Chapter 15

Rapid Selection of High-Affinity Binders Using Ribosome Display

Birgit Dreier and Andreas Plückthun

Abstract

Ribosome display has proven to be a powerful in vitro selection and evolution method for generating high-affinity binders from libraries of folded proteins. It has been successfully applied to single-chain Fv fragments of antibodies and alternative scaffolds, such as *D*esigned *A*nkyrin *R*epeat *P*roteins (DARPins). High-affinity binders with new target specificity can be obtained from highly diverse DARPin libraries in only a few selection rounds. In this protocol, the selection from the library and the process of affinity maturation and off-rate selection are explained in detail.

Key words: Ribosome display, In vitro selection, In vitro translation, *D*esigned *A*nkyrin *R*epeat *P*roteins, Affinity maturation

1. Introduction

Ribosome display is a potent in vitro method to select and evolve proteins or peptides from a naïve library with very high diversity to bind to any chosen target of interest (1–4). The background and mechanism are summarized and discussed in the accompanying chapter (5). We report here the most recent version of the standard protocols for selection from a complex library and affinity maturation using off-rate selection.

The protocol detailed here starts from a library of about 10^{12} DNA molecules in the form of a PCR fragment. Whether this number corresponds to the functional library size, i.e., whether all these 10^{12} molecules are different and at least potentially functional, depends both on the *design quality* of the input library template and the *amount* of the input template used from which this PCR fragment is generated.

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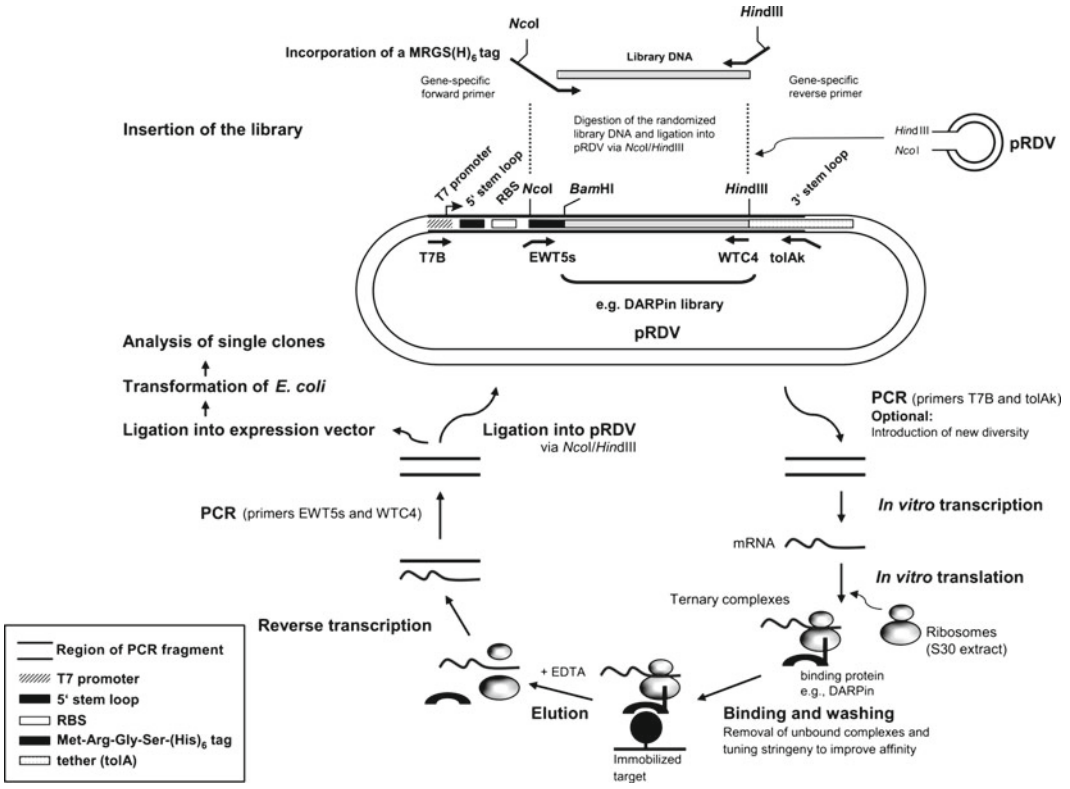

Fig. 1. Scheme of the ribosome display cycle, illustrated for selection of high-affinity DARPins. In ribosome display, all steps of the selection are performed in vitro. The cycle begins with a DNA library (*top*) in the form of a PCR fragment encoding a library of the protein of interest. This cassette is ligated into a vector in vitro which provides a promoter and ribosome-binding site (RBS). The ORF of interest (*light grey*) is fused to an additional protein region (the "*spacer*" or "*tether*," *checkered white*). This *tether or spacer*, here used as an unstructured region from the *E. coli* TolA protein, has the sole function of allowing the protein domain of interest to emerge from the ribosomal tunnel. A PCR is then carried out from the promoter to the middle of the *tether*. Importantly, the PCR fragment does not encode a stop codon at the end. Each member of the library pool is then transcribed from double-stranded DNA into mRNA and is subsequently translated by the ribosomes present in the S30 extract, leading to ternary complexes consisting of ribosomes, mRNA, and the DARPin encoded by that particular mRNA. Since there is no stop codon on the mRNA, the protein is not released from the ribosome. It is believed to be still covalently attached to the tRNA within the ribosome, with the tether in the tunnel, and the domain of interest outside and already folded. Selection can be achieved by binding the protein–ribosome–mRNA complexes to the desired immobilized target, followed by removal of unbound or nonspecifically bound protein by stringent washing. Affinity can be increased by addition of an excess of nonlabeled target (off-rate selection) (see Subheading 3.12.2). Particular selectivity in binding can be achieved by adding an unwanted target as a competitor. Selection for other properties, such as stability, requires other selection pressures at this step (see Note 25). Binders can be easily recovered by destruction of the protein–ribosome–mRNA complex using EDTA and recovery of the genetic information of the binders by RT-PCR using the inner primers WTC4 (annealing to the sequence encoding the C-terminus of the DARPin sequence, which can be replaced by a primer specific for other library folds) and EWT5s (pRDV-specific primer overlapping with the RBS and beginning of the Met-Arg-Gly-Ser-$(His)_6$ tag). The inner primer set is used to amplify the selected clones, which often is not possible with the outer primer set due to incomplete synthesis or degradation of the mRNA. For further selection rounds, the PCR product pool is subcloned into pRDV via the restriction endonucleases, *Nco*I and *Hin*dIII, followed by a second PCR with the outer primers, T7B and tolAk. T7B introduces the T7 promoter sequence and part of the stabilizing 5′ stem loop sequences that are part of the pRDV vector. The tolAk primer binds in the sequence of the tolA spacer region and introduces a stabilizing 3′ stem loop. If further diversity is required, an error-prone PCR can be included at this step. The amplified PCR product then serves as template for in vitro transcription, initiating the next round of selection. At the end of the selection rounds (typically, 2–5), the resulting PCR product pool can be directly subcloned via the restriction endonucleases, *Bam*HI and *Hin*dIII, into an expression vector in order to screen for binders.

A key feature of ribosome display, in contradistinction to most other selection technologies (5), is that it incorporates PCR into the procedure and thus allows a convenient incorporation of a diversification ("randomization") step. Thereby, ribosome display allows refinement and affinity maturation not only of preexisting binders (6–9), but also of the whole pool during selection from a complex library, if desired. This is one of the major advantages of ribosome display over other selection strategies. The diversity of the library members can be easily manipulated at any selection step by introduction of additional mutations, e.g., by using DNA shuffling (10) and/or error-prone PCR (11). This additional randomization step can readily be integrated in the protocol (see Subheading 3.12.1). In combination with off-rate selection (see Subheading 3.12.2) where binders to the biotinylated target protein are competed with a molar excess of nonbiotinylated target protein, many initial leads were improved for affinity in the range of low nM to low pM (6–9, 12). The theoretical considerations for designing efficient off-rate selection experiments were recently summarized (13).

We have previously applied the ribosome display protocol to antibody scFv fragments as libraries of folded proteins (4, 14, 15). Here, we give a protocol for the in vitro selection of protein scaffolds with more favorable biophysical properties than antibody fragments. The general workflow is outlined in Fig. 1 (a similar protocol had been published previously (16)). One of the most promising scaffolds are the *D*esigned *A*nkyrin *R*epeat *P*roteins (DARPins) which are devoid of disulfide bonds, highly soluble, and highly stable, and therefore achieve high expression levels in *E. coli* (17). They also fold well in the in vitro translation inherent in ribosome display and are thus readily enriched for binding specificity. Using ribosome display, DARPins have been evolved to bind various targets with affinities all the way down to the picomolar range (8, 9, 18–23).

2. Materials

2.1. General

1. 96-well Maxisorp plates or strips (Nunc).
2. Adhesive plate sealers (Thermo Scientific).
3. Sterile, RNase-free ART filter tips (Molecular Bio Products).
4. Sterile, RNase-free HydroLogix 1.5- and 2.0-mL tubes (Molecular Bio Products).
5. Roche High pure RNA isolation kit (Roche).
6. illustra MicroSpin™ G-50 Columns (GE Healthcare).
7. NucleoSpin® Extract II DNA purification kit (Macherey-Nagel).

2.2. Selection (see Note 1)

1. Tris-buffered saline (TBS): 50 mM Tris, 150 mM NaCl; adjust pH to 7.5 with HCl at 4°C; filter through 0.22-μm pores.
2. TBST: TBS containing 0.05% Tween-20.
3. Stock solutions for wash buffer (WB) and elution buffer (EB):
   (a) 2 M Tris–acetate; adjust pH to 7.5 at 4°C with acetic acid.
   (b) 5 M NaCl.
   (c) 2 M magnesium acetate.
   (d) 250 mM EDTA; adjust pH to 8.0 by NaOH addition.

   Sterile filter all solutions. For alternative buffer composition, see Note 2.
4. WB/Tween-20 (WBT):
   50 mM Tris–acetate, pH 7.5 at 4°C, 150 mM NaCl, 50 mM magnesium acetate, 0.05% Tween-20; adjust pH to 7.5 with acetic acid at 4°C; filter through 0.22-μm pores.
5. EB: 50 mM Tris–acetate, pH 7.5 at 4°C, 150 mM NaCl, 25 mM EDTA; adjust pH to 7.5 with acetic acid at 4°C; filter through 0.22-μm pores.
6. *Saccharomyces cerevisiae* RNA (Fluka): Dissolve to 25 μg/μL in H_2O; aliquot and store at −20°C.
7. 10% BSA in H_2O; filter through 0.22-μm pores and store at −20°C.
8. Neutravidin and/or streptavidin (Pierce):
   1.2 mg/mL (20 μM) in TBS; store at −20°C.
9. Streptavidin-coated MyOne T1 magnetic beads (Invitrogen).
10. Reagents for biotinylation of the target: Either for chemical biotinylation use a NHS-biotin reagent (e.g., from Pierce EZ-link™ SulfoNHS-LC-biotin) or for enzymatic biotinylation use an AviTag together with the *E. coli* biotinylation enzyme BirA (24) (reagents from Avidity).

2.3. Cleanup of mRNA After In Vitro Transcription

1. 6 M LiCl; filter through 0.22-μm pores.
2. 3 M sodium acetate; filter through 0.22-μm pores.
3. 70% EtOH diluted with H_2O and 100% EtOH; filter through 0.22-μm pores.
4. illustra MicroSpin™ G-50 Columns (GE Healthcare).
5. DNAseI (10 U/μL; Roche).

2.4. Reverse Transcription, PCR, and Cloning

1. Primer dissolved to 100 μM in H_2O; aliquot and store at −20°C.

   EWT5s: 5′-TTCCTCCATGGGTATGAGAGGATCG-3′.

   WTC4: 5′-TTTGGGAAGCTTTTGCAGGATTTCAGC-3′.

T7B: 5′-ATACGAAATTAATACGACTCACTATAGGGAGA CCACAACGG-3′.

tolAk: 5′-CCGCACACCAGTAAGGTGTGCGGTTTCAGTT GCCGCTTTCTTTCT-3′.

2. AffinityScript™ Multiple Temperature Reverse Transcriptase (50 U/μL; Stratagene) and 10× buffer; see Note 3.
3. 100 mM DTT in H2O; aliquot and store at –20°C.
4. RNasin® Ribonuclease Inhibitor (20–40 U/μL; Promega).
5. $Vent_R$® DNA Polymerase (2 U/μL; New England Biolabs) and 10× Thermopol buffer; see Note 4.
6. Platinum® Taq DNA Polymerase (5 U/μL; Invitrogen) and 10× polymerase buffer.
7. dNTPs: 5 mM each (Eurogentec); aliquot and store at –20°C.
8. Nucleotide analogs dPTP and 8-oxo-dGTP (Jena Biosciences) at 100 μM in H_2O.
9. Dimethyl sulfoxide (DMSO; Fluka, 41640).
10. Restriction endonucleases: *Bam*HI (20 U/μL), *Hin*dIII (20 U/μL), *Nco*I (10 U/μL), and 10× buffer all from New England Biolabs.
11. T4 DNA ligase (5 U/μL; Fermentas) and 10× ligase buffer.
12. Ribosome display vector pRDV (GenBank accession code AY327136; please note the revised sequence) (18).

2.5. In Vitro Transcription

1. T7 RNA polymerase (20 U/μL; Fermentas), see Note 5.
2. RNasin® Ribonuclease Inhibitor (20–40 U/μL; Promega).
3. 100 mM DTT in H_2O; aliquot and store at –20°C.
4. T7 RNA polymerase buffer (5×): 1 M HEPES, 150 mM magnesium acetate, 10 mM spermidine, 200 mM DTT; adjust pH to 7.6 with KOH; aliquot and store at –20°C.
5. 50 mM NTP mix: 50 mM adenosine 5′-triphosphate (ATP; Sigma–Aldrich), 50 mM uridine 5′-triphosphate (UTP; Sigma–Aldrich), 50 mM guanosine 5′-triphosphate (GTP; Sigma–Aldrich), 50 mM cytidine 5′-triphosphate (CTP; Sigma–Aldrich) in H_2O; aliquot and store at –20°C.

2.6. In Vitro Translation

1. Protein disulfide isomerase (PDI; Sigma–Aldrich): 22 μM in H_2O; aliquot and store at –80°C.
2. Heparin (Sigma–Aldrich): 200 mg/mL heparin in H_2O; do not filter; aliquot and store at –20°C.
3. L-Methionine (Sigma–Aldrich): 200 mM L-methionine in H_2O; do not filter; aliquot and store at –20°C.
4. STOP mix: 1 mL WBT buffer/0.5% BSA plus 12.5 μL heparin.

2.7. S30 Extract

1. *E. coli* strain MRE600 (25) lacking ribonuclease I activity.
2. Incomplete rich medium: 5.6 g KH_2PO_4, 28.9 g K_2HPO_4, 10 g yeast extract, 15 mg thiamine for 1 L medium. Autoclave and add 50 mL 40% glucose (w/v) and 10 mL 0.1 M magnesium acetate, both sterile filtered.
3. S30 buffer: 10 mM Tris–acetate (pH 7.5 at 4°C), 14 mM magnesium acetate, 60 mM potassium acetate. Chill to 4°C before use.
4. Preincubation mix (must be prepared directly before use): 3.75 mL 2 mM Tris–acetate (pH 7.5 at 4°C), 71 µL 3 M magnesium acetate, 75 µL amino acid mix (10 mM of each of the 20 amino acids; Fluka), 300 µL 0.2 M ATP, 50 units pyruvate kinase (Fluka), 0.2 g phosphoenolpyruvate trisodium salt (Fluka); add to 10 mL H_2O.
5. Dialysis tubing with a MW cutoff of 6,000–8,000 Da (Spectrum Laboratories).

2.8. PremixZ

1. Set up premixA (the final concentration is fivefold lower in the final volume of the in vitro translation reaction, see Subheading 3.4): 250 mM Tris–acetate (from a 2 M stock solution, pH 7.5 at 4°C), 18 µM anti-ssrA oligonucleotide (5′-TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCGACTA-3′) from a 200 µM stock solution, 1.75 mM of each amino acid except for methionine, 10 mM ATP from a 1 M stock solution, 2.5 mM GTP from a 0.2 M stock solution, 5 mM cAMP (Sigma–Aldrich) from a 0.4 M stock solution, 150 mM acetyl phosphate (Sigma–Aldrich) from a 2 M stock solution, 2.5 mg/mL *E. coli* tRNA from strain MRE600 (Roche) from a 25 mg/mL stock solution, 0.1 mg/mL folinic acid (Sigma–Aldrich from a 10 mg/mL stock solution).
2. Set up an in vitro translation reaction (see Subheading 3.4), and use the above premixA, but titrate the optimal concentration of the following components for the final premixZ composition to achieve optimal performance of each newly generated S30 extract. Optimize the final concentrations for the in vitro translation in the order shown below.

   Magnesium acetate (MgAc) usually in the range of 7–15 mM from a 0.2 M stock solution, potassium glutamate (KGlu) usually in the range of 180–220 mM from a 2 M stock solution, and PEG-8000 usually in the range of 2–15% (w/v) from a 40% stock solution. Adjust the premixA with the optimal composition of MgAc, KGlu, and PEG to obtain the premixZ (we are usually using concentrations of 21.4 mM MgAc, 481 mM KGlu, and 7% PEG-8000 in the premixZ). Aliquot the premixZ and flash freeze in liquid nitrogen. Long-time storage should be at −80°C, but the premixZ is stable for several months at −20°C and can be frozen several times. If not noted otherwise, reagents are purchased from Sigma–Aldrich.

2.9. β-Lactamase Assay

Used to test the activity of the S30 extract and to optimize the premixZ.

1. Prepare β-lactamase mRNA from the pRDV template DNA encoding the double Cys→Ala mutant of β-lactamase (26) using PCR with the T7B and tolAk primers (Fig. 1) (see Subheading 3.2), followed by in vitro transcription and purification of mRNA (see Subheadings 3.2 and 3.3).
2. Set up in vitro translation reactions containing 2 μg RNA, 0.5 μL 200 mM methionine, 10 μL S30 extract, and 8.2 μL premixZ and add to 22 μL H_2O. For optimization of the activity of the S30 extract, use premixA and adjust the concentration of magnesium acetate, potassium acetate, and PEG-8000.
3. Incubate at 37°C for 10 min.
4. Add 88 μL STOP mix.
5. Use 5 μL of stopped in vitro translation for the activity assay with the chromogenic substrate nitrocefin (Glaxo Research, obtained from Oxoid Ltd.) (27).
6. Dilute nitrocefin 1:20 in β-lactamase buffer (100 mM sodium phosphate buffer, pH 7.0) from a stock solution (1 mg nitrocefin dissolved in 500 μL DMSO and stored at −20°C). For one reaction, use 20 μL diluted nitrocefin together with 5 μL translation plus 175 μL β-lactamase buffer in a 200 μL reaction.
7. Measure OD_{486nm} immediately. Follow the kinetics for approximately 12 min measuring at least once every minute.

2.10. DARPin Expression and Binding Analysis of Single Clones

1. *E. coli* strain XL-1 blue (Stratagene).
2. Expression plasmid pDST67 (22, 28), which is a derivative of pQE30 (QIAGEN).
3. 2×TY media: 5 g NaCl, 16 g tryptone, 10 g yeast extract per liter. Adjust pH to 7.2 with NaOH.
4. 96-well deep well plates (ABgene).
5. TBS: 50 mM Tris, 150 mM NaCl; adjust pH to 7.4 with HCl.
6. TBST: TBS containing 0.05% Tween-20.
7. 10% BSA in H_2O.
8. Mouse-anti-RGS(His)$_4$ antibody (QIAGEN).
9. Goat-anti-mouse IgG coupled to alkaline phosphatase (Sigma–Aldrich).
10. pNPP substrate (*p*-nitrophenyl phosphate disodium salt; Fluka): 1 M stock in pNPP buffer (50 mM $NaHCO_3$, 50 mM $MgCl_2$); aliquot and store at −20°C.
11. B-PER II detergent solution (Pierce).

3. Methods

3.1. Insertion of the Library

The ribosome display vector pRDV is used to ligate the library of interest using gene-specific primers and insertion via the restriction endonuclease sites *Bam*HI and *Hin*dIII as indicated in Fig. 1 (18, 29) or *Nco*I and *Hin*dIII. The general elements that need to be present on a template used for ribosome display are the T7 RNA polymerase promoter sequence to initiate efficient transcription and a ribosome binding site (RBS) for docking of the ribosome to initiate translation. The PCR fragment (between the primers T7B and tolAk, Fig. 1) that serves as the template for transcription ends without a stop codon in the ORF. At both the 5′ and 3′ ends of the mRNA, stabilizing stem loops are incorporated to protect the mRNA from exonuclease degradation (1, 30). The absence of a stop codon in the resulting mRNA prevents termination of translation. The fact that the library is fused in frame to a spacer (or tether) sequence (e.g., derived from the *E. coli tolA* gene) allows the nascent protein chain to exit the ribosome and fold outside of the ribosomal tunnel. The original pRDV contains an N-terminal FLAG tag instead of an N-terminal MRGS$(His)_6$ tag as shown here for the case of the DARPin libraries (18). Both tag variants lead to efficient initiation of in vitro translation and yield ternary complexes in good yields.

3.2. Transcription of PCR Products

1. To obtain a length-defined fragment of DNA as template for in vitro transcription, use the outer primers T7B and tolAk in the following PCR reaction to introduce the T7 RNA polymerase promoter sequence, RBS, the stabilizing 5′ and 3′ stem loops and the tolA spacer sequence:

5.0 µL	10× Thermopol buffer
2.0 µL	dNTPs (final concentration 200 µM each)
2.0 µL	DMSO (final concentration 5%)
0.5 µL	T7B primer (final concentration 1 µM)
0.5 µL	tolAk primer (final concentration 1 µM)
5.0 µL	Library DNA [either of the initial library or of the amplified DNA after selection which has been ligated to pRDV (see Note 6)]
0.5 µL	Vent DNA polymerase (2 U/µL)
Add to 50 µL with H_2O	

2. Perform a hot start to increase specificity and use the following cycling parameters (see Note 7): 3 min at 95°C, 25 cycles: 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, final extension 5 min at 72°C.

3. Verify the product on an agarose gel.
4. For in vitro transcription, set up the following reaction on ice:

20.0 µL	5x T7 polymerase buffer
14.0 µL	NTPs (final concentration 7 mM each)
4.0 µL	T7 RNA polymerase (20 U/µL)
2.0 µL	RNasin (40 U/µL)
22.5 µL	PCR product without further purification
Add to 100 µL with H_2O	

5. Incubate the transcription for 2–3 h at 37°C (see Note 8).

3.3. Cleanup of Template mRNA for In Vitro Translation

1. In order to remove all impurities from the reaction, the RNA needs to be purified. This can be performed in two ways (see Note 9).

3.3.1. LiCl precipitation

1. A LiCl precipitation can be performed to purify the RNA product. For this purpose, add 100 µL ice-cold H_2O and 200 µL ice-cold 6 M LiCl to the 100 µL translation reaction and vortex.
2. Incubate on ice for 30 min, and then centrifuge at 20,000 × *g* at 4°C for 30 min.
3. Discard the supernatant and wash the pellet with 500 µL ice-cold 70% EtOH ensuring that the pellet is not disturbed.
4. Remove supernatant and dry pellet in a Speedvac apparatus.
5. Completely dissolve the pellet in 200 µL ice-cold H_2O and centrifuge at 20,000 × *g* at 4°C for 5 min to remove remaining precipitates.
6. Transfer 180 µL supernatant to a new tube without disturbing the pellet. Add 20 µL 3 M NaOAc and 500 µL ice-cold 100% EtOH, vortex.
7. Incubate at –20°C for at least 30 min. Vortex and centrifuge at 20,000 × *g* at 4°C for 30 min and discard supernatant.
8. Wash the pellet with 500 µL ice-cold 70% EtOH, dry the pellet in a Speedvac apparatus, and resuspend pellet in 30 µL H_2O.

3.3.2. Gel filtration

1. For purification of the RNA, small gel filtration columns (e.g., illustra MicroSpin™ G-50 Columns) can be used.
2. Vortex the column to resuspend the material and break off the bottom of the column.

3. Place the column into a 1.5-mL tube and centrifuge at 735 × *g* for 1 min to pack the column material.
4. Place the column into a collection tube, apply 50 µL sample from the transcription reaction, and centrifuge at 735 × *g* for 1 min.
5. Optional: DNAse I treatment before loading the column (see Note 10): Take 43 µL of the transcription reaction and add 2 µL of DNAse I solution (10 U/µL) plus 5 µL 10× dilution buffer supplied with the enzyme. Incubate for 10–15 min at room temperature, and then apply the sample to the column.
6. Aliquot RNA and immediately flash freeze in liquid nitrogen. Store at –80°C.
7. Determine the RNA concentration of a 1:100 dilution by OD_{260nm}. If the transcription worked well, a yield of 3–8 µg/µL for RNA after LiCl/EtOH precipitation (total yield from a 100 µL reaction: 90–240 µg) or 1–3 µg/µL from the illustra MicroSpin™ G-50 Columns (total yield from a 50 µL reaction: 50–150 µg) should be obtained.

3.4. In Vitro Translation

1. For one in vitro translation reaction, set up the following mix on ice:

2.0 µL	200 mM methionine
41 µL	premixZ with optimized composition
x µL	In vitro-transcribed RNA (total 10 µg; see Note 11)
50 µL	S30 extract and add to 110 µL with H_2O (for preparation of the S30 extract, see Subheading 3.11)
Add 0.625 µL PDI if your library scaffold requires the formation of disulfide bonds	

2. Mix carefully by pipetting up and down and incubate the reaction at 37°C for 10 min, the time found optimal for DARPins. The incubation time and temperature must be optimized for each library based on different constructs.
3. Stop the reaction by addition of 440 µL ice-cold STOP mix.
4. Mix by pipetting up and down and centrifuge at 20,000 × *g* at 4°C for 5 min. Transfer 500 µL supernatant to a fresh tube and use 100 µL per well when performing selection in plates or 250 µL per tube when performing selections in solution for either the target-containing or control reaction (see Subheading 3.5).

3.5. Selection (see Note 12)

3.5.1. Target Protein Preparation

Express and purify the target by methods of your choice, but the target for selection must be of excellent purity and homogeneity. To immobilize the target for capturing the ternary complexes, it is recommended to biotinylate the target. This is the method of immobilization found to be most robust by far to stringent washing, including washing with detergents. The advantage of immobilizing biotinylated targets is that it is very general, and works equally well for proteins, peptides, oligonucleotides, and small molecules. Furthermore, by avoiding any direct binding to plastic surfaces, the structure of the target is maintained. Finally, the nonbiotinylated version of the target is a convenient competitor in off-rate selections and in the specificity screening of single clones. Biotinylation can be achieved in two ways (see Note 13).

1. Fuse the target to an AviTag and biotinylate it in vivo or in vitro using the *E. coli* biotinylation enzyme BirA (24) following the guidelines posted on the Avidity Web page (http://www.avidity.com).
2. Alternatively, biotinylate surface lysine amino acid residues using NHS-biotin reagents from Pierce following the manufacturer's instructions.

3.5.2. Selection in Plates

1. Coat wells of a 96 well Maxisorp plate with 100 µL of a 66 nM neutravidin or streptavidin solution in TBS and close with an adhesive plate sealer (see Notes 14 and 15). Store overnight at 4°C or for 1 h at room temperature. Invert plate and shake out solution, dry on paper towels, and wash wells three times with 300 µL TBS.
2. Block the wells with 300 µL 0.5% BSA in TBST per well, and seal and incubate on an orbital shaker for 1 h at room temperature. Shake out blocking solution and dry on paper towels.
3. Immobilize 100 µL biotinylated target at a concentration of 100–200 nM (see Note 16) in TBST/0.5% BSA and TBST/0.5% BSA only for control wells. Seal and incubate on an orbital shaker at 4°C for 1 h. Wash plate three times with 300 µL ice-cold TBST and once with 300 µL ice-cold WBT. Remove WBT only when the stopped translation reaction can be added to the wells (see Subheading 3.4).
4. Add the stopped in vitro translation, seal the plate, and incubate the binding reaction at 4°C for 1 h. Wash the wells with 300 µL ice-cold WBT containing 0.1% BSA for eight to ten times. Use two fast washes removing the buffer immediately, followed by incubations starting at 5 min and extending to 15 min in later rounds. In these longer incubations, binders with fast off-rates dissociate and subsequently are washed away.
5. For elution of the RNA, add 100 µL EB containing EDTA to release the mRNA from the captured protein–mRNA–ribosome complexes and freshly add *S. cerevisiae* RNA (final concentration

50 μg/mL) to block the surface of the tubes and perhaps to act as competing substrate for any residual RNases. Incubate at 4°C for 10 min and add to 400 μL lysis buffer of the High Pure RNA purification kit on ice. Repeat the elution step and collect the second elution in the same tube. After vortexing, the RNA is stable and can be processed at room temperature until elution from the column (see Subheading 3.6).

3.5.3. Selection in Solution

1. Starting from the stopped and centrifuged in vitro translation reaction (see Subheading 3.4), divide the reaction into two aliquots of 250 μL and add 250 μL of STOP mix. Add each of the 500 μL of the diluted stopped translation reactions to 40 μL of streptavidin-coated magnetic beads that were washed two times with 500 μL TBS and blocked with 500 μL TBST/0.5% BSA for 1 h in a 2-mL tube as preselection step (see Note 15). Rotate head over end at 4°C for 1 h.
2. Transfer the supernatant to a blocked 2-mL tube and add to 100–200 nM of biotinylated target (omit target in the control reaction) and incubate rotating at 4°C for 1 h (see Note 17).
3. Transfer the supernatant to a blocked tube containing 40 μL of streptavidin-coated magnetic beads and capture the ternary complexes rotating at 4°C for 30 min. Wash with 500 μL ice-cold WBT containing 0.1% BSA as indicated above (see Subheading 3.5.2, step 4). Separate captured complexes using a magnetic separator between each washing step.
4. Proceed with the elution and purification of RNA as described for the selection on plates (see Subheading 3.5.2, step 5).

3.6. Recovery of Eluted RNA

1. Apply the lysis buffer/eluate mixture from Subheading 3.5.2, step 5, on the column of the High Pure RNA isolation kit (see Note 18; *Optional*: As a positive control, also purify 2 μL of the input RNA from the in vitro transcription diluted in 200 μL EB) and centrifuge at 8,000 × *g* for 1 min. Discard the flow-through.
2. Add 100 μL diluted DNAse I solution (1.8 U/μL) directly onto the column filter and incubate at room temperature for 15 min (see Note 19). Add 500 μL wash buffer 1 and centrifuge at 8,000 × *g* for 1 min. Discard flow-through.
3. Wash with 500 μL wash buffer 2, centrifuge, and discard flow-through.
4. Add 100 μL wash buffer 2 and centrifuge at 13,000 × *g* for 2 min to remove any residual EtOH.
5. Elute with 50 μL elution buffer and incubate for 2 min before centrifugation at 8,000 × *g* for 1 min into a fresh 1.5-mL RNAse-free tube.
6. Freeze the remaining sample of eluted RNA in liquid nitrogen and store at −80°C (see Notes 11 and 20).

3.7. Reverse Transcription of DARPin-Encoding mRNA

1. Transfer two times 12.5 µL of eluted RNA to fresh 1.5-mL tubes (see Note 21).
2. Denature the eluted RNA at 70°C for 10 min and chill on ice.
3. Set up the following RT mix (total of 7.75 µL) per RT reaction on ice:

0.25 µL	WTC4 primer (final concentration 1.25 µM)
0.5 µL	dNTPs (final concentration 125 µM of each nucleotide)
0.5 µL	RNasin (40 U/µL)
0.5 µL	AffinityScript™ Multiple Temperature Reverse Transcriptase (50 U/µL)
2.0 µL	10× AffinityScript buffer
2.0 µL	DTT (final concentration 10 mM)
2.0 µL	H_2O

4. Distribute 7.75 µL RT mix per RT reaction to the 12.25 µL samples of denatured RNA.
5. Incubate at 50°C for 1 h.
6. Use 2–5 µL as template for PCR using the inner primers, WTC4 and EWT5s (see Subheading 3.8).
7. Freeze the rest of the cDNA in liquid nitrogen and store at −20°C.

3.8. Amplification of cDNA Coding for DARPins

The standard protocol for Vent DNA polymerase (NEB) is shown below. If another DNA polymerase or primers are used, the reaction conditions might have to be adapted.

1. Set up the following reaction mix per sample:

2–5 µL	cDNA from Subheading 3.7, step 6
5.0 µL	10× Thermopol buffer (NEB)
2.0 µL	dNTPs (final concentration 200 µM of each nucleotide)
2.5 µL	DMSO (final concentration 5%)
0.5 µL	WTC4 primer (final concentration 1 µM)
0.5 µL	EWT5s primer (final concentration 1 µM)
0.5 µL	Vent DNA Polymerase (2 U/µL)
Add to 50 µL with H_2O (see Note 22)	

2. Perform a hot-start PCR to reduce unspecific amplification. Use the following cycling parameters: 3 min at 95°C, 25 cycles:

30 s at 95°C, 30 s at 55°C, 45 s at 72°C, final extension 5 min at 72°C (see Note 23).

3. Verify the product on an agarose gel (see Note 24).

3.9. Incorporation of Promoter Elements, RBS, tolA Spacer, and RNA-Stabilizing Stem Loops

1. Purify PCR products from Subheading 3.8, step 3, either by excision of the according bands from the agarose gel and subsequent purification or in later rounds, when only one single band is observed, by direct purification over commercially available columns, e. g., of the NucleoSpin extract II kit. Elute in a small volume of 20 µL.
2. Digest ≥150 ng of the PCR product with the corresponding restriction enzymes, e.g., *Nco*I and *Hin*dIII for DARPin selections, in a final volume of 30 µL at 37°C for 2 h (see Note 25).
3. Purify digested PCR product using the NucleoSpin® Extract II DNA purification kit. Elute in 15 µL elution buffer supplied with the kit.
4. Ligate the PCR fragments into the ligation-ready pRDV plasmid using 100 ng of digested pRDV and the digested PCR product with a molar ratio of vector to insert of 1:5–7 in a final volume of 10 µL. Add 1 U of T4 DNA ligase and 1 µL ligase buffer. Incubate for 30–60 min at room temperature. Use this ligation as PCR template with the T7B and tolAk primers (see Subheading 3.2) or perform an error-prone PCR to increase diversity (see Subheading 3.12.1).

3.10. Initial Analysis of Selected Individual Library Members in a 96-Well Format (28)

1. After RT-PCR (see Subheadings 3.7 and 3.8), prepare the DARPin pool after enrichment has been observed for subcloning into a prokaryotic expression plasmid using the endonucleases *Bam*HI and *Hin*dIII. Enrichment is indicated by a much stronger PCR band recovered from a well with immobilized target than from a control well without immobilized target.
2. Ligate the PCR fragment into pDST67 (22, 28) as fusion with the sequence coding for an N-terminal MRGS$(H)_6$ tag for purification.
3. After transformation of *E. coli* XL1-Blue, pick single clones and inoculate in deep 96-well plates in 1 mL 2×TY/1% glucose/amp (100 µg/mL) and grow overnight at 37°C while shaking at 540 rpm on an orbital shaker.
4. Transfer 100 µL of each culture to 900 µL fresh media and grow for 1 h at 37°C.
5. Induce with 0.5 mM IPTG (add 100 µL media containing 5.5 mM IPTG) and grow for an additional 3–5 h at 37°C.
6. Harvest cells by centrifugation at 400×*g* for 10 min, and discard supernatant.
7. Resuspend pellet in 50 µL B-PER II detergent and lyse cells for 15–30 min on an orbital shaker.

8. Add 1 mL TBST/0.1% BSA and centrifuge to remove debris.
9. Use 10 to 100 µL of the crude extract for ELISA (see step 13). If high-affinity binders are expected, e.g. after affinity maturation, using 10 µL of a 1:100 predilution of the extract with TBST/0.1% BSA can give you a better indication of the affinity of the binders.
10. For ELISA, coat wells with 100 µL of 66 nM neutravidin in TBS for 1 h at room temperature or overnight at 4°C. Wash two times with 300 µL TBS. Dry plate on paper towels after each step.
11. Block with 300 µL TBST/0.5% BSA for 1 h at room temperature.
12. Invert plate and shake out liquid and immediately add 100 µL of the biotinylated target (10–100 nM) in TBST/0.1% BSA. Incubate for 1 h at 4°C or room temperature on an orbital shaker (see Note 26). Wash three times with 300 µL TBST.
13. Add 100 µL DARPin extract from step 9. Incubate for 1 h at 4°C or room temperature on an orbital shaker. Wash three times with 300 µL TBST.
14. Add 100 µL mouse-anti-RGS(His)$_4$ antibody in a 1:5000 dilution. Incubate for 1 h at 4°C or room temperature on an orbital shaker. Wash three times with 300 µL TBST.
15. Add 100 µL goat-anti-mouse antibody coupled to alkaline phosphatase in a 1:20,000 dilution. Incubate for 1 h at 4°C or room temperature on an orbital shaker. Wash three times with 300 µL TBST.
16. Add 100 µL pNPP substrate solution. Incubate until color development and determine OD_{405nm}.

3.11. Preparation of S30 Extract (31–33)

1. Grow a 100-mL culture of *E. coli* MRE600 in incomplete rich medium overnight at 37°C.
2. Transfer 10 mL of the overnight culture in 1 L of fresh media in a 5-L baffled shaker flask and grow until OD_{600nm} of 1.0–1.2 at 37°C while shaking. This procedure can be scaled up to your needs and 1-L culture usually yields 10–15 mL of S30 extract. The S30 extract is stable for years when stored at –80°C.
3. Chill cultures for 10 min on an ice water bath with gentle shaking.
4. Centrifuge cells at 3,500×*g* at 4°C for 15 min and discard supernatant.
5. Wash the pellet three times with 50 mL of ice-cold S30 buffer per 1-L culture. It is best to resuspend cells with plating beads or on a magnetic stirrer using a sterile magnetic stir bar.
6. Freeze the cell pellet in liquid nitrogen and store for a maximum of 2 days at –80°C or continue immediately.

7. Resuspend the cell pellet (use 50 mL ice-cold S30 buffer per 1 L of culture), centrifuge at 4,000 × *g*. Discard supernatant and resuspend pellet in 4 mL S30 buffer per g wet cells (typically, 1 L of culture yields 1.5–2.0 g cell pellet).
8. Lyse the cells by one single passage through a French press applying 1,000 psi or an EmulsiFlex at ~17,000 psi.
9. Centrifuge cells at 20,000 rpm (SS-34 in a Sorvall centrifuge) at 4°C for 30 min. Transfer supernatant to clean centrifuge bottle(s) and repeat this step.
10. Add 1 mL of preincubation mix to each 6.5 mL of cleared supernatant (usually, 1-L culture yields 8–10 mL of S30 extract) and slowly shake at 25°C for 1 h. In this time, all endogenous mRNAs are translated and cellular nucleases degrade mRNA and DNA (34).
11. Dialyze the S30 extract (MW cutoff of 6,000–8,000 Da) against a 50-fold volume of S30 buffer at 4°C three times for 4 h.
12. Centrifuge S30 extract at 6,000 × *g* in a tabletop centrifuge at 4°C for 10 min. If the library members and target are devoid of disulfide bonds, 1 mM DTT can be added to the extract. Aliquot at 4°C in suitable volumes (e.g., 55 µL is sufficient for one in vitro translation reaction, 110 µL for two) since it should not be refrozen to guarantee best activity. Flash freeze in liquid nitrogen and store at −80°C.

3.12. Affinity Maturation

To increase the affinities of the library members, it is best to select for those having the lowest dissociation rate constant (off rate) from the target (7–9, 35–37). This off-rate selection can be applied for the improvement of known binders (after mutagenizing the gene for defined binders and thus creating a new library), but also during the initial selection from the original library. In this off-rate selection step, an excess of nonbiotinylated target is added after the binding reaction to the biotinylated target has already been equilibrated for >1 h. Any fast dissociating binder is immediately occupied by nonbiotinylated target and thereby prevented from being captured with biotinylated target on streptavidin or neutravidin. Conversely, any high-affinity binder with a slow off rate retains its biotinylated target and thus can be captured. The optimal duration of competitor incubation and the excess concentrations depend on the expected off rates. Considerations as to which parameters to choose have been recently discussed (13). As a general guideline, we recommend to perform the affinity maturation over several selection blocks (usually, three blocks seem sufficient) each containing a round of randomization (see Subheading 3.12.1), a round of off-rate selection (see Subheading 3.12.2), and a low-stringency round (see Subheading 3.5.3) for recovery of rare tight binders from a high background of unselected library members. For the off-rate selections in block 1, we recommend to start with a

modest stringency that can be increased in block 2 and 3, for example: for the first off-rate selection, use a 2-h incubation with a 10- to 100-fold excess of competitor, later use a 1,000- to 10,000-fold excess of competitor. Then, proceed with washing and elution of the bound ternary complexes as above.

3.12.1. Introduction of Additional Diversity Applying Error-Prone PCR

1. Set up PCR reactions on template DNA from Subheading 3.9, step 4, introducing different mutational rates using various concentrations of the nucleotide analogs dPTP and 8-oxo-dGTP in the range of 1 to 20 μM (see Note 27):

1 μL	pRDV_DARPin template (10 ng/μL)
4 μL	dNTPs each (final concentration 250 μM)
1–20 μM	dPTP and 8-oxo-dGTP, each
0.5 μL	T7B primer (final concentration 1 μM)
0.5 μL	tolAk primer (final concentration 1 μM)
5 μL	10× polymerase buffer
3 μL	$MgCl_2$ (final concentration 1.5 mM)
0.2 μL	Platinum® Taq DNA polymerase
Add to 50 μL with H_2O	

2. Apply the following cycling parameters (must be adapted according to primers and template): 3 min at 95°C, 25 cycles: 30 s at 95°C, 30 s at 50°C, 1 min at 72°C, final extension 5 min at 72°C.
3. Verify the product on an agarose gel.
4. Mix PCR products in equimolar amounts to serve as template for the in vitro transcription (see Subheading 3.2, step 4).

3.12.2. Competition with Non-labeled Target (Off-Rate Selection)

This protocol describes a selection strategy to enrich binders with a slow off rate.

Ribosomal complexes are incubated with low amounts of biotinylated target (in solution or immobilized) before adding unbiotinylated target in large excess as competitor. Considerations for selection conditions have recently been published (see ref. 13, Note 28).

1. For prepanning to remove all sticky ribosomal complexes, e.g., containing misfolded DARPins after randomization, add two times 500 μL diluted and stopped translation mix (*from* Subheading 3.4, step 4; see Subheading 3.5.3, step 1) to 20–50 μL of BSA-blocked streptavidin magnetic beads in a blocked, RNAse-free, 2.0-mL tube. Remember to set up two reactions, one containing the target and one not containing the target as negative control.

2. Incubate at 4°C for 30–60 min with head-over-end rotation.
3. After separation of the magnetic beads on a magnetic stand, remove the supernatant carefully and transfer the translation mix to a BSA-blocked 2.0-mL tube. Add biotinylated target in the range of 0.1–10 nM to the selection reaction (see Note 28) and buffer only to the tube containing the control.
4. Allow for equilibration of the DARPin/target complexes at 4°C with head-over-end rotation for 1–14 h.
5. For competition of the complexed DARPins, add a large excess of nonbiotinylated target. The ratio varies dependent on the expected affinity of the binders in the pool (see ref. 13, Note 28).
6. Incubate at 4°C with head-over-end rotation for 1–14 h.
7. Add the binding reactions to 20–50 μL fresh streptavidin magnetic beads previously blocked with BSA in a blocked 2.0-mL tube and capture the complexes remaining on the biotinylated target during a 30-min incubation rotating at 4°C.
8. Wash with 500 μL ice-cold WBT containing 0.1% BSA in each step as indicated (see Subheading 3.5). Separate captured complexes using a magnetic separator between each washing step.
9. Proceed with the elution and purification of RNA as described (see Subheading 3.5.2, step 5).

4. Notes

1. Use RNAse-free water, chemicals, and consumables. Most commercially available water is RNase free or can be generated using a membrane microfiltration system, e.g., MilliQ from QIAGEN, to produce ultrapure water. Alternatively, you can use 0.1% diethylpyrocarbonate (DEPC) which reacts with histidine residues, but also other nucleophilic groups, and therefore inactivates RNases, but for the same reason cannot be used for, e.g., Tris buffers. Chemicals used for RNA should be kept separate from the common chemical shelf and handled only with gloves and a flamed spatula to avoid RNase contamination. Purchase only RNase-free plastic consumables. If necessary, you can bake glass bottles and pipettes at 180°C for 6 h.
2. The buffer composition may be adjusted to the requirements of the library and target, but it is important that the wash buffer contains 50 mM Mg^{2+} to stabilize the ribosome. It is recommended to test buffer conditions with a known binder to ensure stability of the nascent chain complex.
3. Different reverse transcriptases (AffinityScript™ Multiple Temperature Reverse Transcriptase, SuperScript™ II

(Invitrogen, No. 18064-022), ThermoScript™ (Invitrogen, No. 12236-014) and QuantiTect (QIAGEN, No. 205310)) were tested for efficiency on DARPin sequences. With exception of QuantiTect, the yield obtained was comparably high with all other reverse transcriptases.

4. Previously Phusion™ High-Fidelity DNA Polymerase (New England Biolabs) has also been used (31, 35). Different DNA polymerases were tested, e.g., Vent$_R$® DNA Polymerase (New England Biolabs), Herculase® II Fusion DNA Polymerase (Stratagene), and Expand High Fidelity PCR System (Roche Diagnostics). The DNA polymerase mix from the Expand Hi Fidelity PCR System gave the lowest yield of PCR product while Herculase II gave the highest amount of side products. Therefore, we now routinely use Vent$_R$® DNA Polymerase for amplification of DARPin sequences. Since the yield was highest with the Herculase II, it might be a good alternative to increase the yield of PCR product or for amplification of other library scaffolds.
5. Use the homemade RNA polymerase buffer (see Subheading 2.5) as indicated for maximum yield of RNA. Commercial buffers have not worked very well at this step when the PCR product is directly used without further purification.
6. In round one, ensure that the number of molecules actually exceeds the library size, but no more members than ribosomes present in the translation reaction can be displayed. Nonetheless, under standard conditions as described here, ribosomes should be in excess. In a newly constructed library, the diversity cannot be higher than the number of DNA molecules used in this step. What limits the functional library in ribosome display is also discussed elsewhere (5). In later rounds, an enrichment is obtained, and it is generally sufficient to use ~50 ng of pRDV_DARPin template.
7. The PCR products can be used without additional purification. We highly recommend to use nonpurified PCR product at this step, since purified PCR product generally yields a greatly reduced amount of mRNA.
8. Optionally, the transcribed RNA can be analyzed on a denaturing formaldehyde agarose gel following standard procedures (38). The mRNA product should give a sharp band. A smear or no product indicates RNase contamination, which needs to be eliminated and the step repeated. If the band is sharp but the yield is lower than expected, obtain more starting DNA template by not purifying the PCR product that is used as template, as the quality is usually sufficient even without purification (see Note 7), and do use the homemade RNA polymerase

buffer (see Subheading 2.4) for better transcription yield. If the products are not of the expected size, optimize the PCR conditions depending on your template and primers.

9. In our experience, both protocols are yielding high-quality RNA as template for the in vitro translation, but the quality might be still higher using LiCl precipitation (see Subheading 3.3.1). Considerations on which protocol to use might also be the final concentration that is usually obtained (using gel filtration the sample is usually more dilute) or the time it takes (LiCl precipitation is performed over a time frame of 3 h while the purification using gel filtration (see Subheading 3.3.2) can be performed in 10 min).
10. In some cases, the template DNA itself might bind unspecifically to the target, e.g., if the target is highly positively charged, and then it is recommended to remove this contamination by DNAse I treatment before the actual selection. Always freeze small aliquots of DNAse I and store at −20°C. Do not refreeze or vortex solutions containing DNAse I because the enzyme is very sensitive to denaturation.
11. Always freeze RNA immediately after use; only thaw when needed to avoid degradation.
12. For the selection, some general considerations need to be pointed out. Always use the same target preparation through all of the selection and screening rounds, ensure its quality, and account for its stability over the duration of the experiment. If the target denatures, epitopes present in the native protein will vanish, and such binders will be lost. Account for high diversity, especially in the first round, by using sufficient amounts of the starting library. Start selections with a higher number of DNA template molecules than the diversity of the library. Be aware that no matter how large the library is the limitation of molecules that can be displayed depends on the number of ribosomes present in the translation reaction. Fortunately, under the conditions used here, more ribosomes than input DNA molecules are used (5). To extract all putative binders in the library, use a larger surface area to immobilize the target in the first round. The first round should, in general, not be highly selective; it is more important to capture the full diversity of binders, as a binder lost at this stage can never be recovered. In general, it is recommended to perform the selection in duplicates to monitor the selection quality. It is recommended to switch between neutravidin (a chemically modified derivative of avidin) and streptavidin or even switch between selections on immobilized target and target in solution during the selection process to focus selection on binding to the target, rather than on streptavidin/neutravidin or any other surface features. If high-affinity binders in the pM range are needed

(see Subheading 3.12), include the introduction of additional random mutations using error-prone PCR (see Subheading 3.12.1) and increase stringency by applying off-rate selections (see Subheading 3.12.2, Note 27).

Some applications, e.g., for therapy, require high stability of the therapeutic agent (39). Selection for high stability can also be achieved with ribosome display. This is best achieved by first making the whole population unable to fold, by deliberately introducing a reversible destabilization, e.g. by mutating a critical residue, then selecting for compensating mutations, and finally removing the destabilization again. For example, most antibody domains require disulfides for stability, which form only under oxidizing conditions. A destabilization of the antibody fold and increase in aggregation are usually observed when the disulfides are removed (40, 41). Using a reducing environment during the selection, scFv antibody fragments could be evolved that were able to fold under reducing conditions, correlating with conditions in the cytosol, and they showed higher stability than the starting molecule in the absence of the disulfide bonds (35), but also after the disulfide bonds were allowed to form again. Antibody fragments with these improved biophysical property can be used in biomedical applications with disulfides formed, but they also make an intracellular application (as "intrabodies") (42) more feasible. In addition, rational design of the antibody framework (43) could contribute to the development of stability-improved, antibody-based therapeutics.

13. Using the AviTag has the advantage that all biotinylated proteins are labeled uniformly and remote from epitopes which might interfere with later use and are labeled only once, leading to a more homogenous target preparation. Avoid the presence of a Met-Arg-Gly-Ser-$(His)_6$ tag ("RGS-His-tag") on the biotinylated target; rather, use a $(His)_6$ tag for purification, since the detection of DARPins bound to the target is performed using an anti-RGS$(His)_4$ antibody (see Subheading 3.10). Make sure that your target sample is devoid of free biotin. Biotin removal requires an extensive dialysis, for example four times against a 100-fold volume buffer for 4 h each. Nonbiotinylated target can be removed using a monomeric avidin column following the manufacturer's instructions (Pierce, No. 53146).
14. Use one well as nontarget control and two wells with immobilized target in later rounds as mutual controls for enrichment. When starting from the libraries in round one, it is recommended to use a larger surface, e.g., four wells with immobilized target.
15. To remove unspecifically binding ribosomal complexes, it is recommended to use a preselection on BSA-blocked wells coated only with neutravidin or streptavidin, but omitting the

target protein, except for round one, where this "prepanning" should not be performed. For prepanning, the preparation of additional wells and incubation of the ternary complexes from the in vitro transcription for 30–60 min is necessary before transferring the solution to the target-coated or control wells.

16. The amount of target can be reduced in later rounds to 1–20 nM.
17. The amount of target can be reduced to 100 pM, e.g., when performing an off-rate selection, and thus a high amount of competitor can be added. At lower target concentrations, the unspecific binding might prevail over target binding, however, and thus specificity of binding must be carefully controlled.
18. RNA isolation can also be performed with the RNeasy mini kit (QIAGEN) with comparable yield of resulting PCR product.
19. This step is highly recommended to avoid amplification of nonselected template DNA that has been carried over through all steps of the selection procedure. See also Note 10 for handling of DNAse I.
20. The RNA should stable for years at –80°C, but we recommend to immediately proceed with cDNA synthesis and PCR amplification for best recovery of sequences of putative binders.
21. Use one sample without addition of reverse transcriptase as control. The result of the following PCR is a measure for the quality of the selection regarding DNA carryover from the input DNA and putative overcycling (see Notes 19 and 23).
22. Always use one reaction containing no template, but all other components. Appearance of a band in this reaction indicates a contamination in one of the selection/amplification reagents. In our experience, the main candidate is the water used. Replace all the reagents immediately to prevent carryover of DNA of unwanted, unselected clones. To minimize expenses, it is recommended to store aliquots of all the reagents before starting selection.
23. Depending on the round of selection, more or fewer cycles could be advantageous. In the first round, 32–40 cycles are recommended to obtain sufficient product, since only a few clones have the desired properties. After more rounds of selection, specific binders are being enriched; therefore, the output of eluted RNA molecules increases. By lowering the cycle numbers in round 2 to between 28 and 35 and in all following rounds to 25, unspecific amplification can be reduced to a minimum. In addition, note that when the selection pressure increases, for example, after off-rate selection, the yield of PCR product might decrease. In this case, use more cycles.
24. If the quality and amount (<10 ng/μL) of the PCR product was not satisfactory, repeat the PCR. Never reamplify the PCR

product because this might lead to unspecific amplification of unwanted by-products.

25. In parallel, digest the ribosome display vector (pRDV) with the same restriction enzymes, e.g., *Nco*I and *Hin*dIII, for DARPin selections. Purify the plasmid backbone using extraction from a preparative agarose gel. It is recommended to use a larger preparation to last for several selection rounds and/or multiple target selections: Test the quality of the digested plasmid by ligation and transformation and/or PCR on the ligation mix to evaluate the level of religation and therefore quality of the ligation-ready plasmid.
26. The incubation temperature depends on the stability of the target and library scaffold.
27. Add different concentrations of the nucleotide analogs, for example 0, 3, and 10 μM. Up to 20 μM can be used, but the amount of product is greatly reduced at this concentration. The mutational load per kb under the conditions described is 1.5 mutations with 1 μM nucleotide analogs and 3.2 mutations with 3 μM nucleotide analogs. These numbers refer to fresh nucleotides and can vary if the nucleotides are no longer incorporated well, e.g., by hydrolysis of the triphosphate. The use of a low to medium mutational load per selection, but repeating over several rounds, might be beneficial over a high mutational load which might result in a high number of misfolded library members in the pool.
28. Perform one cycle of nonstringent selection, including an error-prone PCR followed by a round of off-rate selection without error-prone PCR. The rationale is that error-prone PCR generates many nonfunctional molecules. First, *all* functional molecules should be recovered by a nonstringent selection, and then from this pool of functional (randomized) molecules the best ones should be recovered. Use these to perform a stringent round using off-rate selection. Here are some general considerations (13): Subsequent selection rounds with modest selection pressure are preferred over high-stringency selection rounds because this leads to higher diversity. Start at 10- to 100-fold excess competitor; increase to 100- to 10,000-fold in later rounds, if feasible. In any case, the highest possible ratio of unbiotinylated:biotinylated target should be used to maximize the selection outcome as there is a greater margin for error in selection time in the presence of competitor (as the optimal selection time is generally unknown). Too long an incubation time eliminates the kinetic selection pressure because the system is at or near equilibrium. An incubation for 2 h is usually a good starting point, but can be increased to 14 h. This longer incubation time should be favored if the amount of target is limited and thus used at very low concentrations (100 pM) and the

highest possible unbiotinylated:biotinylated target ratio is used (e.g., a 1,000- to 10,000-fold access of competitor).

These two rounds should be followed again by a nonstringent round without any additional selection pressure simply to amplify the rare molecules. Perform this cycle of error-prone PCR, off-rate selection, and nonstringent round two to three times before analyzing single clones (see Subheading 3.10). For stringent selections, use 0.1–10 nM (depends on the availability of biotinylated target and expected affinity of the clones), and for nonstringent selections use 100 nM biotinylated target.

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