SUPPORTING INFORMATION

A Novel Regioselective Approach to Cyclize Phage-Displayed Peptides in Combination with Epitope-Directed Selection to Identify a Potent Neutralizing macrocyclic Peptide for SARS-CoV-2

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SUPPLEMENTARY FIGURES

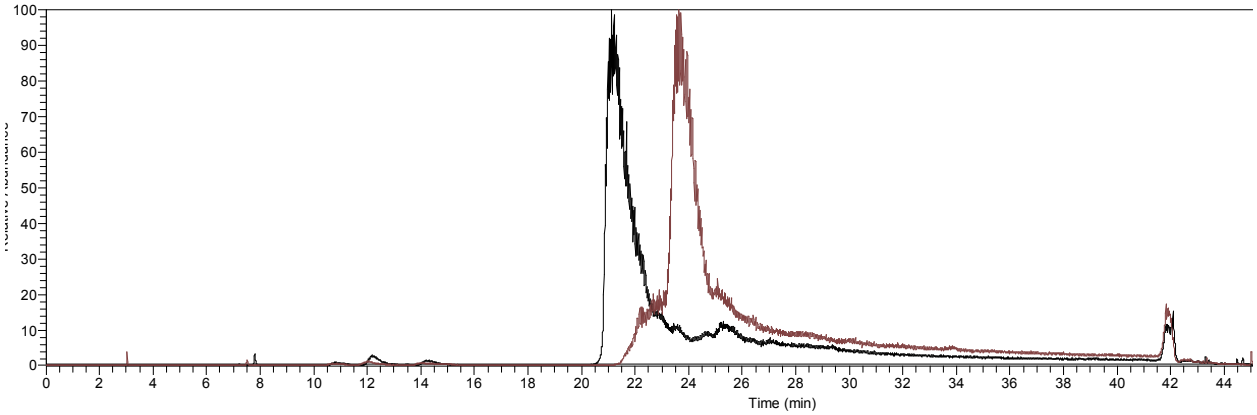


Figure S1: TIC chromatogram for ESI-MS of CA_5C-sfGFP before (black) and after (maroon) reaction with 100 µM of CAmCBT for 3 hours at room temperature.

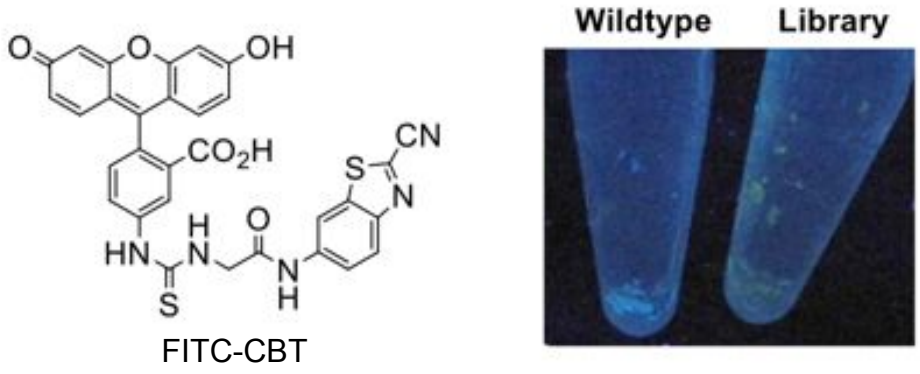


Figure S2: Labelling phages with FITC-CBT. Only the 5mer Library (CX_5C) containing an N-terminal cysteine showed fluorescence when reacted with FITC-CBT.

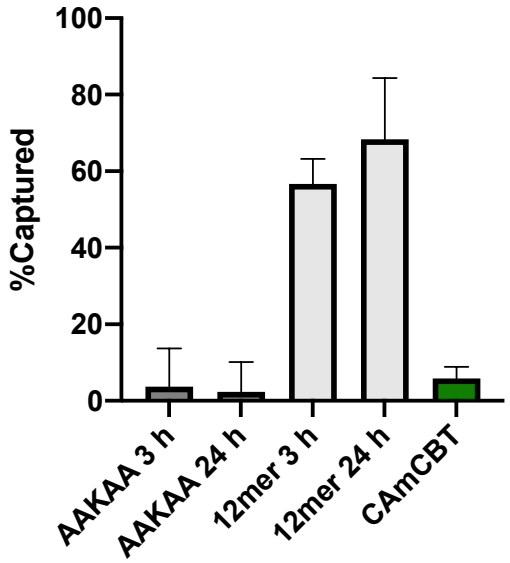


Figure S3: Biotin Capture Assay using Biotin-CBT. Phages were reacted with Biotin-CBT for varying amounts of time (3 or 24 hours) and then captured using streptavidin-coated beads. Phages cyclized with CAmCBT were reacted for 24 hours with Biotin-CBT before capture. AAKAA refers to phages that do not display a library on the N-terminus of pIII. Data is given as the mean ± s.d. of three independent experiments. %Captured was calculated by (phages input – phages eluted)/phages input x 100.

A

p28_12mer_R3top100

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	n	percent
P28S1	C	V	P	E	T	E	L	D	T	TAG	S	G	P	C	194735	47.4536331935404
	C	V	P	E	S	A	R	S	D	D	D	G	E	C	25557	6.22780960550139
P28S2	C	I	P	E	Q	V	L	R	A	D	G	S	S	C	22047	5.37248183951517
P28S3	C	V	P	S	G	A	D	P	TAG	I	A	S	L	C	16700	4.0695081743504
	C	V	P	V	E	V	R	G	P	G	P	T	A	C	14834	3.61479546456969
	C	I	P	E	E	E	L	T	A	TAG	D	S	L	C	9615	2.34301323930414
	C	V	P	D	S	V	TAG	V	N	S	N	V	Q	C	7676	1.87051166145591
	C	V	D	P	F	V	Y	G	G	D	E	TAG	N	C	2676	0.652096040392915
	C	S	G	D	G	I	V	L	S	S	M	G	R	C	2071	0.504667750244292
	C	Q	I	V	W	D	H	P	TAG	N	S	T	L	C	1884	0.459099006016536
	C	I	P	P	E	A	N	G	L	TAG	G	W	R	C	1636	0.398665591211812
	C	I	P	TAG	A	A	D	P	N	S	W	D	L	C	1503	0.366255735691536
	C	V	P	E	A	V	R	G	D	V	G	S	S	C	1331	0.324342238326969
	C	V	P	E	S	E	L	N	S	L	P	R	P	C	901	0.219558494915552

B

p28_12mer_R3enriched

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	n	enrichment
	C	S	G	D	G	I	V	L	S	S	M	G	R	C	2071	1070.66953766001
P28S1	C	V	P	E	T	E	L	D	T	TAG	S	G	P	C	194735	586.571439597557
	C	I	P	TAG	A	A	D	P	N	S	W	D	L	C	1503	387.874774288506
P28S2	C	I	P	E	Q	V	L	R	A	D	G	S	S	C	22047	373.05068109633
	C	V	P	V	E	V	R	G	P	G	P	T	A	C	14834	273.145442533775
	C	V	P	T	I	V	TAG	G	D	S	S	G	D	C	252	259.802243834208
P28S3	C	V	P	S	G	A	D	P	TAG	I	A	S	L	C	16700	256.960049279274
	C	V	P	E	A	V	R	G	D	V	G	S	S	C	1331	228.581869406965
	C	V	P	D	S	V	TAG	V	N	S	N	V	Q	C	7676	219.669976154253
	C	F	R	TAG	L	C	TAG	L	Y	M	A	L	S	C	551	189.082058667524
	C	V	P	E	T	E	L	D	T	L	S	G	P	C	843	173.489120279553
	C	V	P	L	S	V	TAG	A	Q	G	Y	T	A	C	162	166.658585321991
	C	V	P	E	TAG	V	I	S	E	D	T	T	S	C	476	163.208820191909
	C	V	P	E	S	I	Q	E	G	R	Q	TAG	M	C	143	146.994924080523

Figure S4: Next-generation sequencing data for the selection against P28. Sequences were sorted by abundance (A) and enrichment between rounds 1 and 3 of selection (B). Enrichment was calculated by the (%R3-%R1)/%R1.

A

p29_12mer_R3top100

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	n	percent
P29S1	C	V	P	E	S	A	R	S	D	D	D	G	E	C	179599	62.1916802581869
P29S2	C	V	P	E	T	I	I	G	N	TAG	G	V	P	C	8050	2.78756020956912
	C	N	Y	S	A	TAG	P	I	P	C	V	L	M	C	2722	0.942576259682876
P29S3	C	T	F	N	G	W	G	S	G	S	R	TAG	A	C	2311	0.800254862647732
	C	S	G	I	S	D	C	L	F	V	T	A	R	C	1683	0.582790538224203
	C	L	F	L	S	I	A	I	V	S	T	P	S	C	1314	0.455012933586811
	C	Y	S	H	R	F	S	R	N	TAG	G	K	M	C	689	0.238587451477407
	C	F	R	L	F	R	F	Y	T	G	G	E	D	C	525	0.181797404971899
	C	V	P	E	S	V	R	S	D	D	D	G	E	C	489	0.169331297202398
	C	P	K	G	L	L	N	P	Y	F	H	N	Q	C	425	0.147169327834395
	C	F	K	S	R	TAG	G	V	G	R	R	G	V	C	369	0.127777604637392
	C	Y	I	TAG	V	G	D	N	G	H	TAG	F	N	C	363	0.125699920009142
	C	V	V	TAG	R	S	V	A	G	L	TAG	S	Y	C	359	0.124314796923642
	C	H	L	R	TAG	N	C	R	W	R	W	I	V	C	316	0.109424723754515

B

p29_12mer_R3enriched

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	n	enrichment
P29S1	C	V	P	E	S	A	R	S	D	D	D	G	E	C	179599	750.542033291433
	C	V	P	E	S	V	R	S	D	D	D	G	E	C	489	278.312821391841
P29S2	C	V	P	E	T	I	I	G	N	TAG	G	V	P	C	8050	247.545648837044
	C	V	S	E	S	A	R	S	D	D	D	G	E	C	215	244.612501428408
	C	N	Y	S	A	TAG	P	I	P	C	V	L	M	C	2722	171.753805914245
	C	V	P	G	S	A	R	S	D	D	D	G	E	C	106	120.092675122843
P29S3	C	T	F	N	G	W	G	S	G	S	R	TAG	A	C	2311	104.60195177694
	C	V	P	E	S	A	R	S	D	D	D	G	G	C	47	52.6920351959776
	C	I	P	E	S	A	R	S	D	D	D	G	E	C	35	38.9834304650897
	Y	V	P	E	S	A	R	S	D	D	D	G	E	C	32	35.5562792823677
	C	F	P	E	S	A	R	S	D	D	D	G	E	C	27	29.8443606444978
	C	L	F	L	S	I	A	I	V	S	T	P	S	C	1314	24.0182036338704
	C	V	P	E	T	I	I	G	S	TAG	G	V	P	C	17	18.4205233687579
	C	G	S	TAG	F	G	V	V	V	V	Y	C	V	C	291	17.4685369291129

Figure S5: Next-generation sequencing data for the selection against P29. Sequences were sorted by abundance (A) and enrichment between rounds 1 and 3 of selection (B). Enrichment was calculated by the (%R3-%R1)/%R1.

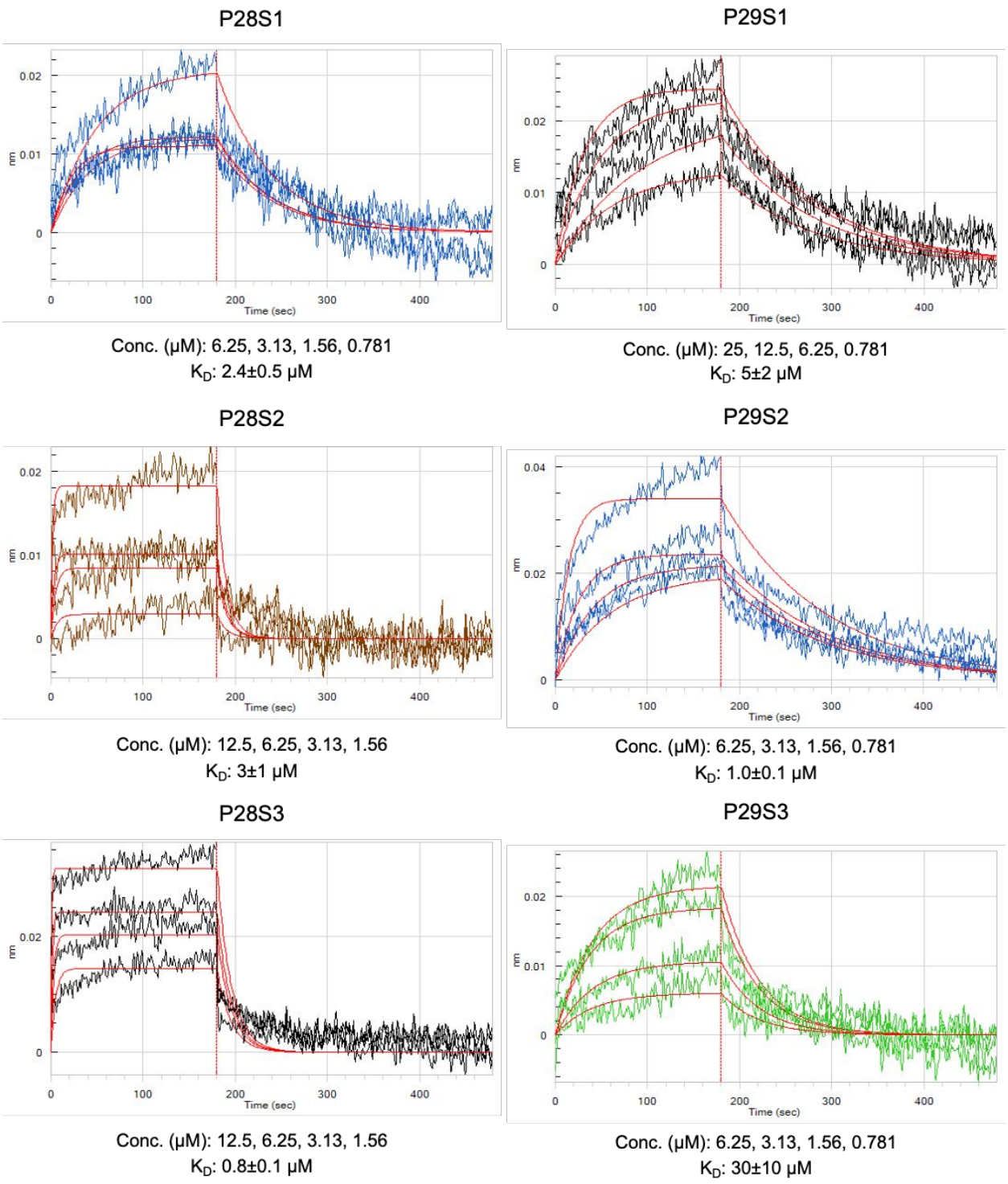


Figure S6: Biolayer interferometry (BLI) traces of the selected peptides against P28 and P29. Concentrations tested are listed below the traces for the corresponding peptides, along with K_D values given as the mean ± s.d. of 3 independent experiments (n=3).

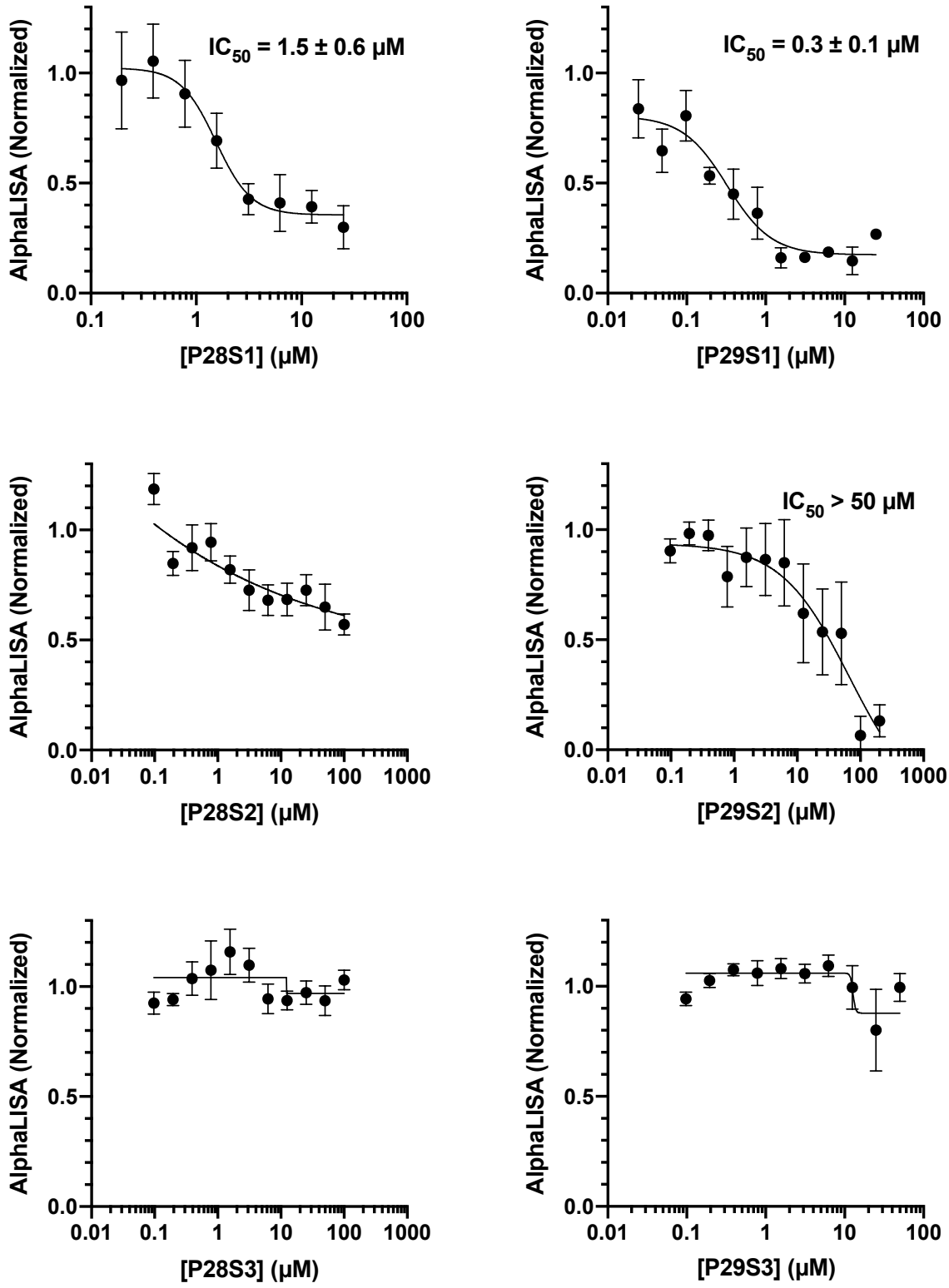


Figure S7: AlphaScreen data for the P28 and P29 selected peptides. Data points and IC_{50} values are given as the mean ± s.d. of three biologically independent experiments (n = 3).

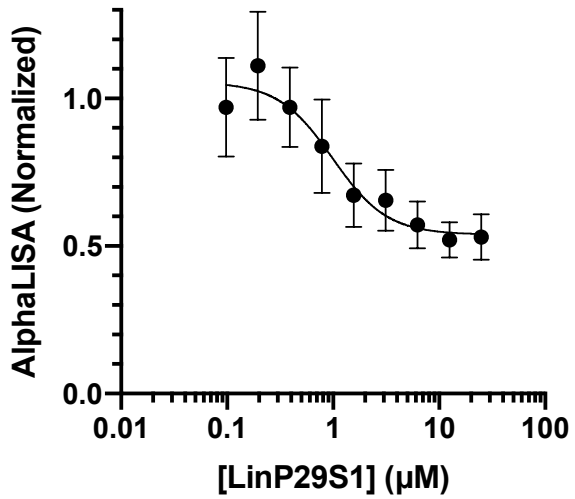
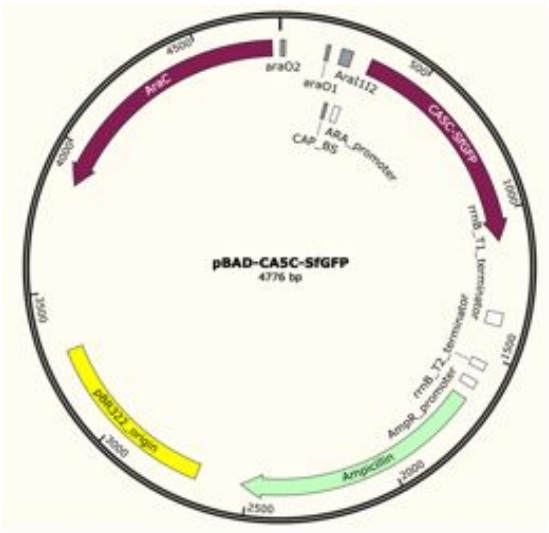


Figure S8: AlphaScreen data for the inhibition of the Spike:ACE2 interaction with LinP29S1. Data points are given as the mean ± sem of three biologically independent experiments (n = 3).



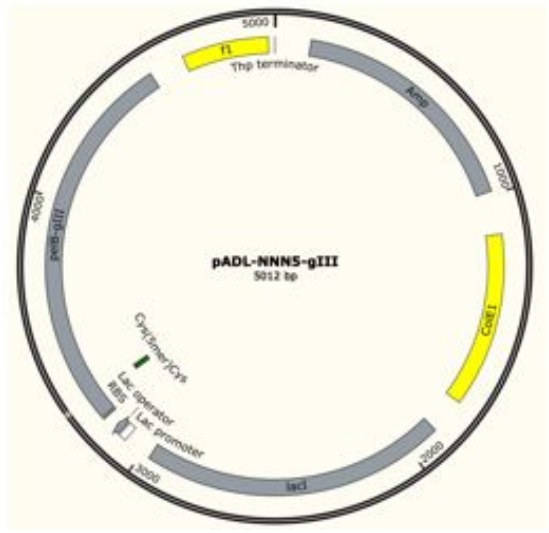


Figure S9: Plasmid constructs for the expression of CA_5C-sfGFP and for the expression of phage libraries.

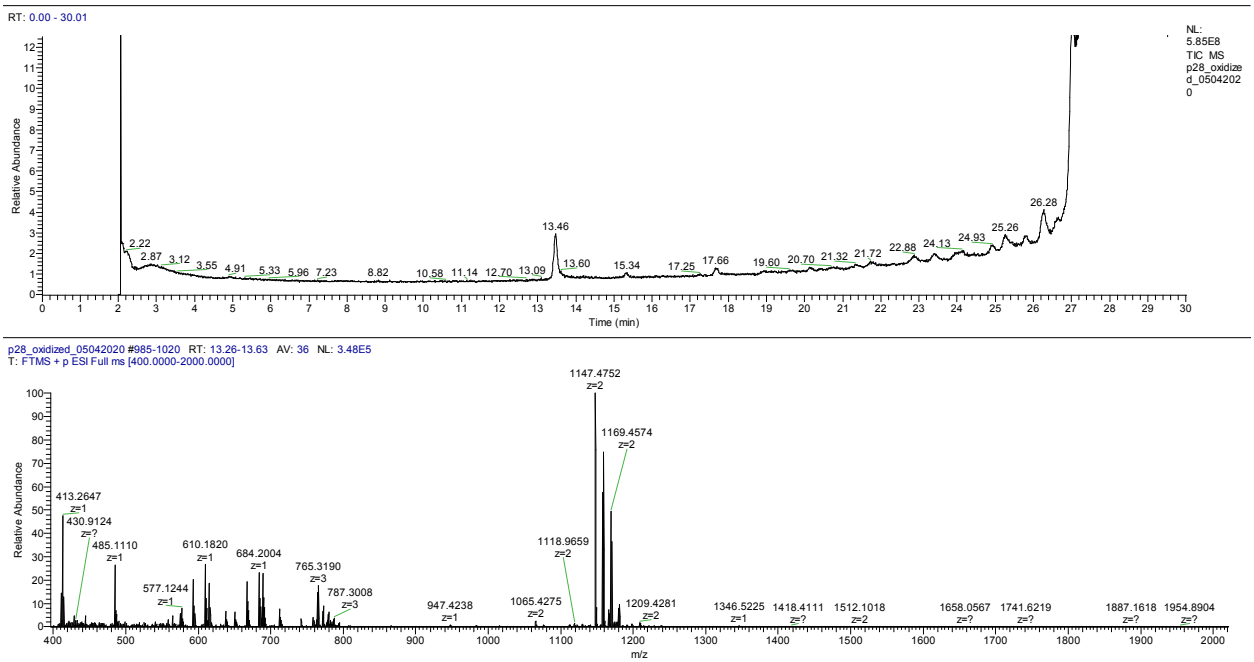


Figure S10: LC-MS data for P28. The TIC is shown on top and extracted data for the isolated peak on bottom.

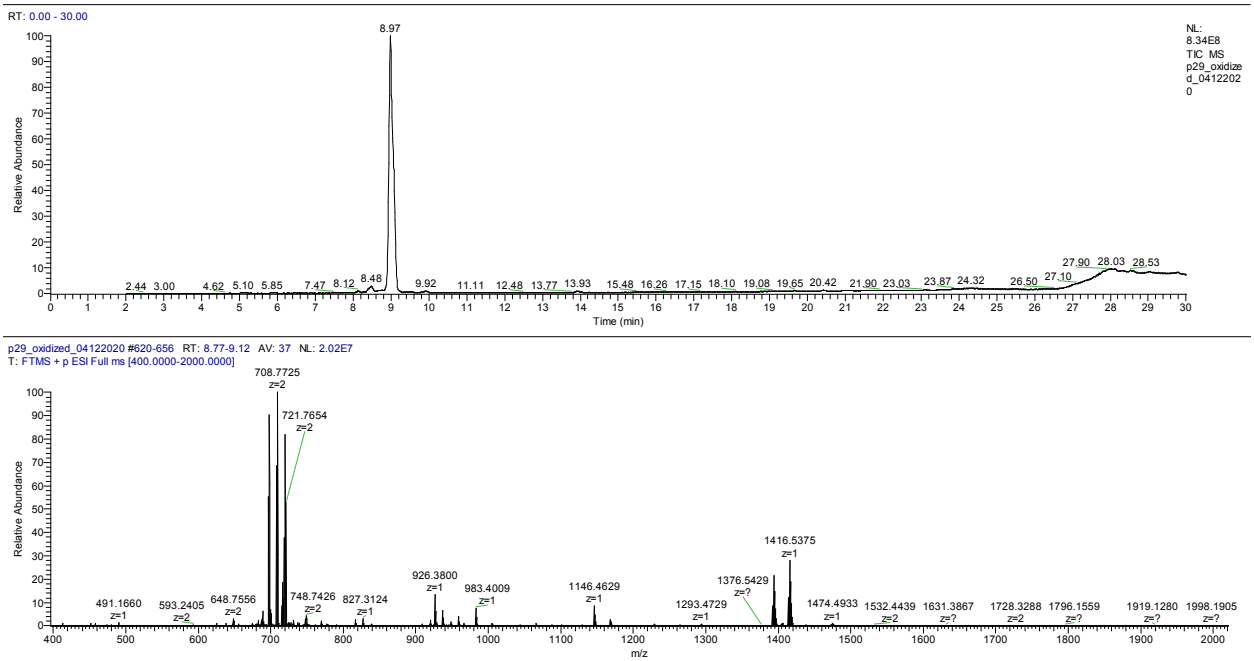


Figure S11: LC-MS data for P29. The TIC is shown on top and extracted data for the isolated peak on bottom.

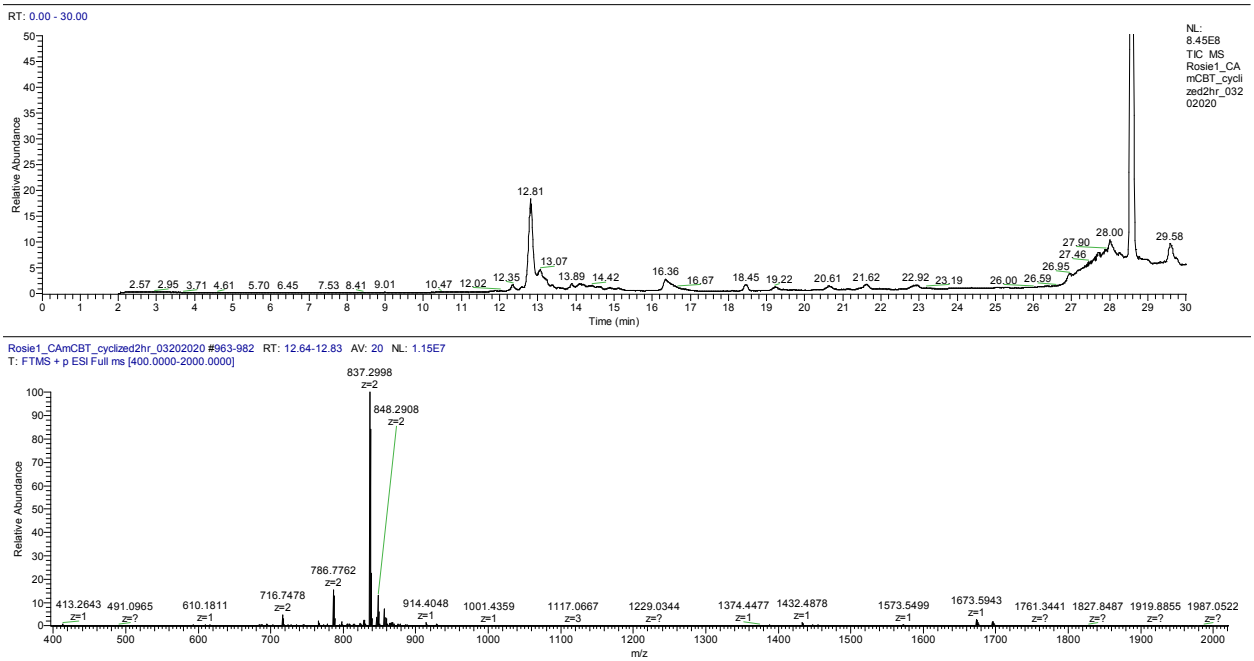


Figure S12: LC-MS data for P28S1. The TIC is shown on top and extracted data for the isolated peak on bottom.

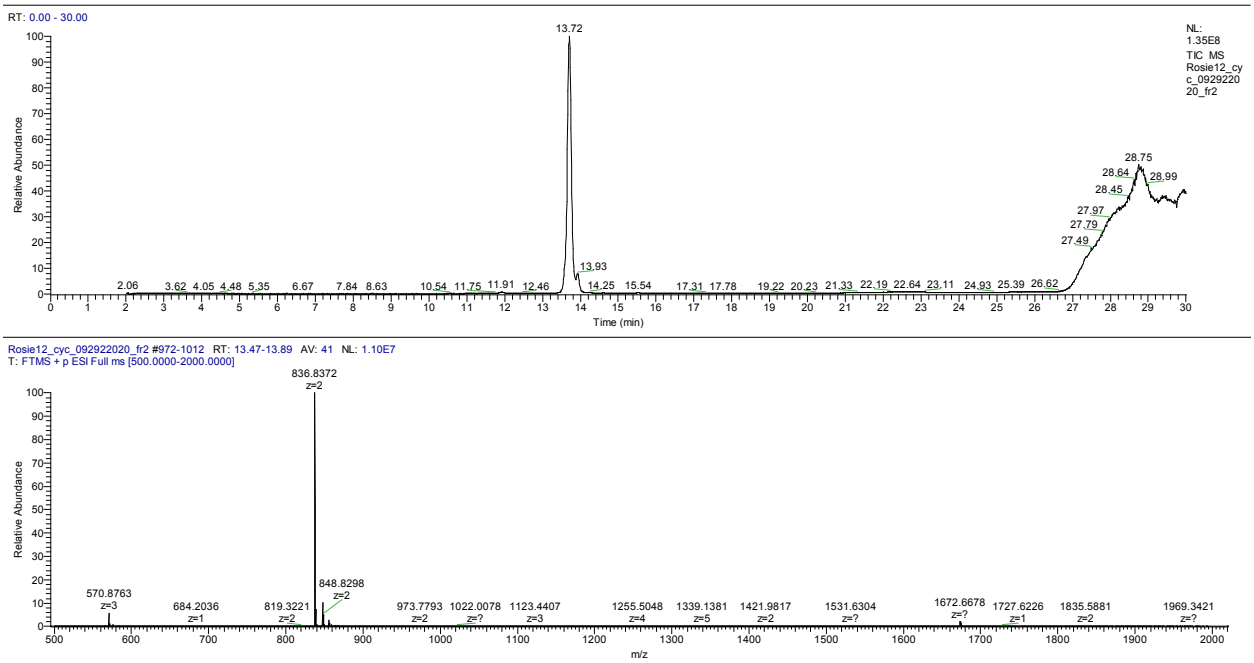


Figure S13: LC-MS data for P28S2. The TIC is shown on top and extracted data for the isolated peak on bottom.

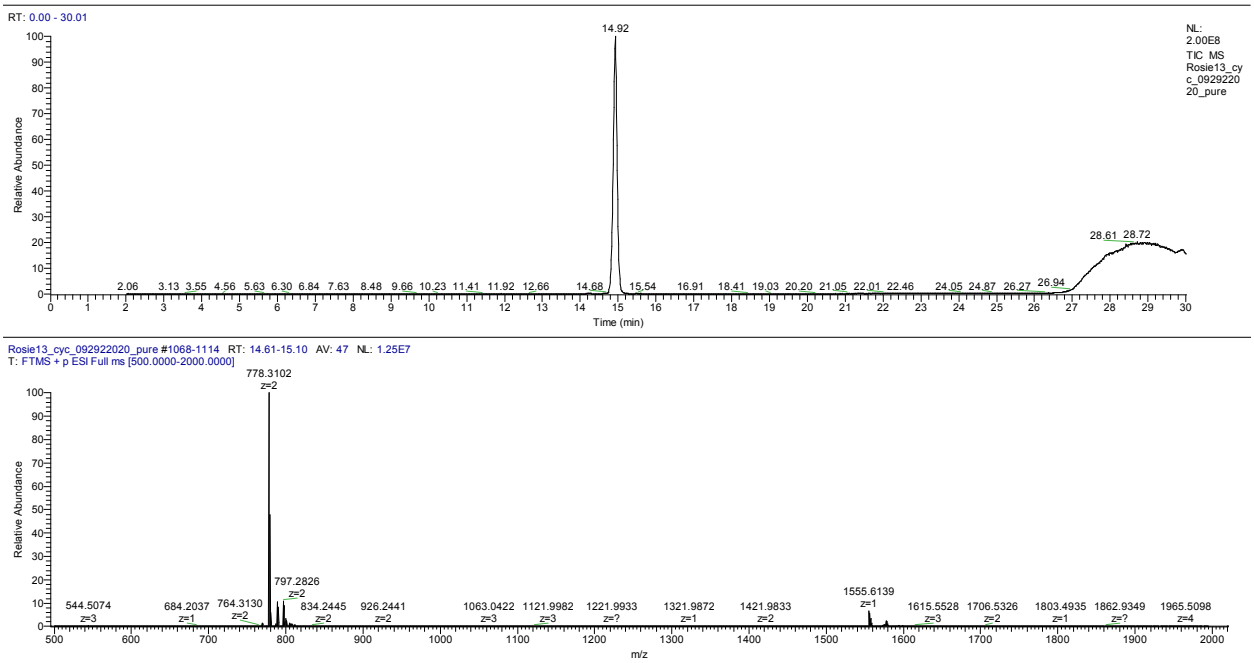


Figure S14: LC-MS data for P28S3. The TIC is shown on top and extracted data for the isolated peak on bottom.

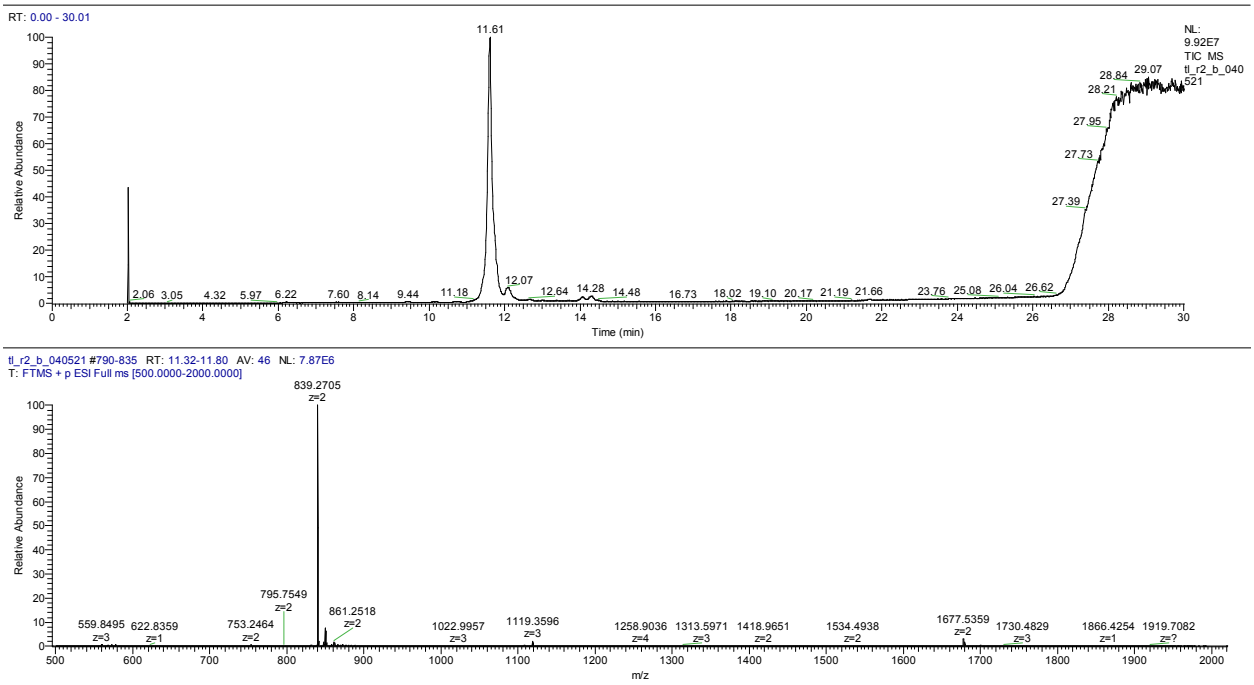


Figure S15: LC-MS data for P29S1. The TIC is shown on top and extracted data for the isolated peak on bottom.

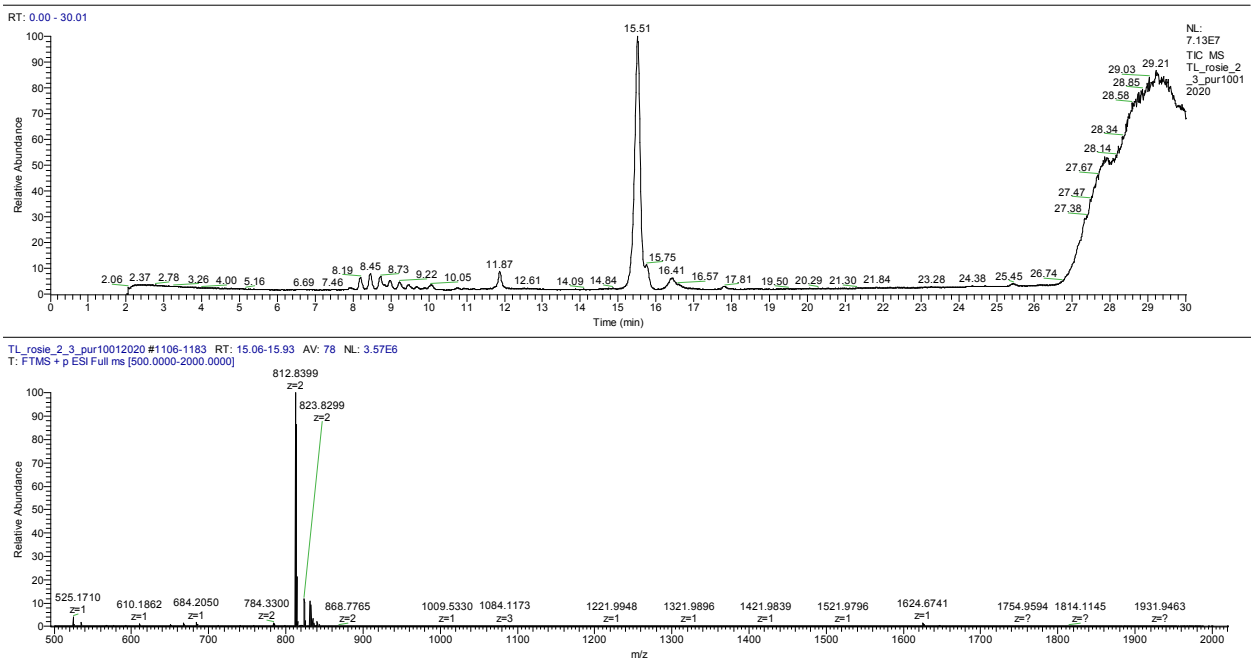


Figure S16: LC-MS data for P29S2. The TIC is shown on top and extracted data for the isolated peak on bottom.

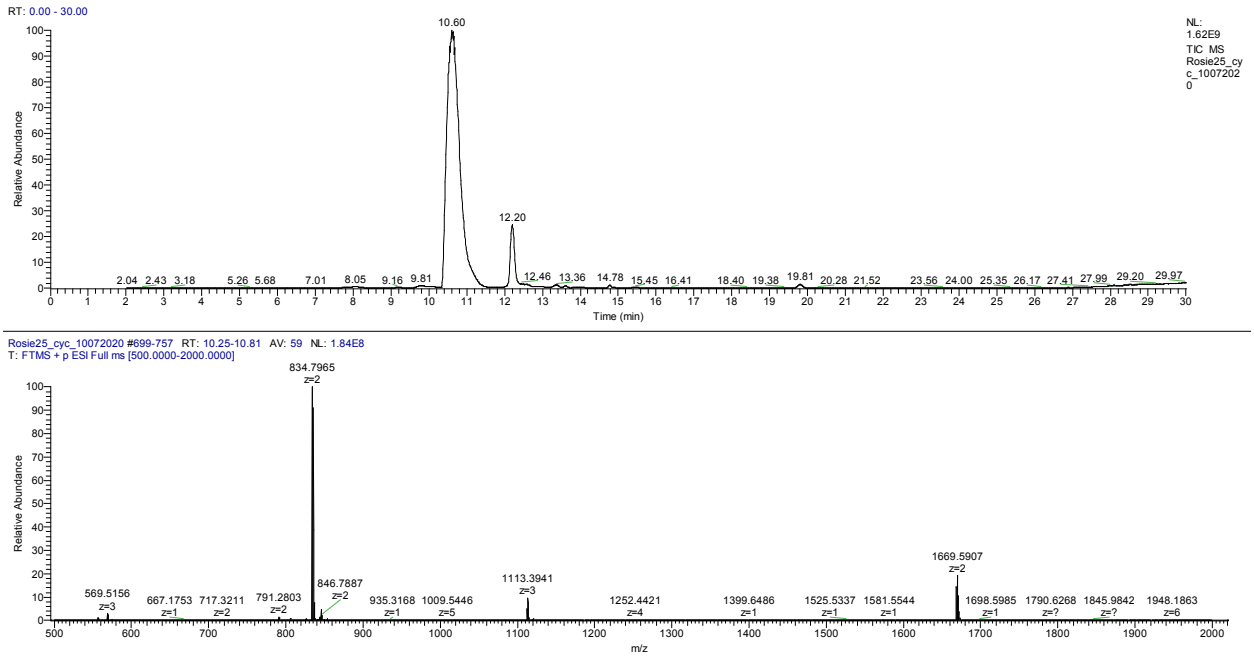


Figure S17: LC-MS data for P29S3. The TIC is shown on top and extracted data for the isolated peak on bottom.

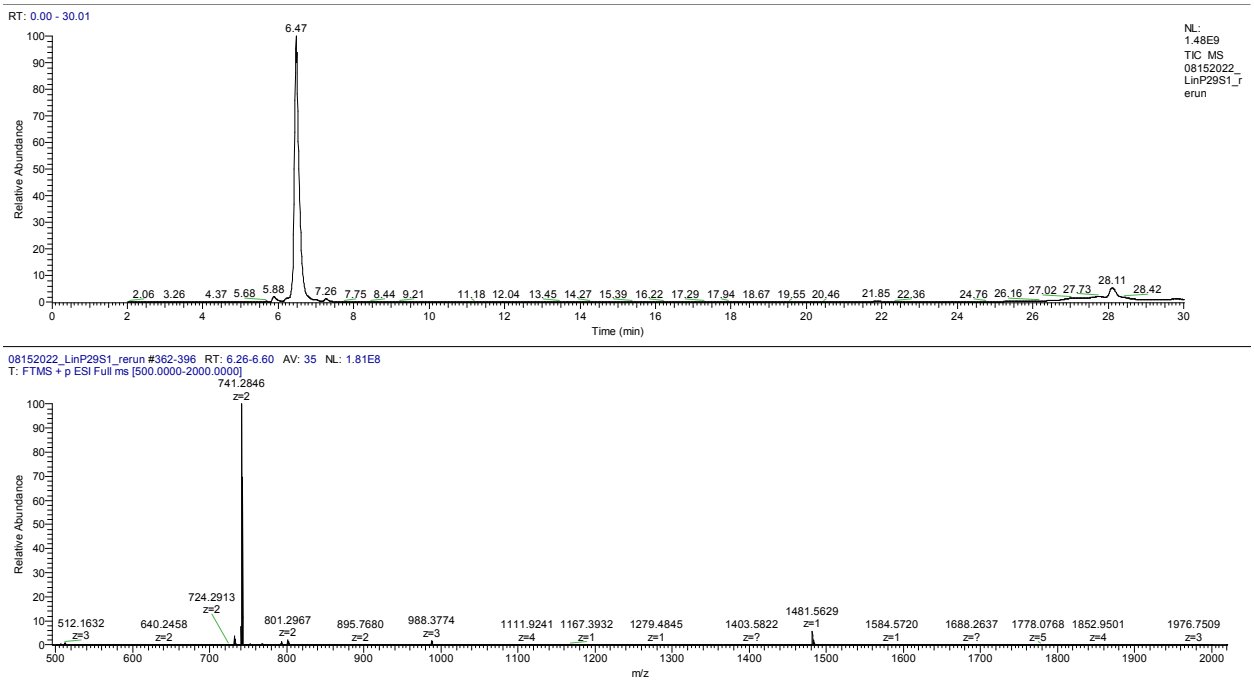


Figure S18: LC-MS Data for LinP29S1. The TIC is shown on top and extracted data for the isolated peak on bottom.

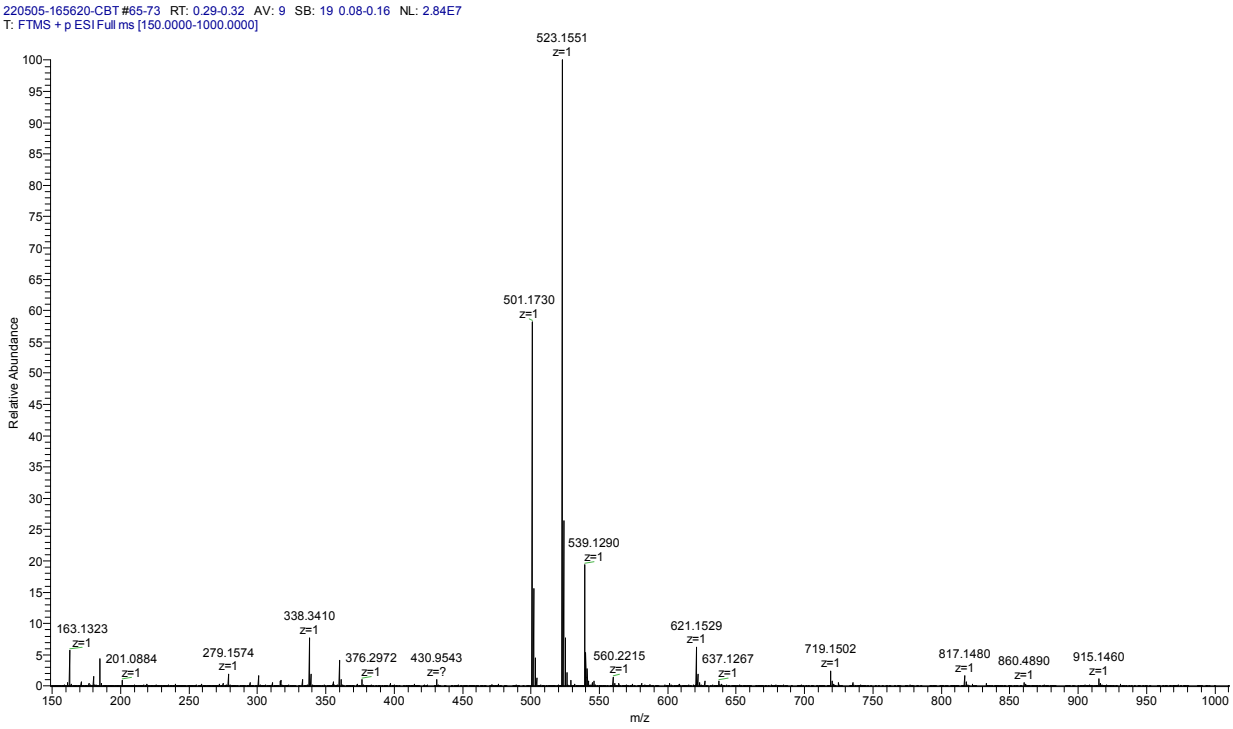


Figure S19: High-Resolution ESI-MS of Biotin-CBT.

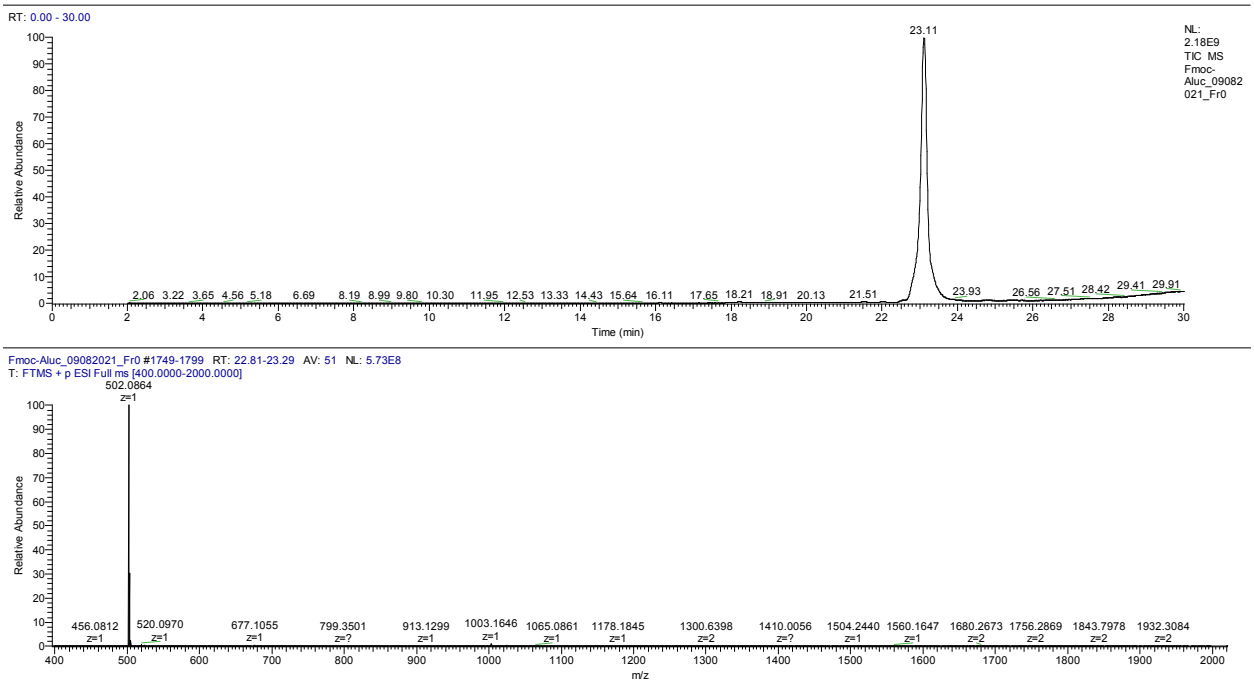


Figure S20: LC-MS data for Fmoc-Aluc. The TIC is shown on top and extracted data for the isolated peak on bottom.

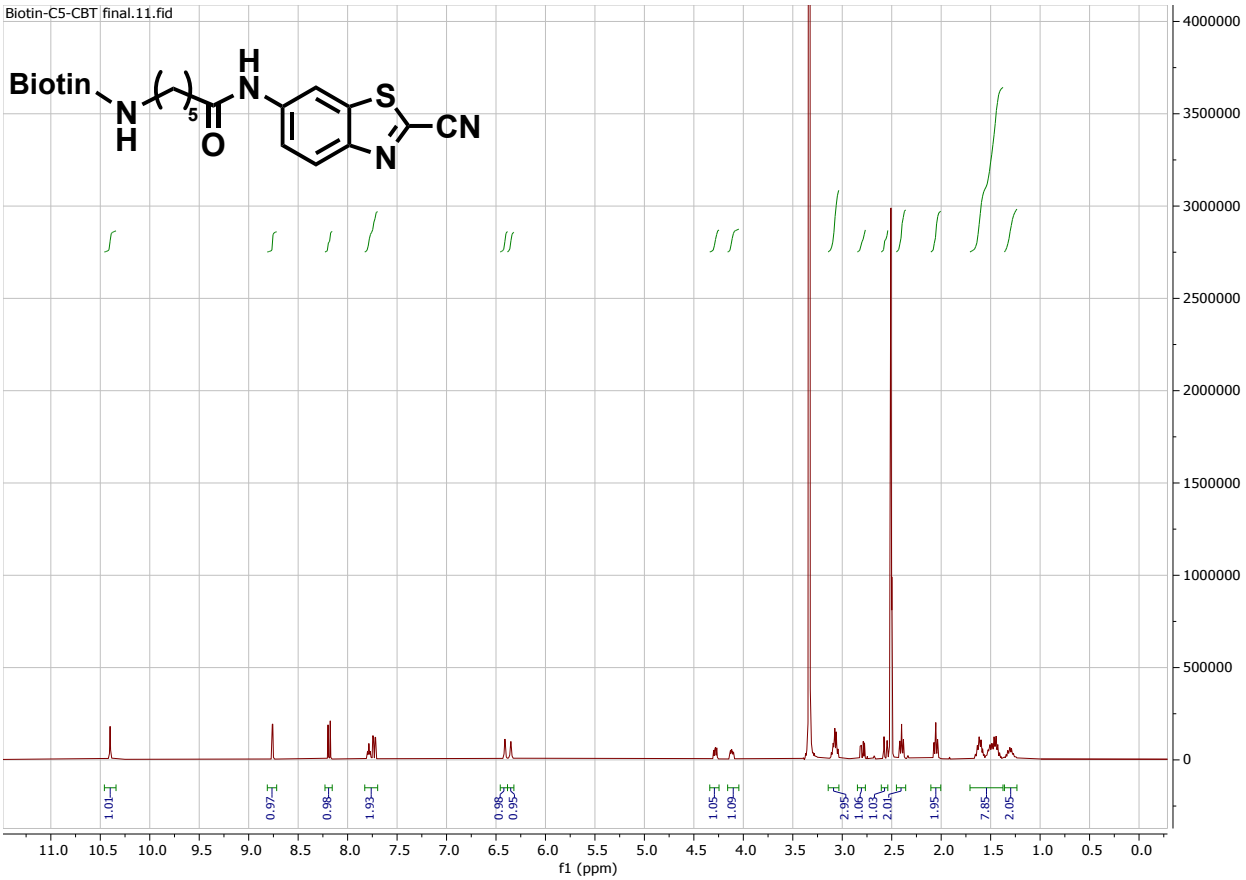


Figure S21: ^{1}H NMR spectra for Biotin-CBT.

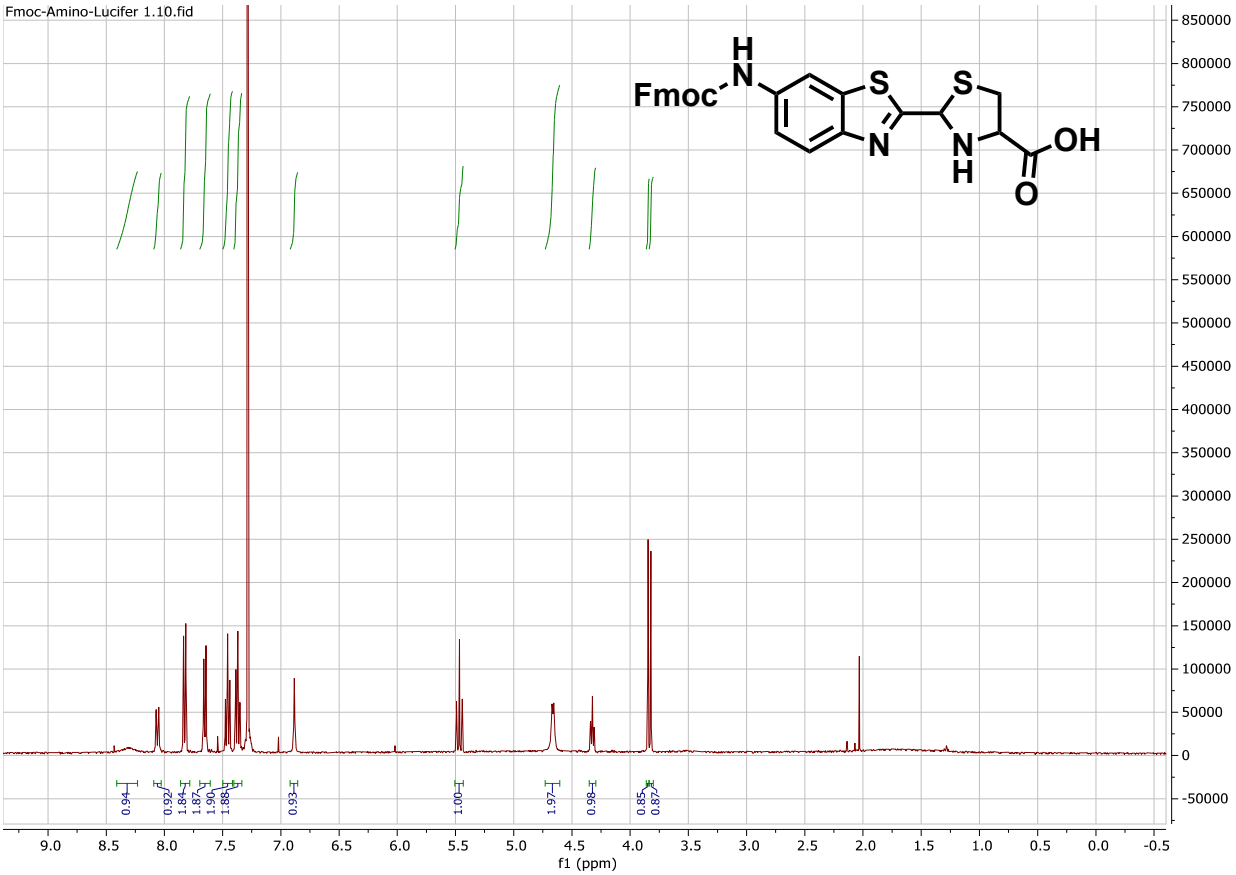


Figure S22: ^{1}H NMR spectra for Fmoc-Aluc.

SUPPLEMENTARY TABLES

Table S1: High-Resolution Mass Spectrometry Data for Synthesized Peptides

Peptide	Sequence	Expected Mass (Da)	Observed Mass (Da)
P28	Biotin-IYQAGSTP**C**NGVEGFN**C**YF	2291.94	2291.94
P29	Biotin-**C**QPTNGVGYQ**C**	1391.54	1391.55
P28S1	C*VPETELDTQSGPC*	1672.60	1672.59
P28S2	C*IPEQVLRADGSSC*	1671.66	1671.67
P28S3	C*VPSGADPQIASLC*	1554.61	1554.61
P29S1	C*VPESARSDDDGEC*	1676.53	1676.54
P29S2	C*VPETIIGNQGVPC*	1623.66	1623.68
P29S3	C*TFNGWGSGSRQAC*	1667.58	1667.59
LinP29S1	CVPESARSDDDGEC	1480.56	1480.56

C: Disulfide linked cysteines; C*: CAmCBT linked cysteines

Table S2: Raw Plaque Counts for SARS-CoV-2 Cytopathic Effect Assay with P29S1

[P29S1] (μM)	Trial 1			Trial 2			Trial 3		
25	0	0	0	0	0	0	0	0	0
12.5	12	14	7	0	0	0	0	0	0
6.25	36	30	35	15	13	11	14	12	10
3.125	49	58	51	41	43	38	43	40	41
1.5625	63	68	66	66	60	58	63	59	61
0.78125	82	80	89	73	71	74	76	70	70
0.390625				85	86	94	83	90	87
0.195313				92	90	87	89	90	84
0.097656				96	97	92	96	93	91
0.048828				94	97	96	94	94	92
Blank	83	91	86	99	94	93	91	97	97

Table S3: Raw Plaque Counts for SARS-CoV-2 Cytopathic Effect Assay with P28S1

[P28S1] (μM)	**Trial 1**		
25	87	79	84
12.5	86	88	81
6.25	81	94	86
3.125	90	87	85
1.5625	90	79	86
0.78125	88	78	93
Blank	83	91	86

SUPPLEMENTARY SCRIPTS

Supplementary Script 1: Amino Acid Analysis

```
library(microseq)
library(RColorBrewer)
library(dplyr)
library(stringr)
library(gplots)
NNK7Ffilt <- readFastq("/Users/traehampton/Documents/Research/Sequencing Results/Next Gen Sequencing/22005Wns_N22008/22005Wns_ZNRF3-R1_S1_L001_R1_001.fastq")
NNK7Rfilt <- readFastq("/Users/traehampton/Documents/Research/Sequencing Results/Next Gen Sequencing/21510Wns_N21170/21510Wns_12mer-negneg_S4_L001_R2_001.fastq")
#Define the following variables
libraryseq <- "GCCCAG.{54}GCGGCG.{6}" #change this regex to match specific library
beginning <- 19 #beginning of library in DNA string
lib <- 14 #number of codons in the library region
end <- beginning+lib*3
initialcodon <- beginning%/%3*4-1
endcodon <- initialcodon + lib*4
del <- beginning%/%3 #number of codons before library
aa <- c("A","C","D","E","F","G","H","I","K","L","M","N","P","Q","R","S","T","V","W","Y","TAG")

#slices out matches that contain start followed by 24 bases to reverse primer
NNK7Ffilt21 <- gregexpr(libraryseq,NNK7Ffilt[,2],extract = TRUE)
NNK7Rrevcomp <- reverseComplement(NNK7Rfilt[,2],reverse = TRUE) #gives reverse complement of reverse reads
NNK7Rcompfilt21 <- gregexpr(libraryseq,NNK7Rrevcomp,extract = TRUE)

#this compares the forward and reverse strands, only allowing for one mismatch in the primers, no mismatches allowed in the library region
n <- length(NNK7Ffilt21)
NNK7Fgood <- vector()
for(i in c(1:n)){
  if(NNK7Ffilt21[[i]][1] == NNK7Rcompfilt21[[i]][1]){
    NNK7Fgood[i] <- NNK7Ffilt21[[i]][1]
```

```
  }
  else{
    split <- strsplit(c(NNK7Ffilt21[[i]][1],NNK7Rcompfilt21[[i]][1]), split = "")
    diff <- which(split[[1]] != split[[2]])
    if(length(diff) < 2 && length(diff) > 0){
      for(x in c(1:length(diff))){
        if(diff[[x]] < beginning || diff[[x]] > end){
          NNK7Fgood[i] <- NNK7Ffilt21[[i]][1]
        }
        else{
          NNK7Fgood[i] <- ""
        }
      }
    }
    else{
      NNK7Fgood[i] <- ""
    }
  }
}
NNK7Fgood <- as.data.frame(NNK7Fgood)
NNK7Fgood <- NNK7Fgood[!apply(is.na(NNK7Fgood) | NNK7Fgood == "", 1, all),]

#this separates nucleotides into codons
codons <- gsub("(...)", "\\1 \\2", NNK7Ffilt21)

#this creates dataframe of sequences with reads organized by frequency
seqcount <- as.data.frame(sort(table(codons), decreasing = TRUE))

#this generates a matrix that contains amino acids in library region
l <- length(codons)
AAs <- matrix(0,l,lib)
AA <- gregexpr("\\s(TT[TC])",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "F"
      }
    }
  }
AA <- gregexpr("(\\sTT[AG])|(\\sCT[GACT])",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "L"
      }
    }
  }
AA <- gregexpr("(\\sTC[GCAT])|(\\sAG[TC])",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "S"
      }
    }
  }
AA <- gregexpr("\\sTA[TC]",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "Y"
      }
    }
  }
AA <- gregexpr("\\sTAG",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "TAG"
      }
    }
  }
AA <- gregexpr("\\sTAA",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- NA
      }
    }
  }
AA <- gregexpr("\\sTG[TC]",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "C"
      }
    }
  }
AA <- gregexpr("\\sTGA",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- NA
      }
    }
  }
AA <- gregexpr("\\sTGG",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "W"
      }
    }
  }
AA <- gregexpr("\\sCC[GCAT]",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "P"
      }
    }
  }
AA <- gregexpr("\\sCA[CT]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "H"
      }
    }
  }
AA <- gregexpr("\\sCA[AG]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "Q"
      }
    }
  }
AA <- gregexpr("(\\sCG[GCAT])|(\\sAG[GA])",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "R"
      }
    }
  }
AA <- gregexpr("\\sAT[CAT]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "I"
      }
    }
  }
AA <- gregexpr("\\sATG",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "M"
      }
    }
  }
AA <- gregexpr("\\sAC[GCAT]",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "T"
      }
    }
  }
AA <- gregexpr("\\sAA[CT]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "N"
      }
    }
  }
AA <- gregexpr("\\sAA[AG]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "K"
      }
    }
  }
AA <- gregexpr("\\sGT[GACT]",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "V"
      }
    }
  }
AA <- gregexpr("\\sGC[GACT]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "A"
      }
    }
  }
AA <- gregexpr("\\sGA[TC]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "D"
      }
    }
  }
AA <- gregexpr("\\sGA[AG]",codons,useBytes = FALSE)
  l <- length(AA)
```

```
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "E"
      }
    }
  }
AA <- gregexpr("\\sGG[GACT]",codons,useBytes = FALSE)
  l <- length(AA)
  for(a in c(1:l)){
    l2 <- length(AA[[a]])
    for(b in c(1:l2)){
      value <- AA[[a]][b]
      if(value > initialcodon && value < endcodon){
        AAs[a,(value%/%4 - (del-1))] <- "G"
      }
    }
  }
AAs <- as.data.frame(AAs)
#this gives unique amino acid sequences
UniqueAAs <- AAs %>% group_by_all() %>% count()
UniqueAAs <- UniqueAAs[order(-UniqueAAs$n),]
UniqueAAs <- UniqueAAs[apply(UniqueAAs,1,function(row) all(row != 0)),]
UniqueAAs <- na.omit(UniqueAAs)

#this counts sequences that have TAG codons, sequences that have more than one are only
 counted once
TAGreg <- regexpr("\\sTAG",codons)
TAGtable <- table(TAGreg)
percentTAG <- sum(TAGtable[2:length(TAGtable)])/length(codons)*100#percent of sequences
 containing TAG
```

```
#this creates a matrix of amino acid sequences that do not contain TAG codons
TAGpos <- which(AAs == "TAG")
TAGrow <- TAGpos%%nrow(AAs)
AAsnoTAG <- AAs[-TAGrow,]

#this creates heatmap for amino acid frequency per library position, change scale according to
 values
AAtable <- apply(AAs,2,function(x) table(factor(x,levels=aa)))
AAtable <- as.matrix(AAtable/length(codons))
colnames(AAtable) <- c(1:lib)
heatmapcolors <- colorRampPalette(brewer.pal(9,"Blues"))(100)
sc <- seq(0.0,0.6,by=0.006)
AAheatmap <- heatmap.2(AAtable, Rowv = NA, Colv = NA, col = heatmapcolors, density.info =
 "none", scale = "none", trace = "none", breaks = sc,  xlab = "Position in Library", ylab =
 "Codon", margins = c(3,4), dendrogram = "none")

#this creates projected heatmap based on NNK randomized codons
randomAAs <- matrix(0,21,lib,dimnames = list(rownames(AAtable),c(1:lib)))
randomAAs[c("A","G","P","T","V"),] <- 2/32
randomAAs[c("C","H","Q","N","K","Y","D","E","W","I","M","TAG","F"),] <- 1/32
randomAAs[c("L","S","R"),] <- 3/32
NNKheatmap <- heatmap.2(randomAAs, Rowv = NA, Colv = NA, col = heatmapcolors,
 density.info = "none", scale = "none", trace = "none", breaks = sc,  xlab = "Position in Library",
 ylab = "Codon", margins = c(3,4), dendrogram = "none")

#this creates heatmap showing bias from random, change scale with respect to range of values
lscale <- seq(-1,4,by=5/100)
librarybias <- (AAtable - randomAAs)/randomAAs
Biasheatmap <- heatmap.2(librarybias, Rowv = NA, Colv = NA, col = heatmapcolors,
 density.info = "none", scale = "none", trace = "none", breaks = lscale,  xlab = "Position in
 Library", ylab = "Codon", margins = c(3,4), dendrogram = "none")
librarybias <- as.data.frame(librarybias)
```

```
#this creates heatmap for AAsnoTAG
AAnoTAGtable <- apply(AAsnoTAG,2,function(x) table(factor(x,levels=aa)))
AAnoTAGtable <- as.matrix(AAnoTAGtable/nrow(AAsnoTAG))
colnames(AAnoTAGtable) <- c(1:lib)
heatmapcolors <- colorRampPalette(brewer.pal(9,"Blues"))(100)
sc <- seq(0.0,0.3,by=0.003)
AAheatmap <- heatmap.2(AAnoTAGtable, Rowv = NA, Colv = NA, col = heatmapcolors,
 density.info = "none", scale = "none", trace = "none", breaks = sc,  xlab = "Position in Library",
 ylab = "Codon", margins = c(3,4), dendrogram = "none")

#writes csv files for uniqueAAs and bias heatmaps change path to make file
path <- "/Users/traehampton/Documents/Research/Sequencing Results/Next Gen
 Sequencing/21510Wns_N21170"
write.csv(UniqueAAs, paste(path,"/12mernegnegUniqueAAs.csv", sep = ""), row.names = F)
write.csv(TAGtable, paste(path,"/12mernegnegTAGtable.csv", sep = ""), row.names = T)
write.csv(librarybias, paste(path,"/12mernegnegLibraryBias.csv", sep = ""), row.names = T)
```

Supplementary Script 2: Enrichment of Peptides

```
#this gives overall enrichment of each peptide sequence by comparing Round 1 and Round 4
 Library
library(prodlim)
path <- "/Users/traehampton/Documents/Research/Sequencing Results/Next Gen
 Sequencing/21510Wns_N21170"
lib <- 7
match <- row.match(UniqueAAsR1[,1:lib],UniqueAAsR3[,1:lib])
matchseq <- which(is.na(match)==FALSE)
percentenriched <- (UniqueAAsR3[match[matchseq],lib+1]/sum(UniqueAAsR3[,lib+1])-
 UniqueAAsR1[matchseq,lib+1]/sum(UniqueAAsR1$n))/(UniqueAAsR1[matchseq,lib+1]/sum(Un
 iqueAAsR1$n))
enrichedseq <- UniqueAAsR3[match[matchseq],]
enrichedseq$enrichment <- percentenriched[,1]
enrichedseq <- enrichedseq[order(-enrichedseq$enrichment),]
enrichedseq <- as.data.frame(enrichedseq)
```

```
UniqueAAsR1$percent <- UniqueAAsR1$n/sum(UniqueAAsR1$n)*100
UniqueAAsR3$percent <- UniqueAAsR3$n/sum(UniqueAAsR3$n)*100
```