

## Supplementary Information

**DNA sequence with the ecotin fusion protein sequence (NdeI and XhoI restriction sites are highlighted):**

**cat atg** aag acg atc tta cct gct gtg ctt ttc gct gct ttt gca acg act agt gcc tgg gcc gca gaa tct gtt cag cct ctg gaa aaa  
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cca cgc gga tcc gaa ttc gag ctg cgt cga caa gct tgc ggc cgc **ctc gag**

**DNA sequence of the expressed nanobodies:**

Nb20:

ggt tct cag gtt cag ctg gtt gaa tct ggt ggt ggt ctg gtt cag gcg ggt ggt tct ctg cgt ctg tct tgc gcg gtt tct ggt gcg ggt gcg  
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ab8:

gaa gtt cag ctg gtt gaa tct ggt ggt ggt ctg gtt cag ccg ggt ggt tct ctg cgt ctg tct tgc gcg gcg tct ggt ttc acc ttc gac gac  
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acc tac tac tgc gcg cgt gac aac ctg ggt tac cgt ccg tct gaa aac ctg tac ggt atg gac gtt tgg ggt cag ggt acc acc gtt acc  
gtt tct tct

H11-H4:

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VHH72:

caa gtg caa tta caa gaa agt gga ggt gga ctg gtg caa gct ggt gga tca tta cgt ctg tcc tgc gcg gct tca ggc cgc act ttt agt  
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gtg act gtg agt agt ggc tcc

**DNA sequence of the full spike protein:**

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atc gac cgg ctg aac gag gtg gcc aag aat ctg aac gag agc ctg atc gac ctg caa gaa ctg ggg aag tac gag cag tac atc aag  
tgg ccc

#### **Amino acid sequence of the ecotin fusion protein:**

MKTILPAVLFAAFATTSAAWAAESVQPLEKIAPYPQAEKGMKRQVIQLTPQEDESTLKVELLIGQTLEVD  
CNLHRLGGKLENKTLEGWGYDYVFDKVSSPVSTMMACPDGKKEKKFVTAYLGDAGMLRYSNKLPI  
VVYTPDNVDVKYRVWKAEEKIDNAVVRGSTSGSGHHHHHSAGLVPR|GS

#### **Amino acid sequence of the expressed nanobodies (residual amino acids from the cleaving are underlined):**

Nb20:

GSQVQLVESGGGLVQAGGSLRLSCAVSGAGAHRVGWFRRAPGKEREFVAAIGASGGMTNYLDSVKG  
RFTISRDNANKNTIYLQMNSLKPQDTAVYYCAARDIETAEYIYWGGTQVTVSS

ab8:

GSQVQLVESGGGLVQPGGSLRLSCAASGFTFDDYAMSWVRQAPGKGLEWIGRMYNNGRTSYNPSLKS  
LVTISRDNANKNTLYLQMNSLRAEDTATYYCARDNLGYRPSNLYGMDVWGGTQVTVSS

H11-H4:

GSQVQLVESGGGLMQAGGSLRLSCAVSGRTFSTAAMGWFRQAPGKEREFVAAIRWSGGSAYYADSV  
KGRFTISRDKAKNTVYLMNSLKYEDTAVYYCAQTHYVSYLLSDYATWPYDYWGQGTQVTVSS

VHH72:

MDQVQLQESGGGLVQAGGSLRLSCAASGRTFSEYAMGWFRQAPGKEREFVATISWSGGSTYYTDSVK  
GRFTISRDNANKNTVYLMNSLKPDDTAVYYCAAAGLGTVVSEWDYDYDYWGQGTQVTVSS

#### **Nanobody expression trials under different conditions:**

**Table S1.** VHH72

Number of trials	Nutrition solution	Bacterial strain	Signal seq.	Prom.	Fusion protein	Expression compartment	Temp. (°C)	Yield (mg/L)
1.	Auto induction	<i>BL21 (DE3)</i>	-	T7	-	periplasm	18	0.17
2.	2YT	<i>BL21 (DE3)</i>	-	T7	-	periplasm	18	0
3.	2YT	<i>Shuffle-T7</i>	-	T7	-	periplasm	18	0
4.	2YT	<i>Shuffle-T7</i>	pelB	T7	-	periplasm	18	0.1
5.	2YT	<i>BL21 (DE3)</i>	-	T7	MBP	cytoplasm	18	0
6.	2YT	<i>Shuffle-T7</i>	-	T7	MBP	cytoplasm	18	0
7.	2YT	<i>BL21 (DE3)</i>	-	Tac	GST	cytoplasm	18	0
8.	2YT	<i>Shuffle-T7</i>	-	Tac	GST	cytoplasm	18	0
9.	Auto induction	<i>BL21 (DE3)</i>	-	T7	-	cytoplasm, periplasm	23	0
10.	Auto induction	<i>BL21 (DE3)</i>	-	T7	-	cytoplasm, periplasm	30	0
11.	Auto induction	<i>HB2151</i>	-	T7	-	cytoplasm, periplasm	30	0

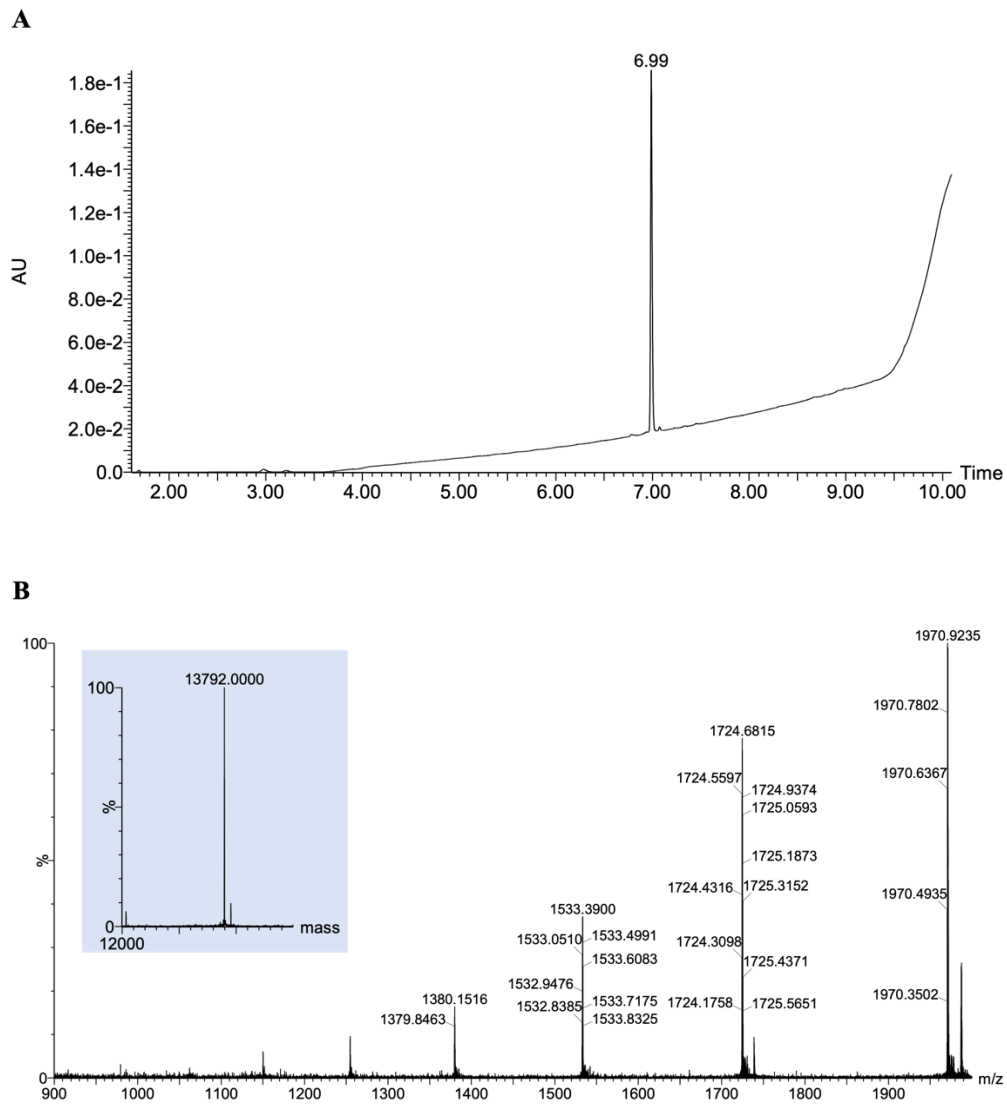
**Table S2.** Nb20

Number of trials	Nutrition solution	Bacterial strain	Signal seq.	Prom.	Fusion protein	Expression compartment	Temp. (°C)	Yield (mg/L)
1.	2YT	<i>Shuffle-T7</i>	-	T7	-	cytoplasm	18	0
2.	2YT	<i>BL21 (DE3)</i>	-	T7	-	cytoplasm	18	0

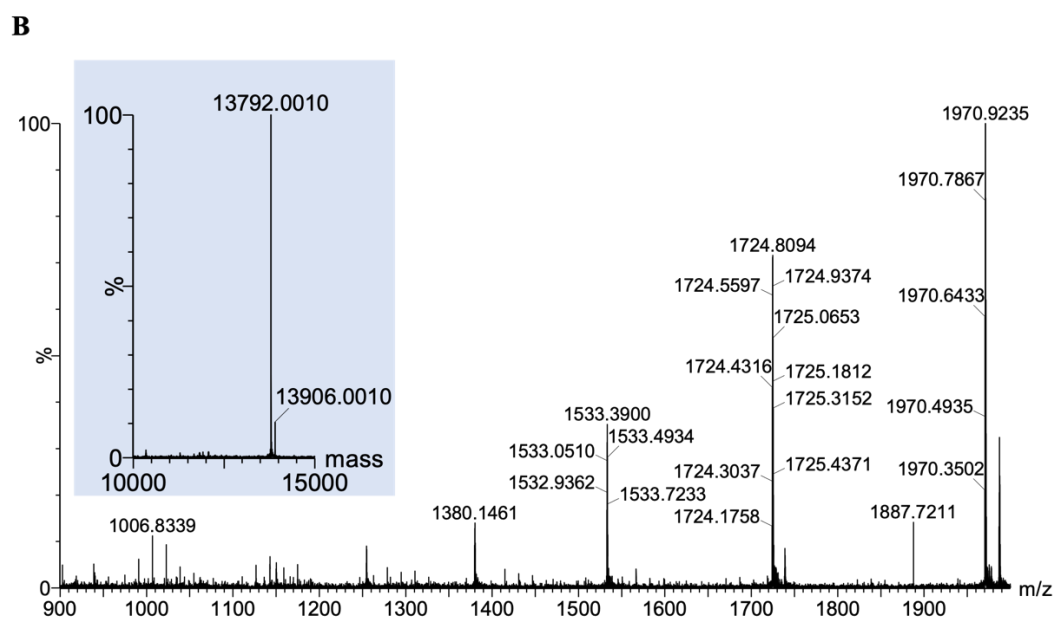
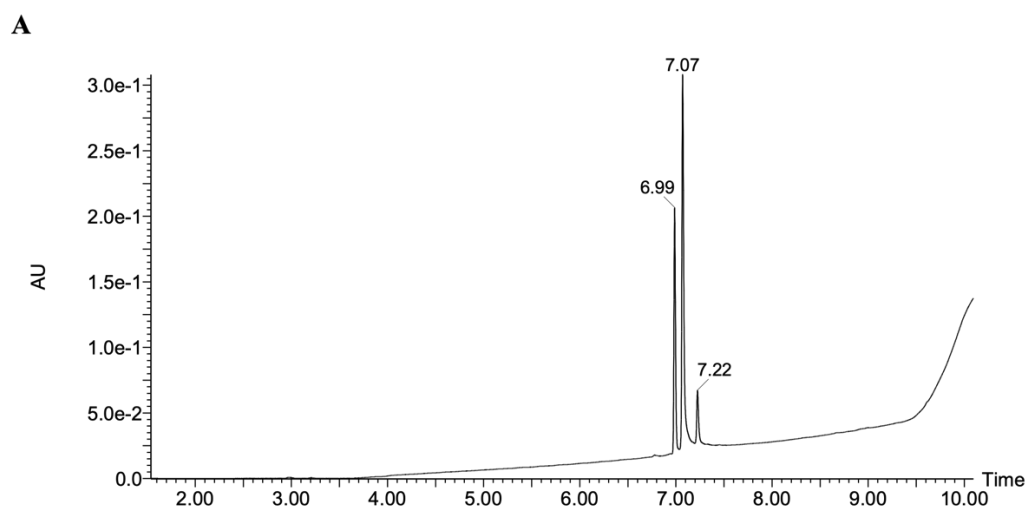
**Table S3.** ab8

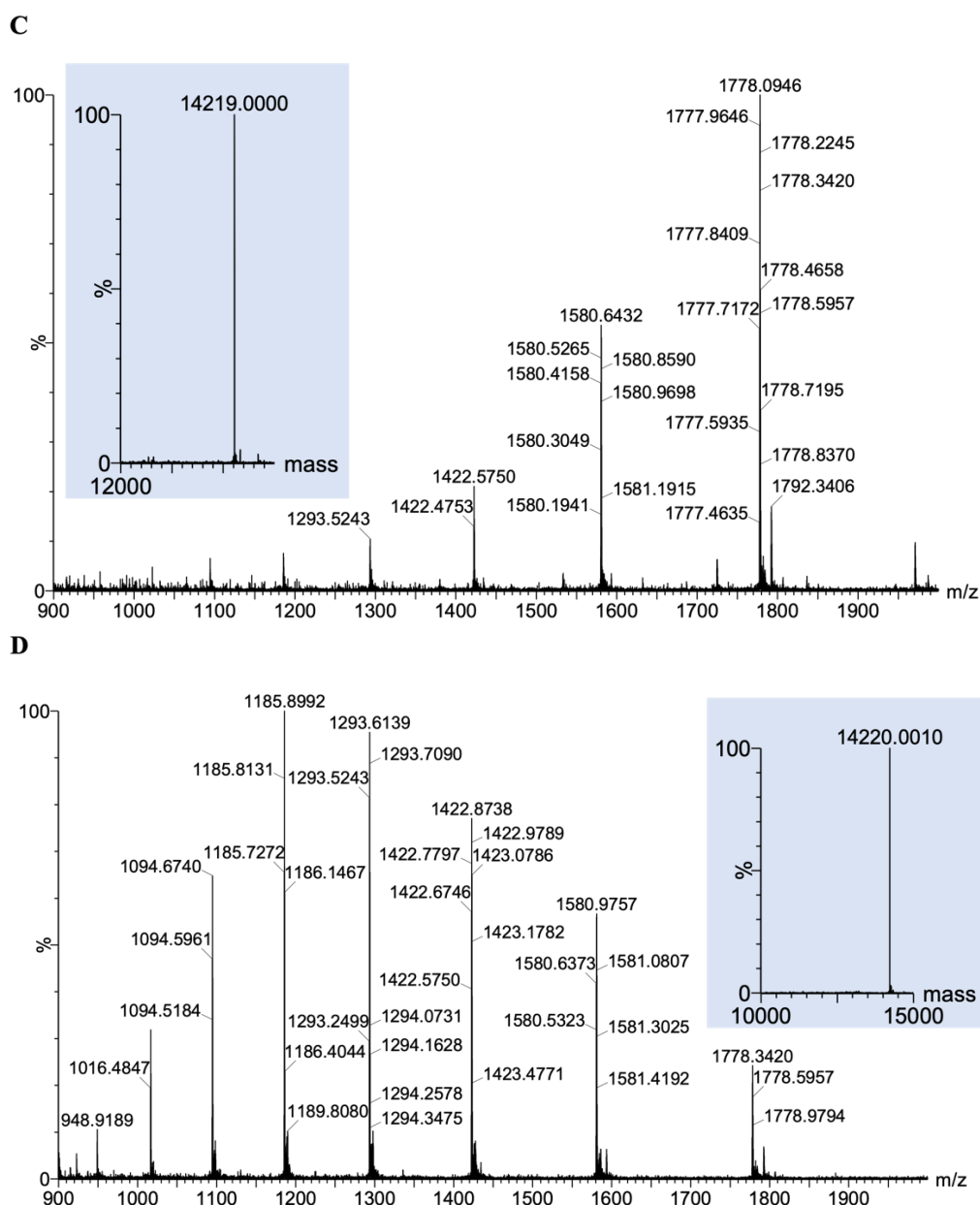
Number of trials	Nutrition solution	Bacterial strain	Signal seq.	Prom.	Fusion protein	Expression compartment	Temp. (°C)	Yield (mg/L)
1.	Auto induction	<i>HB2151</i>	-	T7	-	cytoplasm, periplasm	30	0
2.	2YT	<i>HB2151</i>	-	T7	-	cytoplasm, periplasm	30	0

**Supplemental Figures:**

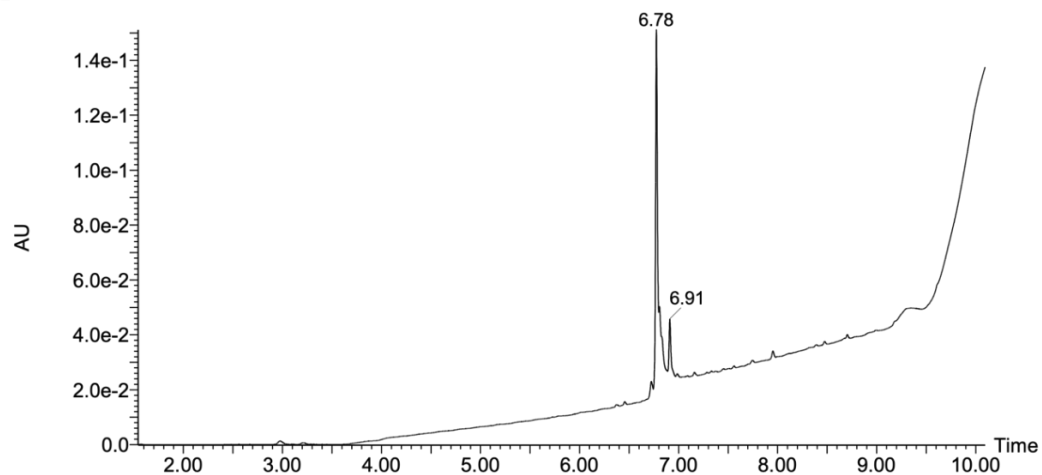
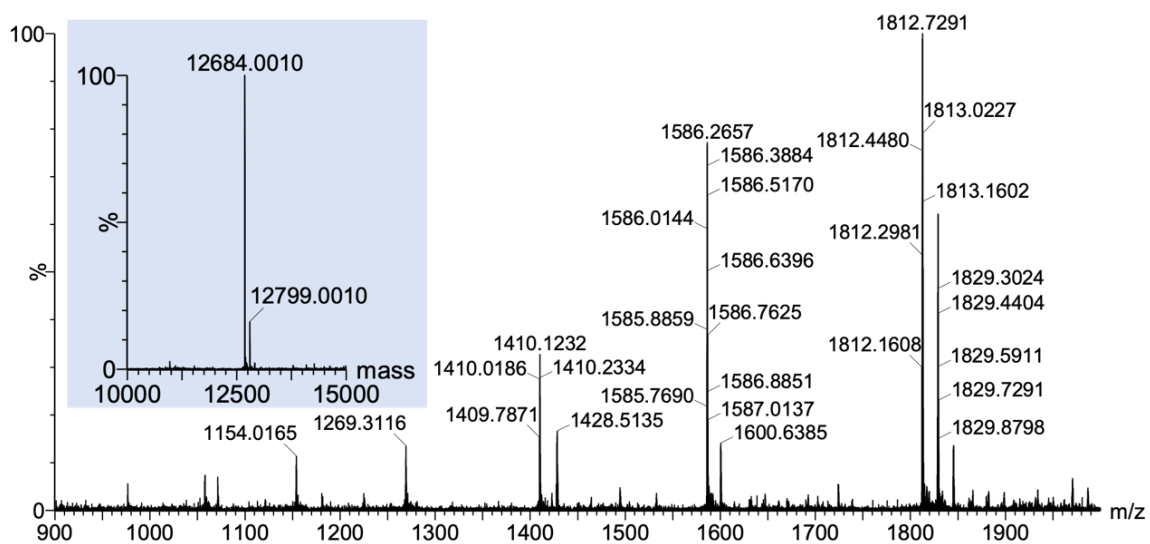


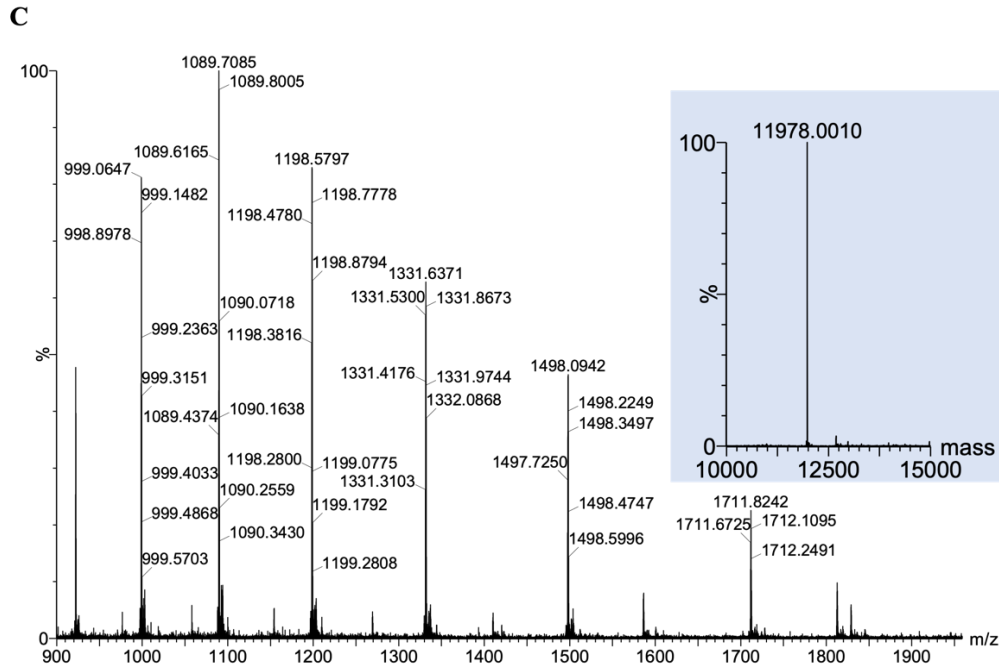
**Figure S1. A)** HPLC chromatogram ( $A_{220\text{nm}}$ ) of the expressed ab8 nanobody. **B)** Mass spectrum and deconvoluted spectrum of the peak  $R_t=6.99$  min.



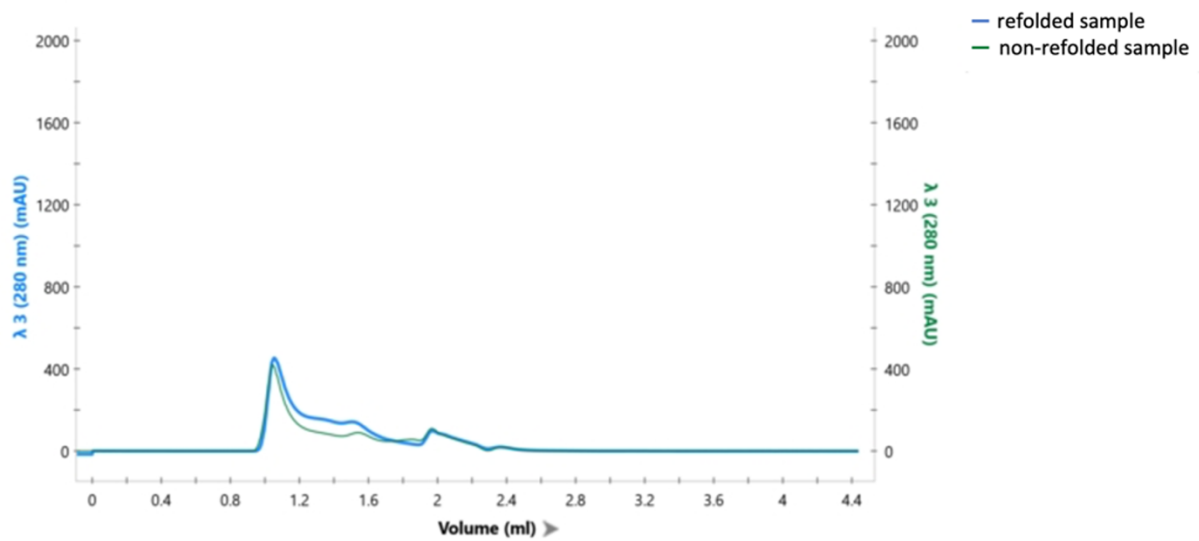


**Figure S2.** **A)** HPLC chromatogram ( $A_{220\text{nm}}$ ) of the expressed H11-H4 nanobody. **B)** Mass spectrum and deconvoluted spectrum of the peak  $R_t=6.99$  min. **C)** Mass spectrum and deconvoluted spectrum of the peak  $R_t=7.07$  min. **D)** Mass spectrum and deconvoluted spectrum of the peak  $R_t=7.22$  min.

**A****B**

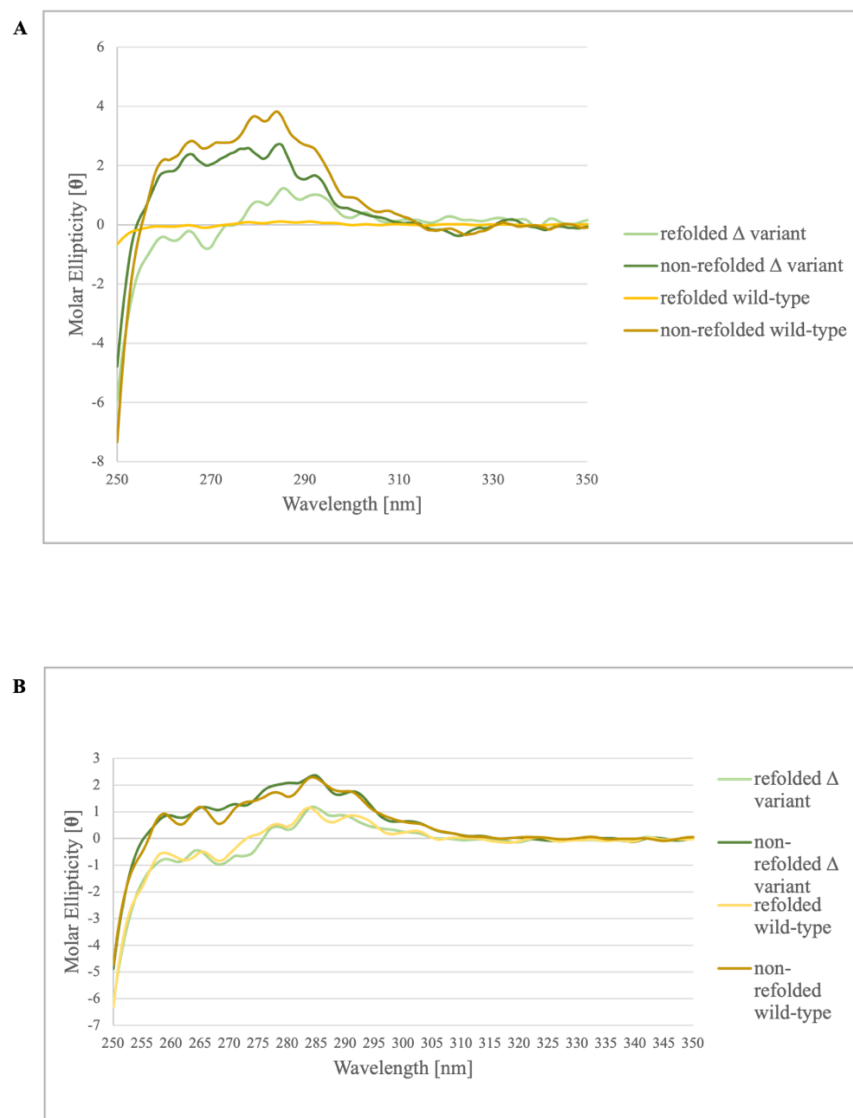


**Figure S3.** A) HPLC chromatogram ( $A_{220\text{nm}}$ ) of the expressed Nb20 nanobody. B) Mass spectrum and deconvoluted spectrum of the peak Rt=6.78 min. C) Mass spectrum and deconvoluted spectrum of the peak Rt=6.91 min.

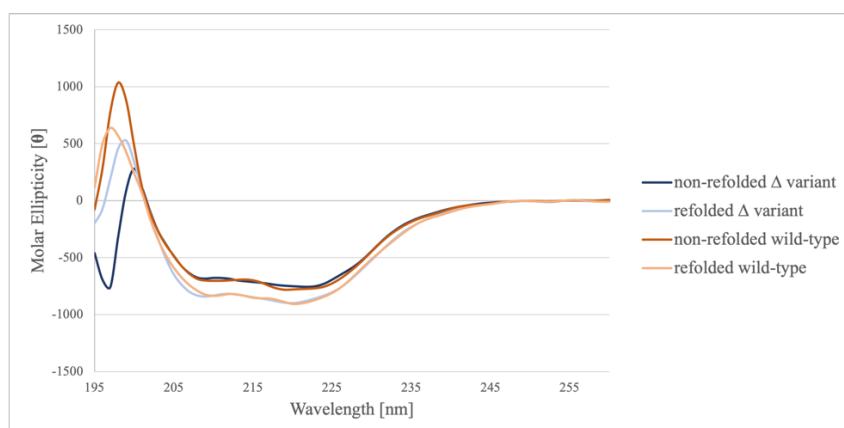


**Figure S4.** Comparison of chromatograms from the size-exclusion chromatography of refolded (blue) and non-refolded (green) spike RBD delta variants.

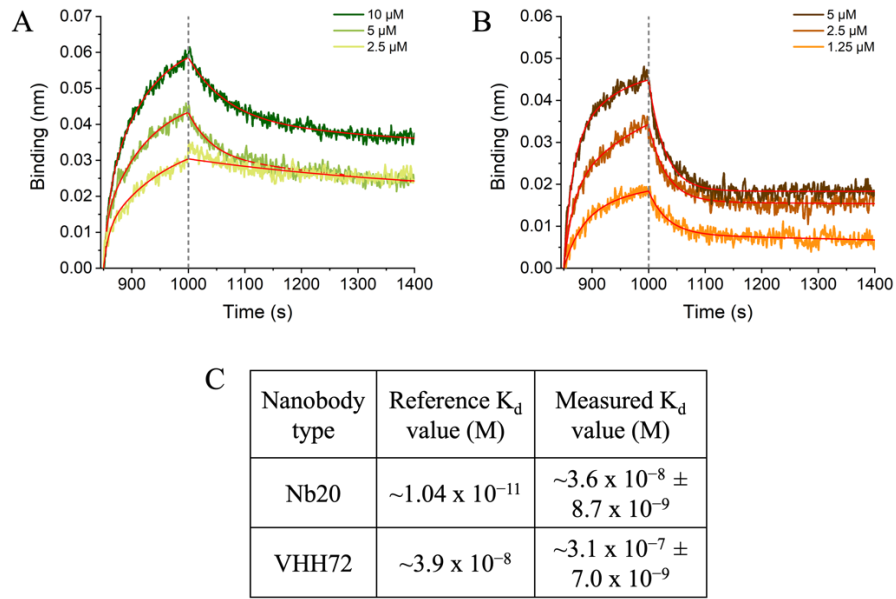




**Figure S5.** Comparison of spike RBD delta and wild-type variants after a refolded and non-refolded purification process by NUV-ECD measurements at **A)** 8 $\mu$ M and **B)** 16  $\mu$ M concentrations.



**Figure S6.** Comparison of spike RBD delta and wild-type variants after a refolded and non-refolded purification process by FUV-ECD measurements at a concentration of 16  $\mu$ M.



**Figure S7.** Binding of **A)** VHH72 and **B)** Nb20 anti-spike antibody variants to the receptor-binding domain of the spike protein fused to the maltose-binding protein at different concentrations. Table **C** contains the collected reference [18,20] and measured  $K_d$  values.