

Ribosome display: selecting and evolving proteins *in vitro* that specifically bind to a target

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Ribosome display is an *in vitro* selection and evolution technology for proteins and peptides from large libraries¹. As it is performed entirely *in vitro*, there are two main advantages over other selection technologies^{2,3}. First, the diversity of the library is not limited by the transformation efficiency of bacterial cells, but only by the number of ribosomes and different mRNA molecules present in the test tube. Second, random mutations can be introduced easily after each selection round, as no library must be transformed after any diversification step. This allows facile directed evolution of binding proteins over several generations (Box 1). A prerequisite for the selection of proteins from libraries is the coupling of genotype (RNA, DNA) and phenotype (protein). In ribosome display, this link is accomplished during *in vitro* translation by stabilizing the complex consisting of the ribosome, the mRNA and the nascent, correctly folded polypeptide (Fig. 1). The DNA library coding for a particular library of binding proteins is genetically fused to a spacer sequence lacking a stop codon. This spacer sequence, when translated, is still attached to the peptidyl tRNA and occupies the ribosomal tunnel, and thus allows the protein of interest to protrude out of the ribosome and fold. The ribosomal complexes are allowed to bind to surface-immobilized target. Whereas non-bound complexes are washed away, mRNA of the complexes displaying a binding polypeptide can be recovered, and thus, the genetic information of the binding polypeptides is available for analysis. Here we describe a step-by-step procedure to perform ribosome display selection using an *Escherichia coli* S30 extract for *in vitro* translation, based on the work originally described and further refined in our laboratory¹. A protocol that makes use of eukaryotic *in vitro* translation systems for ribosome display^{4–7} is also included in this issue⁸.

MATERIALS

REAGENTS

NeutrAvidin (Pierce)	150 mM magnesium acetate, 10 mM spermidine, 200 mM DTT)
<i>Saccharomyces cerevisiae</i> RNA (Fluka)	Tris-buffered saline, TBS (50 mM Tris-HCl (pH 7.4) at 4 °C, 150 mM NaCl)
RNase inhibitor RNasin (Promega)	TBS with Tween, TBST (TBS with 0.05% (500 µl/l) Tween-20)
Reverse transcriptase (for example, Stratascript from Stratagene or ThermoScript from Invitrogen)	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)
DNA polymerase for PCR (for example, Vent polymerase from NEB or Phusion High-Fidelity DNA polymerase from Finnzymes)	Elution buffer, EB (50 mM Tris-acetate pH 7.5 at 4 °C, 150 mM NaCl, 50 mM EDTA)
dNTPs (5 mM of each dNTP; Eurogentec)	10 × MOPS (0.2 M MOPS (pH 7), 50 mM sodium acetate, 10 mM EDTA)
NTPs (50 mM; Sigma)	10 × TBE buffer (890 mM Tris-buffered saline, 890 mM boric acid, 20 mM EDTA)
T7 RNA polymerase (NEB)	QIAquick PCR purification and gel extraction kit (Qiagen)
Heparin (Fluka)	High pure RNA isolation kit (Roche)
T4 DNA ligase (MBI Fermentas)	
Shrimp alkaline phosphatase (SAP; USB)	
[³⁵ S]methionine for radioactive binding assays (PerkinElmer)	
5 × T7 RNA polymerase buffer (1 M Hepes-KOH (pH 7.6),	

For a full list of materials, see **Supplementary Methods** online.



Template generation

PROCEDURE

1| To amplify a diverse library of interest, design primers to introduce, for example, a *Nco*I site just before the start methionine and a *Hind*III site just after the coding sequence according to **Figure 2** and amplify the library by PCR.

The *Nco*I restriction site (5'-CCATGG-3') is preferentially set such that the bases ATG within the recognition sequence encode the start methionine. The primer might introduce an additional restriction site, for example, *Bam*HI in the primers used here (**Supplementary Methods**), a few bases downstream (for example, between an N-terminal tag and the selected protein) to facilitate moving the selected constructs in and out of different expression vectors. The *Hind*III restriction site between protein of interest and spacer introduces lysine and leucine residues. There should be no stop codons present in the construct.

▲ CRITICAL STEP

Construct preparation

2| Prepare the vector plasmid pRDV from 2–4 ml of bacterial culture⁹.

The template for *in vitro* transcription is best generated by ligating a PCR fragment, which codes for the library of interest, into the vector pRDV. The T7 promoter, the ribosome binding site and the spacer sequence are contributed by the vector and thus introduced into the construct via this ligation.

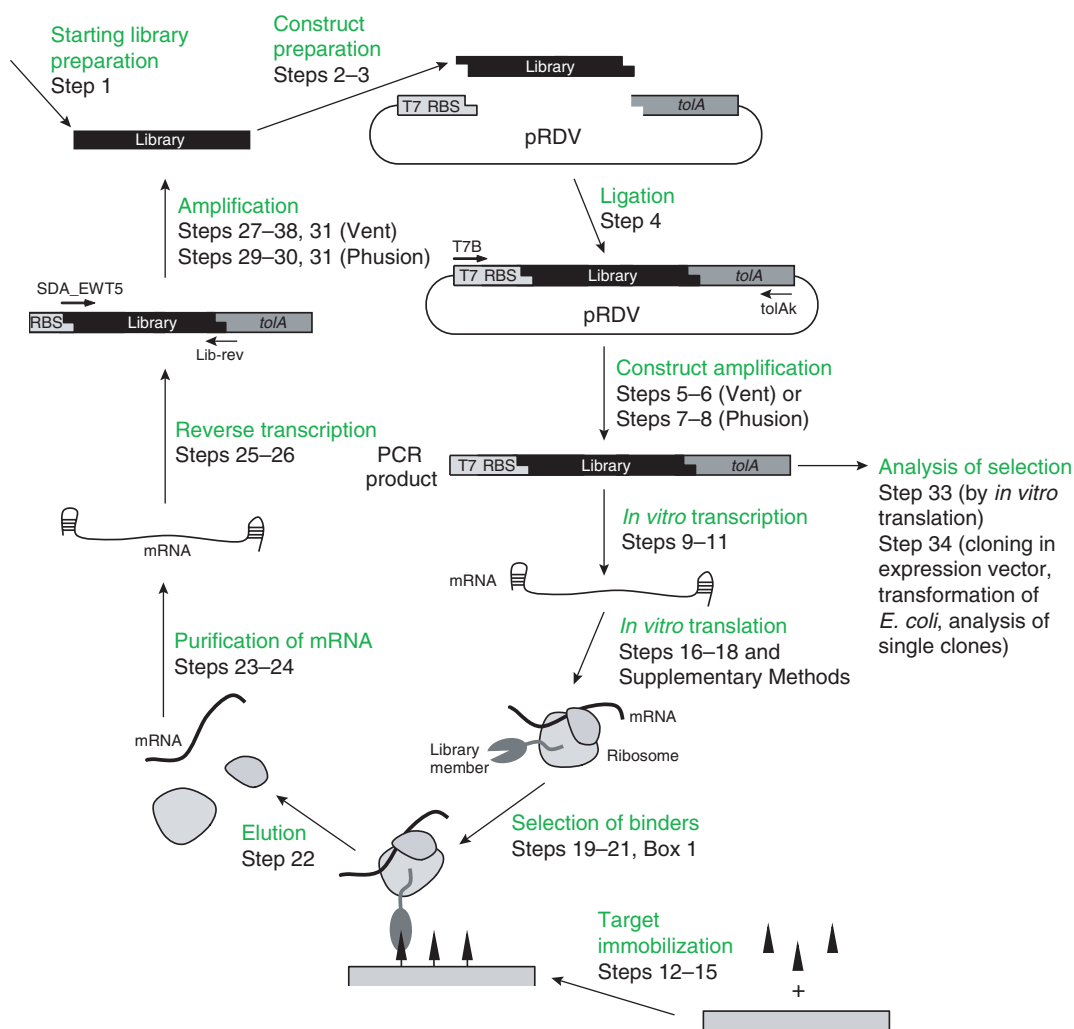


Figure 1 | Overview of the ribosome display selection. A DNA library in the form of a PCR product ('library'; see **Fig. 2** for construct details), coding for binding proteins, is ligated into the ribosome display vector pRDV, thereby genetically fusing it to a *tolA* spacer sequence in-frame, and providing a promoter and translation initiation region at the 5' end. The final ribosome display construct is obtained by PCR amplification of both flanking regions and the library insert from the ligated vector. *In vitro* transcription of this PCR product yields mRNA that is used for *in vitro* translation. The ribosome stalls at the end of the mRNA and does not release the encoded and properly folded protein because of the absence of a stop codon. The mRNA-ribosome-protein ternary complexes are used for affinity selection on an immobilized target. mRNA of bound complexes is recovered after washing, reverse transcribed and amplified by PCR. Thereby the selected pools of binders can be used directly for the next cycle of ribosome display or analysis of single clones after cloning into expression vectors.

3| Digest pRDV (Step 2) and the library generated by PCR (Step 1) with *NcoI* and *HindIII* according to the enzyme manufacturer's instructions. For vector dephosphorylation add 1 µl of SAP to the pRDV digest reaction.

4| Analyze the digested PCR product and vector on a 1.5% agarose gel and isolate the proper bands by gel extraction. Set up three ligation reactions, mixing the digested library and the digested pRDV in molar ratios of 5:1, 2:1 and 1:1, and perform the ligation according to the ligase manufacturer's instructions. Heat-inactivate the reactions by incubating for 20 min at 65 °C.

Follow Steps 5–6 to amplify the products using Vent DNA polymerase or alternatively follow Steps 7–8 when using Phusion DNA polymerase, which in some cases gives better results than amplification with Vent. Then proceed to Step 9.

5| To amplify the constructs including the promoter and the 3' spacer from the three ligation products using Vent DNA polymerase, set up the following reaction for each of the three ligation reactions set up in Step 4:

Primer T7B (100 µM)	0.5 µl
Primer tolAk (100 µM)	0.5 µl
dNTP mix (containing 5 mM each dNTP)	1 µl
Vent DNA polymerase (2 U/µl)	1 µl
10 × ThermoPol buffer	5 µl
DMSO	2.5 µl
Ligation product (Step 4)	5 µl
Water	34.5 µl

▲ CRITICAL STEP

6| Amplify the ligated libraries using hot-start PCR to minimize side reactions as follows:

Cycle number	Denaturation	Annealing	Polymerization
1	5 min at 94 °C		
2–35	30 s at 94 °C	30 s at 55 °C	60 s at 72 °C
36	5 min at 72 °C		

■ PAUSE POINT PCR products can be stored at –20 °C for at least one year.

➔ TROUBLESHOOTING

▲ CRITICAL STEP

7| To amplify the constructs from the three ligation products (Step 4) using Phusion DNA polymerase, set up three amplification reactions as follows:

Primer T7B (100 µM)	0.25 µl
Primer tolAk (100 µM)	0.25 µl
dNTP (containing 5 mM each)	2 µl
Phusion DNA polymerase (2 U/µl)	0.5 µl
5 × Phusion HF buffer	10 µl
DMSO	2.5 µl
Ligation product (Step 4)	5 µl
Water	29.5 µl

Ligation

Construct amplification



In vitro
transcription

8| Amplify the ligated libraries using hot-start PCR to minimize side reactions as follows:

Cycle number	Denaturation	Annealing and polymerization
1	1 min at 98 °C	
2–35	10 s at 98 °C	45 s at 72 °C
36	5 min at 72 °C	

■ **PAUSE POINT** PCR products can be stored at –20 °C for at least one year.

▲ **CRITICAL STEP**

9| Use the best of three PCR products obtained in Steps 6 or 8 without further purification as template for *in vitro* transcription. Place the components for the *in vitro* transcription on ice, mix as follows and incubate the reaction for 3 h at 37 °C.

5 × T7 RNA polymerase buffer	20 µl
NTP mix (50 mM each NTP)	14 µl
T7 RNA polymerase (50 U/µl)	4 µl
RNasin (40 U/µl)	2 µl
Water	37.5 µl
PCR product from Step 6 or 8	22.5 µl

A high-yield in vitro transcription results in the reaction becoming turbid. With the provided protocol, approximately 0.1 mg mRNA is obtained after 2–3 h in a 100-µl transcription reaction with 1–2 µg of PCR product (from Step 6 or 8) as a template¹⁰. We recommend using the T7 polymerase buffer as described in the reagent list, as the results with the commercial buffer were not reproducible in our hands.

10| Add 100 µl of ice-cold RNase-free water and 200 µl of 6 M LiCl, and incubate for 30 min on ice. Centrifuge for 30 min at 4 °C at 20,000 r.p.m. in a standard cooled table-top centrifuge (about 14,000g) and discard the supernatant. Wash the pellet with 70% ethanol, and dry it by leaving the lid of the tube open, or dry it briefly in a Speed Vac apparatus. Completely redissolve the pellet in 200 µl of RNase-free water and centrifuge for 5 min at 14,000g at 4 °C.

▲ **CRITICAL STEP**

11| Transfer 180 µl of this supernatant to 500 µl of 100% ice-cold ethanol, add 18 µl of 3 M sodium acetate and incubate for at least 30 min at –20 °C or overnight. Centrifuge for 30 min at 14,000g at 4 °C, and discard the supernatant. Wash the pellet with 500 µl ice-cold 70% ethanol and dry it in a Speed Vac. Thoroughly resuspend the pellet in 40 µl of RNase-free water and measure the concentration. Dilute the mRNA with RNase-free water to a final concentration of 2.5 µg/µl and freeze 4-µl aliquots in liquid nitrogen.

■ **PAUSE POINT** The mRNA is stable for several months to years at –80 °C.

➔ **TROUBLESHOOTING**

▲ **CRITICAL STEP**

Target
immobilization

12| Wash a Maxisorp microtiter plate or strip three times with TBS and dry it. Add 100 µl of a 66 nM solution of NeutrAvidin in TBS into the wells, and incubate overnight at 4 °C or for 1 h at 37 °C. *It is recommended to prepare two wells per antigen to have duplicates in selection. If the target molecule is not biotinylated it can be directly immobilized as mentioned for NeutrAvidin. Note, however, that in this case the target protein might denature at least partly during direct coating, whereas a peptide or hydrophilic target might not stick to the plate, or an epitope can become inaccessible.*

▲ **CRITICAL STEP**

13| Wash the coated plates 3× with TBS and add 300 µl of TBS containing 0.5% BSA. Incubate the plate for 1 h at room temperature (18–23 °C) on a shaker to allow the BSA to block all remaining hydrophobic surfaces of the plate. As a control surface, similarly block microtiter wells that have not been coated with the target molecules.

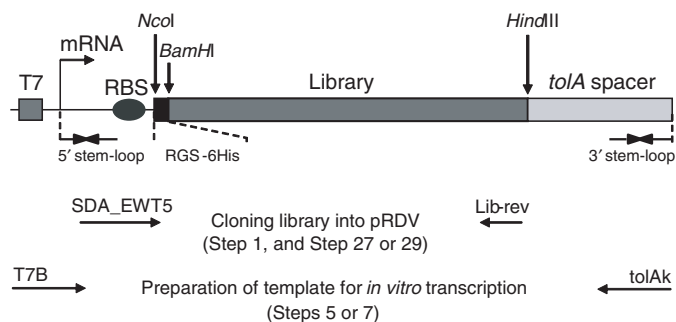


Figure 2 | The construct for ribosome display using *E. coli* ribosomes. A T7 promoter and a ribosome binding site (RBS) are necessary for *in vitro* transcription and translation. The coding sequence starts with Met-Arg-Gly-Ser-6His (the RGS-6His tag, where the initiator Met is cleaved off; or any other tag can be used), followed by the DNA library of the binding proteins and a spacer at the C terminus. The stop codon has been removed from the coding sequence. At the mRNA level, the construct is protected against RNases by 5' and 3' stem-loops. Fusion of the promoter and the spacer sequence can best be achieved by cloning into an appropriate vector providing these sequence elements and subsequent amplification by PCR. The oligonucleotides used for the cloning of the library into pRDV and for the generation of the template for *in vitro* transcription are indicated at the bottom.

14| Discard all solutions from the plate and tap the plate upside-down on a paper towel to get rid of all remaining solution. Add 200 μ l of ~250 nM biotinylated target in TBS containing 0.5% BSA and incubate the plate for at least 1 h at 4 $^{\circ}$ C. Remove all of the remaining buffer, and wash 3 \times with TBST. *The volume can probably be reduced to 100 μ l, as only 100 μ l NeutrAvidin has been coated. The concentration of the target protein may be reduced in subsequent selection rounds to 10 nM. Using low concentrations, longer incubations may be necessary before washing. The composition of the washing solution (TBS and TBST) may have to be adjusted for certain target proteins, such as membrane proteins.*

15| Wash the coated plates 3 \times with ice-cold WBT; then fill the wells with WBT and place the plate on ice or at 4 $^{\circ}$ C.

16| Carry out the *in vitro* translation with an *in vitro* translation system (made in-house or obtained from one of various providers: Promega, Roche, Amgen and others) according to the manufacturer's protocol. Then stop the translation reaction (Step 18).

*Alternatively follow the protocol for the preparation of an E. coli S30 translation extract and for the translation reaction described in **Supplementary Methods**, and then continue with Step 17. Note that several commercial translation extracts contain substantial concentrations of DTT, which preclude their use with binding proteins that are dependent on disulfides (such as antibodies), or subsequent selection on disulfide-containing immobilized targets, as such proteins may lose their conformational integrity when reduced.*

17| When using the translation mix described in **Supplementary Methods**, combine 13 μ l of ultrahigh purity water, 2 μ l of 200 μ M methionine, 41 μ l of Premix Z (thaw on ice and vortex before pipetting), 50 μ l S30 extract (add last). Add 10 μ g of dissolved mRNA (aliquot of 4 μ l from Step 11) to give 110 μ l translation mix and incubate for 10 min at 37 $^{\circ}$ C.

The duration of the translation should be optimized for each template; usually times between 5 and 10 min give optimal yields.

18| Stop the translation by transferring 100- μ l of the reaction into 400 μ l of ice-cold WBT containing 0.5% BSA and 12.5 μ l/ml heparin (from a 200 mg/ml stock solution), and place it on ice for 1–2 min. Centrifuge the translation mix for 5 min at 14,000g at 4 $^{\circ}$ C and transfer to a new ice-cold tube.

It is important to not allow the reaction to warm up during the entire selection.

19| For affinity selection, remove WBT from the ice-cold microtiter wells as in Step 15.

➔ **TROUBLESHOOTING**

20| Add aliquots of 150 μ l of the stopped translation reaction (Step 18) to two target-coated wells and to two wells lacking the target. A prepanning step on identically coated wells except lacking the target or on unrelated targets can be included to increase the specificity of the selected protein binders.

***In vitro* translation and complex formation**

Selection of binders



RNA elution

RNA purification and work-up of the selection round

21| Gently shake the microtiter plate for 1 h in the cold room. Beat the plate dry and wash five times with WBT to remove unbound complexes.

▲ CRITICAL STEP

22| To elute the mRNA, add 100 µl ice-cold EB containing 50 µg/ml *S. cerevisiae* RNA and incubate for 10 min on ice with gentle shaking, and repeat this step once (total elution volume 200 µl). Purify the eluted mRNA immediately.

23| Isolate the eluted mRNA from Step 22 using the High Pure RNA isolation kit according to the manufacturer's instructions.

▲ CRITICAL STEP

24| Elute the purified mRNA in 30 µl of RNase-free water and immediately denature at 70 °C for 10 min. Chill the mRNA samples on ice for 1–2 min after denaturation.

25| Mix the reverse transcription reactions on ice as follows. The amounts given are per eluted well (Step 20).

Reverse primer, usually Lib-rev (100 µM)	0.25 µl
dNTP (5 mM each)	0.5 µl
RNasin (40 U/µl)	0.5 µl
Stratascript reverse transcriptase (50 U/µl)	0.5 µl
10× Stratascript buffer	2 µl
DTT (100 mM)	2 µl
RNase-free water	2 µl

26| Add 12.25 µl of the eluted mRNA (Step 22) to the reverse transcription mix (7.75 µl) and incubate the mixture for 1 h at 50 °C in a PCR machine with hot lid to avoid any evaporation. Freeze the rest of the RNA in liquid nitrogen. Store the remaining mRNA as backup for the workup of the mRNA. It can be stored at –20 °C for several weeks.

BOX 1 AFFINITY MATURATION

Affinity maturation requires to introduce a step of diversity generation and to tailor the selection pressure to 'reward' higher affinity.

Introducing diversity

As ribosome display is performed entirely *in vitro* it is very convenient to introduce diversity into selected pools between different selection rounds. This can be easily done when using nonproofreading polymerases¹⁶. This effect can be further enhanced by error-prone PCR and DNase I shuffling^{29–31}. These randomization steps are best introduced after RT-PCR. The amplification of templates by PCR in the presence of dNTP-analogs such as 8-oxo-guanosine or dPTP (ref. 31) has proven to be a convenient method to introduce mutations. The mutational load can be adapted by increasing the number of amplification cycles or by increasing the concentration of dNTP analogs. The optimal mutational load depends on the library, especially its tolerance to mutations, and the final application. As many mutations will lead to the loss of activity of the mutated proteins, it can also be beneficial to recombine mutations by DNase shuffling after randomization by error-prone PCR^{29–31}.

Increasing selection pressure

Affinity is essentially determined by the ligand-receptor dissociation rate constant (off rate), whereas the association rate constants (on rates) typically fall into a relatively narrow window^{32,33}. To select for very high affinities, off-rate selection has been applied successfully^{3,23,34,35}. In off-rate selection, the ribosomal complexes formed after translation are first equilibrated with biotin-labeled antigen. After equilibration for about 1–4 h, a very large molar excess of competitive nonbiotinylated antigen is added (the higher the excess, the better). Every complex that dissociates from its biotinylated antigen will be captured by the competitive antigen and will therefore not be recovered when binding to streptavidin-coated surfaces. The selection pressure is correlated with the excess of antigen used for selection and the incubation time with competitor antigen. Ribosomal complexes were shown to survive the incubation for up to 10 d (ref. 22), and this is probably not the limit.

Follow Steps 27–28 to amplify the reverse transcription products using Vent DNA polymerase or alternatively follow Steps 29–30 when using Phusion DNA polymerase. Then proceed to Step 31.

► **TROUBLESHOOTING**

27| To amplify the reverse transcription products using Vent DNA polymerase, set up the amplification reaction as follows:

Primer SDA_EWT5 (100 μM)	0.5 μl
Reverse primer, usually Lib-rev (100 μM)	0.5 μl
dNTP (containing 5 mM each)	1 μl
Vent DNA polymerase (2 U/μl)	1 μl
10× ThermoPol buffer	5 μl
DMSO	2.5 μl
Reverse transcription reaction products	5 μl
Water	34.5 μl

▲ **CRITICAL STEP**

28| Perform hot-start PCR to minimize side reactions using the same thermocycling program as in Step 6. Adjust the number of PCR cycles to the selection round. In the first round, start with about 45 cycles and reduce by about 5 cycles per round.

Aliquots of these samples should be saved for use as controls.

■ **PAUSE POINT** PCR products can be stored at –20 °C for at least one year.

▲ **CRITICAL STEP**

29| To amplify the reverse transcription products using Phusion DNA polymerase, set up the amplification reaction as follows:

Primer SDA_EWT5 (100 μM)	0.25 μl
Reverse primer, usually Lib-rev (100 μM)	0.25 μl
dNTP (containing 5 mM each)	2 μl
Phusion DNA polymerase (2 U/μl)	0.5 μl
5× Phusion HF buffer	10 μl
DMSO	2.5 μl
Reverse transcription reaction products	5 μl
Water	29.5 μl

▲ **CRITICAL STEP**

30| Perform hot-start PCR to minimize side reactions using the same thermocycling program as in Step 8. Adjust the number of PCR cycles to the selection round as in Step 28.

Aliquots of these samples should be saved for use as controls.

■ **PAUSE POINT** PCR products can be stored at –20 °C for at least one year.

▲ **CRITICAL STEP**

31| Verify the quality of the PCR product by analysis on a 1.5% agarose gel.

The presence of a diffuse band or other side products indicates that the quality of the PCR product is not very good because the constructs are not of defined length. Purification from the gel followed by a second PCR is normally sufficient to yield high quality DNA. At this step the success of the selection can be estimated by comparing the PCR product obtained from a target-containing well with that from a non-target-containing well.

32| To carry out an additional round of affinity selection on the isolated pools, couple the PCR product from Step 31 to the promoter and the spacer again by ligating it into pRDV, repeating Steps 2–6.

In case the reverse transcription was amplified using primers that anneal to the spacer (for example, T7B and toIAk), recommended only for later rounds, these steps can be skipped and the PCR product (Step 31) can be used directly for in vitro transcription (Step 9).

Preparation of the next selection round and controls



Analysis of selection

33 | Analysis of the selected pools can be done either by *in vitro* translation (still analyzing the whole pool) or by analyzing single clones (Step 34). To analyze the pool by *in vitro* translation (independent of a subsequent PCR), translation is carried out with [³⁵S]methionine as in Step 16. The radioactive translation mix can then be tested in radioimmuno assay for binding specificity. A particularly informative test is to use the soluble target as competitor. Even though it is of course possible to reclone the pool into a vector not containing a spacer and translate this pool *in vitro*, it is more informative to analyze such a mixture after transformation of bacteria at the level of single clones.

34 | Digest and ligate the selected pools into a suitable expression vector and transform *E. coli* to obtain individual clones. Testing for binding can often be done in similar assay setups as for the selection round. In most cases, crude extract ELISAs can be carried out. The specificity of the binders might be analyzed by comparing the binding to the specific antigen with binding to unrelated antigens. Alternatively, by analyzing the degree to which the specific binding can be inhibited by adding the purified nonbiotinylated target molecule in solution as a competitor, the affinity of the analyzed binders can be estimated by measuring the level of inhibition with several inhibitor concentrations.

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Step 6 There is a low yield of DNA after amplification. (Also Steps 8, 28 and 30.)	Modify PCR conditions when using longer amplicons or when using different primer pairs.
Step 11 There is a low yield of mRNA.	The amount of DNA used for <i>in vitro</i> transcription might have been too low. Use at least 1 µg of DNA for <i>in vitro</i> transcription.
Step 19 The selection is not specific enough.	Carry out a 'prepanning step'. The 150-µl aliquots of the stopped translation reaction are added first to a microtiter well, which is identical to the target well except lacking the target (for example, containing NeutrAvidin, BSA and any fusion partners of the target if applicable). Incubation is carried out as with the target (1 h in the cold room), and the supernatant is then transferred to a well containing the target in the presence of the same molecules. If necessary, the prepanning step can also be carried out twice. It is advantageous to only carry out pre-panning only in later rounds, as in the beginning it is most essential to not lose the binding molecules, and tolerate lower selection stringency. Sometimes it can be advantageous to reduce the incubation time with the target to 30 min to reduce unspecific sticking to the surface.
Step 26 There are faint or smeary PCR bands after reverse transcription (RT)-PCR.	Add the polymerase only after having heated the PCR reaction to 94 °C (hot-start PCR). For better quality we store the DNA polymerase at -80 °C in very small aliquots (for example, 10 µl), such that for every RT PCR a new aliquot can be used.

CRITICAL STEPS

Step 1 In many cases it is useful to insert a DNA sequence just after the 5' restriction site, which is kept constant for the entire library (such as an RGS-6His tag or a Flag tag). On the DNA level, this facilitates amplification with specific primers, and on the RNA level, it results in more homogenous *in vitro* translation of the different library members because of identical starts of translation. On the protein level, this allows detection when needed.

Step 5 The quality of the PCR product is crucial for the efficiency of the subsequent *in vitro* transcription. Make sure that the PCR product used as template in Step 5 appears as a sharp band on agarose electrophoresis and corresponds to ideally 5–10 ng per 50 µl reaction (minimum 0.5–1 ng per 50 µl reaction) starting material.

Step 6 The quality of PCR products should be analyzed by gel electrophoresis, and a single clearly visible band should be obtained without any smear, indicating that the library consists of uniformly sized molecules. If necessary, either repeat the PCR step or cut out the desired band from the gel and purify it before going on to the next step. Note that this does not apply if the library contains molecules with diversity in size.

Step 8 See note for Step 6.



Step 10 It is crucial to completely dissolve the pellet, as failure to do so may reduce the yield of mRNA dramatically.

Step 11 The quality of the mRNA can be checked best by agarose gel electrophoresis. Cast an agarose gel (typically 1.5%) and add 2% (wt/vol) 1 M guanidinium thiocyanate. Denature 5 µg mRNA by incubation for 10 min at 70 °C in 15.5 µl of loading buffer (10 µl formamide, 3.5 µl 37% formaldehyde, 2 µl 5 × MOPS) and analyze it by gel electrophoresis. The band should be sharp and clearly visible.

Step 12 For a successful selection it is important that the target molecule is immobilized in a conformation relevant for the future application. The direct immobilization of target proteins on a MaxiSorp plate could lead to partial denaturation. Beside the immobilization via NeutrAvidin after biotinylation, specific antibodies may be used in some cases for the immobilization. Note, however, that in these cases the antibody is specifically blocking its epitope on the target protein for the selection of new binding proteins. Also note that binders can be generated against all molecules present on the plate and/or fused to the target. During preselection (prepanning) these molecules must be present and during binder analysis recognition of the target must be tested in comparison to recognition of any of the auxiliary molecules.

Step 21 The stringency of selection can be increased by increasing the number of washing steps and the duration of the washing steps. In later rounds, competitors constituting undesired targets can be added during incubation. For later selection rounds, the number of washing cycles and the incubation time should be increased.

Step 23 The most critical steps in ribosome display are actually those involving handling of DNA and especially RNA. As the procedure involves working with extremely small amounts of RNA right after the initial selection, the usual precautions of working with RNA must be taken very seriously: use RNase-free reagents, work with gloves only, carefully clean bench and pipettes, work with RNase-free tubes and tips; while working with RNA, keep samples on ice; consider the use of RNase inhibitors.

Step 27 We recommend using a reverse primer, which is specific for the amplification of the library module rather than the spacer (for example, primer Lib-rev from Step 1), and the forward primer SDA_EWT5 for greater specificity and higher yield. Any incomplete presence of the spacer region (because of a lack of synthesis or degradation) would not affect PCR by such 'inner' primers (**Figs. 1 and 2**). In favorable cases it is also feasible to directly use a primer that is specific for the spacer¹¹, such as toIAk, and the generic forward primer T7B, thereby directly restoring the final transcription template. This saves the ligation step into pRDV, which would otherwise be needed to reattach the spacer to the library for subsequent selection rounds. In this case, the N-terminal promoter and ribosome binding site would have to be attached by overlap PCR¹¹.

Step 28 Extension time may need to be adapted for longer amplicons. In early selection rounds, increase the number of amplification cycles to as many as 45 cycles, whereas in later selection rounds (when enrichment for specific binding has occurred), as few as 20 cycles are sufficient. Adapt the annealing temperature according to the primer used. See also note for Step 6.

Step 29 See note for Step 27.

Step 30 See note for Step 6 and for Step 28.

COMMENTS

Ribosome display has been used for the selection of peptide ligands^{4,12–14} and proteins, such as single-chain Fv antibody fragments and alternative binding scaffolds with specificity and affinity to haptens^{1,6,7,15,23}, peptides¹⁶, proteins^{17–20} and nucleic acids²¹. Additionally, ribosome display has been used for improvement of the affinity^{17–20,22,23} and the stability²³ of existing protein ligands, and for enrichment for soluble, folded proteins²⁴. Using transition-state analogs or enzyme inhibitors that bind reversibly or irreversibly to their enzyme (suicide substrates), ribosome display can also be used for the selection for enzymatic activity^{25,26}.

One of the key advantages of ribosome display is that the diversity of the library is not limited by the transformation efficiency of bacterial cells. The diversity of ribosome display libraries is only limited by the number of different protein-displaying ribosomal complexes present in the reaction volume,



and the functional diversity is given by the number of ribosomal complexes that display a functional protein. This number is limited by the number of functional ribosomes or different mRNA molecules, whichever is smaller. An extremely conservative estimate, representing a lower limit, of the number of active complexes with folded protein can be obtained from the number of mRNA molecules that could actually be isolated after one round of ribosome display of a unique antibody scFv fragment, which was determined as 2.6×10^{11} per milliliter of reaction²⁷. This number includes all mechanical losses of material upon selection and mRNA isolation, incomplete folding of the antibody fragment, or owing to complexes with incompletely translated protein chain.

It should also be noted that PCR errors in the initial preparation of the starting library in the ribosome display format increase the library diversity over that of cloned starting material, and this diversification continues in every round. Also, the absence of any cells in the amplification process between rounds prevents faster growing clones from dominating the selection, as is observed in other selection technologies such as phage display, and after three rounds of selection, still high diversity is observed among the isolated sequences^{17,20}.

The present protocol uses ligation into a ribosome display vector (pRDV)⁹, instead of overlap extension as in our earlier protocols^{1,11,28}. Although both can be used, the pRDV secures that the upstream and downstream region are always correct and not changed by PCR errors. Also, the overlap PCR method requires two subsequent PCR steps, which can create problems with some templates. Note that the pRDV ligation step does not involve transformation of the ligation product, which would otherwise decrease diversity.

The presented protocol has been optimized for *E. coli* S30 translation systems. At minimum, with eukaryotic translation systems the translation initiation region needs to be changed⁵, but it may be necessary to optimize other steps as well, as for example, described in the accompanying protocol⁸.

Note: Supplementary information is available on the Nature Methods website.

SOURCE

The protocol is based on the work of Hanes *et al.*^{1,11}, as refined by Schaffitzel *et al.*²⁸ and Amstutz *et al.*⁹ and further evolved in subsequent projects.

ACKNOWLEDGMENTS

We thank R. Skirgaila for originally suggesting and testing Phusion polymerase in ribosome display, as well as A. Batyuk, D. Ferrari, T. Huber, P. Martin Killias, P. Parizek, N. Sainz-Pastor and S.R. Wyss-Stoekle for experimentally checking the protocol in detail and for many helpful suggestions and discussions, as well as former members of the Plückthun laboratory for establishing the protocol.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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