Supplemental Information

Multifactorial determinants of protein expression in prokaryotic open reading frames

Malin Allert, J. Colin Cox, & Homme W. Hellinga

Table of Contents

Supplemental Data

Figure S1

Relating to Figure 2 and supplementary Table S1, we provide the raw images of Coomassie-stained SDS-PAGE gels of 285 designed genes used to experimentally determine expression scores in this work. Variants are named by the protein, a number, and an allele letter (see also Table S1). The assigned expression score (0, 1, 2, or 3) is provided underneath the allele name. Purified expression reactions were run on NuPage 4-12% Bis-Tris gels (Invitrogen; NP0321) along with a protein ladder (BenchMark Pre-stained Protein Ladder (Invitrogen; 10748-010)). Arrows denote the protein band of interest. Expression reaction volumes are indicated in each title.

Figure S1A – ttAST, 7 calculation conditions, replicate 1 (100 μl)

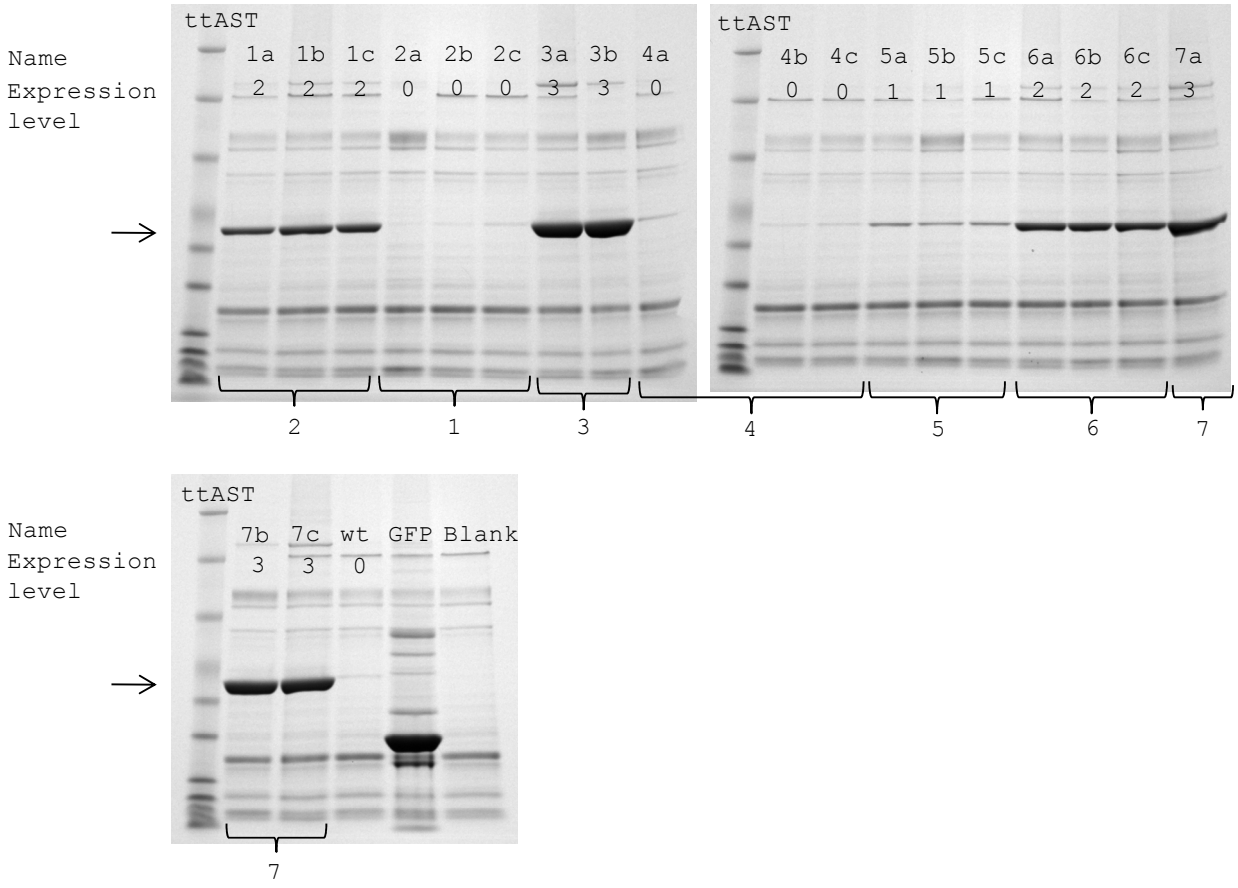


Figure S1B – ttAST, 7 calculation conditions, replicate 2 (100 μl)

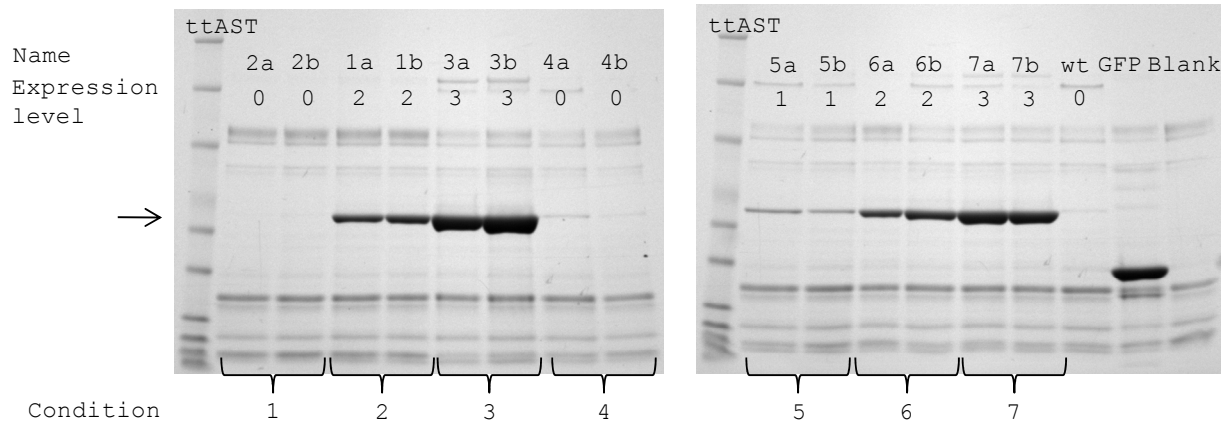


Figure S1C – ttAST, 7 calculation conditions, replicate 3 (100 μl)

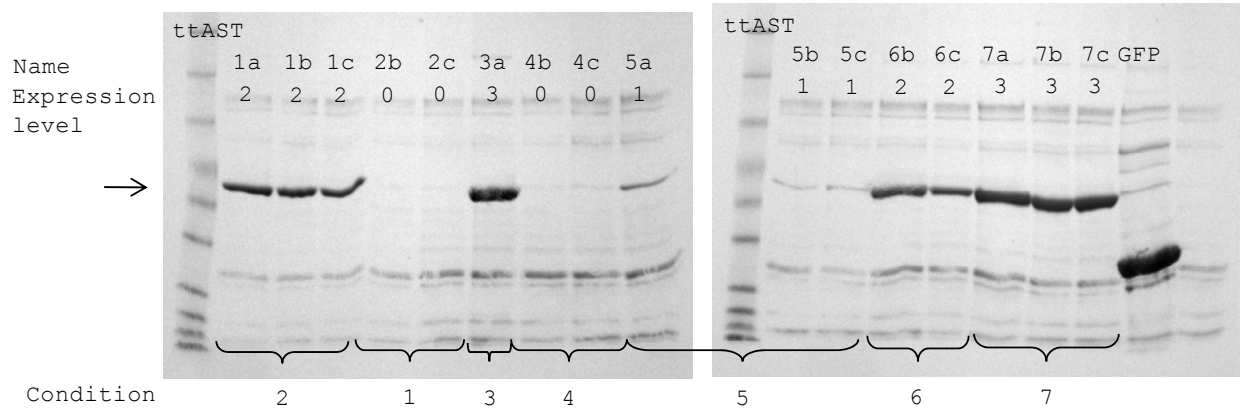


Figure S1D – ttAST, (50 μl)

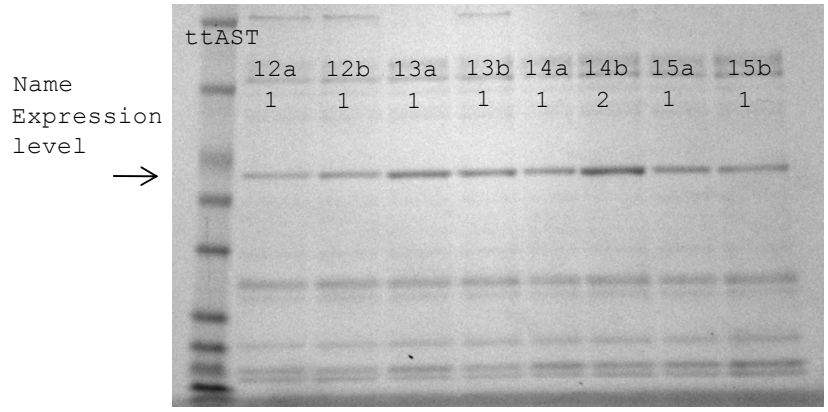


Figure S1E – ttAST, replicate 1 (100 µl)

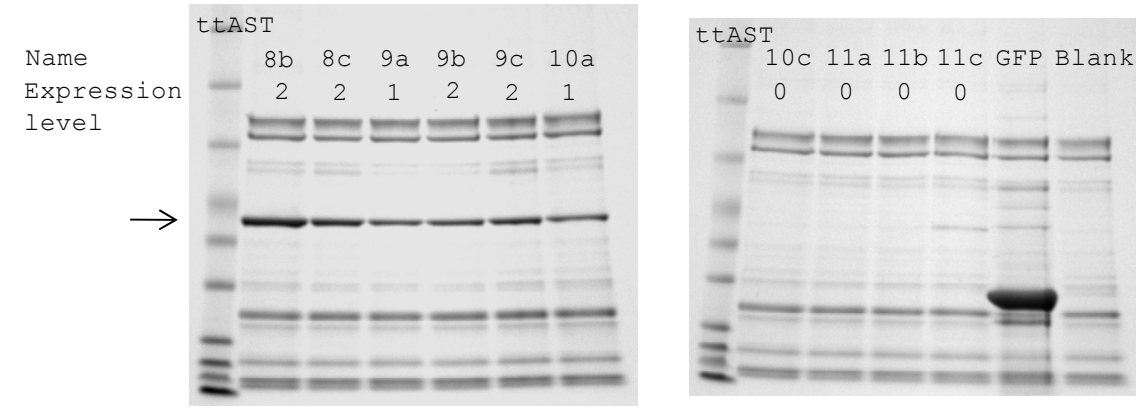
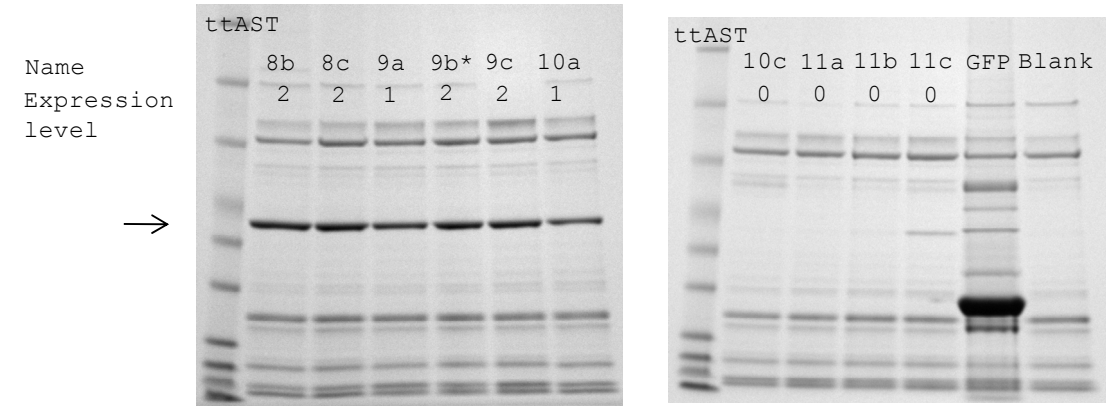
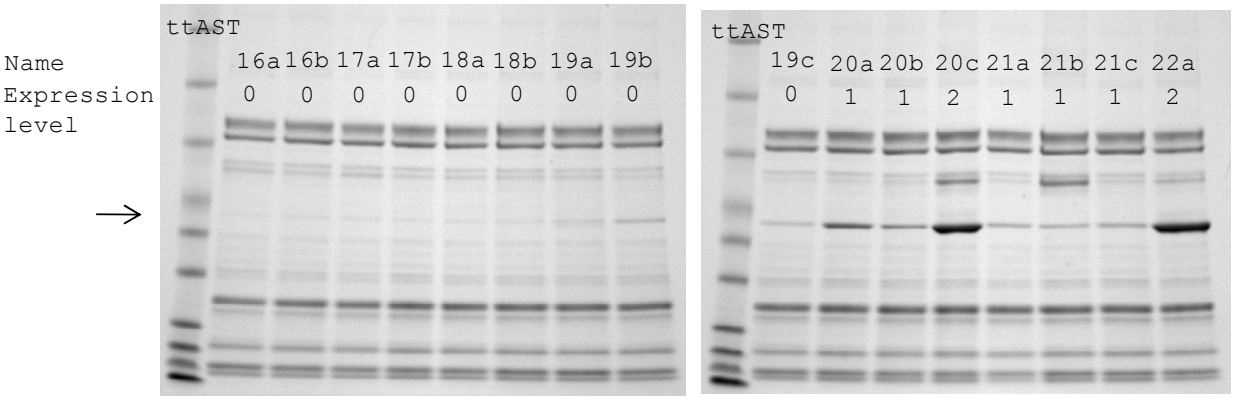


Figure S1F – ttAST, replicate 2 (100 µl)



* Added an additional 0.8 ul H_2O by misstake

Figure S1G – ttAST, replicate 1 (100 μl)



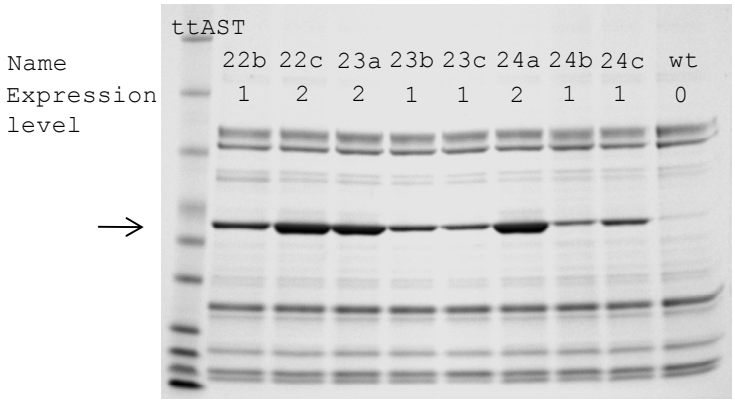


Figure S1H – ttAST, replicate 2 (100 μl)

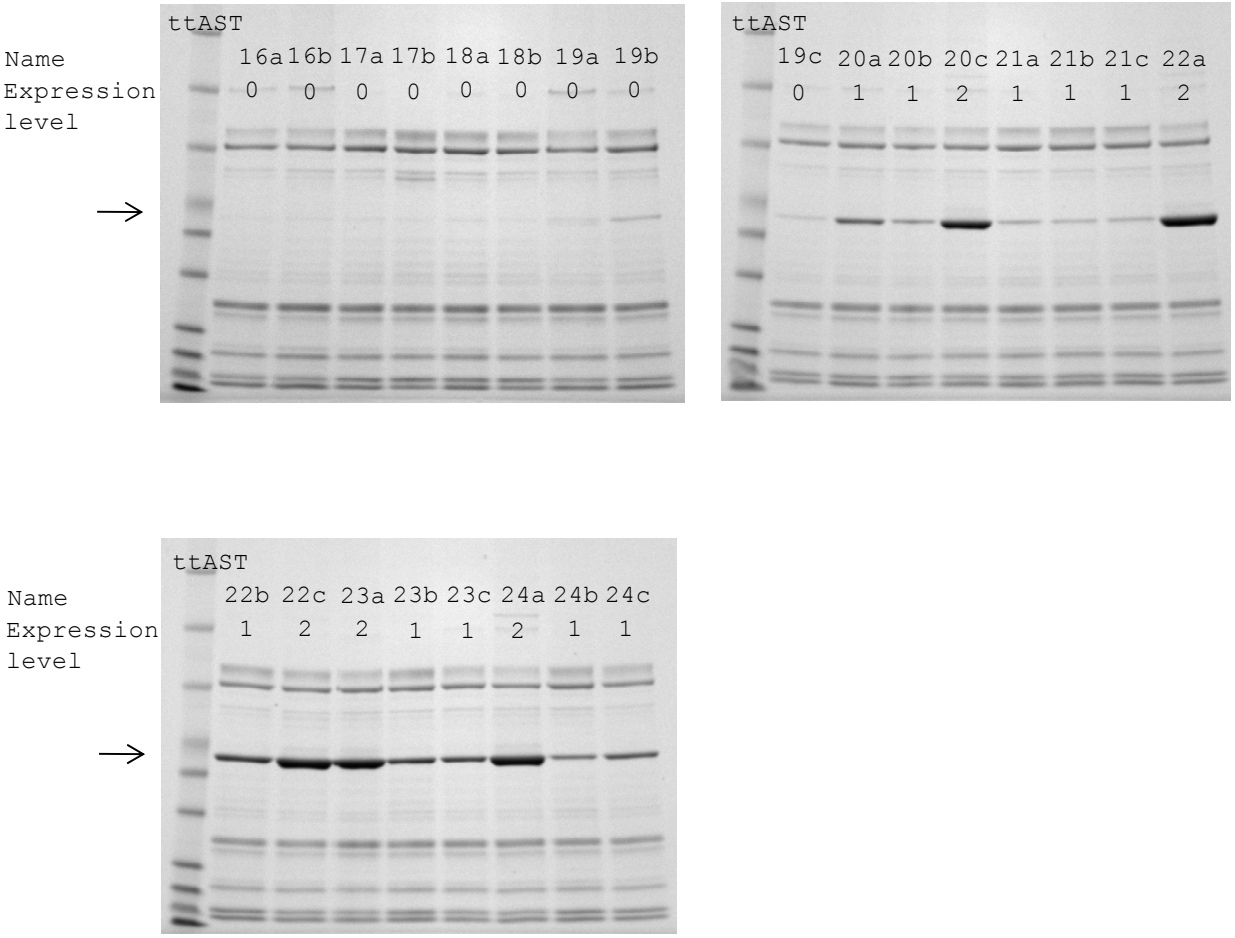
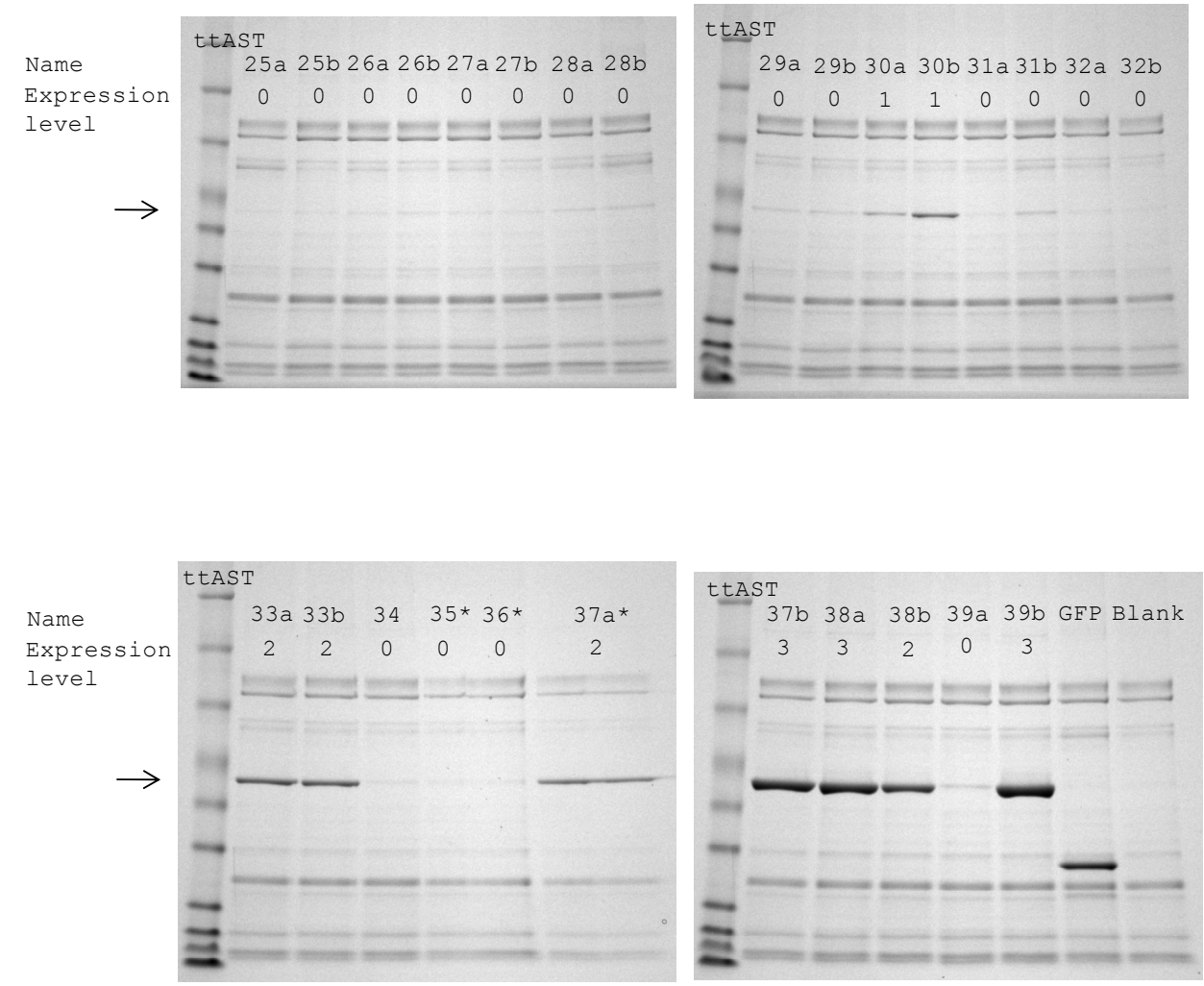
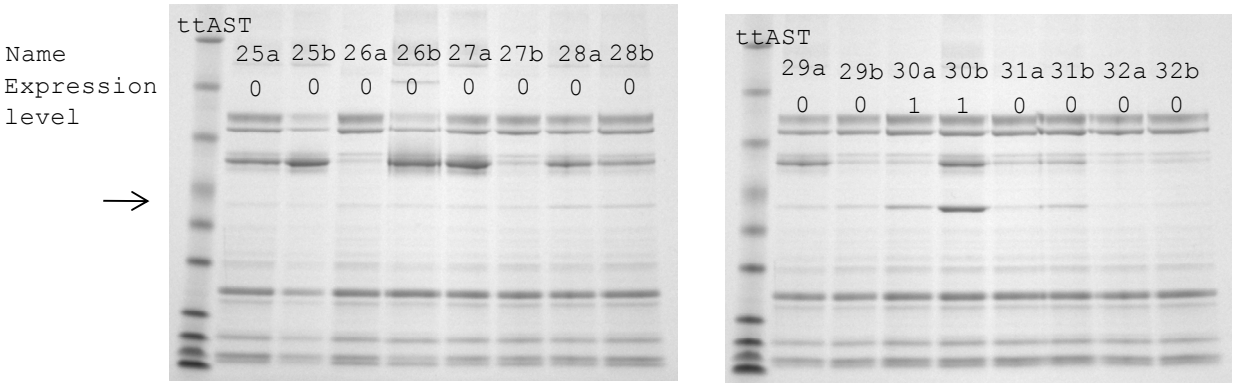


Figure S1I – ttAST, replicate 1 (50 μl)



*End of gel not functioning properly. Sample ttAST.35, ttAST.36 not perfectly separated and ttAST.37a bled out in two lanes.

Figure S1J – ttAST, replicate 2 (100 μl)



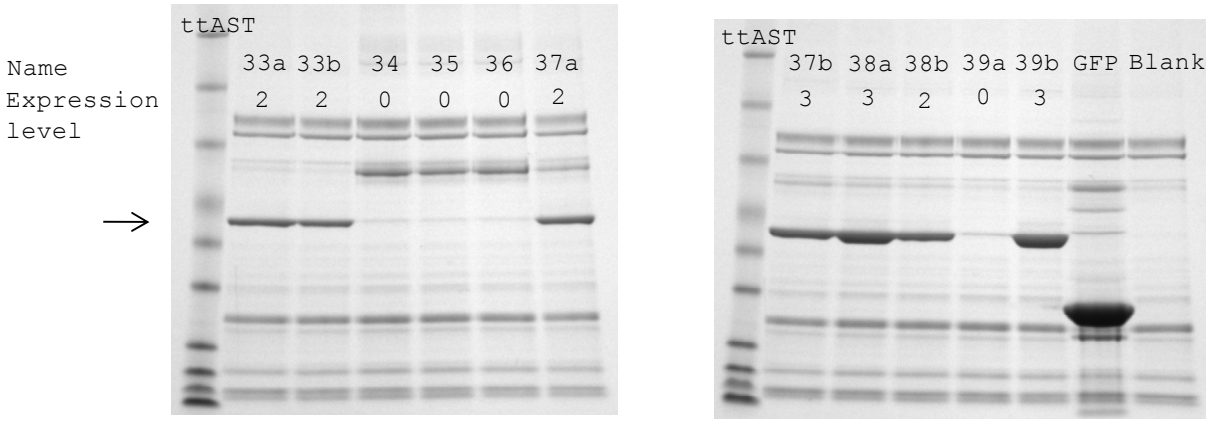
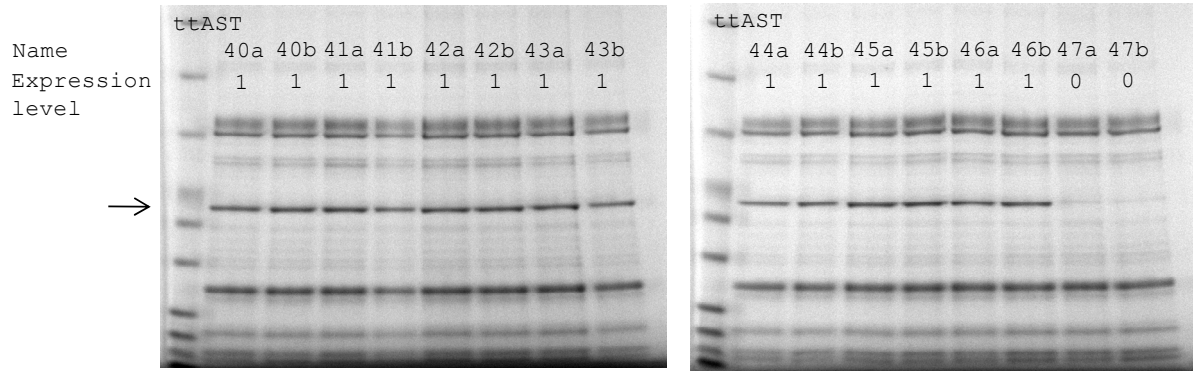
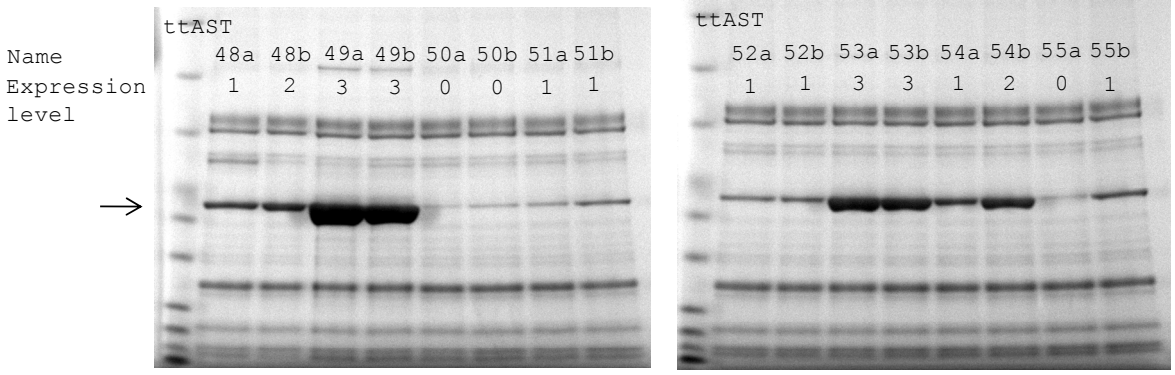
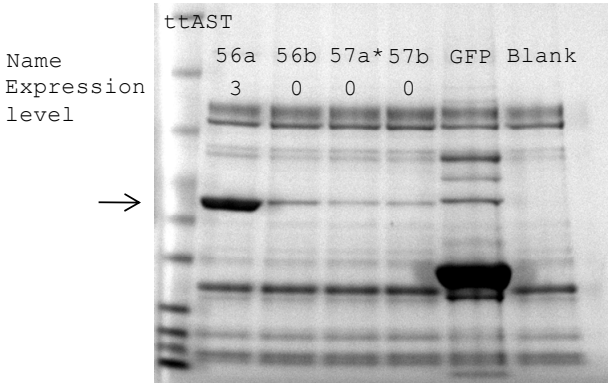


Figure S1K – ttAST, replicate 1 (100 μl)

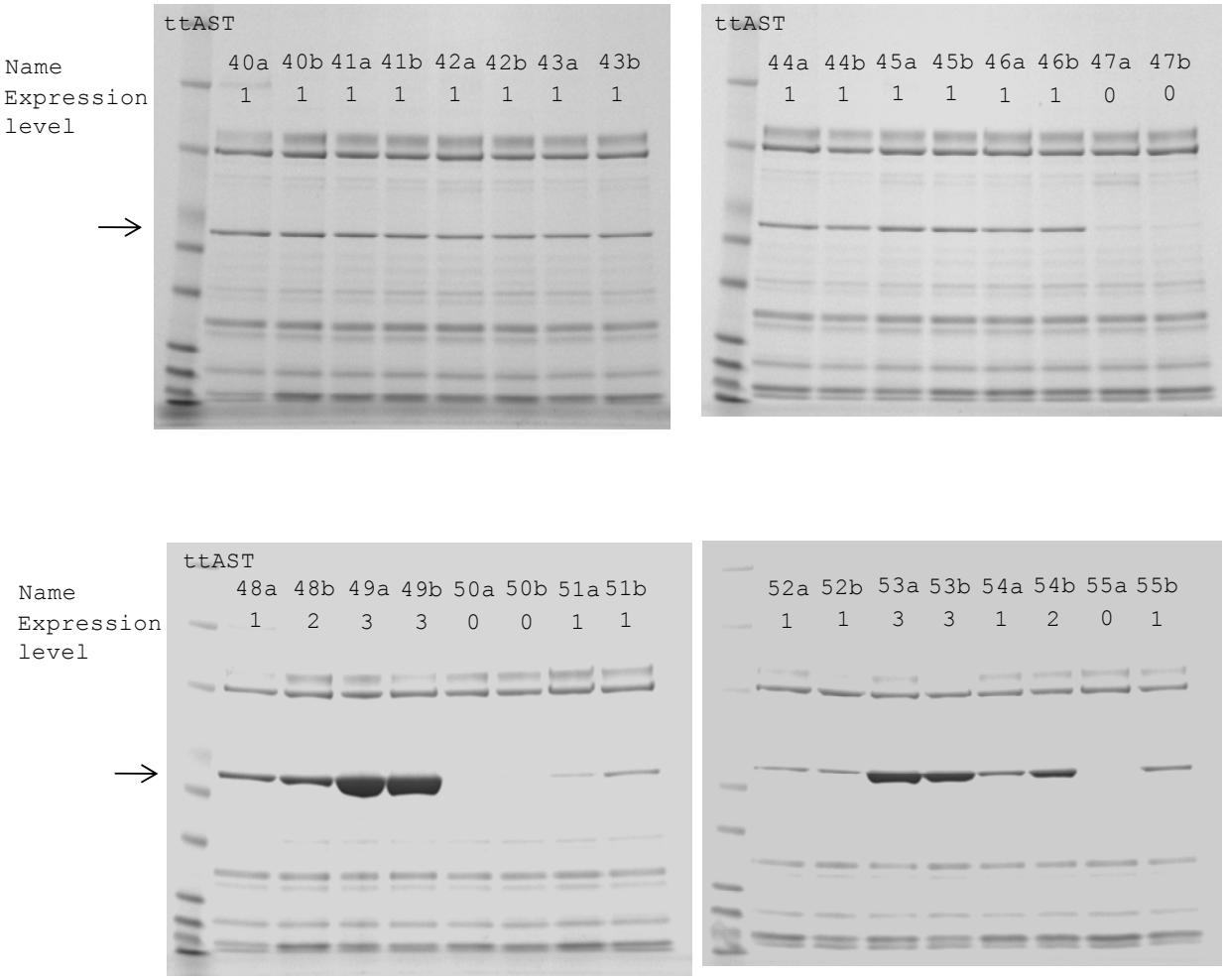






* ttAST.57a has been used as a starting sequence for these OrfOpt calculations.

Figure S1L – ttAST, replicate 2 (100 μl)



* ttAST.57a has been used as a starting sequence for these OrfOpt calculations.

Figure S1M – ggFABP, (50 μl)

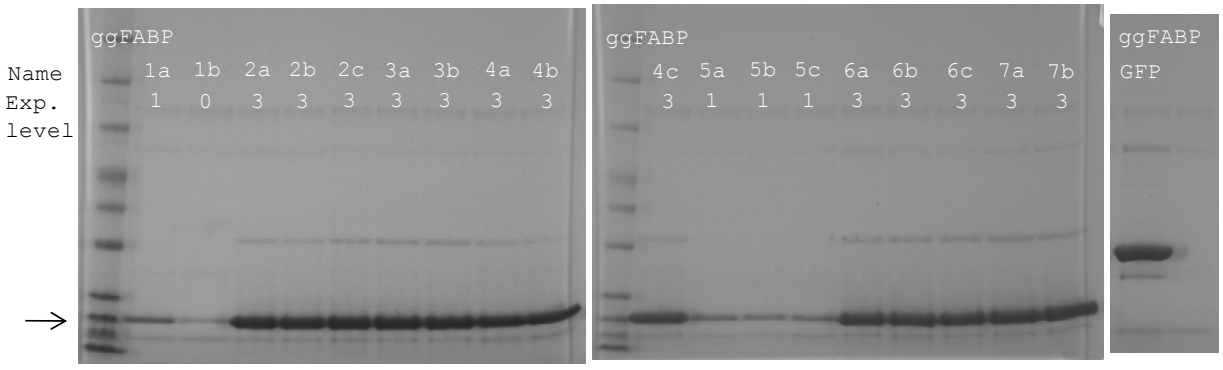
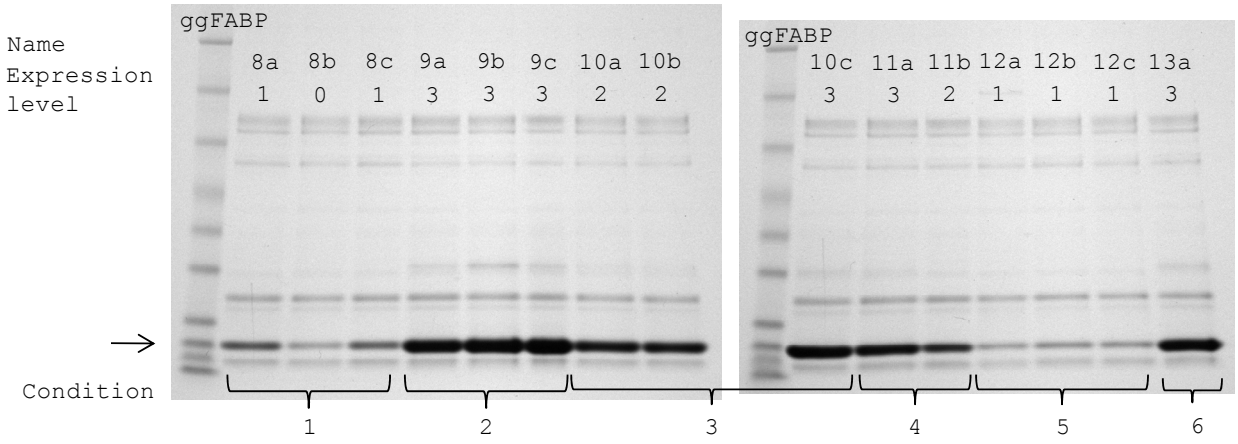


Figure S1N – ggFABP, 7 calculation conditions, replicate 1 (50 μl)



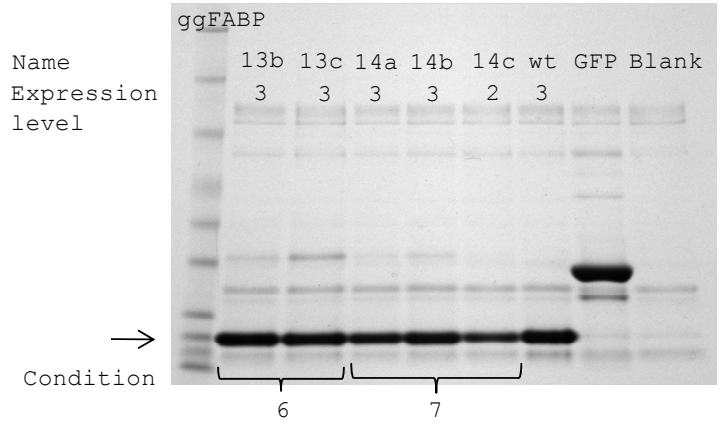
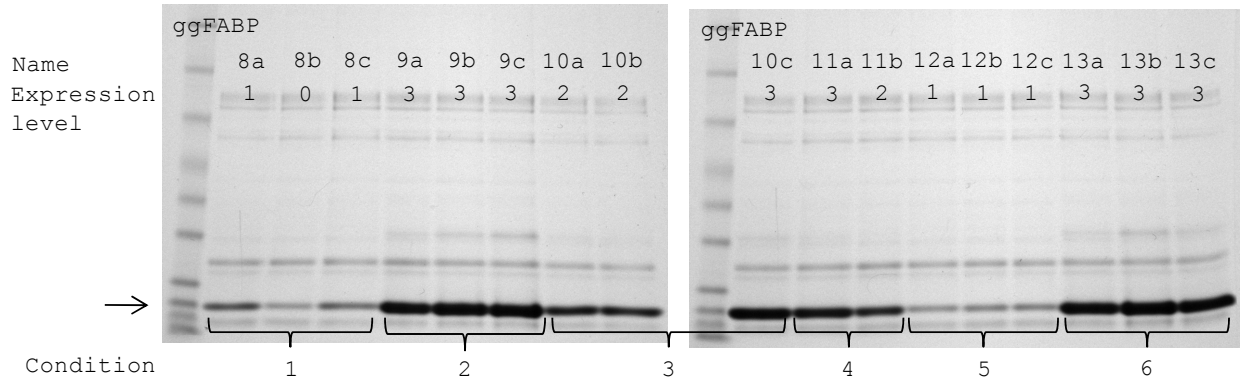


Figure S1O – ggFABP, 7 calculation conditions, replicate 2 (50 μl)



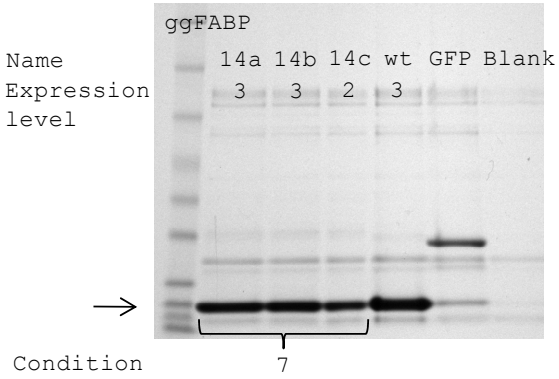


Figure S1P – ggFABP, 7 calculation conditions, replicate 3 (50 μl)

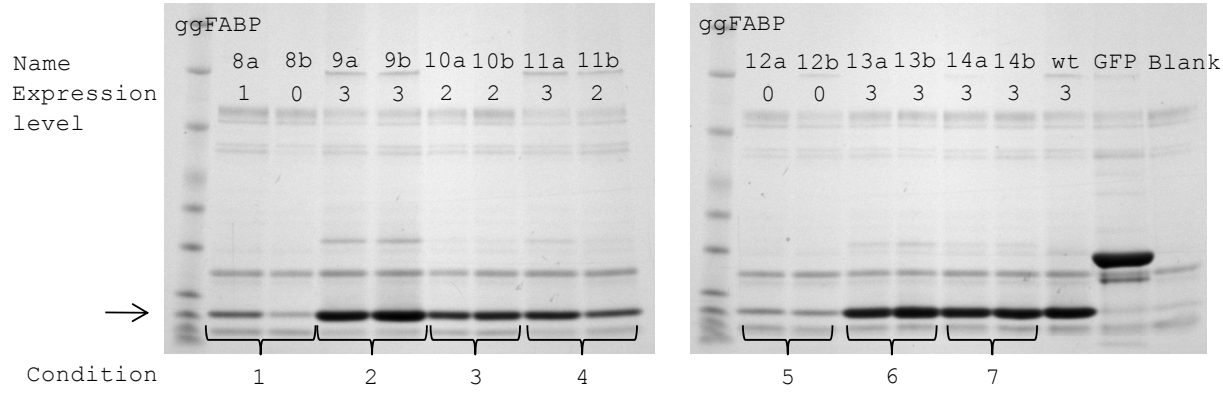


Figure S1Q – lmTIM, 7 calculation conditions, replicate 1 (50 μl)

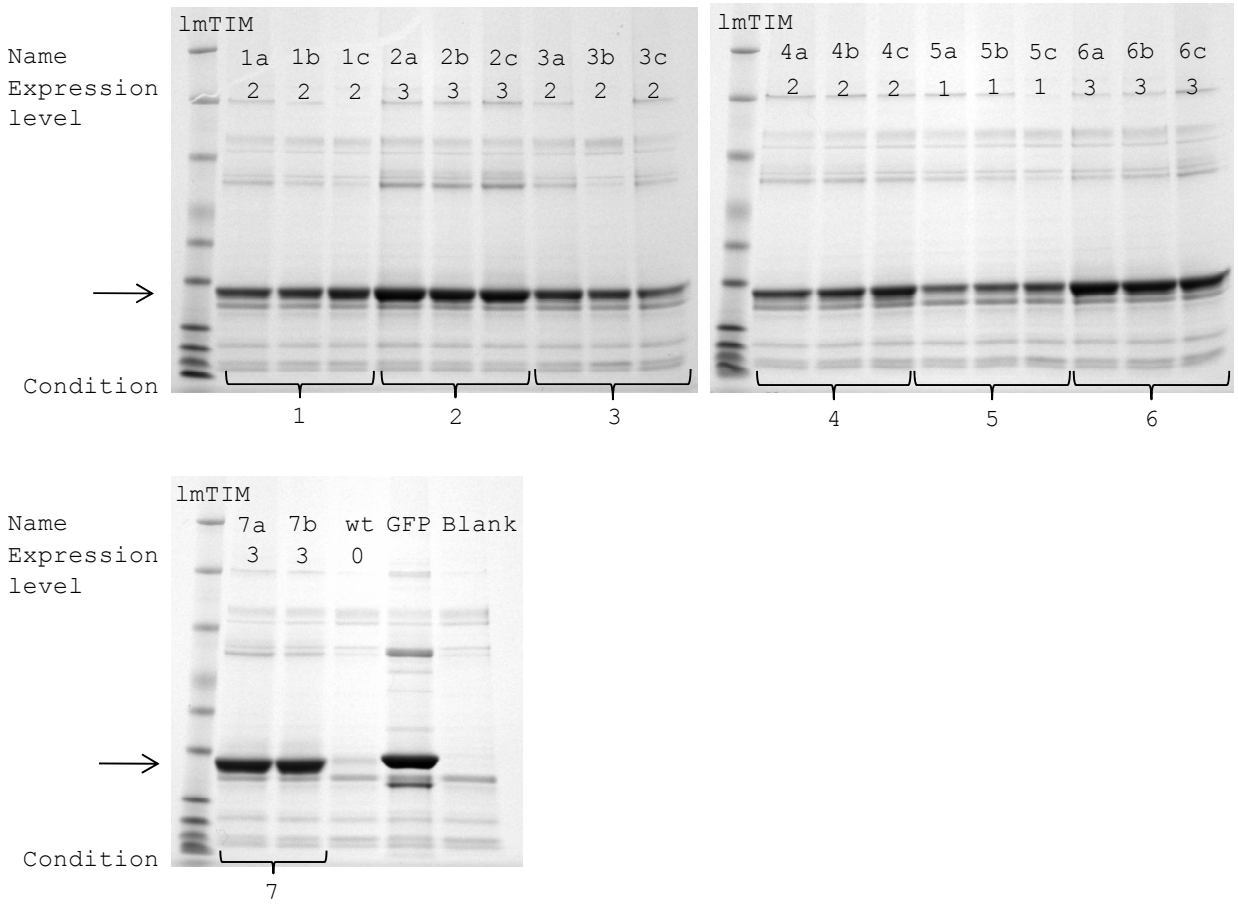


Figure S1R – lmTIM, 7 calculation conditions, replicate 2 (50 μl)

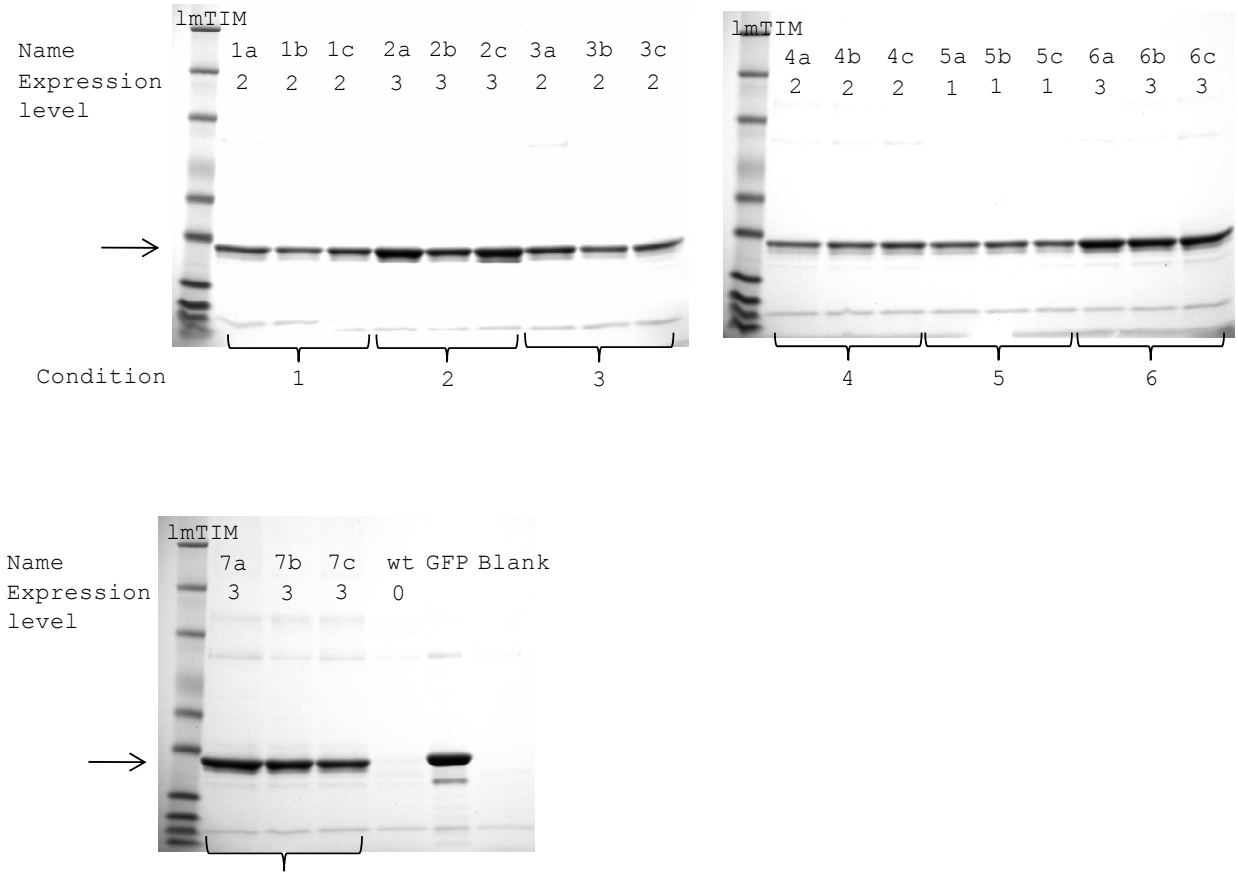


Figure S1S – lmTIM, 7 calculation conditions, replicate 3 (50 μl)

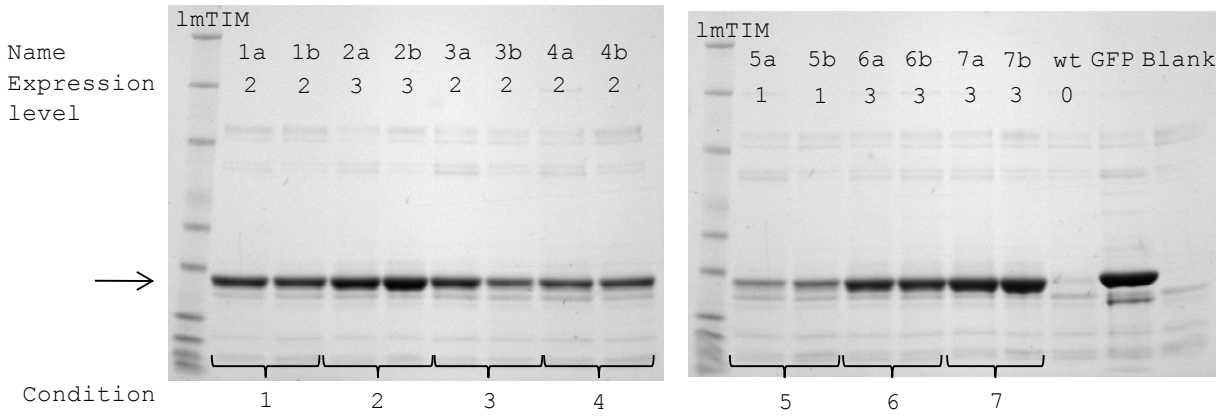


Figure S1T – lmTIM, 7 calculation conditions, replicate 4 (25 μl)

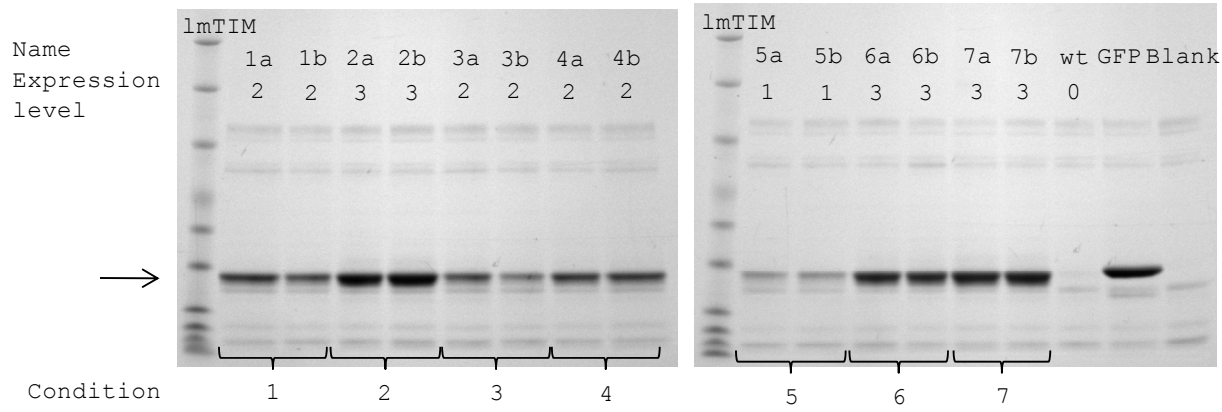


Figure S1U – lmTIM, (50 μl)

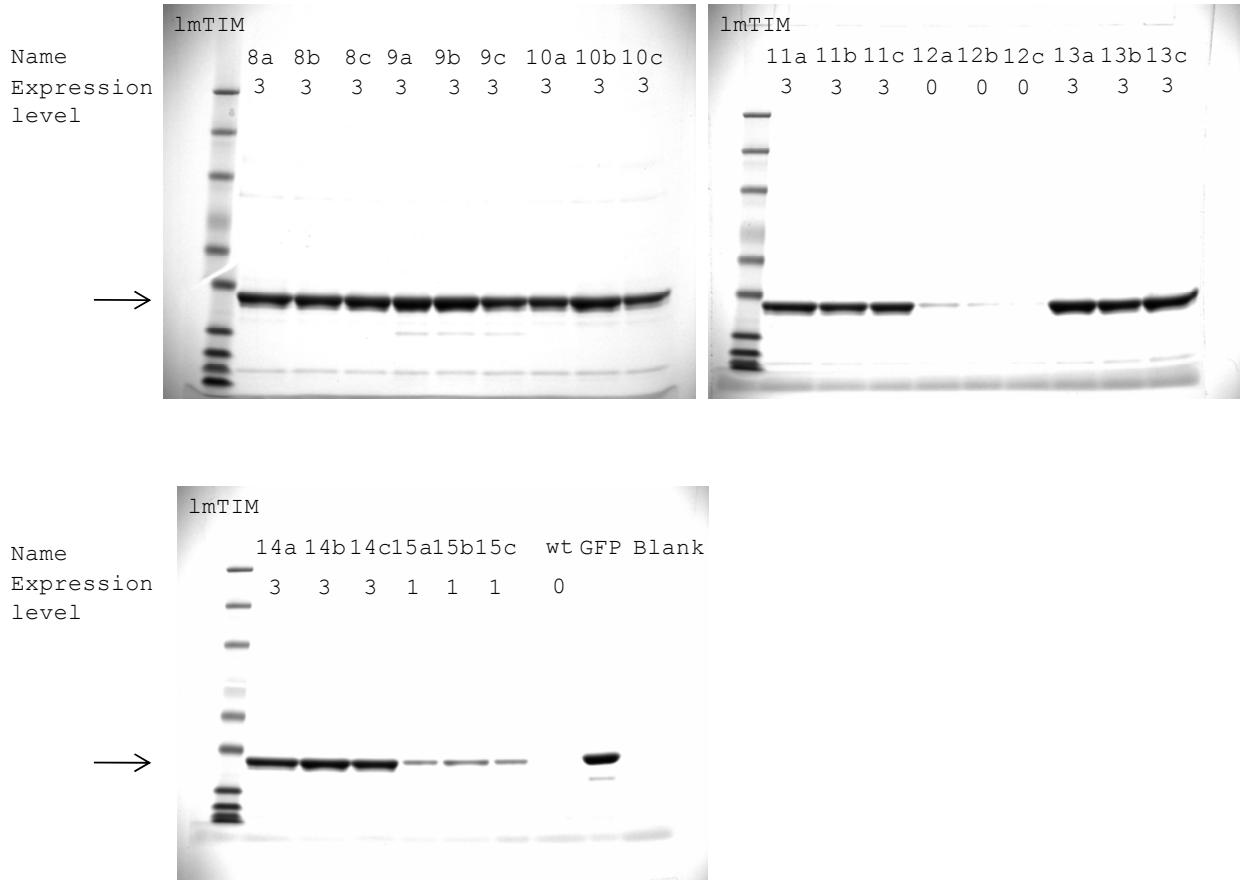


Figure S1V – lmTIM, (50 μl)

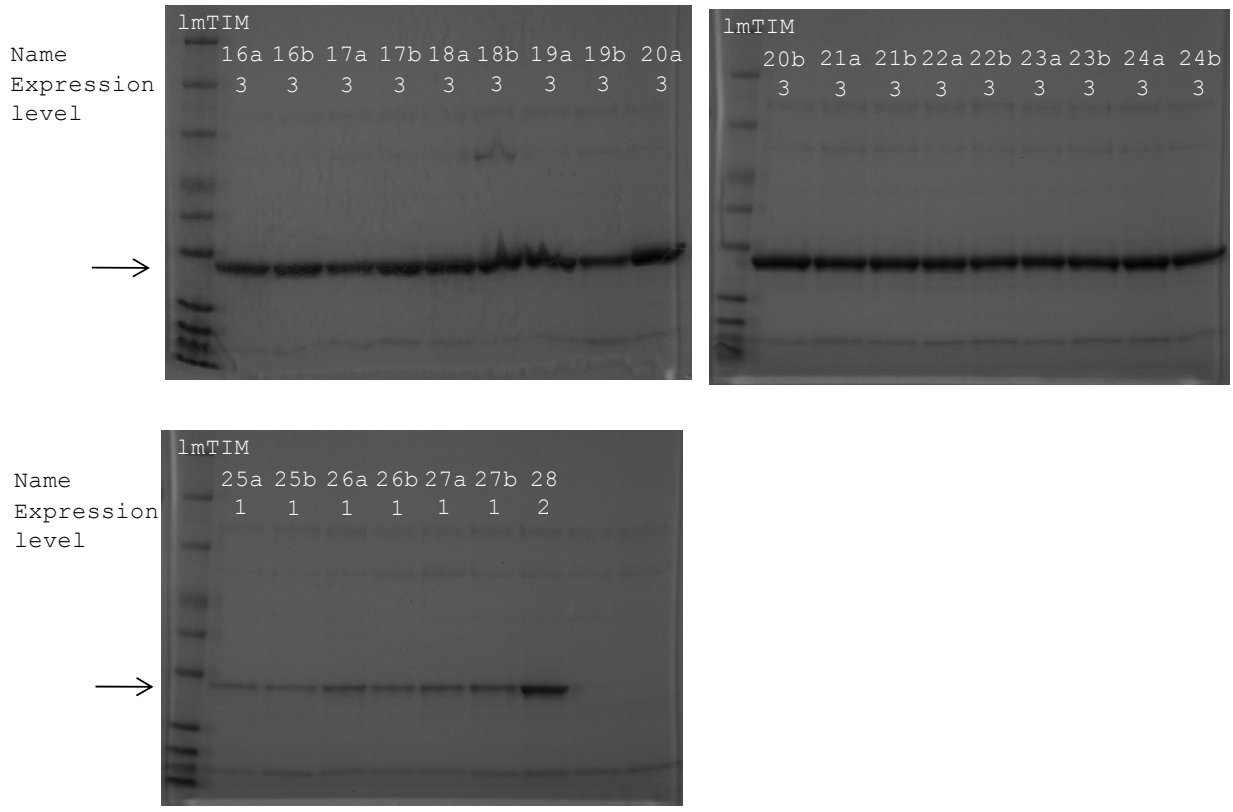
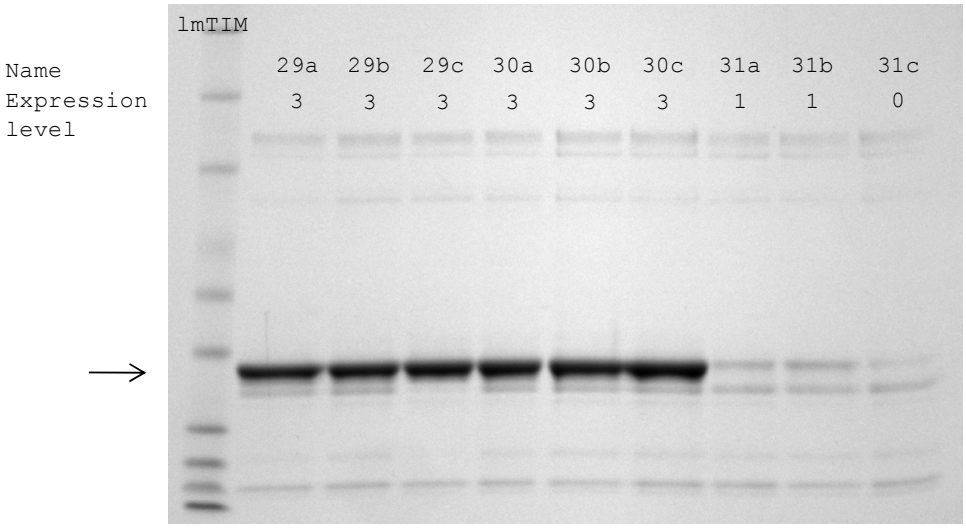


Figure S1W – lmTIM, replicate 1 (50 μl)



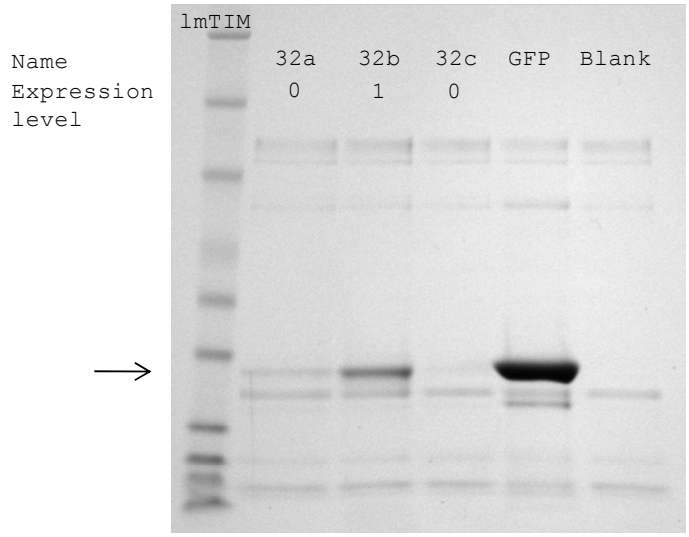


Figure S1X – lmTIM, replicate 2 (50 μl)

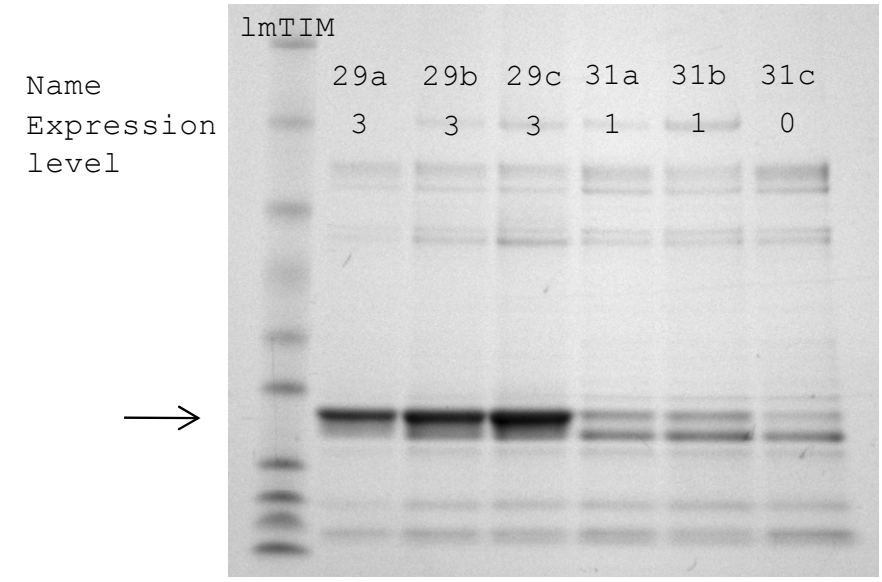


Figure S1Y – lmTIM, replicate 1 (50 μl)

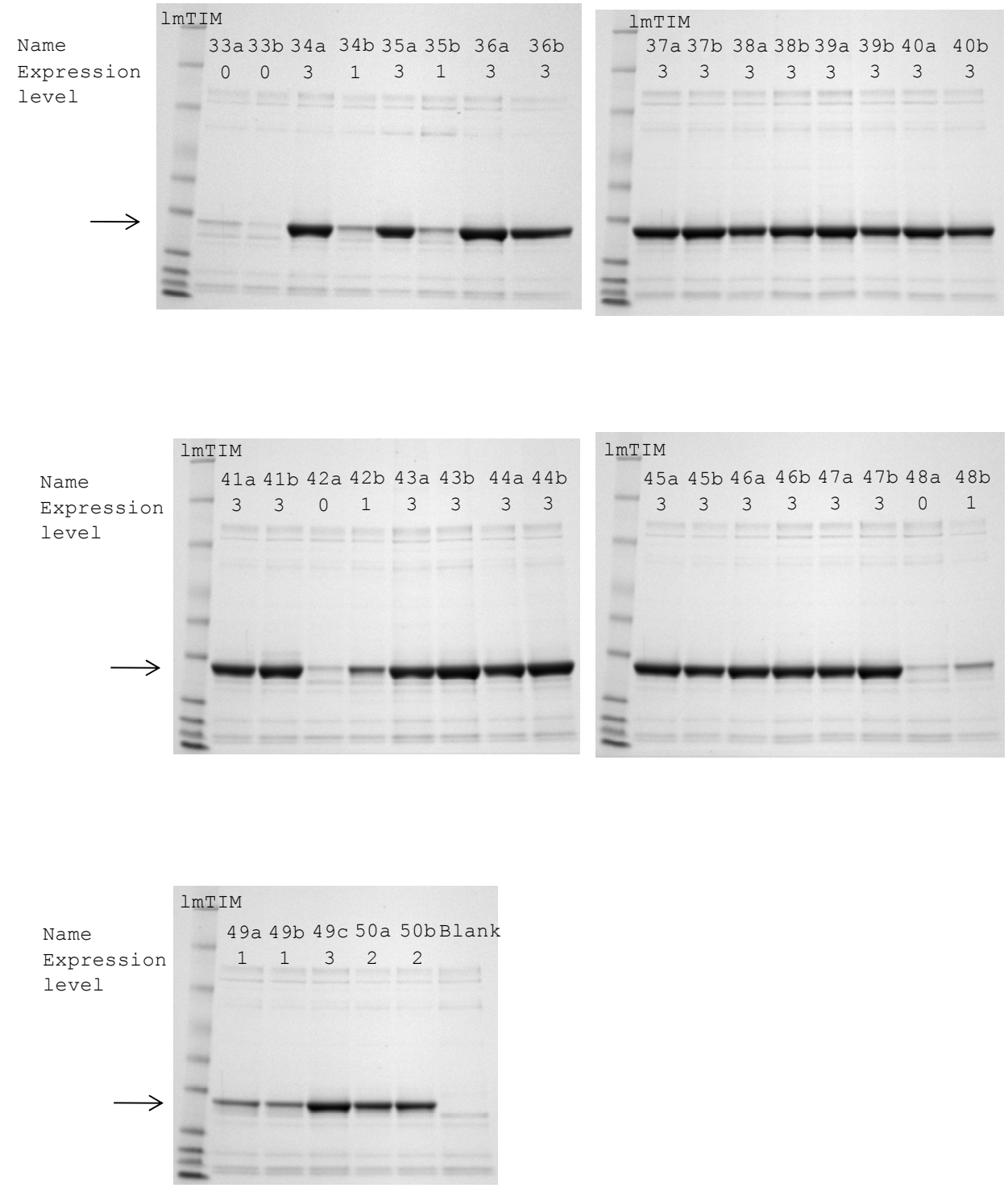
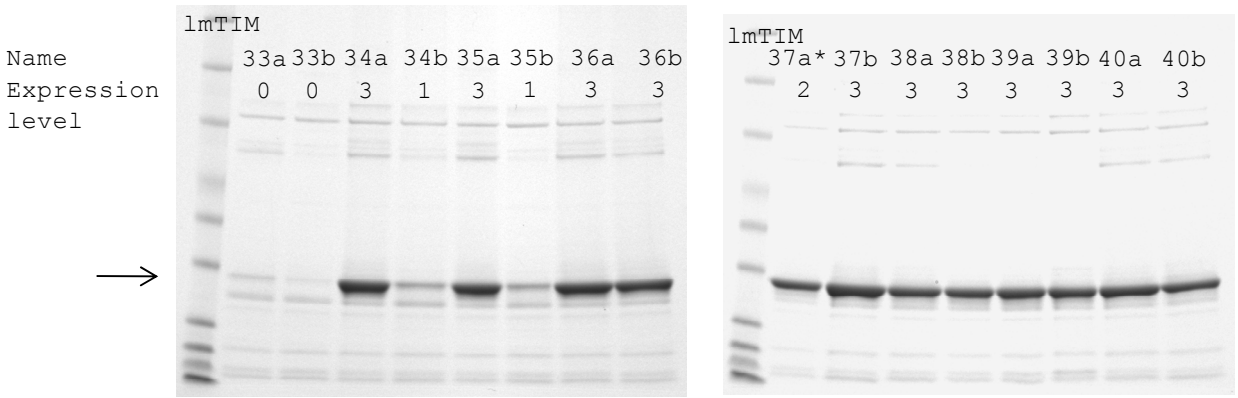
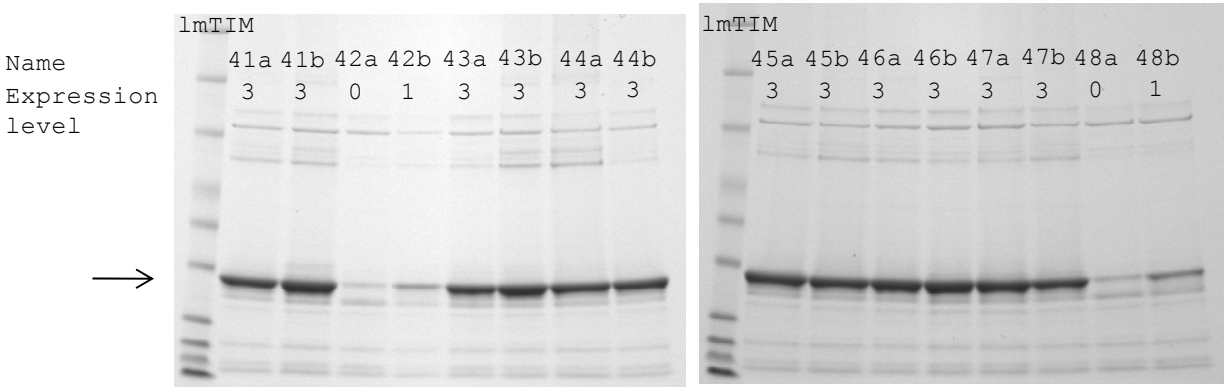
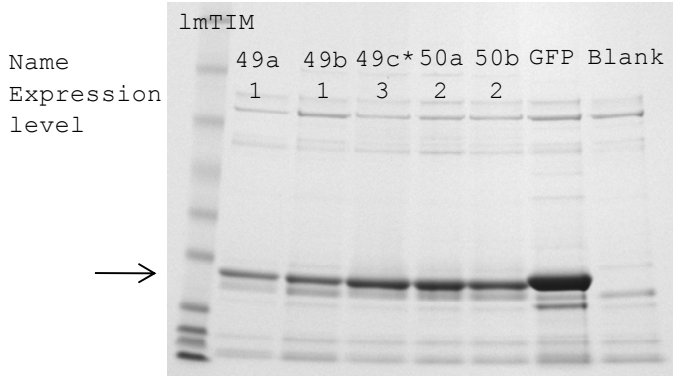


Figure S1Z – lmTIM, replicate 2 (50 μl)



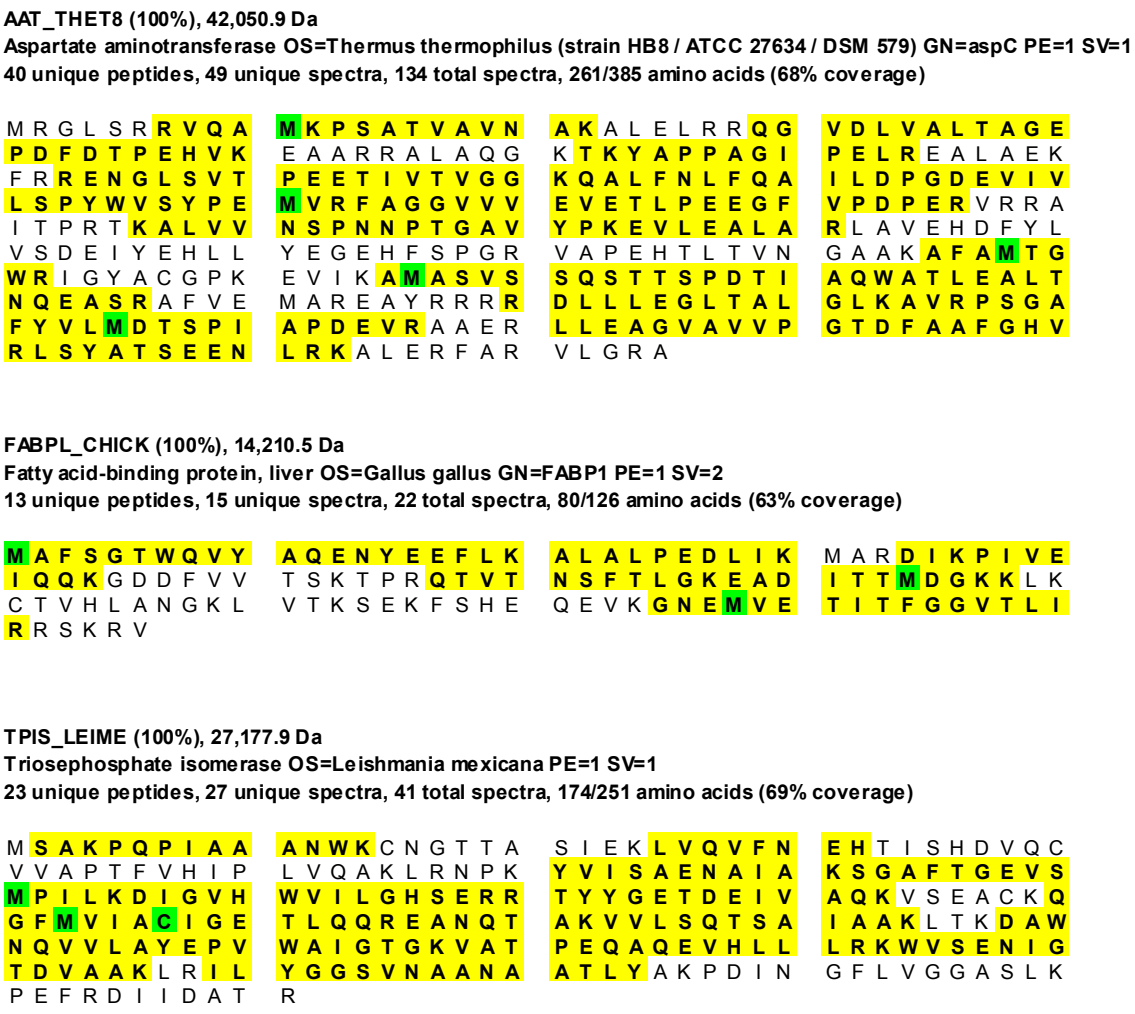




* Behaves as a '3' on gel 6/19/2009; entered as a '3' in scoring file.

Figure S2

Relating to Figure 2, we provide the sequence coverage obtained by LC-MS/MS analysis for aspartate aminostransferase (*Thermus thermophilus*), fatty acid binding protein (*Gallus gallus*) and triose phosphate isomerase (*Leishmania mexicana*). Yellow shading indicates protein regions for which peptides were sequenced by tandem mass spectrometry. Green highlights denote amino acids that were modified during sample processing; carbamidomethylated (Cys) or oxidized (Met).



Supplemental Experimental Procedures

Synthetic oligonucleotide synthesis & gene assembly

Synthetic genes were assembled by PCR from 80-100 mer oligonucleotides.

Synthesis reagents

Oligonucleotides were chemically synthesized on a MerMade 192 DNA synthesizer (BioAutomation Corp., MM-192) using standard phosphoramidite chemistries (Caruthers et al. 1983; Caruthers et al. 1987). Controlled-pore glass (CPG) columns were placed into the synthesis manifold as indicated by the software and were sealed into the manifold with a rubber mallet. Phosphoramidites are solubilized to a concentration of 1 g per 20 ml directly before use. Reagents, part numbers, and vendor information are summarized in the table below.

Reagent	Part number	Source	Description
Deblock	BIO830	EMD Biosciences	dichloroacetic acid (3%) in dichloromethane
Cap A	BIO221	EMD Biosciences	2,6-lutidine (10%), acetic anhydride (10%) in THF
Cap B	BIO345	EMD Biosciences	methylimidazole (16%) in THF
Oxidizer	BIO420	EMD Biosciences	0.02 M iodine in THF (70%), pyridine (20%), water (10%)
Activator	BIO152	EMD Biosciences	0.25M 5-(ethylthio)-1H-tetrazole in acetonitrile
Acetonitrile (wash)	AX0151	EMD Biosciences	acetonitrile, anhydrous
Acetonitrile (diluent)	40-4050-50	Glen Research	acetonitrile, anhydrous
dA phosphoramidite	10-1000	Glen Research	dA-CE phosphoramidite
dC phosphoramidite	10-1015	Glen Research	Ac-dC-CE phosphoramidite
dG phosphoramidite	10-1029	Glen Research	dmf-dG-CE phosphoramidite
dT phosphoramidite	10-1030	Glen Research	dT-CE phosphoramidite
dA CPG column	SCG1-1000-5	Biosearch	5'-DMT-dA(Bz)-Suc, 1000Å, 50 nmol
dC CPG column	SCG1-1100A-5	Biosearch	5'-DMT-dC(Ac)-Suc, 1000Å, 50 nmol
dG CPG column	SCG1-1200F-5	Biosearch	5'-DMT-dG(dmf)-Suc, 1000Å, 50 nmol
dT CPG column	SCG1-1300-5	Biosearch	5'-DMT-dT-Suc, 1000Å, 50 nmol
Water traps	TP-(gram amount)	ChemAssist	Molecular trap pack

Table of reagents and consumables for synthesis of oligonucleotides used to construct synthetic genes.

Synthesis protocol

Prior to the start of synthesis, the CPG columns were rinsed twice with anhydrous, synthesis-grade acetonitrile, capped twice with capping reagents, and again washed twice with acetonitrile. Synthesis used the following operation sequence: deblock (twice), wash (twice), couple (twice), wash, cap, wash, oxidation, wash, cap, and wash (twice). After completion of synthesis, the columns were incubated with deblock three times and washed three times. During all chemical steps reaction steps (deblocking, coupling, capping and oxidation), two short vacuum pulses were applied to the columns approximately at 15 and 30 seconds reaction time. This technique pulls fresh reagent placed on top of the column into the synthesis resin. Reaction order, reagent volume, and reaction times are provided in the following tables.

Table of pre-synthesis reaction sequence

Step	Volume	Incubation time (s)	Vac. pulse
Wash	250 µl acetonitrile	0	N/A
Wash	250 µl acetonitrile	0	N/A
Capping	60 µl cap A reagent 60 µl cap B reagent	45	two
Capping	60 µl cap A reagent 60 µl cap B reagent	45	two
Wash	250 µl acetonitrile	0	N/A
Wash	250 µl acetonitrile	0	N/A

Table of synthesis reaction sequence, per monomer

Step	Volume	Incubation time (s)	Vac. pulse
Deblock	120 µl deblock	50	two
Deblock	120 µl deblock	50	two
Wash	250 µl acetonitrile	0	N/A
Wash	250 µl acetonitrile	0	N/A
Couple	60 µl phosphoramidite 80 µl activator	75	two
Couple	60 µl phosphoramidite 80 µl activator	75	two
Wash	250 µl acetonitrile	0	N/A
Capping	60 µl cap A reagent 60 µl cap B reagent	45	two
Wash	250 µl acetonitrile	0	N/A
Oxidize	95 µl oxidizer	45	two
Wash	250 µl acetonitrile	0	N/A
Capping	60 µl cap A reagent 60 µl cap B reagent	45	two
Wash	250 µl acetonitrile	0	N/A
Wash	250 µl acetonitrile	0	N/A

Table of post-synthesis reaction sequence

Step	Volume	Incubation time (s)	Vac. pulse
Deblock	120 µl deblock	50	two
Deblock	120 µl deblock	50	two
Deblock	120 µl deblock	50	two
Wash	250 µl acetonitrile	0	N/A
Wash	250 µl acetonitrile	0	N/A
Wash	250 µl acetonitrile	0	N/A

Oligonucleotide postprocessing

Oligonucleotides were chemically cleaved from CPG columns by incubation in strong base. 100 µl of ~30% ammonium hydroxide (VWR, EM-AX1303-11) was placed into the CPG column and incubated at room temperature for 15 minutes. After incubation, the ammonium solution was captured in a 2 ml deep-well microplate (Phenix Research, M-1810) in the microplate cleavage apparatus (BioAutomation, MPM-M192-1-029). This process was repeated twice more for a total of three cleavage incubations. Next, the plate was sealed with an adhesive aluminum foil sheet (ISC BioExpress, T-2420-1), placed into a microplate deprotection chuck (BioAutomation, A-MM192-DEPROTECTION CHUCK), and deprotected in ammonia (16 – 20 hours) at 55°C.

After deprotection, the ammonium was removed by evaporation in an evaporative microplate dryer (BioAutomation, 11-80965) at 55°C, 20 l / min air, for 10 – 20 minutes, leaving ~200 µl. Ten volumes (2 ml) of 1-butanol (Sigma Aldrich, B7906) were added to each microwell and the plate was sealed in a thermal microplate sealer (REMP, EasySealer) with an Easy Peel Heat Sealing Film sheet (ISC BioExpress, T-2418-1). The plate was rotated end-over-end on a Mini LabRoller (Labnet International, H5500) for 10 – 15 minutes to mix thoroughly. The precipitated oligonucleotides were pelleted by centrifugation at 2,500 *g* for 10 minutes in a microplate centrifuge (Thermo Fisher Scientific, 11177564). The sealing film was discarded and the plate carefully decanted. The oligonucleotide pellets were dried in the evaporative microplate dryer at 55°C, 20 l / min air, for 5 – 15 minutes. The oligonucleotides were resuspended in 300 µl of TE buffer (10 mM Tris,pH 8.0, 1 mM EDTA).

The oligonucleotides were diluted 200-fold (1 µl stock into 199 µl of water) in a UV-transparent microplate (Costar, 3635), and their concentration determined spectrophotometrically in a microplate spectrophotometer (Tecan, GENios). Each oligonucleotide was diluted to a common stock concentration (100 µM) using a Tecan Genesis liquid-handling robot, programmed by custom software program, REARRAYER. Working plates (1 ml microplate, Nalge Nunc, 260252) containing 1 µM oligos were prepared from stock plates.

Synthetic open reading frame creation

The synthetic genes were assembled by PCR from oligonucleotides using liquid-handling robotics, as described (Cox et al. 2007). The following tables indicate the characteristics of the oligonucleotide scaffold system used to create the ORFs in this work.

lmTIM (*Leishmania mexicana* triosephosphate isomerase, E65Q mutant)

Oligo #	Fragment	Oligo pair	Strand	Start nucleotide	End nucleotide
1	A	1	S	184	274
2	A	1	A	335	245
3	A	2	S	123	213
4	A	2	A	397	306
5	A	3	S	62	152
6	A	3	A	459	368
7	A	4	S	1	91
8	A	4	A	521	430
9	B	1	S	675	765
10	B	1	A	826	736
11	B	2	S	614	704
12	B	2	A	888	797
13	B	3	S	553	643
14	B	3	A	950	859
15	B	4	S	492	582
16	B	4	A	1012	921

lmTIM (amino acid sequence GI:12084529) ORFs are encoded as a two fragment system (A and B) comprised of four primer pairs per fragment. Strand indicates sense (S) or anti-sense (A) directionality.

ggFABP (*Gallus gallus* liver basic fatty acid binding protein)

Oligo #	Fragment	Oligo pair	Strand	Start nucleotide	End nucleotide
1	A	1	S	107	188
2	A	1	A	240	159
3	A	2	S	54	136
4	A	2	A	292	211
5	A	3	S	1	83
6	A	3	A	344	263
7	B	1	S	420	501
8	B	1	A	553	472
9	B	2	S	368	449
10	B	2	A	605	524
11	B	3	S	315	397
12	B	3	A	657	576

ggFABP (amino acid sequence GI:56966105) ORFs are encoded as a two fragment system(A and B) comprised of three primer pairs per fragment. Strand indicates sense (S) or anti-sense (A) directionality.

ttAAT (*Thermus thermophilus* aspartate aminotransferase)

Oligo #	Fragment	Oligo pair	Strand	Start nucleotide	End nucleotide
1	A	1	S	178	266
2	A	1	A	324	237
3	A	2	S	119	207
4	A	2	A	382	295
5	A	3	S	60	148
6	A	3	A	440	353
7	A	4	S	1	89
8	A	4	A	498	411
9	B	1	S	646	734
10	B	1	A	792	705
11	B	2	S	587	675
12	B	2	A	850	763
13	B	3	S	528	616
14	B	3	A	908	821
15	B	4	S	469	557
16	B	4	A	879	966
17	C	1	S	114	1202
18	C	1	A	1260	1173
19	C	2	S	1055	1143
20	C	2	A	1318	1231
21	C	3	S	996	1084
22	C	3	A	1376	1289
23	C	4	S	937	1025
24	C	4	A	1434	1347

ttAAT (amino acid sequence GI:5821836) ORFs are encoded as a three fragment system(A,B, and C) comprised of four primer pairs per fragment. Strand indicates sense (S) or anti-sense (A) directionality.

In vitro coupled transcription and translation system

The *in vitro* protein expression system described here is derived from the work of the James Swartz lab at Stanford University (Kim and Swartz 2001; Jewett and Swartz 2004c; Jewett and Swartz 2004a; Jewett and Swartz 2004b; Liu et al. 2005).

S30 lysate preparation

BL21 Star (DE3) cells (Invitrogen, C6010-03) were plated on a LB agar plate and incubated overnight at 37°C. A fresh colony was used to inoculate 75 mL 2xYT-PG medium (16 g / l tryptone (Sigma Aldrich, T7293), 10 g / l yeast extract (Sigma Aldrich, 70161), 5 g / l NaCl (Sigma Aldrich, 71376), 22 mM NaH_2PO_4 (Sigma Aldrich, S5011), 40 mM Na_2HPO_4 (Sigma Aldrich, S5136), and 100 mM glucose (Sigma Aldrich, G7021) in a 250 ml baffled flask, and grown to stationary phase under vigorous aeration overnight at 37°C. This seed culture was used to inoculate 1.5 l 2xYT in 6 l baffled flasks (1:100 inoculum:medium), and grown at 37°C to 30% completion of logarithmic growtn (~ 2.5 hours at 115 rpm; growth curve was established independently to determine appropriate stopping point). T7 RNA polymerase expression was induced by addition of 0.25 mM IPTG. The culture was grown

further to ~0.75% (an additional ~ 1.75) and then chilled immediately on ice for 15 minutes. The cells were harvested by centrifugation in a pre-chilled rotor at 4°C (20 min, 5,000 *g*). Exhausted medium was removed by decanting, and the pellet was resuspended in 75 mL S30 buffer (10 mM Tris-acetate, (pH 8.2; Sigma Aldrich, T1258), 14 mM magnesium acetate tetrahydrate (Sigma Aldrich, M5661), 60 mM potassium acetate (Sigma Aldrich, P1190), and 2 mM dithiotheitol (Sigma Aldrich, D9779)) using vigorous agitation with a microcentrifuge tube vortexer, or a cordless drill with a plastic spatula attached as the bit (VWR, 53800-005). The resuspended cell slurry was pelleted at 4°C for 10 min at 5,000 *g* in sterile conical 250 ml flasks, decanted and flash-frozen in liquid nitrogen after determination of wet weight. Following overnight storage at -80°C, the frozen cell pellets were thawed on ice for ~1 hr and resuspended in S30 buffer supplemented with 5 mM DTT (1 ml buffer per g of wet cell paste) and lysed by French press at (17,000 psi). A clarified lysate was made in two successive centrifigutions (4°C, 30,000 *g*, 30 min each). Endogenous *E. coli* mRNA was removed in a simplified run-off reaction by incubating at 37°C for 80 minutes, rotating end-over-end on a Mini LabRoller (Labnet International, H5500) in the dark. The lysate was dialyzed (6 – 8 kDa MWCO; Spectra/Por, 132-650 dialysis tubing) against 80 volumes S30 (4°C, 1 hr), and centrifuged (4°C, 4,000 *g*, 10 min) to remove precipitates. This cell-free extract was aliquoted into microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at -80°C.

Reaction mix formulation

Reaction mix was prepared at 4X final concentration.

Reagent	Part number	Source	Description
PEP	108294	Roche	phosphoenol-pyruvate monopotassium salt
NAD	N6522	Sigma Aldrich	β-Nicotinamide adenine dinucleotide hydrate
CoA	C4282	Sigma Aldrich	coenzyme A hydrate
Putrescine	D13208	Sigma Aldrich	1,4-Butanediamine
Spermadine	S0266	Sigma Aldrich	N-(3-Aminopropyl)-1,4-diaminobutane
Oxalate	O0501	Sigma Aldrich	potassium oxalate monohydrate
Magnesium glutamate	49605	Sigma Aldrich	L-glutamic acid hemimagnesium salt tetrahydrate
Ammonium glutamate	G1376	Sigma Aldrich	L-glutamic acid ammonium salt
Potassium glutamate	G1501	Sigma Aldrich	L-glutamic acid potassium salt monohydrate
Folinate	F7878	Sigma Aldrich	folinic acid calcium salt
tRNAs	109550	Roche	tRNA from *E. coli* MRE 600
Rifampicin	R3501	Sigma Aldrich	rifampicin
rATP	A2383	Sigma Aldrich	adenosine 5′-triphosphate disodium salt
rCTP	C1506	Sigma Aldrich	cytidine 5′-triphosphate disodium salt
rGTP	G8877	Sigma Aldrich	guanosine 5′-triphosphate sodium salt hydrate
rUTP	U6750	Sigma Aldrich	uridine 5′-triphosphate trisodium salt hydrate
Alanine	05129	Sigma Aldrich	L-alanine
Arginine	11009	Sigma Aldrich	L-arginine
Asparagine	11009	Sigma Aldrich	L-asparagine
Aspartate	11149	Sigma Aldrich	L-aspartic acid
Cysteine	30089	Sigma Aldrich	L-cysteine

Glutamate	49449	Sigma Aldrich	L-glutamic acid
Glutamine	49419	Sigma Aldrich	L-glutamine
Glycine	50049	Sigma Aldrich	Glycine
Histidine	53319	Sigma Aldrich	L-histidine
Isoleucine	58879	Sigma Aldrich	L-isoleucine
Leucine	61819	Sigma Aldrich	L-leucine
Lysine	62929	Sigma Aldrich	L-lysine monohydrochloride
Methionine	64319	Sigma Aldrich	L-methionine
Phenylalanine	P5482	Sigma Aldrich	L-phenylalanine
Proline	81709	Sigma Aldrich	L-proline
Serine	84959	Sigma Aldrich	L-serine
Threonine	89179	Sigma Aldrich	L-threonine
Tryptophan	93659	Sigma Aldrich	L-tryptophan
Tyrosine	93829	Sigma Aldrich	L-tyrosine
Valine	94620	Sigma Aldrich	L-valine

Table of reagents included in the reaction master mix.

250 ml solution of 50 mM amino acid stock solution was prepared as a suspended mixture first, excepting tyrosine due to its poor solubility at near-neutral pH: 1.46 g valine, 2.53 g tryptophan, 2.07 g phenylalanine, and 1.64 g isoleucine were dissolved in 200 ml water and incubated at 37°C with mixing or agitation for 15 min to facilitate dissolution. Next, 1.64 g leucine and 1.52 g cysteine were added followed by incubation and mixing at 37°C for 15 min. Then, 1.87 g methionine, 1.11 g alanine, 2.18 g arginine, 1.65 g asparagine, 1.66 g aspartate, 1.84 g glutamate, 0.94 g glycine, and 1.83 g glutamine were added, followed by adjustment of the pH by addition of 1.0 ml of 10N KOH (Sigma Aldrich, P5958). Finally, 1.94 g histidine, 2.28 g lysine, 1.44 g proline, 1.31 g serine, and 1.49 g threonine wereadded and dissolved, and the final solution volume brought up to 250 ml by addition of water. The mixture was divided into six aliquots and stored at -80°C.

Solution	[Stock]	Sol'n Volume	Amount	Final pH	Acid/base
PEP	1 M	35 ml	7.22 g	6.8 – 7.3	10N KOH
NAD	50 mM	7 ml	232 mg	6 – 7	10N KOH
CoA	40 mM	7 ml	215 mg	7.3	10N KOH
Putrescine	100 mM	10 ml	88.2 mg / 100.5 µl	7.3	glacial HOAc
Spermidine	100 mM	15 ml	218 mg / 236 µl	7.3	glacial HOAc
Oxalate	1 M	15 ml	2.76 g	8.4	unbuffered
Mg^{++} glutamate	1 M	50 ml	19.43 g	7.3	10N KOH
NH_4^+ glutamate	1.5 M	50 ml	12.32 g	7.3	10N KOH
K^+ glutamate	3.5 M	250 ml	177.83 g	8.2	unbuffered
Folinate	10.8 mg / ml	15 ml	162 mg	7.0 – 7.5	unbuffered
tRNAs	34 mg / ml	5 ml	172 mg	7.2	dissolved in 10 mM K_2PO_4, pH 7.2
Rifampicin	1mg / ml	50 ml	50 mg	6 – 7	10N KOH
rATP	500 mM	5 ml	1.38 g	7.3	10N KOH
rCTP	500 mM	5 ml	1.32 g	7.3	10N KOH

rGTP	500 mM	5 ml	1.31 g	7.3	10N KOH
rUTP	500 mM	5 ml	1.38 g	7.3	10N KOH
19 amino acids	50 mM	250 ml	Various	n/a	10N KOH

Table of stock concentrations, volumes, and pH used to construct the 4X reaction master mix solution. Solutions that are not completely consumed in one batch of reaction mix are stored at -80°C. Putrescine and spermidine are incubated at 37°C in order to change phase into liquid form and are pipetted rather than weighed. Solutions listed as "unbuffered" are not pH-corrected with acid or base due to the solutions' weak buffering capacity, or because it was specifically left unbuffered in previous protocols.

250 mL of 4X reaction master mix was prepared by combining the amino acid stock with powdered tyrosine, mixing, addition of glutamate salts, mixing, addition of folinic acid, tRNAs, PEP, NAD, coenzyme A, oxalic acid, putrescine, spermadine, and rifampicin, mixing, addition of the four ribonucleotides (amounts of components given below). The solution was aliquoted (while mixing to ensure even distribution of insoluble flakes of tyrosine), flash frozen in liquid nitrogen, and stored at -80°C.

Solution	Stock	Amount to add	4x conc.	1x conc.
19 amino acids	50 mM	40 ml	8 mM	2 mM
Tyrosine	n/a	371 mg	8 mM	2 mM
Water	n/a	37.3 ml	n/a	n/a
Mg^{++} glutamate	1 M	10 ml	40 mM	10 mM
NH_4^+ glutamate	1.5 M	6.7 ml	40 mM	10 mM
K^+ glutamate	3.5 M	50 ml	700 mM	175 mM
Folinate	10.8 mg / ml	3.14 ml	136 µg / ml	34 µg / ml
tRNAs	34 mg / ml	5 ml	682 µg / ml	171 µg / ml
PEP	1 M	33.3 ml	120 mM	30 mM
NAD	50 mM	6.67 ml	1.33 mM	0.33 mM
CoA	40 mM	6.67 ml	1.08 mM	0.27 mM
Oxalate	1 M	2.7 ml	10.8 mM	2.7 mM
Putrescine	100 mM	10 ml	4 mM	1 mM
Spermadine	100 mM	15 ml	6 mM	1.5 mM
Rifampicin	1 mg/ml	15 ml	40 µg / ml	10 µg / ml
rATP	500 mM	2.5 ml	5 mM	1.25 mM
rCTP	500 mM	2 ml	4 mM	1 mM
rGTP	500 mM	2 ml	4 mM	1 mM
rUTP	500 mM	2 ml	4 mM	1 mM

Table of stock volumes used to create the 4X reaction master mix.

DNA template

The invariant 5' UTR contains a T7 RNA polymerase promoter (Chamberlin et al. 1970; Davanloo et al. 1984) and Shine-Delgarno ribosome binding site (Shine and Dalgarno 1974; Curry and Tomich 1988). The ATG codon starts immediately following the 3' end. The promoter is denoted in bold text; the ribosome binding site is italicized. lmTIM alleles 29-50b and ttAST alleles 25a-57b contained a 5' terminal PstI restriction endonuclease recognition sequence, indicated with parentheses.

5′–(GCACTGCAG)CGGCGTAGAGGATCGAGATCTCGATCCCGCGAAAT**TAATACGACTCACTATA**GGGG
AATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTA*AGAAGGAG*ATATACC–3′.

The invariant 3’ segment contains a gly-gly-ser fusion linker (in bold), poly-histidine purification tag (in italics), and two stop codons (underlined) as well as a T7 transcriptional terminator stem-loop structure (Mertens et al. 1996). lmTIM alleles 29-50b and ttAST alleles 25a-57b contained a 3’ terminal EcoRI restriction endonuclease recognition sequence, indicated with parentheses.

5′–**GGCGGCTCC***CACCATCACCATCACCAT*TAATGAGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGC
TGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG
(GAATTCGTA)–3′

Supplemental References

Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY. 1987. Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. *Methods Enzymol* **154**: 287-313.

Caruthers MH, Beaucage SL, Becker C, Efcavitch JW, Fisher EF, Galluppi G, Goldman R, deHaseth P, Matteucci M, McBride L et al. 1983. Deoxyoligonucleotide synthesis via the phosphoramidite method. *Gene Amplif Anal* **3**: 1-26.

Chamberlin M, McGrath J, Waskell L. 1970. New RNA polymerase from Escherichia coli infected with bacteriophage T7. *Nature* **228**(5268): 227-231.

Cox JC, Lape J, Sayed MA, Hellinga HW. 2007. Protein fabrication automation. *Protein Sci* **16**(3): 379-390.

Curry KA, Tomich CS. 1988. Effect of ribosome binding site on gene expression in Escherichia coli. *DNA* **7**(3): 173-179.

Davanloo P, Rosenberg AH, Dunn JJ, Studier FW. 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci USA* **81**(7): 2035-2039.

Jewett MC, Swartz JR. 2004a. Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. *Biotechnol Bioeng* **86**(1): 19-26.

-. 2004b. Rapid expression and purification of 100 nmol quantities of active protein using cell-free protein synthesis. *Biotechnol Prog* **20**(1): 102-109.

-. 2004c. Substrate replenishment extends protein synthesis with an in vitro translation system designed to mimic the cytoplasm. *Biotechnol Bioeng* **87**(4): 465-472.

Kim DM, Swartz JR. 2001. Regeneration of adenosine triphosphate from glycolytic intermediates for cell-free protein synthesis. *Biotechnol Bioeng* **74**(4): 309-316.

Liu DV, Zawada JF, Swartz JR. 2005. Streamlining Escherichia coli S30 extract preparation for economical cell-free protein synthesis. *Biotechnol Prog* **21**(2): 460-465.

Mertens N, Remaut E, Fiers W. 1996. Increased stability of phage T7g10 mRNA is mediated by either a 5'- or a 3'-terminal stem-loop structure. *Biol Chem* **377**(12): 811-817.

Shine J, Dalgarno L. 1974. The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc Natl Acad Sci USA* **71**(4): 1342-1346.