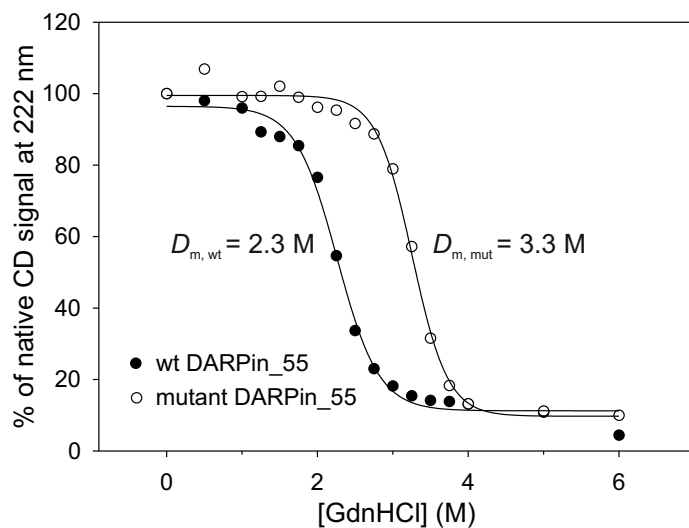
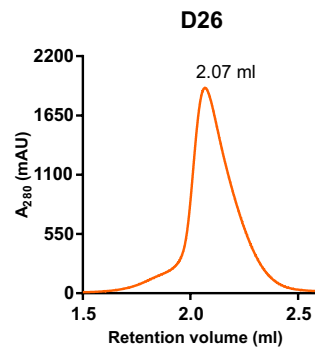
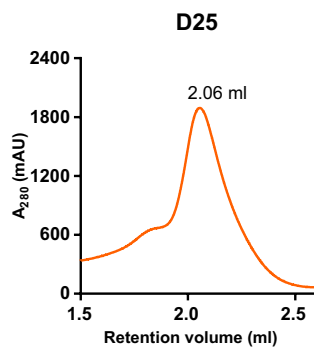
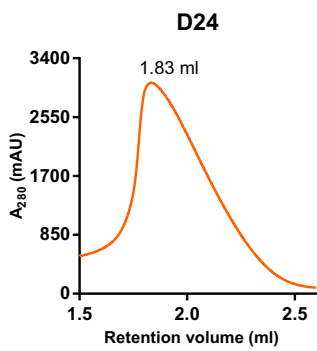
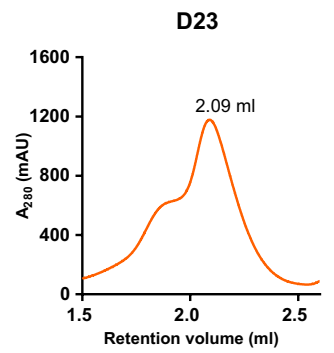
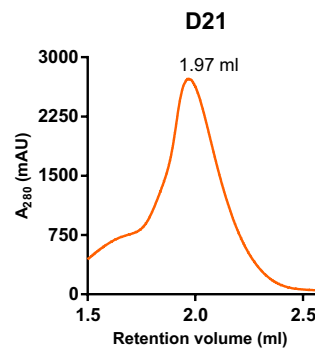
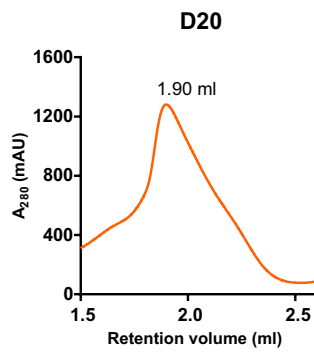
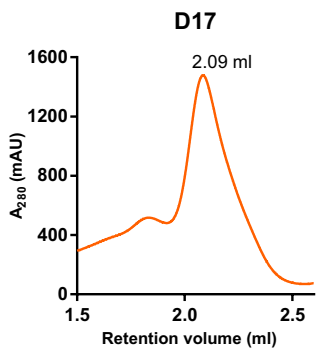
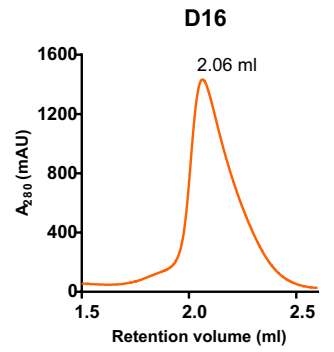
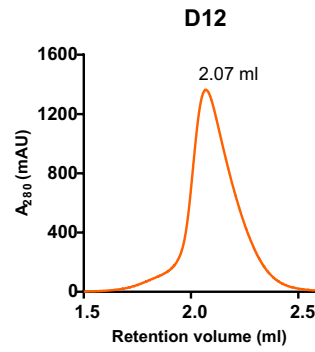
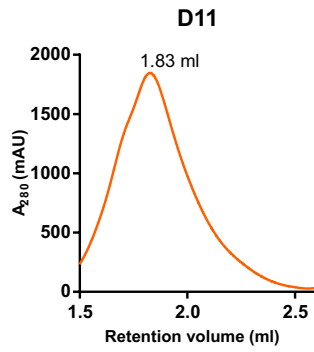
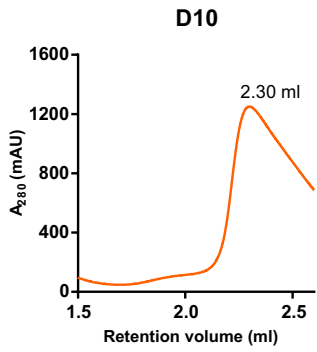
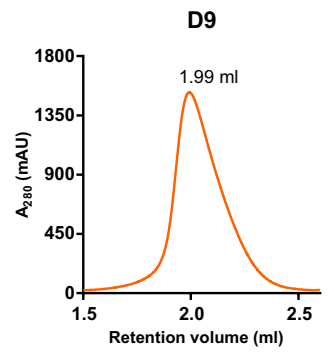
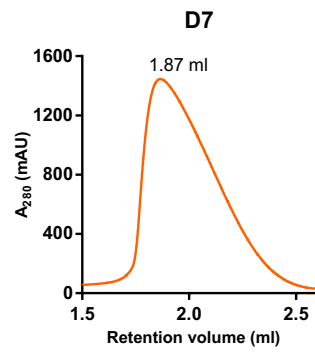
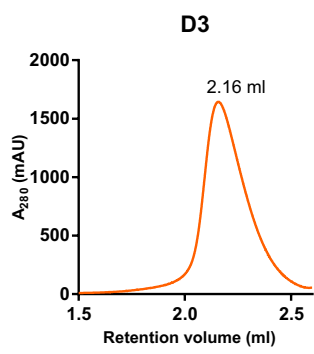
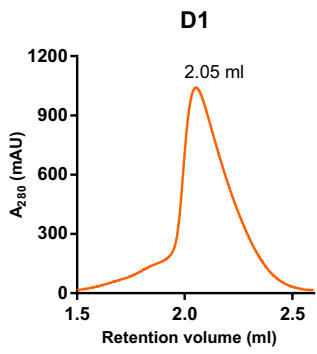
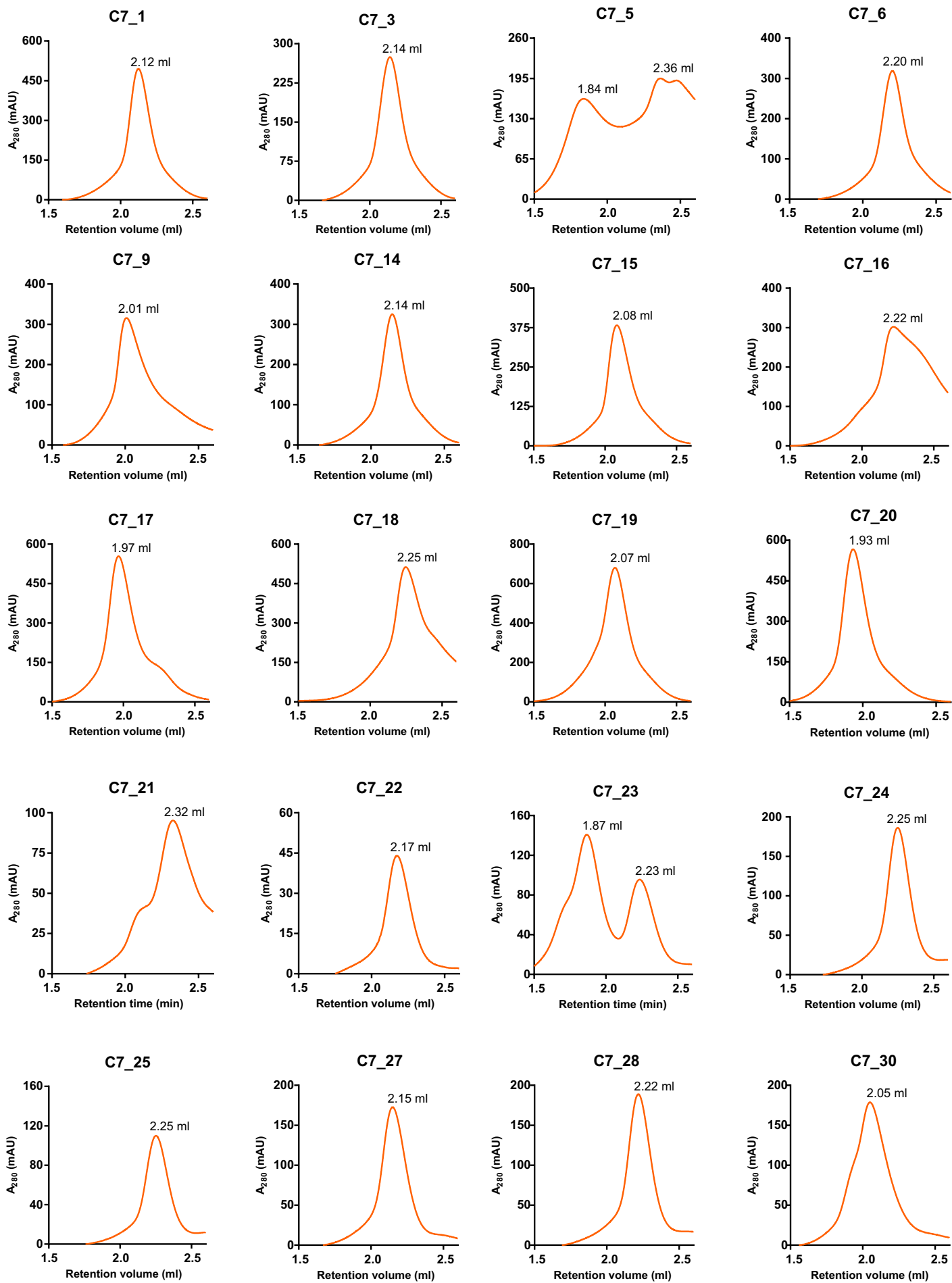
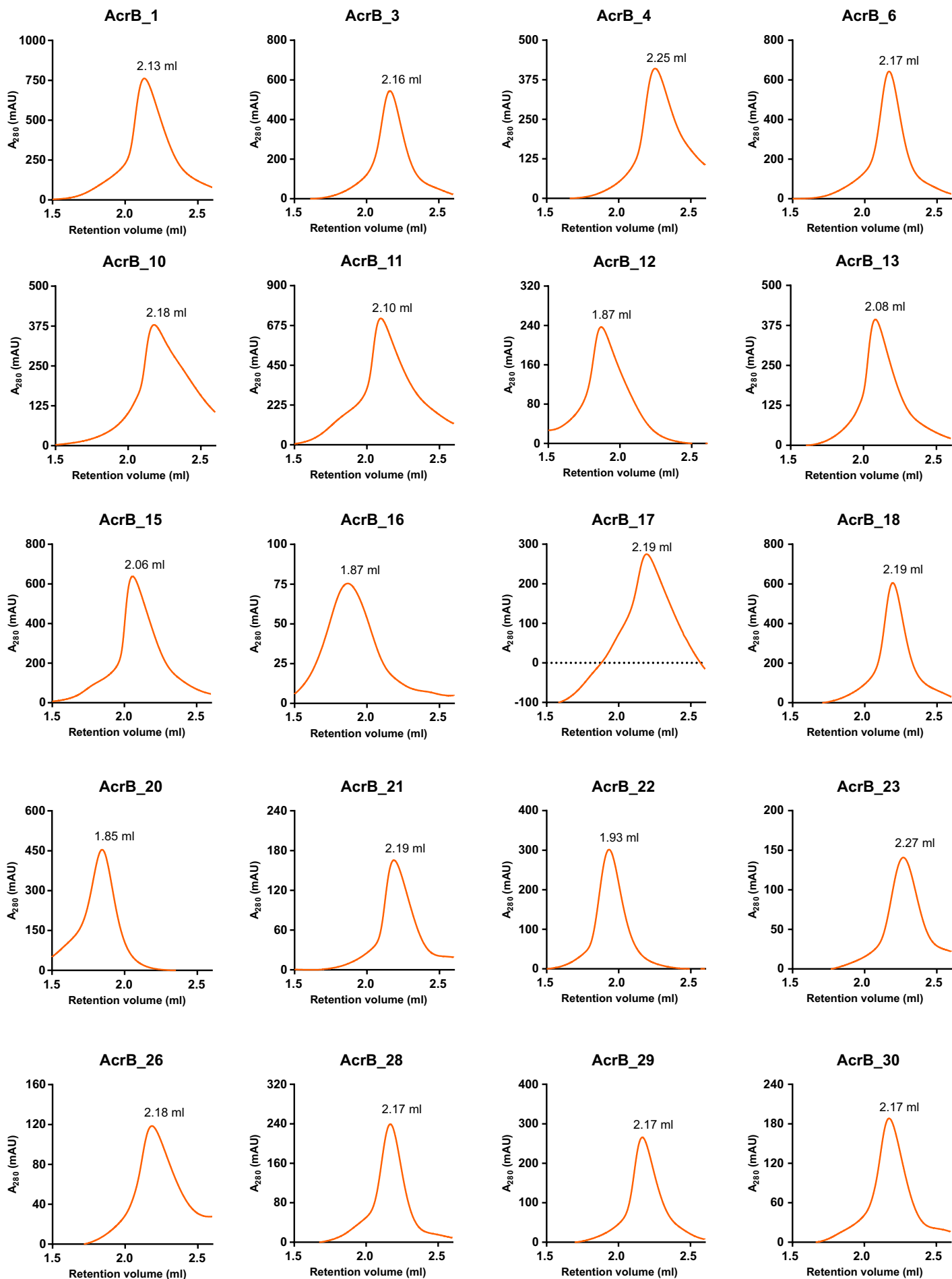


A**B**

Supporting Information Figure S1: Biochemical analysis of surface entropy reduction mutations in DARPin_55. Glutamate of ankyrin repeat position 21 and lysine of position 25 (Figure 1C) were mutated to aspartate and alanine, respectively, in all three internal repeats of DARPin_55²⁹. The resulting 6-fold mutant was compared to wild type DARPin_55 by SEC using a Superdex 200 10/300 GL column (A) and equilibrium unfolding using GdnHCl (B).



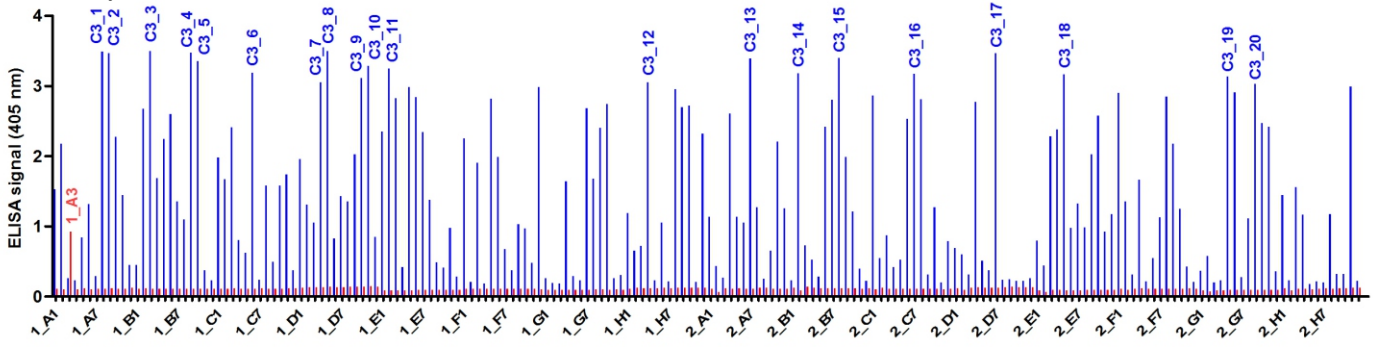




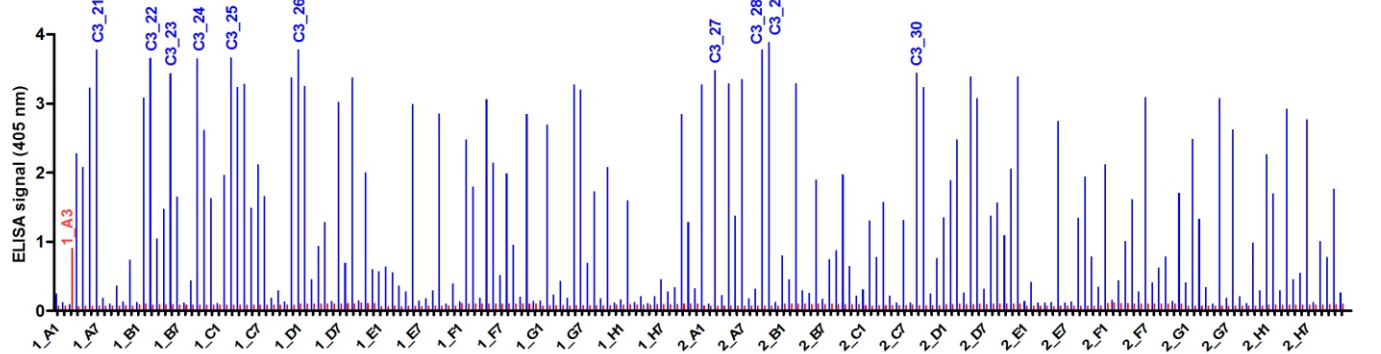
Supporting Information Figure S3: Analytical SEC profiles of all DARPs of the new library appearing in this study.

A

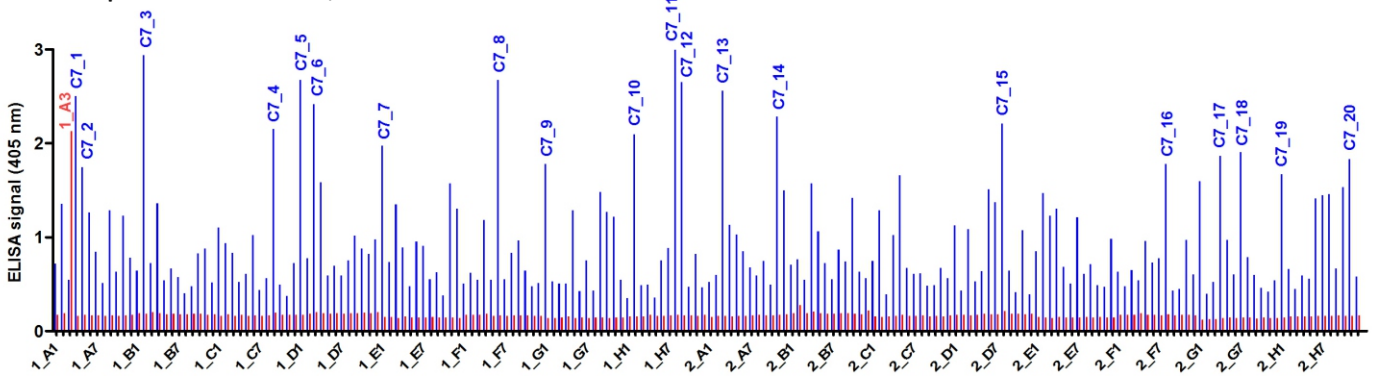
Caspase-3 binders, after selection round 3



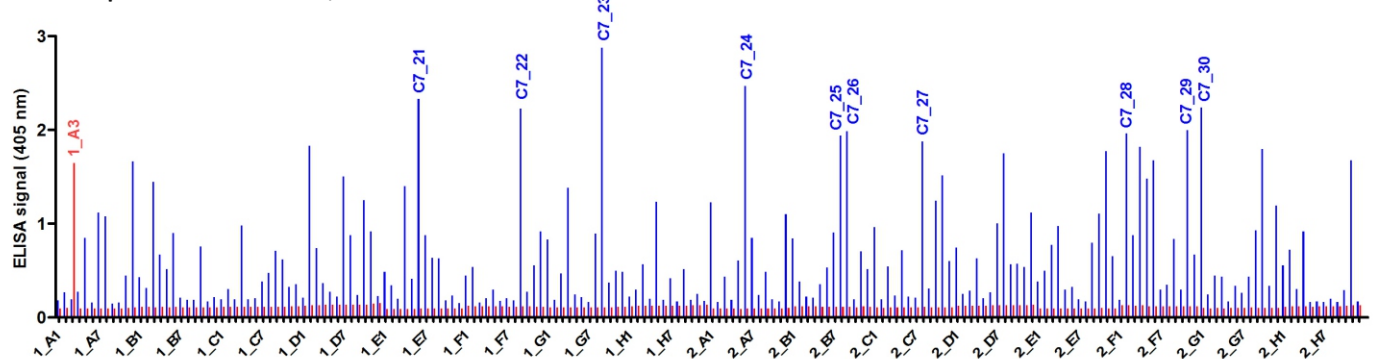
Caspase-3 binders, after selection round 4

**B**

Caspase-7 binders, after selection round 3

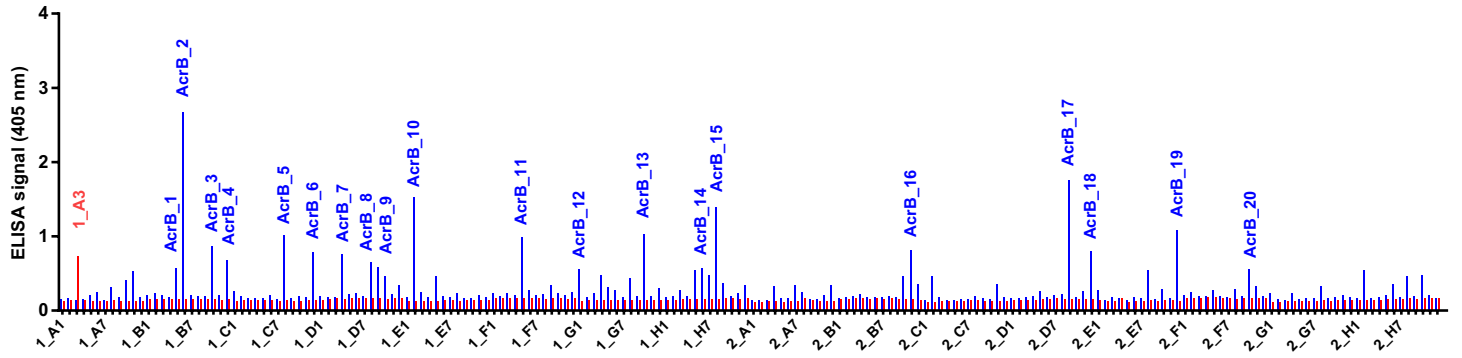


Caspase-7 binders, after selection round 4

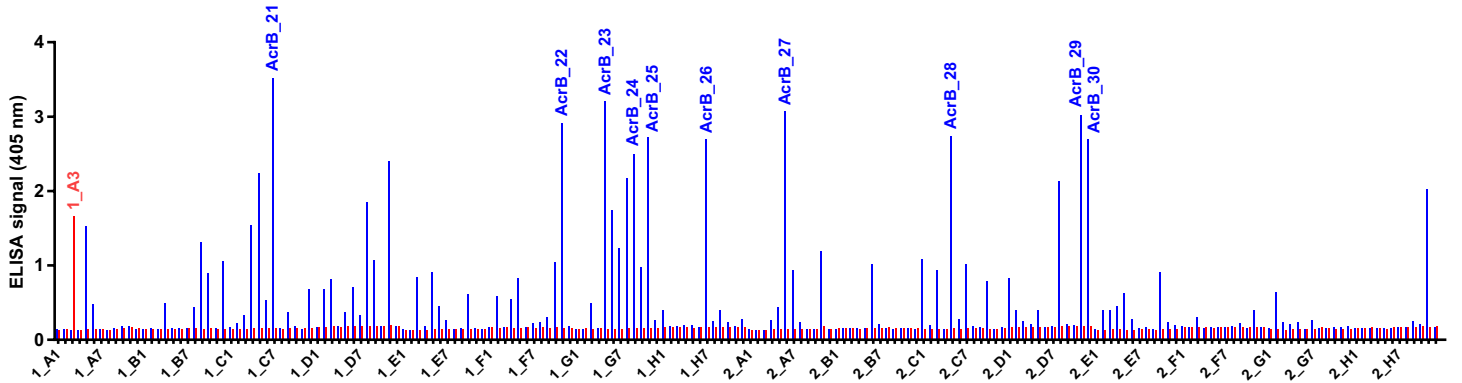


C

AcrB binders, after selection round 3



AcrB binders, after selection round 4




Supporting Information Figure S4: Crude cell extract ELISAs. DARPins selected against caspase-3 (A), caspase-7 (B) and AcrB (C) were probed for binding against the respective target and the control protein MBP.

		10	20	30	40	50	60	70																							
N3C	MRGSHHHHHGSDLGKKLLDAASAGQDDEVRLMANGADVNAS	X	X	X	G	X	T	P	L	H	X	X	X	G	H	L	E	I	V	D	V	L	L	A	Z	G	A	D	V	N	A
C3_1		DRQ	A	A	A	A	A	T	S	I	I	Y	E	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C3_2		DNW	A	A	A	A	A	S	S	S	S	Y	Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	D
C3_3		DND	A	A	A	A	A	S	S	S	S	Y	Y	Y	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
C3_4		DHA	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_5		DWH	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_7		TQT	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_8		TEH	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_9		NWH	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_10		DWN	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_11		SYA	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_12		TTN	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_13		DTN	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_15		TAT	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_16		NQY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_17		DHN	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_18		DSY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_19		SAY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_20		TDS	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_21		TDE	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_22		DYY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_23		SEY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_24		DKH	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_25		SDY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_26		TNA	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_27		DNS	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_28		DKY	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_29		DMN	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_30		SNW	A	A	A	A	A	S	S	S	S	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

N-Cap
Repeat 1

		80	90	100	110	120	130	140	150																																
N3C	S	X	X	X	G	X	T	P	L	H	X	X	X	G	H	L	E	I	V	D	V	L	L	A	Z	G	A	D	V	N	A	N	X	X	X	G	K	T	P	F	
C3_1		TYY	E	A	R	N	N	T	Q	W	A	S	T	S	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C3_2		TNY	E	A	Y	N	N	T	T	A	S	T	Q	S	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_3		TDY	E	L	Y	H	H	T	N	W	Y	V	K	N	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_4		TDY	E	L	Y	H	H	T	S	W	H	A	R	H	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_5		TEY	E	A	A	N	N	T	T	H	Y	S	S	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C3_7		TAY	E	A	Q	N	N	T	N	W	Y	V	K	T	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_8		TDY	E	V	Y	T	T	N	Y	T	W	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_9		TYY	E	A	E	T	H	T	T	E	W	S	V	A	R	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_10		TQY	E	A	Q	A	I	N	N	T	V	Y	T	S	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C3_11		TYY	E	V	Y	S	N	N	N	T	Q	H	N	V	R	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_12		TAY	E	V	R	Y	N	N	N	T	Q	W	N	S	S	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C3_13		TYY	E	A	Y	A	N	N	N	S	H	W	D	A	K	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C3_15		TYY	E	A	Y	T	N	N	N	T	N	W	Y	V	A	Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_16		TWH	E	V	Y	R	N	N	N	T	D	W	Y	V	A	N	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_17		TWY	E	V	Y	S	N	N	N	T	A	W	T	V	S	E	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_18		TNY	E	V	Y	K	N	N	N	T	T	W	Y	V	H	H	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C3_19		TAY	E	V	T	R	H	N	N	T	A	W	S	S	R	D	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_20		TYY	E	V	Y	R	Y	N	N	T	N	W	N	T	R	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
C3_21		TNY	E	V	N	A	N	N	N	T	A	Y	R	A	R	W	F	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
C3_22		THY	E	L	T	N	N	N	N	T	N	N	W	Y	A	R	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_23		TAY	E	T	N	N	N	N	N	S	N	W	A	S	A	W	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C3_24		TAY	E	A	N	S	Y	N	N	T	N	N	W	Q	S	Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_25		TAY	E	V	Y	S	Y	N	N	T	S	Q	W	A	S	A	R	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_26		TDY	E	V	Y	A	Y	N	N	T	Q	W	N	V	A	Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_27		NHY	E	A	S	K	N	N	N	T	S	N	W	A	V	R	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_28		TDY	E	A	T	S	A	N	N	T	A	W	R	V	Y	A	V	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_29		TDY	E	V	Y	T	N	N	N	T	H	W	T	A	T	Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
C3_30		TDY	E	V	Q	E	H	N	N	T	A	W	N	V	K	Y	T	Y	Y																						

	160	170	180	
N3C	DLAIDNGNEDIAEVLQKAAKLGSGSMEQKLI	SEEDLNE		188
C3_1			187
C3_2			188
C3_3N.....		188
C3_4			188
C3_5			188
C3_7			188
C3_8			188
C3_9			188
C3_10			188
C3_11			188
C3_12V.....		188
C3_13			188
C3_15	N.....			188
C3_16			188
C3_17			188
C3_18			188
C3_19			188
C3_20			188
C3_21			188
C3_22			188
C3_23			188
C3_24G.....		188
C3_25T.....		188
C3_26			188
C3_27			188
C3_28			188
C3_29			188
C3_30			188



C-Cap

Supporting Information Figure S5: Sequence alignment of caspase-3 specific DARPins. A TXYGE motif in the β -turn of the second internal repeat suggests a single binding epitope on caspase-3 for all these binders.

		10	20	30	40	50	60	70																																
N3C	MRGSHHHHHGSDLGKKLLDAASAGQDDEVRLMANGADVNAS	X	X	X	G	X	T	P	L	H	X	A	X	X	G	H	L	E	I	V	D	V	L	L	A	Z	G	A	D	V	N	A								
C7_1		D	Y	A	W	W	T	I	S	W	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N							
C7_3		D	A	Y	W	W	T	I	S	W	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N							
C7_5		N	R	R	Y	Y	V	V	S	A	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N						
C7_6		D	A	W	R	R	S	S	T	W	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N					
C7_7		D	W	T	H	R	S	S	R	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N				
C7_9		D	Y	A	Q	Q	L	L	H	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N			
C7_14		D	S	W	R	R	A	A	H	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N			
C7_15		S	W	E	H	L	L	A	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N		
C7_16		N	Q	Q	W	W	A	T	E	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_17		D	W	Y	W	W	T	I	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_19		D	N	S	W	W	V	V	A	W	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_20		D	S	W	R	R	V	V	Y	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_21		D	T	Y	E	E	V	V	W	R	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_22		N	W	E	Y	Y	L	L	W	W	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_23		S	W	E	Y	Y	L	L	Y	A	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_24		D	A	W	Q	Q	L	L	Q	W	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N
C7_25		D	S	W	I	I	A	A	Y	A	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_26		N	S	W	W	W	L	L	W	K	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_27		D	S	W	S	S	S	S	S	N	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_28		D	A	W	Q	Q	L	L	Q	W	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N
C7_29		N	W	E	Y	Y	V	V	Y	Y	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	
C7_30		S	D	N	E	E	A	A	H	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	H	N	


		80	90	100	110	120	130	140	150																																				
N3C	S	X	X	X	G	X	T	P	L	H	X	A	X	X	G	H	L	E	I	V	D	V	L	L	A	Z	G	A	D	V	N	A	N	X	X	X	G	K	T	P	F				
C7_1	D	H	T	Y	Y	V	V	Q	Q	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_3	D	H	T	Y	Y	V	V	Q	Q	I	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_5	D	Q	W	W	W	V	V	Q	Q	I	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_6	D	W	Y	D	D	L	L	T	Q	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_7	D	W	T	N	N	S	S	R	S	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_9	D	T	W	W	W	A	A	T	S	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_14	D	W	Y	E	E	L	L	N	T	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_15	N	Q	E	E	L	L	D	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
C7_16	D	S	Y	S	S	S	W	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C7_17	D	K	A	S	S	V	W	H	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C7_19	S	T	W	I	I	T	Y	T	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C7_20	D	W	Y	E	E	L	L	D	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C7_21	D	R	S	Y	Y	A	A	T	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_22	D	T	Y	Y	Y	L	L	H	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_23	N	Q	E	A	A	L	L	Y	Q	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
C7_24	D	W	Y	S	S	S	A	K	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_25	D	W	Y	T	T	L	L	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_26	D	W	T	S	S	V	T	Q	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_27	D	W	Y	N	N	A	A	A	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_28	D	W	Y	S	S	S	A	K	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_29	D	N	W	A	A	L	L	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
C7_30	D	Q	W	W	S	S	H	E	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

	160	170	180	
N3C	DLAIDNGNEDIAEVLQKAAKLGSGSMEQKLI	SEEDLNE		188
C7_1	188
C7_3	188
C7_5	188
C7_6	188
C7_7	188
C7_9	188
C7_14	188
C7_15	188
C7_16	188
C7_17	188
C7_19S.....	188
C7_20	188
C7_21I.....	188
C7_22	188
C7_23	188
C7_24N.....	188
C7_25G.....	188
C7_26V.....	188
C7_27	188
C7_28	188
C7_29	188
C7_30V.....	188


C-Cap

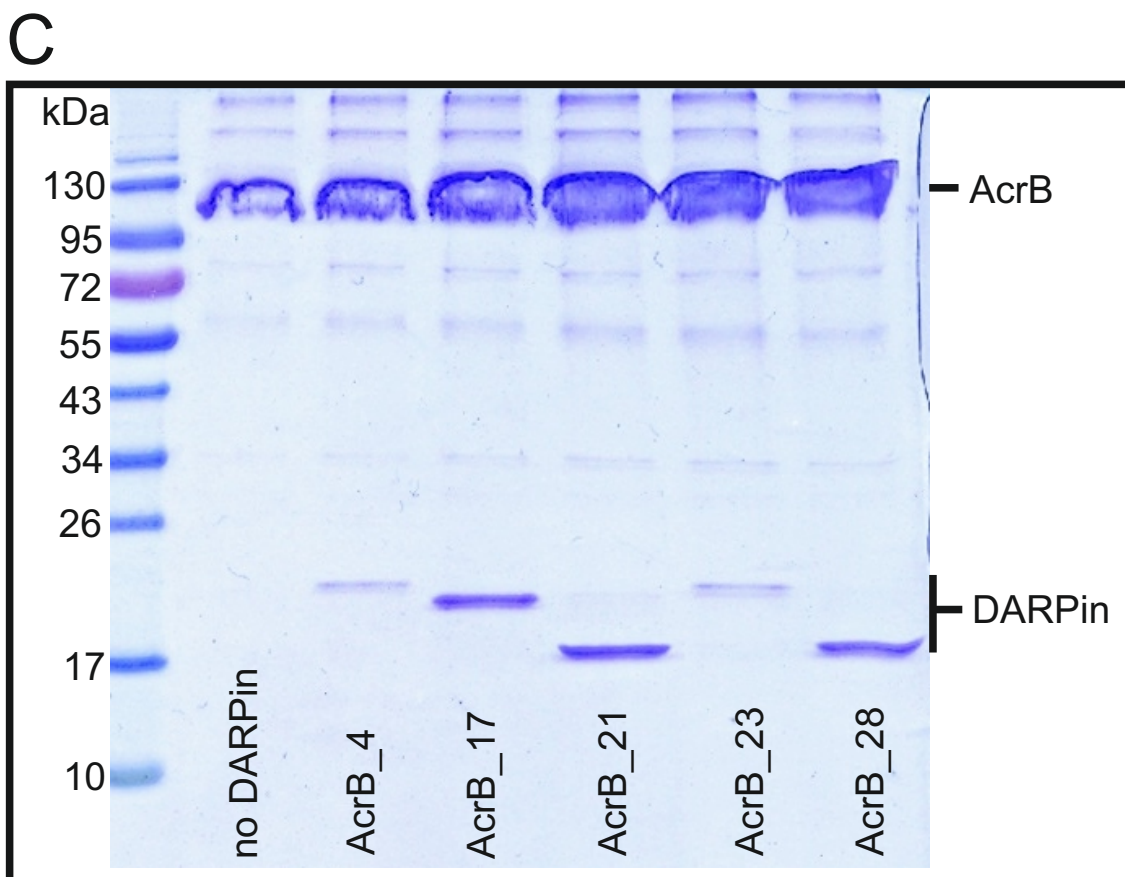
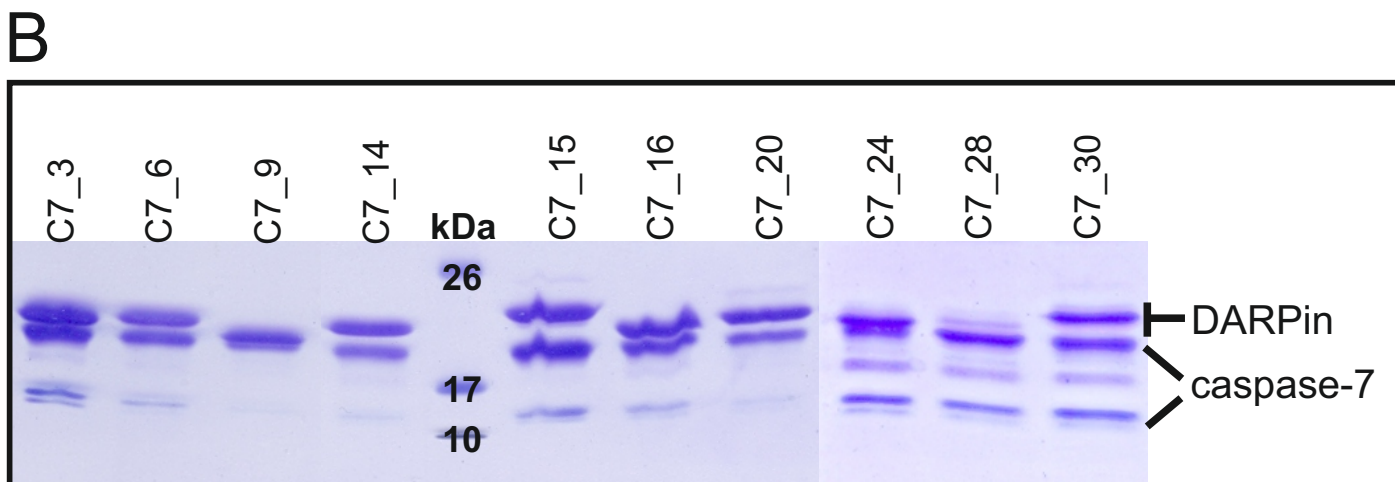
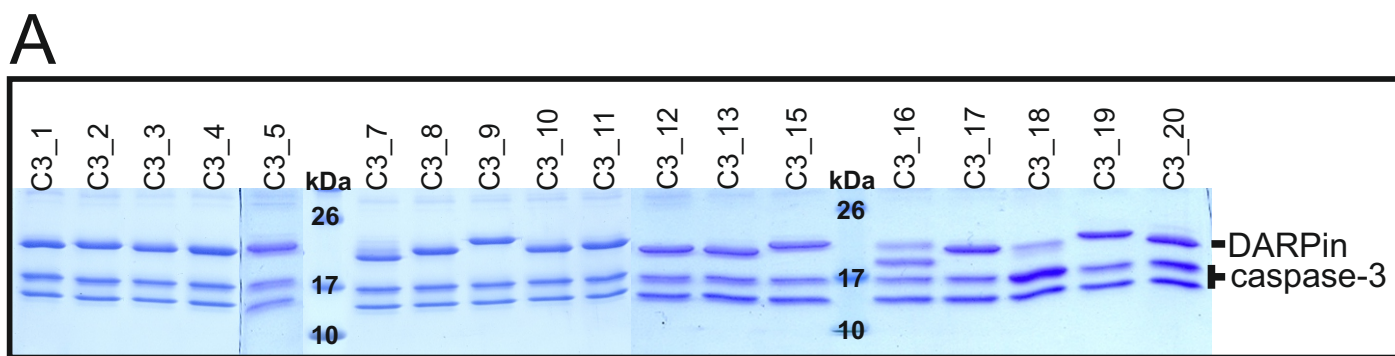
Supporting Information Figure S6: Sequence alignment of caspase-7 specific DARPins.

	150	160	170	180	
N3C	TPFDLAIDNGNEDIAEVLQKAAKLGSGSMEQKLISEEDLNE				188
AcrB_1				188
AcrB_3				188
AcrB_4				188
AcrB_5				188
AcrB_6				188
AcrB_7				188
AcrB_9				188
AcrB_10E.....				188
AcrB_11				188
AcrB_12				188
AcrB_13				188
AcrB_15				188
AcrB_16				188
AcrB_17				188
AcrB_18				188
AcrB_20				187
AcrB_21				188
AcrB_22				189
AcrB_23N.....				188
AcrB_25	...E.....				188
AcrB_26				189
AcrB_27E.....				188
AcrB_28				188
AcrB_29				188
AcrB_30				188

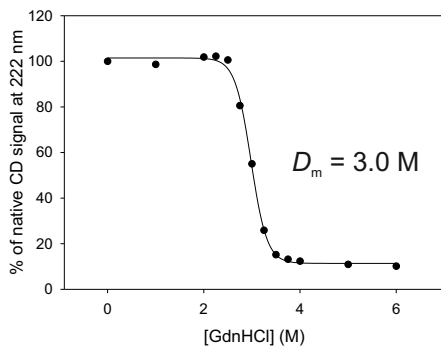
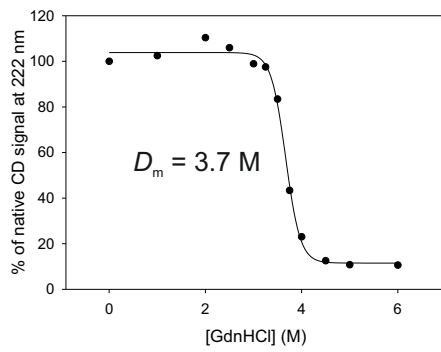
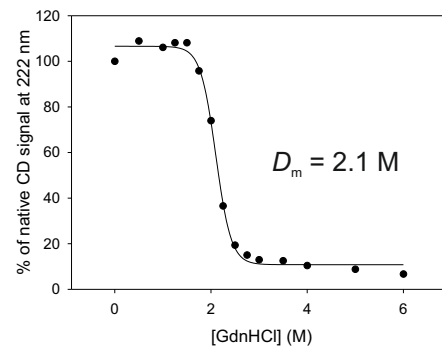
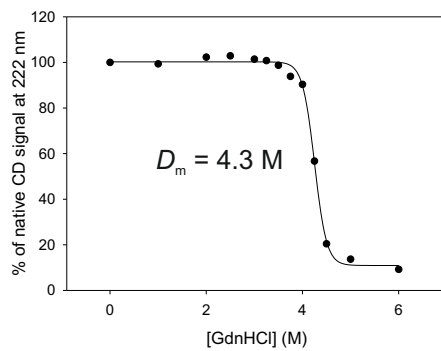
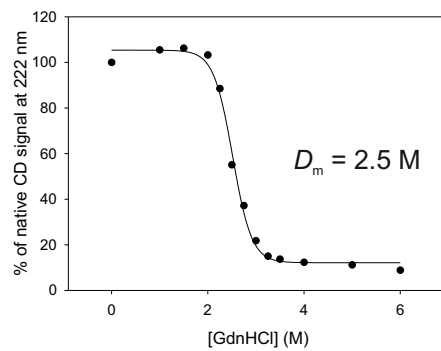
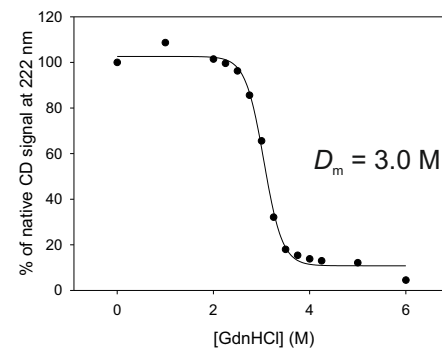
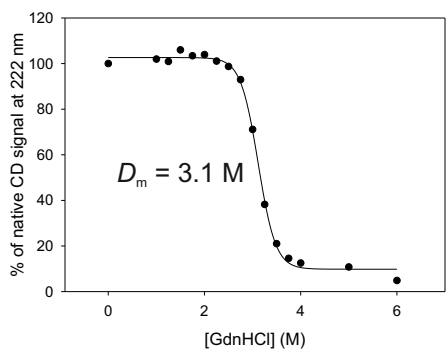
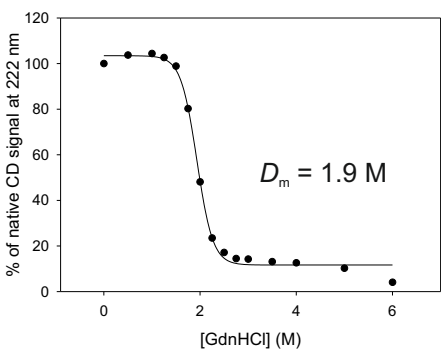
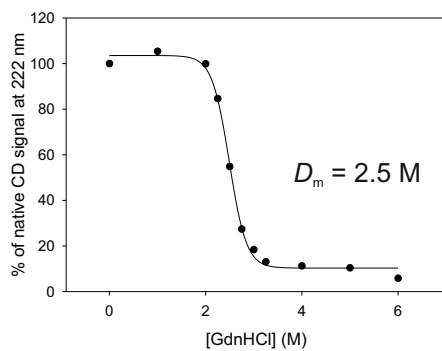
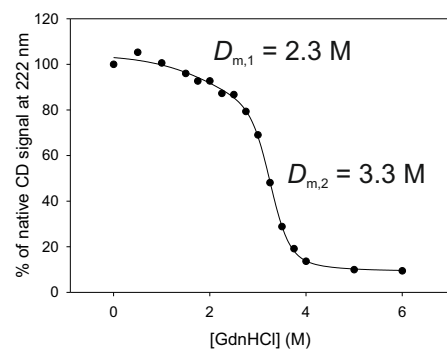
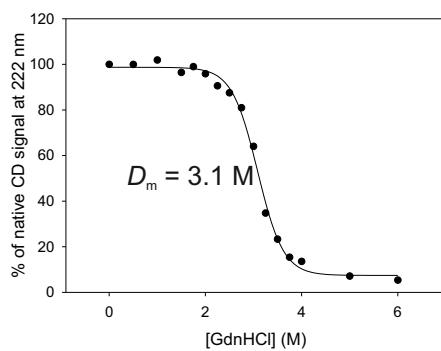
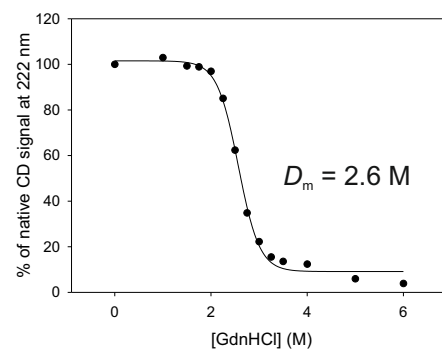


C-Cap

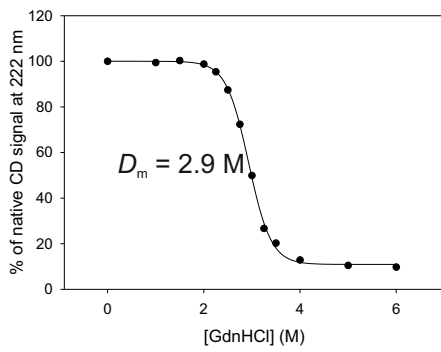
Supporting Information Figure S7: Sequence alignment of AcrB specific DARPins.



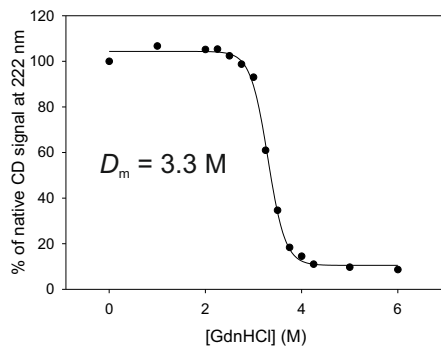
Supporting Information Figure S8: DARPins specific for caspase-3 (A), caspase-7 (B) and AcrB (C) were co-purified by SEC with their targets (example in Figure 6B) and the complex peak fractions were analyzed by SDS-PAGE. The double band for C3_16 in (A) can be explained by incomplete denaturation of this DARPin during SDS-PAGE. AcrB_4 and AcrB_23 in (C) appear to partially dissociate from AcrB during SEC analysis, owing to their modest affinity (Figure 6F).

D1**D3****D7****D9****D10****D11****D12****D16****D17****D20****D21****D23**

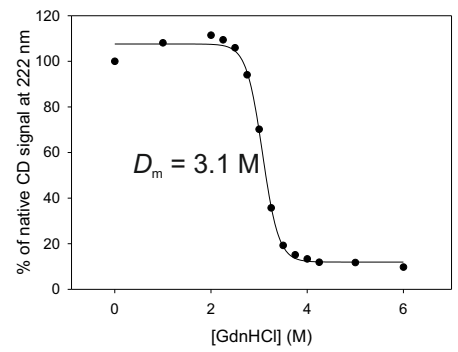
D24



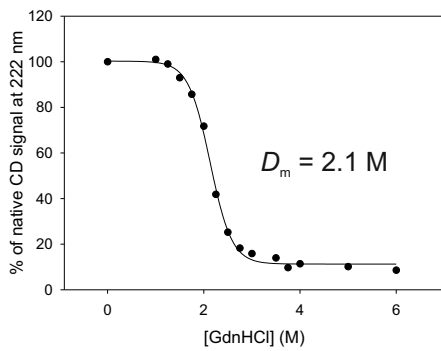
D25



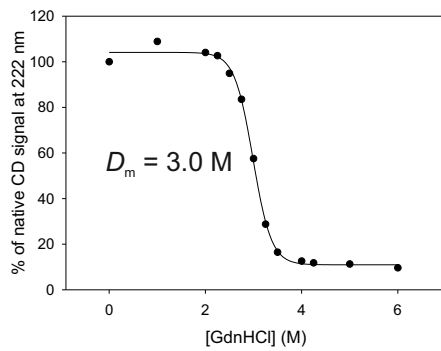
D26



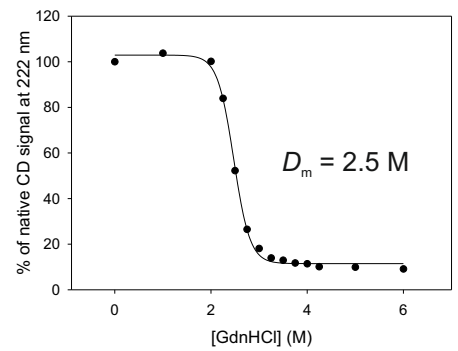
C3_11



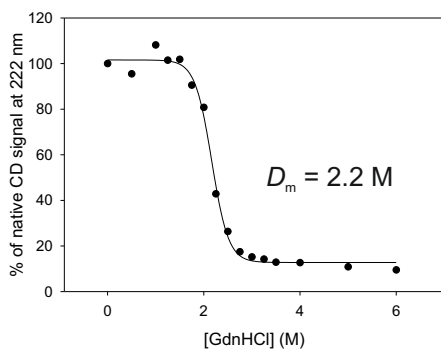
C3_13



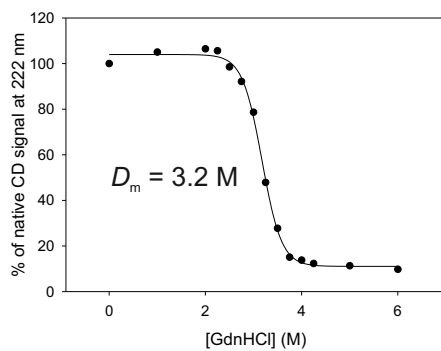
C3_15



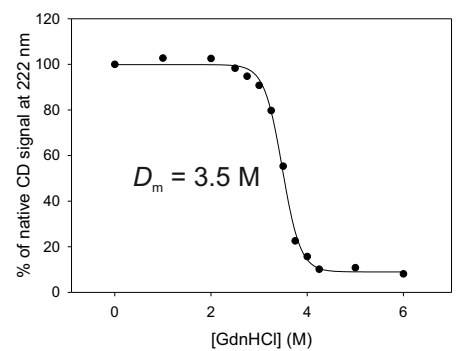
C3_19



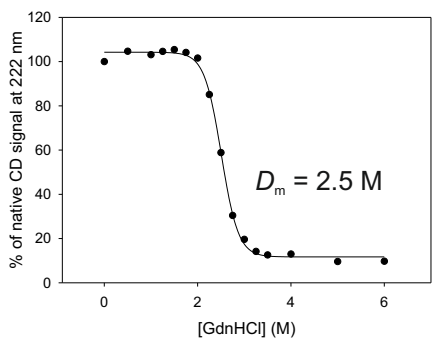
C3_20



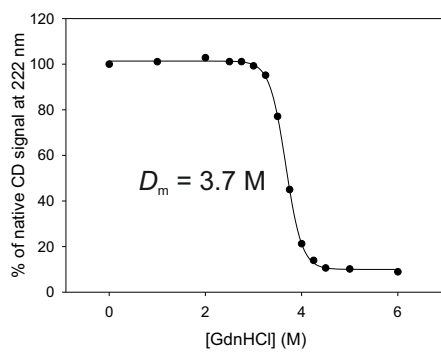
C7_14



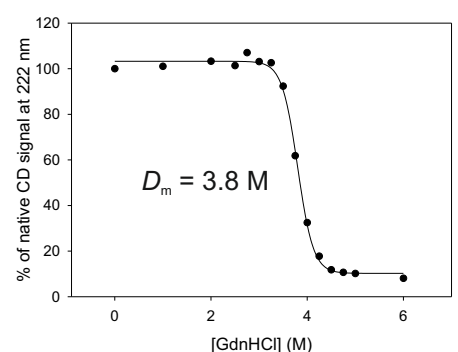
C7_16



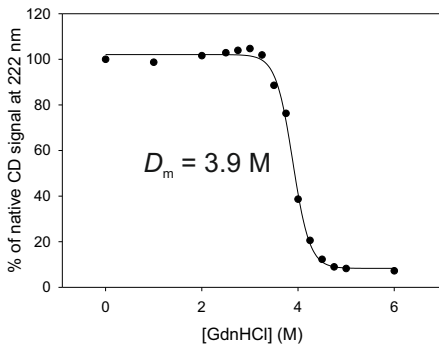
C7_20



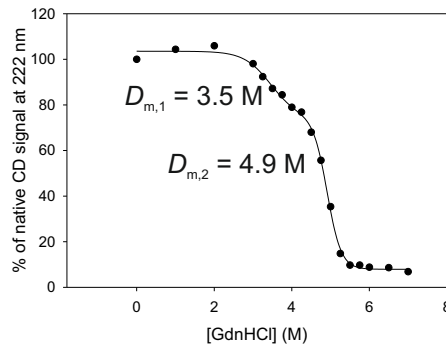
C7_24



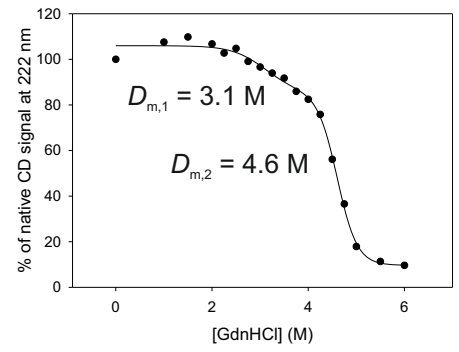
C7_28



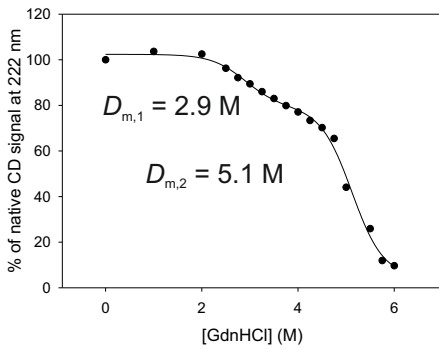
off7 (1SVX)



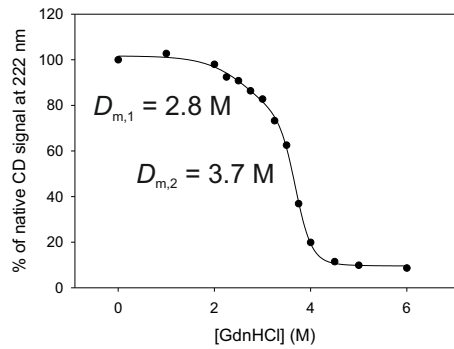
1108_19 (2J8S)



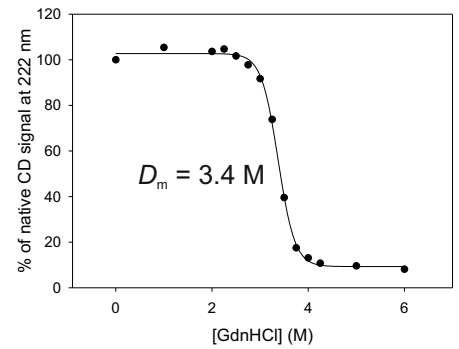
DARPin#2 (3NOC)



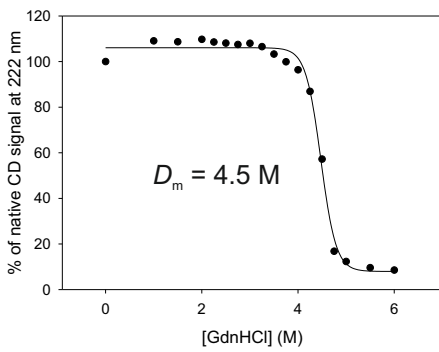
DARPin#3 (3NOG)



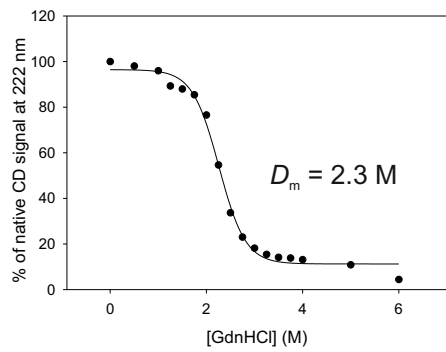
α -LmrCD#3



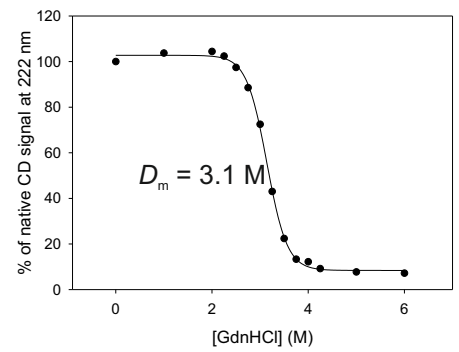
α -LmrCD#4



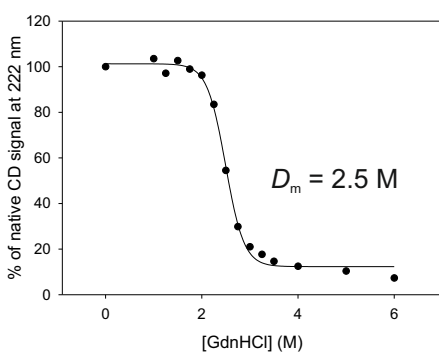
DARPin_55



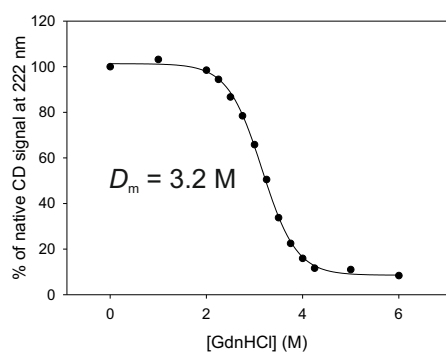
D3.4 (2XZD)



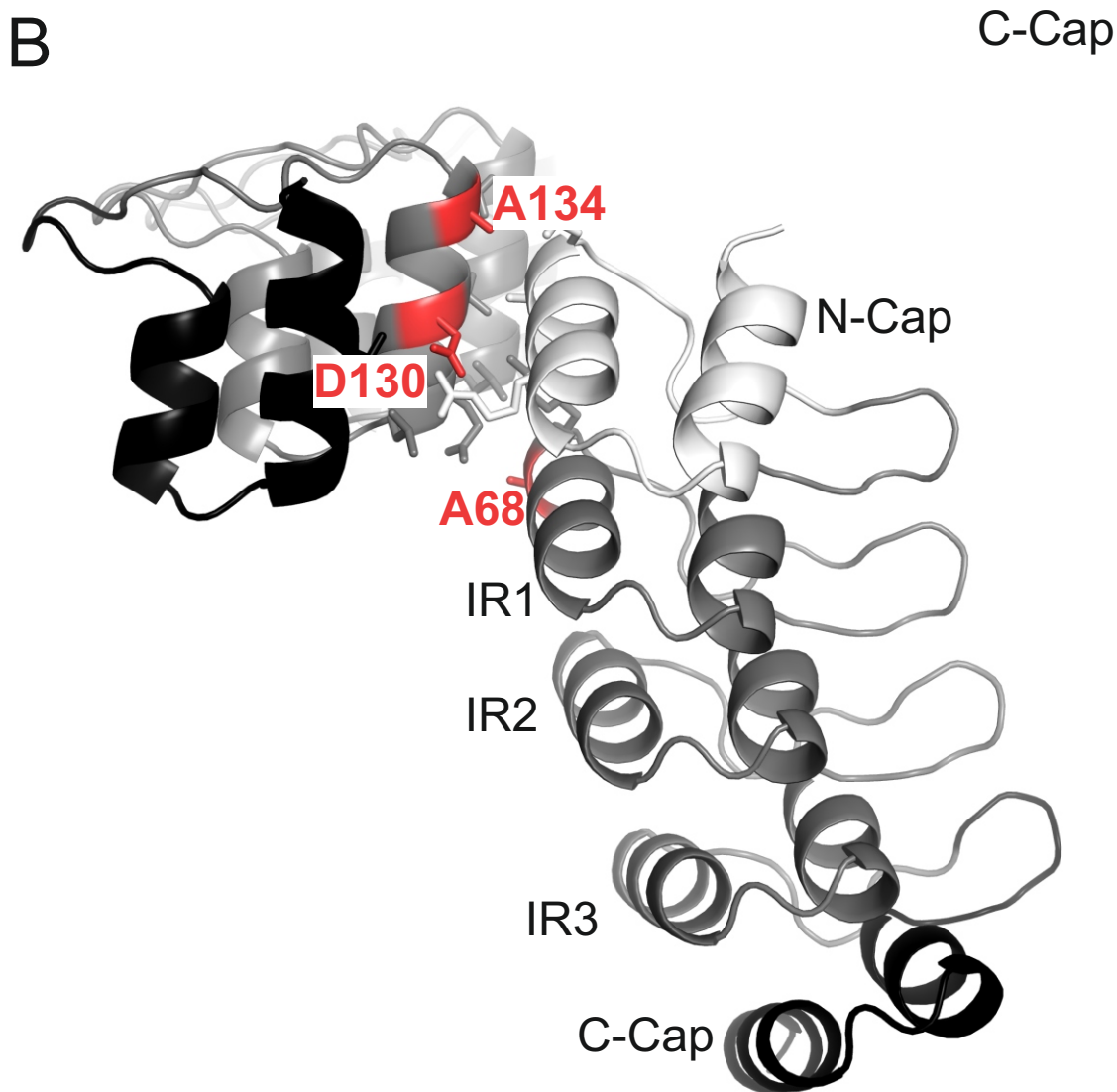
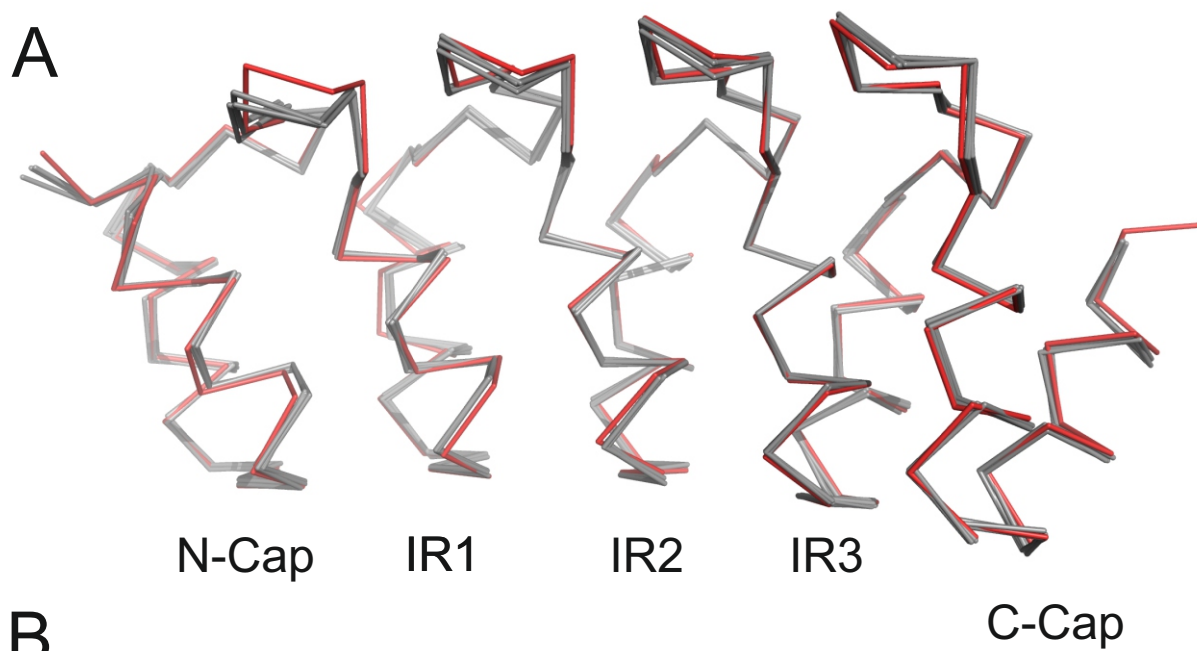
D7.18



D8.14 (2Y1L)



Supporting Information Figure S9: Equilibrium unfolding curves of original and new DARPins. 25 new DARPins (15 unselected DARPins (denoted by a “D” before the number), 5 DARPins each specific for caspase-3 and caspase-7 (denoted by “C3_” and “C7_” before the number)) and original DARPins off7 (PDB 1SVX, against MBP)¹², 11_0819 (PDBs 2J8S and 4DX5, against AcrB)^{28,36}, DARPIn#2 and #3 (PDBs 3NOC and 3NOG, against AcrB)⁴², α -LmrCD#3 and #4 (against LmrCD)³⁰, DARPIn#55 (against MsbA)²⁹, D3.4 (PDB 2XZD, against caspase-3)⁴⁰, D7.18 (against caspase-7, to be published) and D8.14 (PDB 2Y1L, to be published) were analyzed by GdnHCl unfolding. Monophasic or biphasic sigmoidal equations were fitted to the experimental data (Equations 1 and 2, see Materials and Methods) to determine midpoints of denaturation (D_m for monophasic and $D_{m,1}$ and $D_{m,2}$ for biphasic unfolding curves).



Supporting Information Figure S10: (A) DARPin C7_16 (red) is superimposed with a full-consensus DARPin containing an optimized C-cap (2XEE, Kramer et al. 2010, all four chains of the asymmetric unit are shown in grey). (B) Analysis of DARPin-mediated crystal contact of the C7_16-caspase-7 complex structure. The crystal contact interface involves low-entropy residues A68, A130 and D134 (highlighted in red), which were lysines and glutamates, respectively, in the original DARPin library.