Supplementary Information

mRNA display pipeline for protein biosensor construction

Zhenling Cui [1][2][3] , Cagla Ergun Ayva [2][3], Yi Jin Liew [4], Zhong Guo [1][2][3] , Roxane Mutschler [2][3], Birgit Dreier [5], Maria M Fiorito [2][3] , Patricia Walden [2][3] , Christopher B Howard [6] , Fernanda Ely [7] , Andreas Plückthun [5] , Carel Pretorius [8][9] , Jacobus PJ Ungerer [8][9] , Ashley M Buckle [10][a] and Kirill Alexandrov [1][2][3]*

[1] ARC Centre of Excellence in Synthetic Biology, Brisbane, Queensland 4001, Australia

[2] Centre for Agriculture and the Bioeconomy, Queensland University of Technology, Brisbane, Queensland 4001, Australia

[3] School of Biology and Environmental Science, Queensland University of Technology, Brisbane, Queensland 4001, Australia

[4] CSIRO Health & Biosecurity, Westmead, New South Wales 2145, Australia

[5] University of Zurich, Department of Biochemistry, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

[6] Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Queensland 4072, Australia

[7] Maltexco S.A, Santiago 806, Chile

[8] Department of Chemical Pathology, Pathology Queensland, Brisbane, Queensland 4006, Australia

[9] Faculty of Health and Behavioural Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

[10] PTNG Consulting, Melbourne, Victoria 3071, Australia

[a] Present address: Replay, 5555 Oberlin Drive, San Diego CA 92121, US

*Correspondence to: kirill.alexandrov@qut.edu.au

Supplementary methods and results

Molecular Cloning, Expression, and Purification of Transaminase Proteins

Genes encoding transaminases were synthesized with an N-terminal His-tag, Avi-tag and SUMO-tag (Table S1) and inserted into a pET28a plasmid by Gene Universal. The sequence-verified transaminase plasmid and BirA plasmid (MBI, Singapore) were co-transformed into BL21(DE3) cells and plated onto LB agar plates containing kanamycin and chloramphenicol antibiotics and grown overnight at 37 °C. The next day, the transformed cells were each grown at 37 °C in 6 liter LB culture supplemented with the appropriate antibiotics until an optical density OD600=0.8. Subsequently, 0.3 mM IPTG and 25 mg of biotin per liter of LB culture were added to induce protein expression and biotinylation, respectively. The cells were grown at 18 °C overnight and were harvested by centrifugation at 4000 rpm for 15 min. The cell pellets were resuspended in lysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1 mM AEBSF and DNAse I, and disrupted using a CFII cell disrupter (Constant Systems, UK) at 26 kPsi. Following centrifugation at 20,000 rpm, the supernatant was collected and loaded onto a Ni-NTA HisTrap FF crude column driven by an ÄKTA PURE (Cytiva) system for recombinant protein purification. The binding buffer used for purification contained 50 mM sodium phosphate, 300 mM NaCl and 20 mM imidazole (pH 8.0), and the elution buffer contained 50 mM NaH2PO4, 300 mM NaCl and 500 mM imidazole (pH 8.0).

Proteins eluted from the HisTrap column were subjected to further purification by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column (Cytiva). Prior to protein sample loading,

the column was pre-equilibrated with storage buffer containing 20 mM Tris/HCl (pH 7.2) and 100 mM NaCl. After size-exclusion chromatography, the peak corresponding to the correct size of the target protein was collected and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (50 kDa MWCO). The purified proteins were aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C.

Streamlining the mRNA Display Pipeline

To enhance protein display efficiency or facilitate ribosome dissociation, salts are typically added after translation in eukaryotic mRNA display systems [1], whilst EDTA is routinely supplemented after translation in prokaryotic systems [2,3]. However, both salt and EDTA can affect downstream reverse transcription efficiency. When using salt, purification of the mRNA-puromycin-protein conjugate (mRPP) is required, whereas when using EDTA, optimization of the Mg^{2+} concentration is necessary for chelation of the excess EDTA before reverse transcription. A desalting step is typically performed before the downstream panning procedure. We systematically examined the effects of salt, EDTA and incubation time on the formation of mRPP complexes (Figure S3A). It was observed that salt slightly enhanced the mRPP complex formation. Following salt incubation, optimization of the EDTA concentration to chelate the extra Mg^{2+} before reverse transcription was undertaken. Unfortunately, addition of EDTA could not rescue the reverse transcription and could not alleviate the requirement for desalting or purification step (Figure S3C). However, we observed that a 60-minute translation slightly improved protein display efficiency compared to a 30-minute translation. We established that the translation reaction could be directly used for reverse transcription without any prior treatment (Figure S3B). Consequently, the incubation step was omitted, and the translation reaction was directly used for reverse transcription. This streamlined the selection procedure, enabling completion of a selection round in 7 hours.

mRNA Display Binder Selection against *mAST and ALT*

The selection campaigns against mAST and ALT were performed concurrently, following a similar approach as the one used for cAST with some variations. In the mAST binder selection campaign, the R1 selection yielded only a small amount of mRNA transcripts from the heat-based cDNA elution. The remaining denatured Dynabeads were PCR amplified and produced good amounts of transcripts which were used for R2 selection. In R2, the Dynabeads, without denaturation, were directly used for PCR amplification resulting very limited amount of mRNA transcripts. These mRNAs were ligated to puromycin oligos, together with 80 pmol of naïve mRNA-PuL, were used for R3 selection. In R3 and the following R4 and R5 selection, the heat-denatured Dynabeads were used as templates for PCR amplification. During the ALT binder selection campaign, R1 yielded a substantial amount of mRNA transcripts from the heat-based cDNA elution. In R2, the undenatured Dynabeads were directly used as PCR templates. R3 employed heat-based cDNA elution of transcripts for PCR. In R4, 67% of the PCR products originated from undenatured Dynabeads and 33% from denatured Dynabeads served as templates for *in vitro* transcription. In R5, the cDNA amplified from denatured Dynabeads was used as a template for *in vitro* transcription. The amount of cDNA generated in each selection round was consistent with the qPCR results.

Cloning of the Binders into Expression Vectors in Fusion with CaM Binding Peptide (CaM-BP) and BLA-CaM Protein

Two gene fragments, His-[protein_insertion]-CaM-BP and BLA-CaM-[protein_insertion]-His (Table S1), were synthesized and cloned into pET28a plasmids via NcoI and HindIII restriction enzymes by Gene Universal. The sequence-verified plasmids were double-digested with BamHI and SalI. These linearized plasmid backbones were separated on a 1% agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The cDNA pool from round 5 and round 6 selections were PCR-amplified using either primer pair 215/226 or primer set 224/225, respectively. The PCR products were then separated on a 2%

agarose gel and subsequently purified as previously mentioned. The 215/226 amplified PCR products and double digested pET28a-His-insertion-CaM-BP vector were assembled using a Gibson assembly master mix (NEB), incubated for 1 hour at 50 °C before being transformed into TOP10 Competent Cells (Thermo Fisher Scientific). Similarly, the 224/225 amplified PCR products were assembled into the double digested pET28a-BLA-CaM-insertion-His vector using Gibson assembly.

Ninety-five single colonies for each selection were individually picked and placed into two separate 96-well plates. One plate contained 200 µl of LB medium supplemented with 50 µg/ml of kanamycin in each well, whilst the other plate contained 40 µl of UltraPure DNase/RNase-Free Distilled Water (no. 10977023, Thermo fisher Scientific) in each well. The plate containing the cells on LB medium was cultured at 37 °C for 10-14 hours before being supplemented with 20% glycerol and stored at -80 °C. The plate containing the cells in water was heated at 95 °C for 8 min and subsequently used for colony PCR. For the CaM-BP constructs, colony PCR was performed using primer pair 222/223 with an annealing temperature of 50 °C and 30 cycles of amplification. For the BLA-CaM constructs, PCR amplification was carried out using primer pair 224/T7 terminator with an annealing temperature of 68 °C and 30 cycles of amplification. Sequencing was conducted at Macrogen, using primer 222 for the CaM-BP constructs and T7 terminator for the BLA-CaM constructs.

Recombinant Expression and Purification of the Potential FN3con Binders

The plasmids containing the potential binders, which were identified from sequencing analysis, were isolated from the TOP10 cells using QIAprep Spin Miniprep Kit (Qiagen). These purified plasmids were then transformed into competent cells BL21(DE3) (Thermo Fisher Scientific). The transformed cells were cultured in LB broth supplemented with kanamycin and incubated with shaking at 37 °C. Once the optical density reached 0.8, protein expression was induced by adding 0.3 mM IPTG followed by an overnight incubation at 18 °C. Subsequently, the cells were lysed, and the protein was purified using HisTrap column, following the same procedure as used for transaminase proteins. The eluted protein was collected and then dialyzed against a buffer containing 20 mM Tris/HCl (pH 7.2) and 100 mM NaCl. The protein aliquots were frozen in liquid nitrogen and stored at -80 °C.

K_d Estimation using ELISA Assay

A Maxisorp plate (Thermo Fisher Scientific) was coated with the cAST protein at 10 µg/ml in PBS and incubated at room temperature overnight. The plate was washed with PBST buffer three times, and then blocked with 2% MPBS for 1 h. The purified binder proteins were serially diluted from 3000 nM to 5 nM in 2% MPBS buffer from columns 1 to 11. After a 1 h incubation, the plate was washed three times with PBST and then incubated 1 h with 100 µl of 1/5000 diluted anti-Flag M2-HRP antibody (Sigma A8592) in each well. Subsequently, the plate was washed again with PBST, and 100 µl of TMB solution (#34028, Thermo Fisher Scientific) was added into each well. The reaction was stopped using 1 M sulfuric acid after a 10-minute incubation or when the blue colour sufficiently developed. The A450 absorbance was measured using a Neo2 plate reader. The absorbance data were plotted against the binder concentrations and fitted using GraphPad Prism to the equation of Specific binding with Hill slope to estimate the K_d.

Biolayer Interferometry Assay

Biolayer interferometry binding data were acquired using an Octet RED8 system (ForteBio) equipped with 8-channels of Octet Streptavidin (SA) biosensors. For each kinetic analysis, the assay was conducted as follows: equilibration using assay buffer for 120 seconds, loading the biotinylated protein for 300 seconds, equilibration using assay buffer for 120 seconds, association with binders for 300 seconds, and dissociation in assay buffer for 600 seconds. Sensor tips were immobilized with 2.5-10 µg/ml of biotinylated transaminases and titrated with FN3con binders from either 1000 nM to 31.25 nM or from 100 nM to 12.5 nM, using a two times serial dilution.

All assays were conducted in assay buffer containing PBS with 0.02% Tween 20 and 0.1% BSA. The data were analysed using ForteBio data analysis 9.0 software.

Ribosome Display of DARPin Library and Identification of cAST Binders

To generate DARPin binders, biotinylated cAST was immobilized on Dynabeads MyOne Streptavidin T1 (Thermo Fisher Scientific) or Sera-Mag neutravidin-coated beads (GE), depending on the selection round. Ribosome display selections were performed essentially as described [4], using a semi-automatic KingFisher Flex MTP96 well platform. The library encodes N3C-DARPins with the originally reported randomization [5] and a stabilized C-cap [6-8]. Additionally, the library is a mixture of DARPins with randomized and non-randomized N- and C- terminal caps, respectively [8, 9], and successively enriched pools were cloned as intermediates in a ribosome display-specific vector [9]. Selections were performed over four rounds with decreasing target concentration and increasing number of washing steps, and the third round included a competition with non-biotinylated cAST to enrich for binders with slow dissociation rates [10].

The final enriched pool of cDNA encoding for putative DARPin binders was cloned as a fusion construct into a bacterial pQE30 derivative vector (Qiagen) with an N-terminal MRGS$(H)_8$ tag and C-terminal Flag tag via unique *BamH*I and *Hind*III sites. The construct contained a T5 *lac* promoter and *lacIq* for expression control. After transformation of *E. coli* XL1-blue, 380 single DARPin clones selected to bind cAST were expressed in 96-well format by addition of 1 mM IPTG and lysed by addition of B-Per Direct detergent plus Lysozyme and Nuclease (Pierce). After centrifugation these crude extracts were used for initial screening to bind cAST using Homogeneous Time Resolved Fluorescence (HTRF). Briefly, binding of the Flag-tagged DARPins to streptavidin-immobilized biotinylated cAST was measured using FRET mediated by donor Streptavidin-Tb cryptate (610SATLB, Cisbio) and acceptor mAb anti Flag M2-d2 (61FG2DLB, Cisbio). Control experiments without biotinylated cAST in the assay allowed for discrimination of Brachyury-specific hits while the assays using biotinylated mAST were performed to exclude binders with cross-reactivity. Experiments were performed at room temperature in white 384-well Optiplate plates (PerkinElmer) using the Taglite assay buffer (Cisbio) at a final volume of 20 µl per well. FRET signals were recorded after an incubation time of 30 minutes using a Varioskan LUX Multimode Microplate (Thermo Fisher Scientific). HTRF ratios were obtained by dividing the acceptor signal (665 nm) by the donor signal (620 nm) and multiplying this value by 10,000 to derive the 665/620 ratio (Table S4). The background signal was determined by using reagents in the absence of DARPins.

From the initial hits of DARPins identified to bind cAST, 32 single clones were sequenced and twenty-three clones with unique sequences were identified (Table S5). These were expressed in a 96-well format, lysed with Cell-Lytic B (Sigma) and purified over a 96 well IMAC column (HisPur™ Cobalt plates, Thermo Fisher Scientific) including washing with high salt (1 M NaCl) and low salt (20 mM NaCl) PBS buffer. Elution was performed with PBS, 400 mM NaCl and 250 mM imidazole. ELISA was performed using streptavidin-coated 384 well plates for immobilizing either biotinylated cAST or biotinylated mAST at a concentration of 50 nM. Detection of DARPins (1:1000 dilution of crude extracts) was performed using a mouse-anti-Flag M2 monoclonal antibody (dilution 1:5000; Sigma, F1804) as primary and a goat-anti-mouse antibody conjugated to an alkaline phosphatase (dilution 1:10000; Sigma, A3562) as secondary antibody. After addition of pNPP (para-nitrophenyl phosphate), absorbance at 405 nm was determined after 30 minutes. Signals at A540 nm were subtracted as background correction (table S5).

The oligomerization state of the positive DARPin binders was analyzed by size exclusion chromatography, and the results are summarized in Table S5. Specifically, IMAC purified DARPins were analyzed at a concentration of 10 µM on a Superdex 75 5/150 GL column (GE Healthcare) using an Äkta Micro system (GE Healthcare) with PBS containing 400 nM NaCl as running buffer. The molecular weight was estimated using β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa) as molecular mass standards. Three DARPins, B11, D11 and F12, that eluted as monomers, displayed strong positive ELISA signal and showed low cross-reactivity towards other transaminases (Table S5), were cloned into expression vectors in fusion with BLA-CaM and CaM-BP. To identify the best biosensor forming pair BLA-CaM-DARPin/ DARPin-CaM-BP combinations were tested for activity in the absence and presence of cAST.

Supplementary Figures

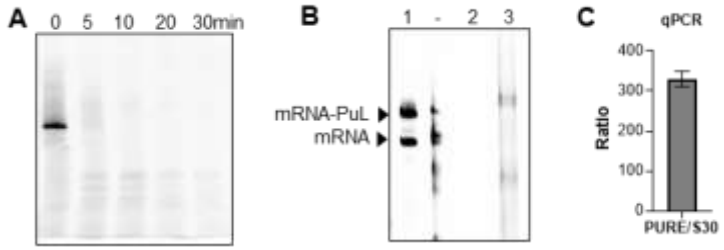


Figure S1. mRNA stability analysis in S30-based and PURE cell-free protein synthesis systems. **A.** The stability of a short mRNA in S30-based CFPS. Flag-mRNA was generated by *in vitro* transcription using Flag-DNA as a template (Table S1). Flag-mRNA and DNA-FAM (6-carboxyfluorescein) (Table S1) in a 1:1.5 ratio were hybridized at 37 °C for 30 minutes, followed by purification using the Monarch RNA purification kit. Purified conjugate (1 μM) was added into the S30-based CFPS and incubated for 5, 10, 20 and 30 minutes. The samples were then resolved on a 6% TBE-Urea gel and scanned for fluorescence. **B.** Testing the stability of mRNA-Puromycin in S30-based and PURE CFPS. The puromycin oligonucleotide linker (PuL-A, Table S1) was phosphorylated using T4 polynucleotide kinase (NEB) and ligated to Flag-mRNA using T4 RNA ligase 1. The ligation efficiency of mRNA and PuL was estimated to be approximately 50% by separating the products on a 6% TBE-urea gel, stained with SYBR Green II and scanned for fluorescence (lane 1). The mRNA-Pu conjugate was then incubated with either S30-based (lane 2) or PURE CFPS (PURExpress, NEB) (lane 3) for 30 minutes and quantified after resolving on the 6% TBE-Urea gel. **C.** A plot of the ratio of the residual mRNA remaining after its incubation in the PURE and S30-based CFPS. After 30 minutes of incubation in either PURE or S30-based CFPS, the samples were subjected to reverse transcription and quantified by qPCR.

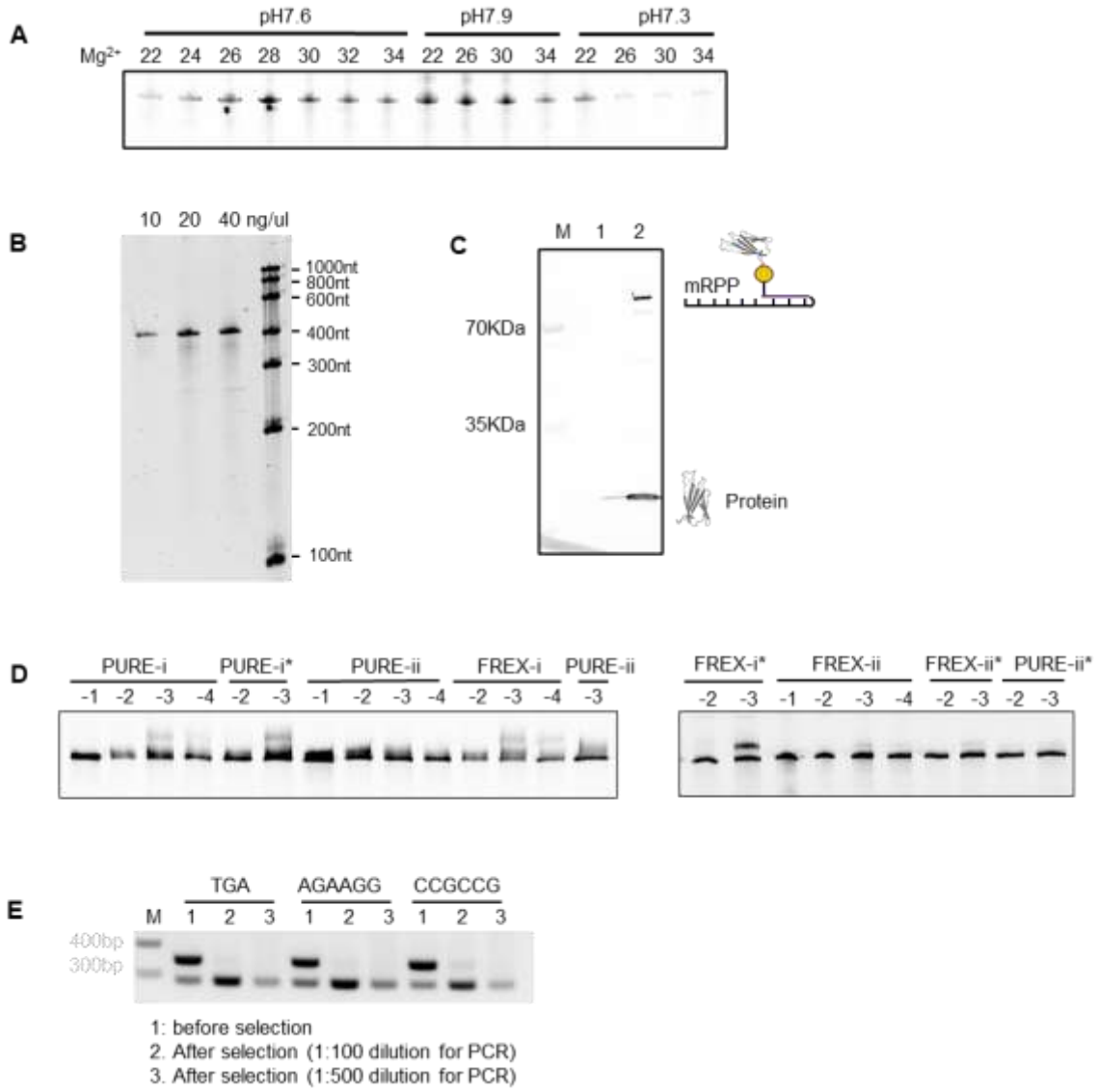


Figure S2: Optimisation of the production and purification of mRNA, mRNA-Pu conjugate (mRP), mRNA-Pu-protein conjugate (mRPP) as well as mRNA display conditions. **A.** Optimization of *in vitro* transcription using different pH and Mg^{2+} concentrations. 40 mM Hepes-KOH buffer at pH 7.9 and 22-26 mM of $Mg(OAc)_2$ were found to be highly favourable conditions by resolving the transcribed mRNA on a 6% TBE-Urea gel stained with SYBR Green II. **B**. Optimization of *in vitro* transcription using different DNA template concentrations. Transcribed mRNA was resolved on a 6% TBE-urea gel and stained with SYBR Green II. The optimal condition was 40 mM Hepes-KOH (pH 7.9), 25 mM $Mg(OAc)_2$, 2 mM spermidine, 40 mM DTT, 5 mM rNTPs, 50 µg/ml T7 RNA polymerase, 50 µg/ml yeast inorganic pyrophosphatase with ~20 ng/µl DNA template incubated at 35 °C for 3 hours. **C.** Visualization of the free and mRNA-displayed protein by Western Blot. mRNA (lane 1) or mRP (lane 2) were translated in PURExpress CFPS and subsequently separated on a 4-12% SDS-PAGE gel and blotted onto a PVDF membrane. Flag-antibody (rabbit) and goat anti-rabbit secondary antibody were used on a Western blot to visualize Flag-tagged proteins. The DNA ladder is shown in lane M. **D.** Fluorescence scanning of the free and the protein displayed mRNA on 4-12% SDS-PAGE gel. The effect of different mRNA purification methods, different puromycin oligonucleotides as well as different translation kits on protein display efficiency were tested. The *in vitro* transcribed mRNA was purified using either a Monarch RNA cleanup kit or using PAGE purification (marked with *). The purified mRNAs were conjugated to two different puromycin oligos with a fluorescein dT in their linker region for easy visualization: one without (i, PuL, Table S1) and the other with (ii, PuL-B, Table S1) 2'OMe modification. The mRP conjugates purified using the

Monarch cleanup kit were then translated using two different PURE systems: PURExpress (PURE) and PUREfrex (FREX). We monitored the mRP (-1), mRP translation at 0 minutes (-2), mRP translation at 30 minutes (-3), and mRP translation at 30 minutes followed by Monarch RNA cleanup kit purification (-4) on a fluorescently scanned 4-12% SDS-PAGE gel. **E.** Selection results of Flag-monobody (Flag-Mb) mRNA using constructs with either a stop codon (UGA) or rare codons (arginine: AGAAGG or proline: CCGCCG) in front of the hybridization site. In addition to the mRNA display constructs with a UGA codon (Figure 2D), constructs of Flag-Mb and Twin-Strep-Mb with two consecutive rare codons of arginine or proline were synthesized and conjugated to PuL. The two mRP conjugates of Flag- and Twin-Strep-Mb constructs with rare codons were mixed at a 1: 10 ratio, and 1 μM of the mixture was subjected to translation, reverse transcription and then selection against Flag-antibody immobilized Dynabeads. The eluted cDNA was subsequently diluted at the specified ratio, PCR amplified and visualized on a 2% agarose gel.

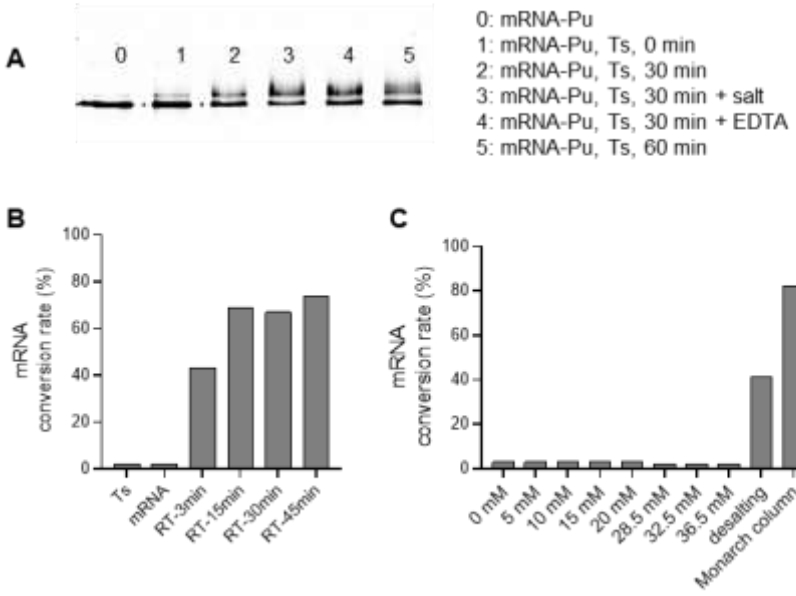


Figure S3: The effect of additives on the formation of mRNA-Pu-protein (mRPP) conjugates and downstream reverse transcription efficiency. **A.** A photograph of a urea-SDS-PAGE gel loaded with mRPP complexes generated under different concentrations of salt, EDTA and incubation time. 1 μM of purified mRP conjugate (lane 0) was translated using PURExpress at 37 °C for the indicated periods of time and subsequently supplemented with specified additives followed by separation and visualization on a 6% urea-SDS-PAGE gel scanned for fluorescence. The loading of the gel is as follows: lane 1: translation of mRP for 0 minutes; lane 2: translation of mRP for 30 minutes; lane 3: translation of mRP for 30 minutes and incubation with salt (375 mM KCl and 32.5 mM $MgCl_2$) at room temperature for 20 minutes; lane 4: translation of mRP for 30 minutes and incubation with 15 mM EDTA at room temperature for 10 minutes; lane 5: translation of mRP for 60 minutes. All reactions were performed in 4 μl volumes. **B.** A plot of mRNA conversion rate in the reverse transcription reactions by using different templates and incubation time. qPCR was used to quantify the produced cDNAs and calculate the mRNA conversion rate. The translation reaction (Ts) and mRNA samples served as no DNA template controls to confirm the absence of contamination. The mRNAs in the translation reaction were reverse transcribed at the indicated times (3, 15, 30, 45 minutes) and the generated cDNAs were then quantified using qPCR. **C.** A plot of the recovery efficiencies of reverse transcription using various methods to chelate the salt additive in the translation reactions. Salt-incubated translation reactions (lane 3, Figure S3A) were treated with varying concentrations of EDTA (0 to 36.5 mM), desalted using a Zeba spin desalting column (Thermo Fisher Scientific) or purified using the Monarch RNA cleanup kit. The resulting samples were then used as templates for reverse transcription and the cDNA generated in each reaction was quantified using qPCR.

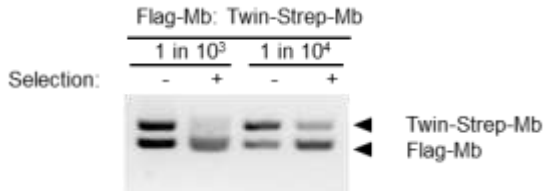


Figure S4: Selection results of Flag-monobody (Mb) mRNA in the second round of mRNA display. The recovered cDNAs from Round 1 of selection (Figure 2D) were subjected to *in vitro* transcription, followed by mRNA purification, puromycin ligation, *in vitro* translation, and reverse transcription. 1 μl of the reverse transcription reaction was reserved as a non-selection control (-), whilst the remaining reaction was panned against the immobilized Flag-antibody Dynabeads (+). After washing, the cDNA remaining on the Dynabeads was eluted, PCR amplified, and visualized on a 2% agarose gel.

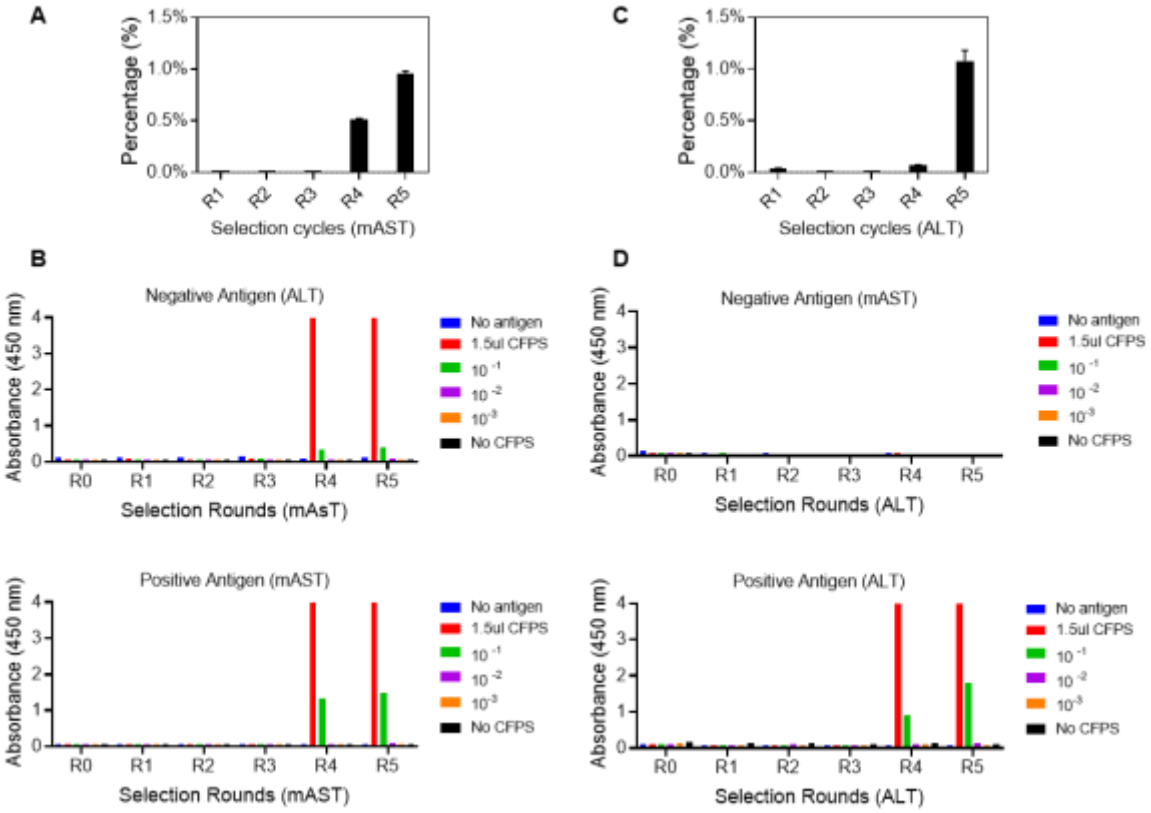


Figure S5. Characterization of the outputs of mRNA display FN3con selection rounds against human mitochondrial aspartate aminotransferase (mAST) and alanine aminotransferase 1 (ALT). **A.** A bar plot of the relative abundance of FN3con cDNA recovered in each round of selection against mAST as quantified by qPCR analysis by comparing the input and output cDNA. **B.** ELISA signals of the serially diluted CFPS reactions primed with cDNA library and outputs of five selection rounds (R1-R5) to monitor the mAST binder enrichment. The interactions were detected using HRP-conjugated anti-Flag antibody. A non-related protein, ALT, served as the negative control protein. **C.** The relative abundance of cDNAs recovered in each round of selection against ALT were quantified by qPCR and analysed as described in (A). **D.** ELISA assays used to monitor the ALT binder enrichment in subsequent selection rounds. The assays were set up as previously described in (B) but used ALT as the target protein and mAST as the non-related negative control protein.

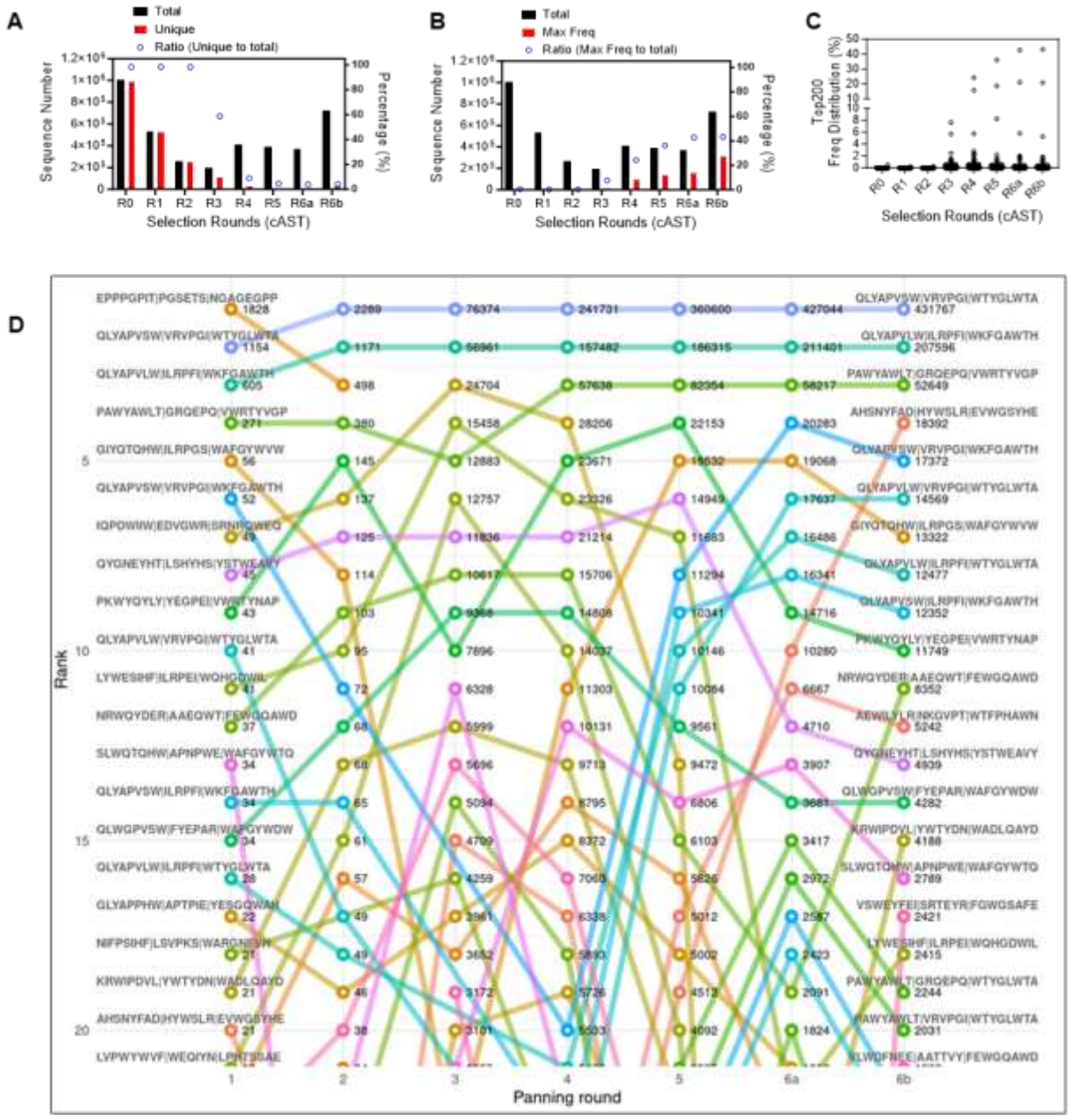


Figure S6. NGS Analysis of the naïve FN3con library and the outputs of six selection rounds against cAST protein. **A.** A bar graph showing the number of unique and total sequences as well as their ratios in the naïve library and in the six selection rounds. **B.** A bar graph showing the frequency of the most enriched sequences and their proportion in total sequences in the naïve library and all six rounds of selection. **C.** Plotting the frequency distribution of the top 200 protein sequences in the naïve library and all six selection rounds. **D.** A bump chart using the top 20 sequences from the first round (R1) and the last round (R6) of selection as anchor points to show the sequence evolution trajectories.

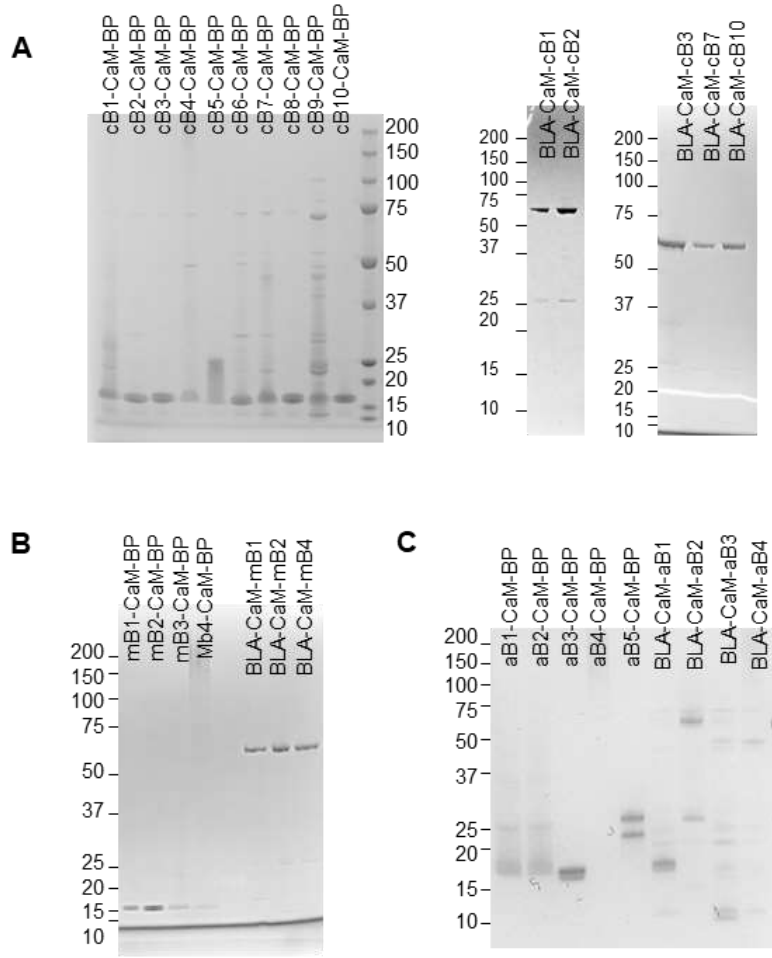


Figure S7. Purified biosensor proteins separated on 4-12% SDS-PAGE gels and imaged after staining with Coomassie Blue. **A.** Fusion proteins of selected cAST binders with either CaM-BP or BLA-CaM. **B.** Fusion proteins of selected mAST binders with either CaM-BP or BLA-CaM. **C.** Fusion proteins of selected ALT binders with either CaM-BP or BLA-CaM.

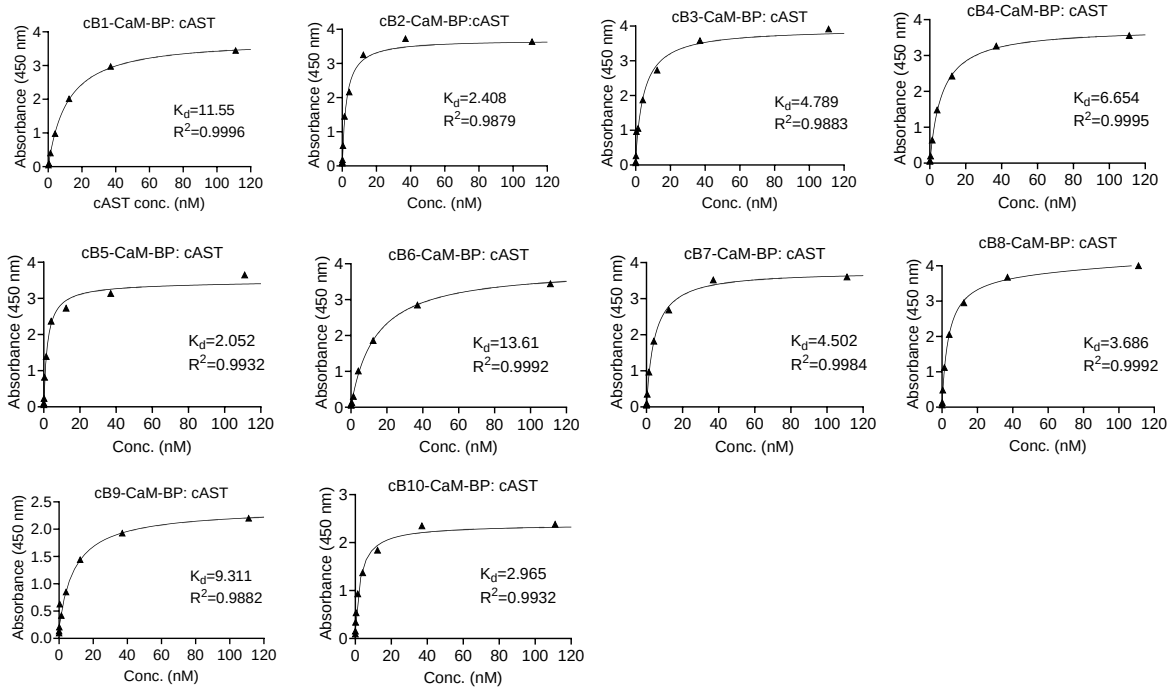


Figure S8. Plots of semi-quantitative ELISA values to obtain the apparent dissociation constant (K_d) of purified protein binders towards the cAST target. The cAST target protein was immobilised at 10 μg/mL on a Maxisorp ELISA plate and then incubated with binder-CaM-BP at concentrations ranging from 0 to 3 μM. HRP conjugated Flag-antibody was used to visualise and quantify the binding events by measuring the absorbance at 450 nM in a Neo2 plate reader. The absorbance data were plotted against the binder concentrations (0-120 nM) and fitted using GraphPad Prism to the equation of one site saturation binding (total binding) to calculate the apparent K_d.

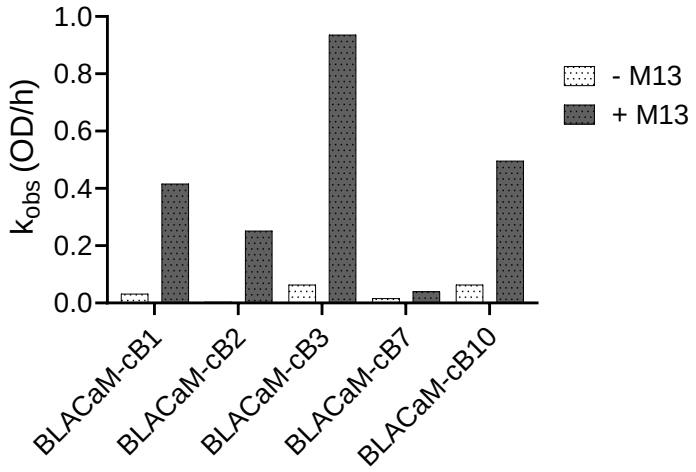


Figure S9. A plot of the catalytic activities of BLA-CaM-binder fusions activated by saturating concentrations of M13 calmodulin binding peptide (CaM-BP). The enzymatic assays were performed in 20 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1 mM $CaCl_2$ with 25 nM of BLA-CaM-binder protein and 300 nM of M13 CaM-BP. After 30 minutes of incubation, 50 μM of nitrocefin was added to the reaction, and the absorbance was monitored at 486 nm using a Neo2 plate reader. The linear phase of the reaction was used to calculate the k_{obs}.

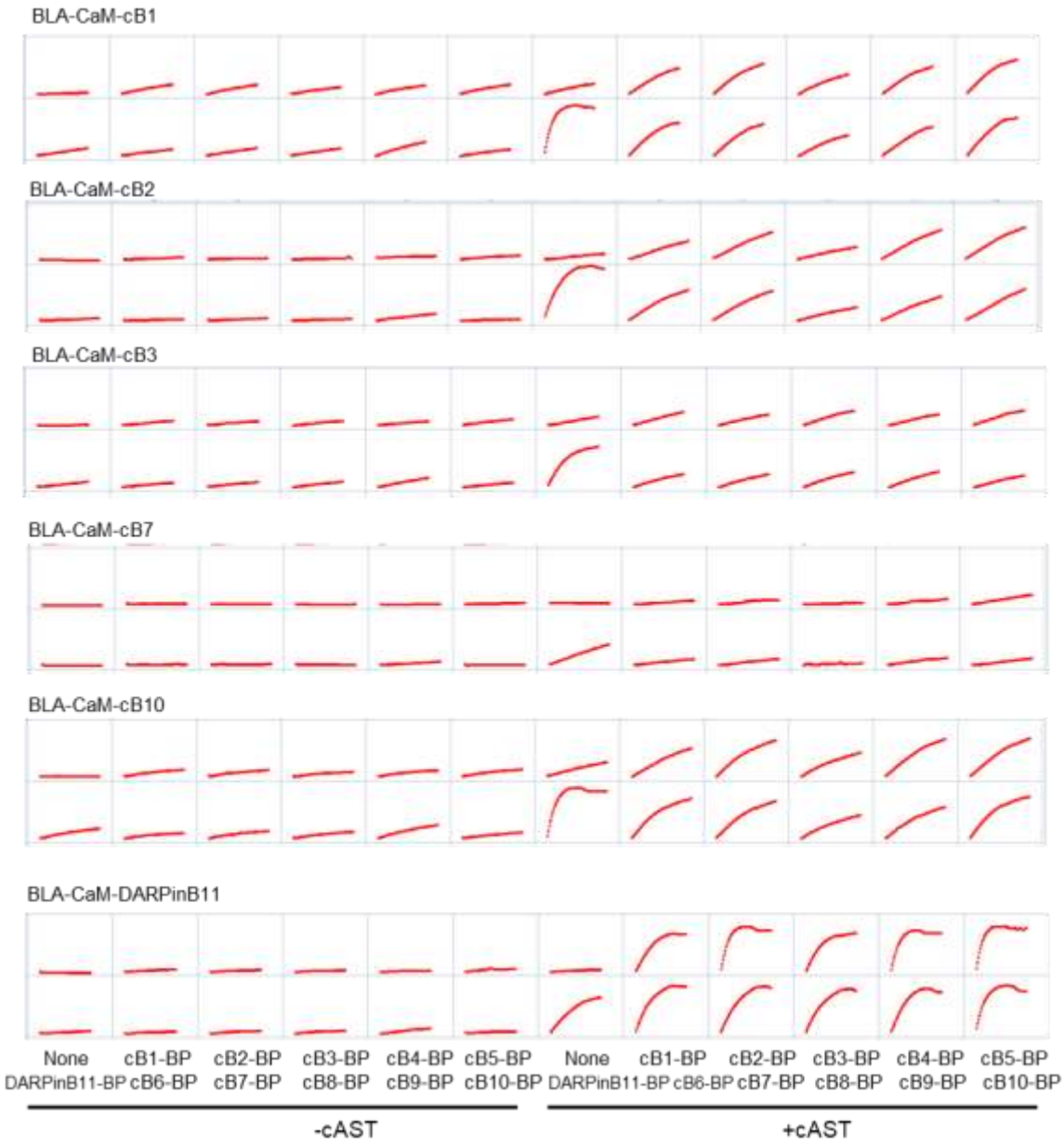


Figure S10. Real-time kinetics of cAST biosensor assays performed with different BLA-CaM-binder and binder-CaM-BP combinations. The enzymatic assays were conducted in 20 mM of Tris-HCl (pH7.2), 100 mM of NaCl, and 1 mM of $CaCl_2$, with 25 nM BLA-CaM-binder protein and 100nM binder-CaM-BP protein in the absence or presence of 200 nM cAST protein. After 30 minutes of incubation, 50 μM nitrocefin was added to the reaction, and the absorbance was monitored at 486 nm using a Neo2 plate reader.

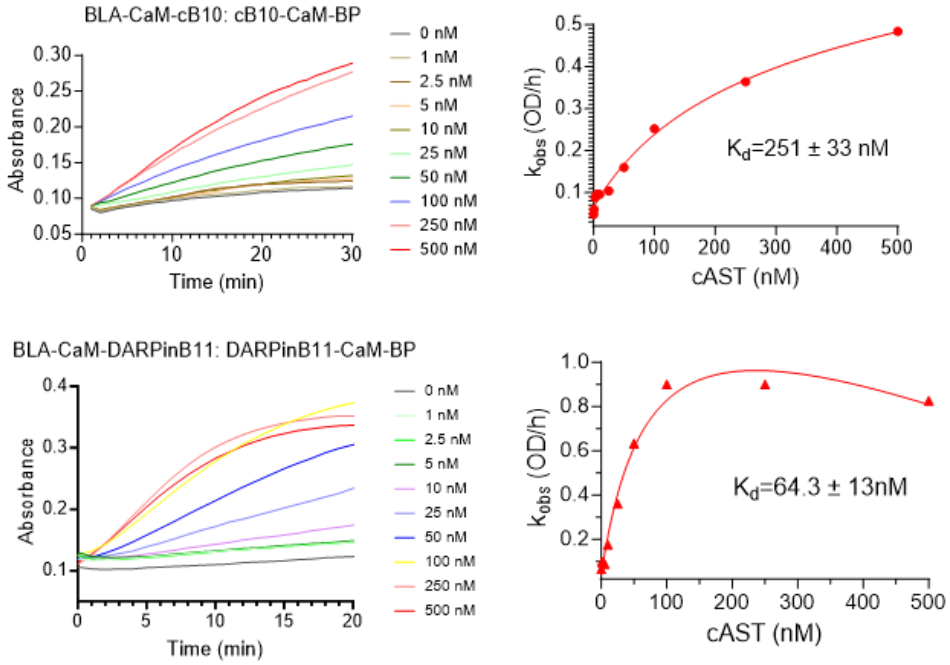


Figure S11. Analysis of real-time reaction kinetics and estimation of derived dissociation constants for the developed cAST biosensors. **A**. Titration of cAST (0-500 nM) in a solution of 20 mM Tris-HCl (pH 7.2), 100 mM NaCl, and 1 mM $CaCl_2$, with 25 nM BLA-CaM-cB10 and 100 nM cB10-CaM-BP. The absorbance at 486 nM was recorded over 30 minutes, and the linear phase of the reaction was used to derive k_{obs}. These values were plotted against their respective cAST concentrations and fitted using nonlinear regression to calculate the apparent K_d. **B**. Titration of cAST (0-500 nM) in a solution of 20 mM Tris-HCl (pH 7.2), 100 mM NaCl, and 1 mM $CaCl_2$, with 25 nM BLA-CaM-DARPinB11 and 100 nM DARPinB11-CaM-BP. The reactions were analysed as described in (A). All experiments were performed in duplicate, with the representative data shown and the K_d values presented as mean ± SD.

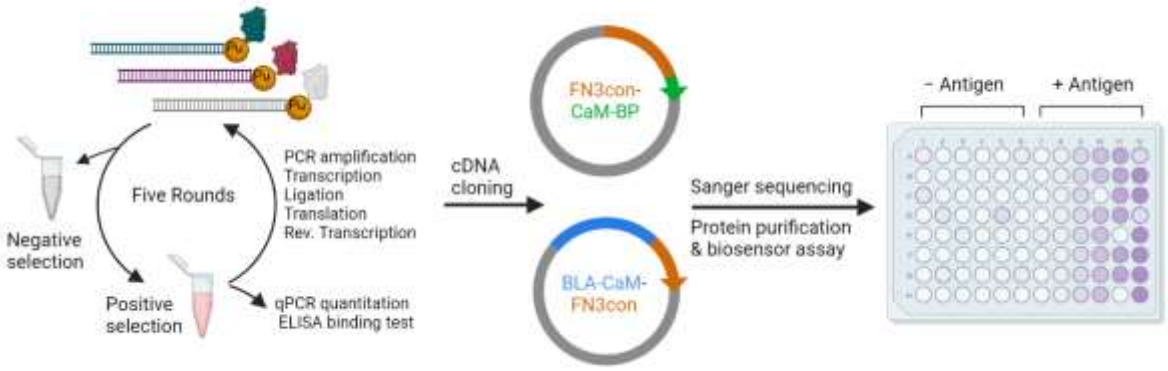
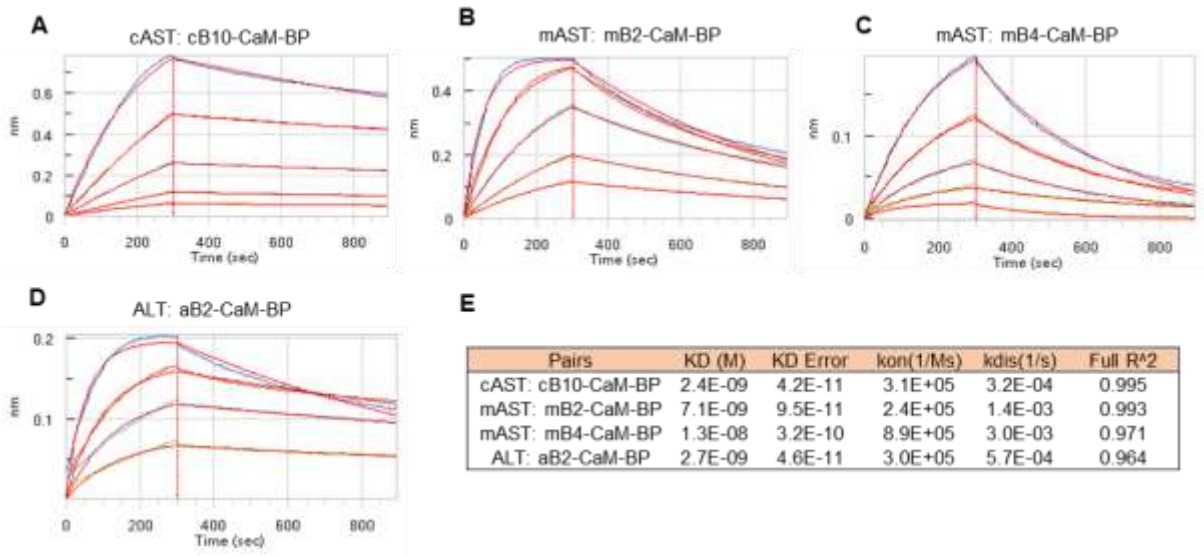


Figure S12: Integrating mRNA display binder selection with biosensor prototyping. The biosensor development process was streamlined by directly cloning cDNAs resulting from mRNA display into bacterial expression vectors, in frame with biosensor components CaM-BP and BLA-CaM. The combinations of different fusion proteins were tested for biosensor function in multiplexed biosensor assays.



E

Pairs	KD (M)	KD Error	kon(1/Ms)	kdis(1/s)	Full R^2
cAST: cB10-CaM-BP	2.4E-09	4.2E-11	3.1E+05	3.2E-04	0.995
mAST: mB2-CaM-BP	7.1E-09	9.5E-11	2.4E+05	1.4E-03	0.993
mAST: mB4-CaM-BP	1.3E-08	3.2E-10	8.9E+05	3.0E-03	0.971
ALT: aB2-CaM-BP	2.7E-09	4.6E-11	3.0E+05	5.7E-04	0.964

Figure S13. Biolayer interferometry (BLI) characterization of the selected protein binders (binder-CaM-BP fusions) against three transaminases. The binding curves are shown in blue, while the fits to a 1:1 binding model provided by the ForteBio data analysis software are depicted in red. **A.** BLI sensorgrams of the binding between cAST and cB10-CaM-BP protein. The BLI streptavidin sensor tips were coated with 5 μg/ml of biotinylated cAST, and cB10-CaM-BP was titrated at concentrations of 40, 20, 10, 5, 2.5 nM (top to bottom). **B.** BLI sensorgrams of the binding between mAST and mB2-CaM-BP protein. Biotinylated mAST was immobilized on the BLI sensor tips at 2.5 μg/ml, and mB2-CaM-BP was titrated at concentrations of 80, 40, 20, 10, 5 nM (top to bottom). **C.** BLI sensorgrams of the binding between mAST and mB4-CaM-BP protein. Biotinylated mAST was immobilized on the BLI sensor tips at 2.5 μg/ml, and mB4-CaM-BP was titrated in at concentrations of 40, 20, 10, 5, 2.5 nM (from top to bottom). **D.** BLI sensorgrams of the binding between ALT and aB2-CaM-BP protein. Biotinylated ALT was immobilized at 10 μg/ml on the sensor tips, and aB2-CaM-BP was titrated at concentrations of 100, 50, 25, 12.5 nM (top to bottom). **E.** A summary of the interaction parameters of the four binding pairs (A, B, C and D) determined by BLI.

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