Supplementary Methods

Primers and Reagent List for Ribosome Display and Preparation of an *in vitro* translation system

1 Reagents for ribosome display

- Agarose (Invitrogen 30391-023)
- Magnesium acetate (MgAc, Sigma M-0631)
- Potassium acetate (KAc, Fluka 60034)
- Sodium chloride (NaCl, Fluka, 71376)
- Tween-20 (Sigma, P-7949)
- NeutrAvidin (Pierce 31000)
- Bovine serum albumin (BSA, Fluka 05476)
- *Saccharomyces cerevisiae* RNA (Fluka 83847)
- Ribonuclease inhibitor RNasin (Promega N211B)
- Reverse transcriptase (e.g. Stratascript, 50 U/µl, Stratagene 600085-51, 10x Stratascript buffer, Stratagene 600085-52; or alternatively, reverse transcriptase Thermoscript (15 U/µl; Invitrogen catalog number 11146-016, 5x Thermoscript buffer delivered with the enzyme)
- DNA-polymerase for PCR (e.g., Vent® polymerase, NEB M0254L, ThermoPol PCR buffer, delivered with Vent® polymerase, or alternatively Phusion™ High-Fidelity DNA Polymerase, Finnzymes F-530S, 5x Phusion™ HF PCR buffer, delivered with Phusion™ polymerase)
- dNTPs (5 mM of each dNTP, Eurogentec NU-0010-50)
- Dimethylsulfoxide (DMSO, Fluka 41640)
- NTPs (50 mM, Sigma)
- Hepes (Sigma H-3375)
- Spermidine (Sigma S-2501)
- T7 RNA polymerase (NEB M0251L)
- Lithium chloride (LiCl, Fluka 62476)
- 100% ethanol (EtOH)
- Sodium acetate (NaAc, Fluka 71180)

- Heparin (Fluka 51550)
- Disodium ethylenediamine tetraacetate (EDTA, Fluka 03680)
- T4 DNA-ligase (MBI Fermentas EL0011)
- Shrimp alkaline phosphatase (SAP, USB PN 70092)
- 4-morpholinopropanesulfonic acid (MOPS, Fluka 69949)
- Boric acid (Fluka 15660)
- Guanidine thiocyanate (Fluka 50990)
- N,N-dimethyl formamide (Sigma Aldrich 27.054-7)
- 37% formaldehyde (Fluka 47629)
- Protein disulfide isomerase should be used[1,2 14] (PDI; Sigma P3818)
- ^{35}S-methionine for radioactive binding assays (PerkinElmer NEG009H)
- Triethylamine (Sigma-Aldrich 90335)
- 5x T7 RNA polymerase buffer (1 M Hepes KOH pH 7.6, 150 mM magnesium acetate, 10 mM spermidine, 200 mM DTT)
- Tris-buffered saline, TBS (50 mM TrisHCl pH 7.4 at 4 °C; 150 mM NaCl)
- TBS with Tween, TBST (TBS with 0.05 % (500 µl/l) Tween-20)
- Washing buffer with Tween, WBT (50 mM Tris-acetate pH 7.5 at 4°C; 150 mM NaCl; 50 mM MgAc; 0.05% Tween-20)
- Elution buffer, EB (50 mM Tris-acetate pH 7.5 at 4°C; 150 mM NaCl; 50 mM EDTA)
- 10 x MOPS (0.2 M MOPS pH 7, 50 mM sodium acetate, 10 mM EDTA)
- 10 x TBE buffer: (890 mM Tris-buffered saline, 890 mM boric acid, 20 mM EDTA).
- QIAquick PCR purification and gel extraction kit (Qiagen 28104 and 28704)
- Roche high pure RNA isolation kit (Roche 1 828 655)

2 Primers for ribosome display

Table 1 | Primers

Name	Sequence (5'-3')	Description
T7B	ATACGAAATTAATACGACTCACTA-TAGGGAGACCACAACGG	Introduces T7 promoter. Overlaps with SDA_EWT5
SDA_EWT5	AGACCACAACGGTTTCCCTCTAGAA-ATAATTTTGTTTAACTTTAAGAAGGA-GATATAT**CC*ATGG***_GTATGAGAGGATCG_	Introduces ribosomal binding site, _Nco_I site (bold). Anneals to N-terminal RGS-6His tag (coding residues, italics).
EWT5	TTCCTCCATGGGT**ATGAGAGGATCG-CATCACCATCACCATCAC**_GGATCCGA-CCTGGG_	Introduces MRGS-His tag (bold) and anneals to the N-terminal part of a DARPin library (italic)[18].
tolAk	CCGCACACCAGTAAGGTGTGCGGTTT-CAGTTGCCGCTTTCTTTCT	Reverse primer, which introduces stem loop on the 3′-part of the ribosome display construct (see **Fig. 2**)

These primers are designed to work with the DARPin library and the pRDV as described in the main text. With different vectors, tags or libraries, primers have to be adapted accordingly.

3 Preparation of an S30 *in vitro* translation extract

Alternatively to the use of commercially available *in vitro* translation kits, bacterial translation extracts may be prepared according to the protocol described here. We describe the preparation of an *E. coli* S30-Extract according to Lesley, Zubay and Pratt, with minor modifications[1; 2; 3; 4]. One liter of *E. coli* culture yields approximately 8 ml extract. It is important that the cells used for extract preparation are harvested in an early logarithmic phase. If the libraries used for selection contain disulfide bonds, one should omit DTT from the extract. If no disulfides need to be formed in the library, and if the target is not sensitive to reduction, 1 mM DTT can be added to the S30 buffer, as this increases the translation efficiency slightly. Each batch of translation extract needs to be optimized in terms of the concentration of magnesium, potassium, PEG8000 and amount of extract in order to optimize the translation yields. To optimize the extracts we routinely monitor the activity of *in vitro* translated β-lactamase after adding increasing amounts of KGlu, MgAc and PEG-8000 in the concentration ranges indicated below.

Material and Equipment

- Baffled flasks
- Shaker at 37°C for *E. coli* culture
- Refrigerated centrifuges (GS-3, SS-34)
- Dialysis tubing MW cut-off 6000-8000 Da.
- Emulsiflex or French press
- 0.2 mm syringe filter (Millipore SLGPR25KS)
- Dialysis tubing with a molecular weight cut-off of 6000-8000 Da (for example: Spectrum Laboratories SpectraPor 132 650)

Reagents and Buffers

- Luria broth base (GibcoBRL, 12795-084)
- Glucose (Fluka 49150)
- Potassium dihydrogen phosphate (KH_2PO_4, Fluka 60230)
- Di-potassium hydrogen phosphate ($K_2HPO_4 \cdot 3H_2O$, Merck 1.05099.1000)
- Yeast extract (GibcoBRL 30393-037)

- Thiamine (Sigma T-4625)
- Tris-acetate (Serva 37190)
- Magnesium acetate (MgAc, Sigma M-0631)
- Potassium acetate (KAc, Fluka 60034)
- L-glutamic acid monopotassium salt monohydrate (KGlu, Fluka 49601)
- 20 natural amino acids (Sigma LAA-21 kit)
- Adenosinetriphosphate (ATP, Roche Diagnostics 519 987)
- Phosphoenolpyruvate trisodium salt (PEP, Fluka 79435)
- Pyruvate kinase (Fluka 83328)
- GTP (Sigma, G-8877)
- cAMP (Sigma A-6885)
- Acetylphosphate (Sigma A-0262)
- *E. coli* tRNA (Sigma R-4251)
- Folinic acid (Sigma 47612)
- PEG 8000 (Fluka 81268)
- 1,4-dithio-treitol (DTT, Promega, V3155)
- Sodium chloride (NaCl, Fluka, 71376)
- ^{35}S-methionine (PerkinElmer NEG009H)
- *E. coli* strain MRE600[5]
- Incomplete rich medium (5.6 g/l KH_2PO_4, 37.8 g/l $K_2HPO_4 \cdot 3H_2O$, 10 g/l yeast extract, 15 mg/l thiamine, after autoclaving add 50 ml 40% (w/v) glucose and 10 ml 0.1 M MgAc sterile filtered)
- 10x S30 buffer (100 mM Tris-acetate pH 7.5 at 4°C, 140 mM MgAc, 600 mM KAc, store at 4°C or chill buffer in ice bath before use)
- 10 ml preincubation mix (must be prepared immediately before use; 3.75 ml 2 M Tris-acetate pH 7.5 at 4°C, 71 μl 3 M MgAc, 75 μl amino acid mix (10 mM of each of the 20 natural amino acids), 0.3 ml 0.2 M ATP, 0.2 g PEP, 50 U pyruvate kinase)
- PremixA (250 mM Tris-acetate pH 7.5 at 4°C, 1.75 mM of each amino acid except methionine, 10 mM ATP, 2.5 mM GTP, mM cAMP, 150 mM acetylphosphate, 2.5 mg/ml *E. coli* tRNA, mg/ml folinic acid, μM α−ssrA DNA (an oligonucleotide with the sequence 5'-TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCGACTA), KGlu (180-

220 mM), MgAc (10-15 mM), PEG-8000 (5-15% w/v), KGlu, MgAc and PEG8000 have to be adjusted to the corresponding extract)

- β-lactamase assay buffer (5.3 mg nitrocefin in 250 μl DMSO and add this to 50 ml 50 mM potassium phosphate buffer (pH 7)[6].

Procedure

1 | Prepare an LB/glucose plate, streak out MRE600 on the plate and let it grow overnight at 37°C

2 | Prepare all chemicals, media and buffers for *E. coli* extract preparation: Autoclave 1 l incomplete rich medium, 500 ml of LB/glucose medium and one 100 ml and one 5 l shake flask. Prepare 50 ml 40% glucose, 10 ml 0.1 M MgAc and 1 l 10x S30 buffer (it will be used as a 10-fold diluted 1x S30 buffer afterwards). Store all the buffers at 4°C.

3 | Prepare an overnight starter culture by shaking 50 ml LB/glucose medium overnight at 37°C inoculated with a colony of MRE 600 from the plate.

4 | On the next day, add 1 l incomplete rich medium into the 5 l shake flask and add by sterile filtration 50 ml 40% glucose and 10 ml 0.1M MgAc (0.2 μm syringe filter).

5 | Inoculate the medium with 10 ml overnight culture (approximately 1%) and let it shake at 37°C to OD_{600} = 1.0 – 1.2. Transfer the culture in an ice-water bath and immediately add 100 g ice (small shovel) to the culture. Cool the culture in the ice-water bath for 5 minutes by gently shaking the flask by hand. Collect the cell pellet by centrifugation at 4°C (15 min, GS-3, 5000 rpm) and wash it at least 3 times with 50-100 ml of cold S30 buffer.

6 | Weigh the pellets (typically 1-1.5 g/l). The cell pellet can now be shock-frozen in liquid nitrogen and stored at -80 °C until further processing. Do not store the pellets longer than a few days. *Do wear gloves during all following steps to avoid RNase contamination.*

7 | Thaw the pellets on ice, resuspending the cells in S30 buffer (50 ml). Centrifuge at 4°C, full speed in a cooled table-top centrifuge to collect the cell pellet. Resuspend the cells in 4 ml/g (wet cell weight) S30 buffer.

8 | Disrupt the cells by one passage through an EmulsiFlex (approximately 17000 psi) or a French press (at 1000 psi). Repeating passages can decrease translation activity. Centrifuge the lysate at 4°C for 30 min (SS-34, 20,000 g). Repeat this centrifugation step with the supernatant.

9 | Transfer the supernatant after the second centrifugation step into a clean flask and add 1 ml of preincubation mix for each 6.5 ml of S30 extract. This solution is slowly shaken for 1 h at 25°C (no foaming should occur). During this time all translation of endogenous mRNA will be finished and the endogenous mRNA and DNA will be degraded by nucleases present in the cell extract.

10 | Dialyze the S30 cell extract at 4°C three times for 4 hours against a 50-fold volume of chilled S30 buffer

11 | Centrifuge the cell extract for 10 min at 4°C at 4,000 g and freeze the supernatant in aliquots in RNAse-free tubes in liquid nitrogen. Store it at -80°C. *The extract can be stored at -80°C for months to years without losing activity, it can even be frozen a second time after thawing. If the extract is thawed more than twice it may lose activity.*

12 | Mix all components to yield PremixA and incubate the premix in a water bath at 37°C to solubilize all components.

13 | Perform in parallel different *in vitro* translations of β-lactamase mRNA for 10 min. Add increasing concentrations of MgAc (10-15 mM), KGlu (180-220 mM), PEG-8000 (5-15% w/v of premix) and amount of extract (30-50 μl for a 110 μl translation reaction). *We use cysteine-free β-lactamase for the optimization of the* in vitro *translation extracts*[6; 7]*, as its concentration can easily be determined by an activity*

assay. However, any other single-domain and well folding protein, whose concentration can be assayed easily, may be used for the optimization.

14 | First optimize the MgAc concentration, then the KGlu concentration and last the PEG-8000 concentration. The translation time relevant for optimization should be around 10 min. Stop translation by diluting the reaction 5 times in WBT.

15 | To detect β-lactamase activity, use a nitrocefin assay[6]. Use 10-20 μl of the stopped translation per ml β-lactamase assay buffer and follow the reaction with a spectrophotometer at 486 nm.

16 | After determining the conditions giving the highest activity, add the chemicals at the optimal concentration to the PremixA stock yielding PremixZ, optimized for this very batch of extract.

17 | Aliquot the PremixZ in RNase-free tubes and shock freeze the samples in liquid nitrogen. *The PremixZ aliquots can be stored at -80°C for several months.*

4 References

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