Chapter 13

Rapid Selection of High-Affinity Antibody scFv Fragments Using Ribosome Display

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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 works entirely in vitro, and this has two important consequences. First, since no transformation of any cells is required, libraries with much greater diversity can be handled than with most other techniques. Second, since a library does not have to be cloned and transformed, it is very convenient to introduce random errors in the library by PCR-based methods and select improved binders. Thus, a true directed evolution, an iteration between randomization and selection over several generations, can be conveniently carried out, e.g., for affinity maturation, either on a given clone or on the whole library. Ribosome display has been successfully applied to antibody single-chain Fv fragments (scFv), which can be selected not only for specificity but also for stability and catalytic activity. High-affinity binders with new target specificity can be obtained from highly diverse 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, Antibody scFv fragments, 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 [1–6]. No cells need to be transformed with the library, and thus the full available diversity can be subjected to selection, and not only the aliquot that ends up in transformed cells, like in most other display methods. A key feature of ribosome display, in contradistinction to most other selection technologies [6], 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, either individual or combined to a small collection [7–12], but also of the

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whole pool directly 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 [13] and/or error-prone PCR [14], as has been done in the above examples. This additional randomization step can readily be integrated in the protocol (*see* Subheadings 3.11.1 and 3.11.2). In combination with off-rate selection (*see* Subheading 3.11.3) where binders to the biotinylated target protein are competed with a molar excess of non-biotinylated target protein, many initial leads were improved for affinity in the range of low nM to low pM [3, 7, 9–11, 15]. The theoretical considerations for designing efficient off-rate selection experiments have been summarized elsewhere [16].

We have originally developed the ribosome display protocol for antibody scFv fragments [5, 8, 17]. We have applied it in the direct selection from libraries [1, 3, 17], where it combines selection with affinity maturation, and in the evolution of single clones [9, 15, 18]. Most antibody fragments require disulfide bonds for proper folding, and thus, it was initially a concern, whether ribosome display might only be feasible for the subset of antibodies that can fold without proper disulfide formation. To answer this question, experiments had been carried out prior to developing the ribosome display protocol for antibodies, in order to investigate native folding of antibody scFv fragments during in vitro translation [19]. The key conclusion was that disulfide isomerization is indeed very important but can be efficiently catalyzed by eukaryotic protein disulfide isomerase (PDI). This finding was the key reason why in our ribosome display protocols transcription and translation are not coupled: transcription can thus be run in the presence of DTT, translation (and folding) in its absence.

Occasionally, it has been surmised that eukaryotic proteins such as antibody fragments should be better produced in vitro by eukaryotic ribosomes than prokaryotic ones. However, a direct comparison gave no evidence for this belief [20].

In recent years we have extended the in vitro selection to protein scaffolds with more favorable biophysical properties than antibody fragments, the *D*esigned *A*nkyrin *R*epeat *P*roteins (DARPins) [21, 22]. DARPins, which are devoid of disulfide bonds, are highly soluble and highly stable and therefore achieve high expression levels in *E. coli* [23] and have thus been found versatile tools for many applications in laboratory research, diagnostics, and therapy [22]. 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 [7, 10, 24–32]. A protocol describing their selection has been published.

Some applications using antibody fragments, e.g., for therapy, require high stability of the therapeutic agent [33–35]. Selection for high stability can also be achieved with display technologies [18, 36–38]. If the stability is already high but an even more stable molecule is desired, this can be achieved by first making the whole population unable to fold by introducing a reversible destabilization, and 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 and increase in aggregation of the antibody fold is usually observed when the disulfides are removed [39, 40]. 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 [18] 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") [41] more feasible. In addition, rational design of the antibody framework [42] could contribute to the development of stability-improved, antibody-based therapeutics.

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 periplasmic expression and purification of scFv fragments of antibodies is not covered in this chapter but has been described elsewhere [43, 44]. The general workflow of ribosome display selections is outlined in Fig. 1.

2 Materials (*See* Note 1)

2.1 Preparation of the Ribosome Display Construct

1. Plasmid DNA of pAK200 (Figs. 2b and 3) [45].
2. DNA encoding the antibody library or a single antibody for affinity maturation.
3. Oligonucleotides SDA, T7B, T3Te, Gene-IIIAB, and ABrev (Table 1, Fig. 2a).
4. Taq DNA polymerase (2 U/μL; Invitrogen) and 10× buffer.
5. dNTPs (5 mM each; Eurogentec).
6. Restriction endonucleases *Sfi*I, *Hin*dIII, and *Nco*I (all from New England Biolabs).
7. T4 DNA ligase (5 U/μL, Fermentas) and 10× buffer.
8. QIAquick Gel Extraction Kit (Qiagen).

2.2 In Vitro Transcription

1. T7 RNA polymerase (20 U/μL; Fermentas) (*see* **Note 2**).
2. RNasin® Ribonuclease Inhibitor (20–40 U/μL; Promega).

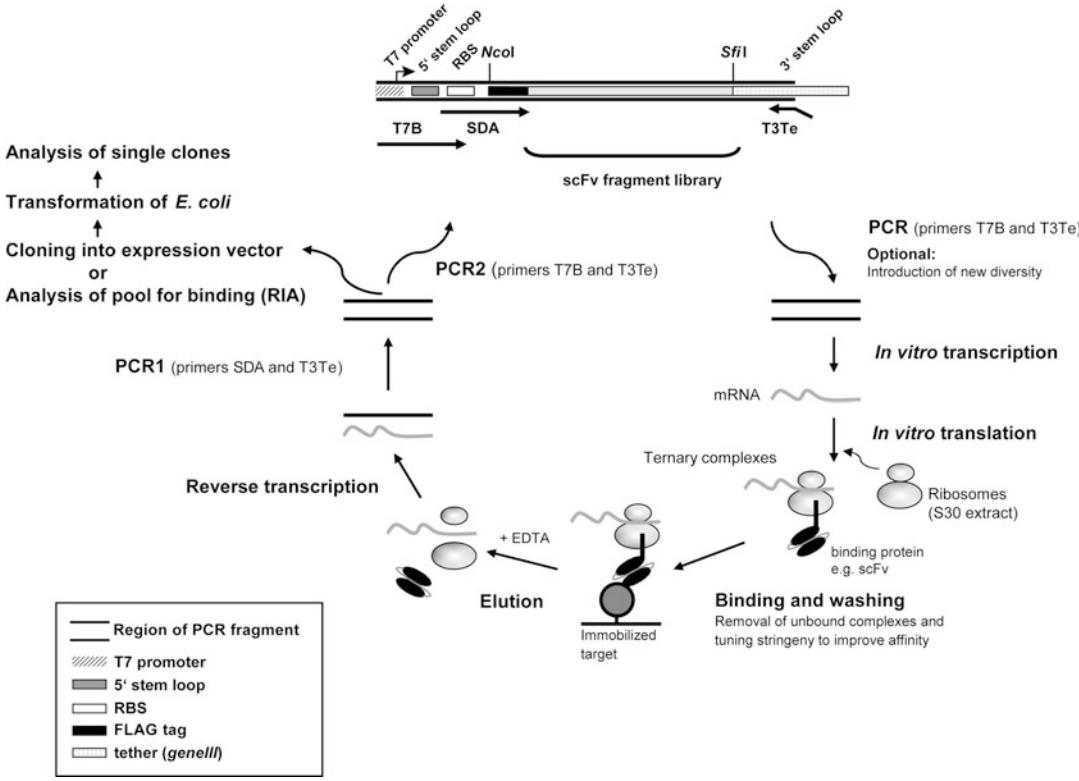

Fig. 1 Scheme of the ribosome display cycle. In ribosome display all steps of the selection are performed in vitro. The cycle begins with a DNA library (top) in the form of a PCR fragment encoding a library of the protein of interest and containing a T7 promoter and ribosome-binding site. The ORF of interest (light gray) 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 CT domain of *gene*III from filamentous phage M13, has the sole function of allowing the protein domain of interest to emerge from the ribosomal tunnel. Therefore, any non-globular polar protein sequence can in principle be used. 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 an *E. coli* S30 extract, leading to ternary complexes consisting of ribosomes, mRNA, and the scFv fragments 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 non-specifically bound protein by stringent washing. Affinity can be increased by addition of an excess of non-labeled target (off-rate selection) (*see* Subheading 3.11.3). 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 [18, 34, 39, 41, 42]. 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 primer set SDA and T3Te. The initial product is then amplified using primers T7B and T3Te to introduce the T7 promoter sequence and part of the stabilizing 5′ stem loop. The T3Te primer binds in the sequence of the *geneIII* 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.

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.

2.3 Clean-Up 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.

3a. 70% EtOH diluted with H_2O and 100% EtOH; filter through 0.22 μm pores

or

3b. illustra MicroSpin™ G-50 Columns (GE Healthcare).

4. DNase I (10 U/μL; Roche).

2.4 In Vitro Translation

1. 22 μM protein disulfide isomerase (PDI; Sigma-Aldrich) in H_2O; aliquot and store at −80 °C.
2. 200 mg/mL heparin in H_2O; do not filter, aliquot, and store at −20 °C.
3. 200 mM L-methionine in H_2O; do not filter, aliquot, and store at −20 °C.
4. STOP mix: 1 mL WBT buffer (*see* Subheading 2.8)/0.5% BSA plus 12.5 μL heparin.
5. S30 extract without DTT (*see* Subheading 2.5).
6. Premix Z (*see* Subheading 2.6).

2.5 S30 Extract

1. *E. coli* strain MRE600 [46, 47] 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.

Fig. 1 (continued) At the end of the selection rounds (typically, 2–5), the resulting PCR product pool can be analyzed by agarose gel electrophoresis (*see* Subheading 3.12.1) or by RIA (*see* Subheading 3.13). In order to analyze single clones for binding, the pool of PCR fragments is either subcloned via the restriction endonucleases *Nco*I and *Hin*dIII (the *Hin*dIII restriction site is introduced by a PCR primer at the 3′ end of the ORF) into the cytoplasmic expression vector pTFT74 [43] or via *Nco*I and *Sfi*I into pAK400 [52] for periplasmic expression (*see* Figs. 2b and 3)

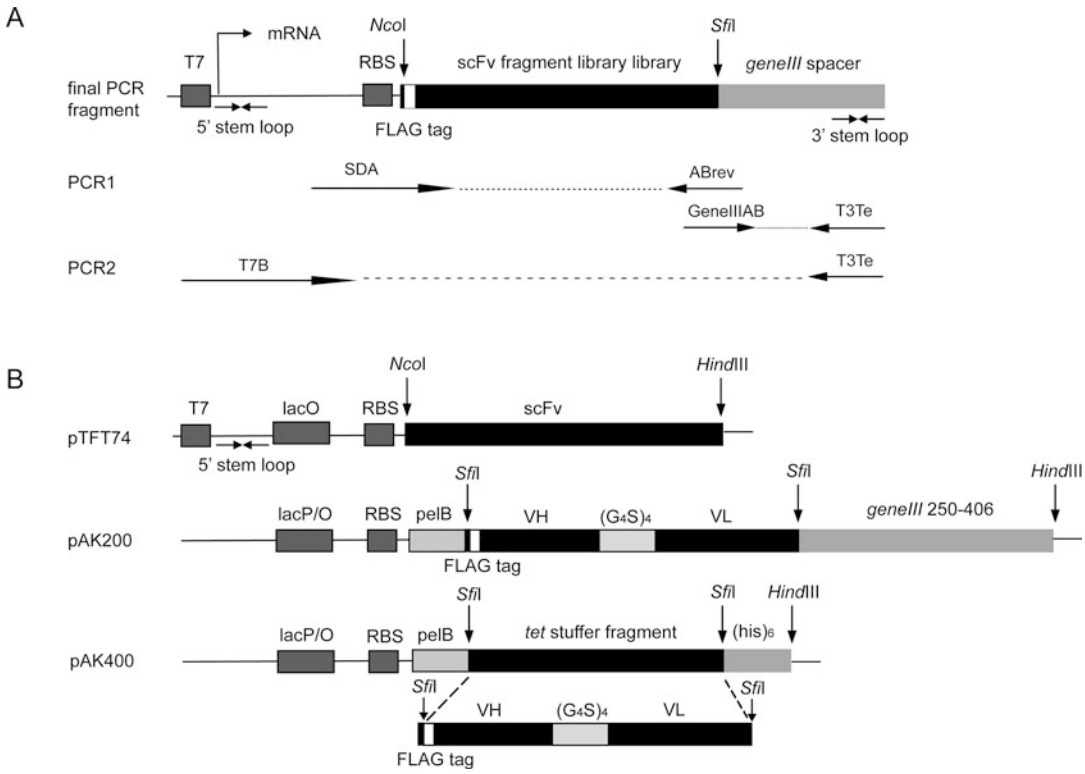

Fig. 2 (**a**) Assembly of the antibody scFv fragment library used in ribosome display. The steps necessary to generate a scFv library from natural or synthetic sources have been described [52, 56]. In addition to the sequence of the antibody fragment, where the stop codon is removed from the coding sequence, the PCR product used in ribosome display must contain a ribosome-binding site (RBS), T7 promoter, a tether (*geneIII*), and 5′ and 3′ stabilizing stem loops. These elements can be incorporated by assembly PCR or ligation. After a selection round, these elements are reassembled using a two-step PCR strategy to produce a PCR fragment that serves as template for the in vitro transcription of the following round of ribosome display. (**b**) Elements of the plasmids used for ribosome display and expression of antibody scFv fragments. T7, T7 promoter; lacO, *lac* operator; lacP/O, *lac* promoter/operator; RBS, ribosome-binding site; pelB, leader (signal) sequence for transport to the periplasm. The location of the restriction sites *Nco*I, *Sfi*I, and *Hin*dIII for cloning is indicated. The plasmid pTFT74 [43] is used for the cytoplasmic expression of scFv fragments as inclusion bodies (requiring subsequent refolding) or for their in vitro transcription and subsequent in vitro translation. Plasmid pAK200 [52] serves as source for the *gene*III fusion, representing the 3′ tether in ribosome display, of the scFv fragments if assembling a new scFv library. Alternatively, the fully synthetic scFv fragment library HuCAL [56] has been converted to the format suitable for ribosome display [3]. Replacement of the *tet* stuffer fragment by the DNA encoding the scFv fragments via the *Sfi*I restriction sites the plasmid pAK400 [52] allows their periplasmic expression and purification via the C-terminal His_6 tag. The FLAG tag is used for the detection of the scFv fragments. VH, sequence of the antibody heavy chain variable domain; VL, sequence of the antibody light chain variable domain; $(G_4S)_4$, glycine-serine linker connecting VH and VL. The opposite domain orientation VH-linker-VL can also be used

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.

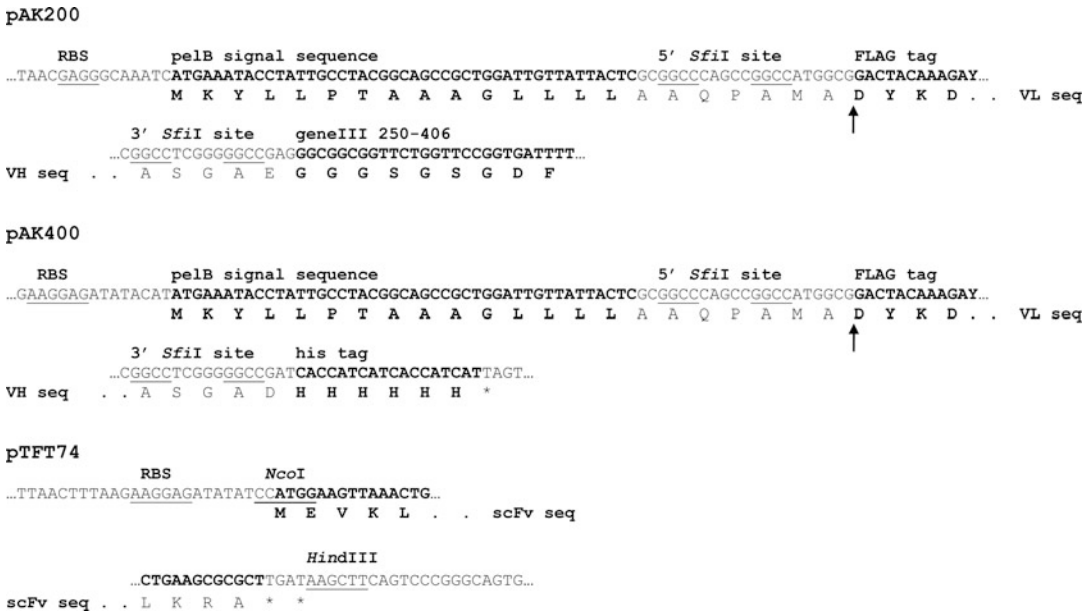

Fig. 3 Annotated sequences of the plasmids used for selection, expression, and purification of antibody scFv fragments. The sequences shown represent the 5′ and 3′ flanking regions of the region coding for the inserted scFv fragment [scFv seq; VL seq, sequence of the light chain variable domain; and VH seq, sequence of the heavy chain variable domain which are spaced by a glycine-serine linker (Fig. 2b)]. Both, pAK200 and pAK400 [52], contain a pelB leader sequence for export of the scFvs into the periplasm and a 5′ and 3′ *Sfi*I restriction site for cloning of the scFv fragments. The pelB signal sequence is processed by signal peptidase and cleaved off at the position indicated by the arrow. For pAK200 this will result in an N-terminal fusion with the *gene*III product suitable for phage display. For periplasmic expression of the scFv fragments from pAK400, their coding sequence is inserted such that it replaces the *tet* stuffer fragment (Fig. 2b) via the *Sfi*I restriction sites. The scFv fragments are expressed with a C-terminal his tag for purification and an N-terminal FLAG tag for detection. For cytoplasmic expression (as inclusion bodies requiring subsequent refolding) or in vitro transcription and in vitro translation, the scFv fragments are cloned into the plasmid pTFT74 [43] via *Nco*I and *Hin*dIII (the *Hin*dIII restriction site is introduced by a PCR primer at the 3′ end of the ORF)

4. Amino acid mix: 10 mM of each of the 20 amino acids (Fluka).
5. Preincubation mix (must be prepared directly before use): 3.75 mL of 2 mM Tris-acetate (pH 7.5 at 4 °C), 71 µL of 3 M magnesium acetate, 75 µL of amino acid mix, 300 µL of 0.2 M ATP, 50 units of pyruvate kinase (Fluka), and 0.2 g of phosphoenolpyruvate trisodium salt. Add to 10 mL of H_2O.
6. Dialysis tubing with a MW cutoff of 6000–8000 Da.

2.6 PremixZ

1. PremixA: 250 mM Tris-acetate (from a 2 M stock solution, pH 7.5 at 4 °C), 18 µM anti-ssrA oligonucleotide (from a 200 µM stock solution; Table 1), 1.75 mM of each amino acid except for methionine, 10 mM ATP (from an 1 M stock solution), 2.5 mM GTP (from a 0.2 M stock solution), 5 mM cAMP (from a 0.4 M stock solution), 150 mM acetyl phosphate (from

Table 1
Oligonucleotides used in ribosome display

Name	Sequence	Purpose
ABrev	Library dependent	Reverse primer encoding the 3′-end of the cDNA library
GeneIIIAB	Library dependent	Forward primer annealing to the 5′-end of the *geneIII* sequence
SDA	5′-AGACCACAACGGTTTCCCTCTAGAAATA-ATTTTGTTTAACTTTAAGAAGGAATATATCC ATGGACTACAAAGA-3′	Introduction of a ribosome-binding site
T7B	5′-ATACGAAATTAATACGACTCACTATAGG-GAGACCACAACGG-3′	Introduction of the T7 promoter as well as the 5′-stem loop
T3Te	5′-GGCCCACCCGTGAAGGTGAGCCTCA-GTAGCGACAG-3′	Encodes the translated early transcription terminator of phage T3
tolAk	5′-CCGCACACCAGTAAGGTGTGCGG-TTTCAGTTGCCGCTTTCTTTCT-3′	Transcription of β-lactamase mRNA
Anti-*ssr*A	5′-TTAAGCTGCTAAAGCGTAGTTTTCGT-CGTTTGCGACTA-3′	Inhibition of the 10Sa RNA peptide tagging system

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 (from a 10 mg/mL stock solution).

The final concentration will be fivefold lower in the final volume of the in vitro translation reaction (*see* Subheading 3.4).

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:

   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 were purchased from Sigma-Aldrich.

2.7 β-Lactamase Assay

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

1. Prepare β-lactamase mRNA as fusion with the tolA spacer from the ribosome display vector pRDV (GenBank accession No. AY327136.1) encoding the double Cys → Ala mutant of β-lactamase [48] using PCR with the primer set T7B and tolAk (Table 1).
2. Set up an in vitro transcription, and purify resulting mRNA (*see* Subheadings 3.2 and 3.3).
3. 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.
4. Incubate at 37 °C for 10 min.
5. Add 88 μL STOP mix.
6. Use 5 μL of stopped in vitro translation for the activity assay with the chromogenic substrate nitrocefin (Oxoid) [49].
7. 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.
8. Measure $OD_{486\ nm}$ immediately. Follow the kinetics for approximately 12 min measuring at least once every minute.

2.8 Selection and Elution of mRNA of Binders (see Note 1)

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; adjust pH to 7.4 with HCl at 4 °C; filter through 0.22 μm pores.
2. PBST: PBS containing 0.1% Tween-20.
3. 2 M Tris; adjust pH to 7.5 at 4 °C with acetic acid.
4. 5 M NaCl.
5. 2 M magnesium acetate.
6. 250 mM EDTA; adjust pH to 8.0 by NaOH addition.
7. WB/Tween-20 (WBT): 50 mM Tris-acetate (pH 7.5 at 4 °C), 150 mM NaCl, 50 mM magnesium acetate, 0.1% Tween-20; filter through 0.22 μm pores.
8. EB: 50 mM Tris-acetate (pH 7.5 at 4 °C), 150 mM NaCl, 25 mM EDTA; filter through 0.22 μm pores.
9. *S. cerevisiae* RNA (Fluka): dissolve to 25 μg/μL in H_2O; aliquot and store at −20 °C.
10. 12% skim milk in PBST or 10% BSA in PBST.

11. 1.2 mg/mL streptavidin and neutravidin.
12. Streptavidin-coated magnetic particles (Thermo Scientific).
13. MaxiSorp Immuno™ tubes (Nunc).
14. Maxisorp 96-well microtiter plates (Nunc).
15. Plate sealers.
16. Reagents for biotinylation of the target: either for chemical biotinylation a NHS-biotin reagent (e.g., from Pierce EZ-link™ Sulfo-NHS-LC-biotin) or for enzymatic biotinylation of an AviTag using the *E. coli* biotinylation enzyme BirA [50] (reagents from Avidity).
17. High pure RNA isolation kit (Roche).

2.9 Reverse Transcription, PCR, and DNase I Shuffling

1. Primer dissolved to 100 μM in H_2O; aliquot and store at −20 °C
   (T3Te, SDA, T7B, and library-specific primer; *see* Table 1).
2. AffinityScript™ Multiple Temperature Reverse Transcriptase (50 U/μL; Stratagene) and 10× buffer.
3. 100 mM DTT in H_2O; aliquot and store at −20 °C.
4. RNasin® Ribonuclease Inhibitor (20–40 U/μL; Promega).
5. Taq DNA polymerase (5 U/μL; Invitrogen) and 10× polymerase buffer.
6. 50 mM $MgCl_2$.
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).
10. Triton-X100.
11. QIAquick Gel Extraction Kit and QIAEX II (Qiagen).
12. DNase I (Roche).

2.10 Analysis of scFv Pools: RIA (Radioimmunoassay) [51]

1. All reagents needed for the in vitro transcription and translation (*see* Subheading 2.2–2.4).
2. Biotinylated target (*see* also Subheading 2.8, **step 13**).
3. [^{35}S]Methionine (10 mCi/mL, 1175 Ci/mmol, New England Nuclear).
4. Liquid scintillation cocktail OptiPhase2 (Wallac).
5. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; adjust pH to 7.4 with HCl at 4 °C; filter through 0.22 μm pores.
6. PBST: PBS with 0.05% (v/v) Tween-20.
7. Milk powder: 4% (w/v) in PBST.

8. 1.2 mg/mL neutravidin in PBS; aliquot and store at −20 °C.
9. 96-well Maxisorp plates or strips (Nunc).
10. Adhesive plate sealers.

2.11 scFv Expression and Binding Analysis of Single Clones

1. Plasmid pTFT74 [43] and/or plasmid pAK400 [52, 53] (Figs. 2b and 3).
2. Restriction enzymes *Nco*I and *Hin*dIII (New England Biolabs).
3. All reagents needed for the in vitro transcription and translation (*see* Subheadings 2.2–2.4).
4. Biotinylated target (*see* Subheading 3.6.1).
5. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; adjust pH to 7.4 with HCl at 4 °C; filter through 0.22 μm pores.
6. PBST: PBS with 0.05% (v/v) Tween-20.
7. Milk powder: 4% (w/v) in PBST.
8. 1.2 mg/mL neutravidin in PBS; aliquot and store at −20 °C.
9. 96-well Maxisorp plates or strips (Nunc).
10. Adhesive plate sealers.
11. Mouse-anti-FLAG tag antibody (M2; Sigma-Aldrich).
12. Goat-anti-mouse antibody coupled to alkaline phosphatase (Sigma-Aldrich).
13. pNPP buffer: 50 mM $NaHCO_3$, 50 mM $MgCl_2$.
14. pNPP substrate (*p*-nitrophenyl phosphate disodium salt; Fluka): 1 M stock in pNPP buffer; aliquot and store at −20 °C.

3 Methods

3.1 Construction of the Library

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 T3Te, Fig. 2a) 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 [2, 30, 54]. 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 allows the nascent protein chain to exit the ribosome and fold outside of the ribosome. The commonly used spacer for ribosome display selections of scFv fragments is derived from the *geneIII* of filamentous phage M13,

covering amino acids 211–299 (Swissprot: P03662), but others have been used, e.g., spacers derived from the *E. coli tolA* gene (amino acids 118–214; Swissprot: P19934) or the *tonB* gene (amino acids 62–229; Swissprot: P02929) (for an overview *see* [5]).

To introduce the 5′ and 3′ elements necessary for ribosome display (promoter and ribosome-binding site on the 5′ end and spacer (tether) with 3′ stem loop on the 3′ end), there are four principal options. One can (i) ligate the elements as restriction fragments, (ii) add the elements by assembly PCR, (iii) add the elements by nested PCR (only possible for the shorter elements at the 5′ end), or (iv) add all elements from a single plasmid, by ligating the ORF library into a plasmid that contains both the necessary 5′ and 3′ elements [51, 55]. In every case, the final in vitro product will contain the ORF library flanked by the necessary upstream and downstream elements, and this final product will serve as a template for transcription.

We will detail two strategies to convert this library into the ribosome display format. In either one, the 5′ elements are added by nested PCR. The methods differ how the 3′ tether (spacer) is added. This can either be achieved by ligating it to a restriction fragment constituting the spacer (*see* Subheading 3.1.1) or by assembly PCR (*see* Subheading 3.1.2).

The scFv library DNA is thus first connected to the 3′ tether, either through ligation or by assembly PCR. It is then directly amplified in two steps by PCR (Fig. 2a), by using in the first step the primer set SDA, which introduces the ribosome-binding site, and T3Te, which encodes the translated early transcription terminator of phage T3, and in the second step, the primer set T3Te and T7B, which introduces the T7 promoter as well as the 5′ stem loop (Table 1; [2]).

The starting material of the 3′ tether is in either case the vector pAK200 [45], from which it can be isolated as a *Sfi*I/*Hin*dIII fragment (Figs. 2b and 3) and fused in frame with the scFv library (*see* Subheading 3.1.1) or from which it can be obtained by assembly PCR (*see* Subheading 3.1.2).

The scFv starting library can in principle come from a synthetic library, an immunized animal, or a randomized single scFv fragment. The construction of large libraries for the selection of binders from immunized mice suitable for ribosome display has been described in detail elsewhere [1, 53], improving upon the original methods and primers described by Krebber et al. [45]. The fully synthetic human Ab library HuCal in the scFv format has also been converted into a format suitable to perform selections using ribosome display [56]. Finally, single antibodies have been brought into this format [9, 15, 18].

3.1.1 Preparation of the Ribosome Display PCR Product by Ligation

1. Digest the plasmid pAK200 [45] with the restriction endonucleases *Hin*dIII and *Sfi*I (Figs. 2b and 3). Separate the 481-bp long *geneIII* spacer by agarose gel electrophoresis, and purify it using the QIAquick Gel Extraction Kit.
2. Prepare the library DNA containing a 3′ *Sfi*I restriction site (omit the stop codon) compatible with the 5′ *Sfi*I restriction site of the *geneIII* spacer DNA (Figs. 2b and 3) either by PCR or digestion from a plasmid encoding for a library [1, 45, 56], and purify.
3. For the ligation combine 150 ng of the library DNA and 450 ng of spacer DNA with 10 units T4 DNA ligase. Ligate overnight at 16 °C.
4. Amplify 5–10 µL ligated library DNA in a reaction volume of 50 µL using the primer set SDA and T3Te (PCR1, Fig. 2a): 94 °C for 4 min; 5 cycles of 94 °C for 30 s, 37 °C for 30 s, 72 °C for 2.5 min; 15–20 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2.5 min; final extension at 72 °C for 10 min.
5. Separate the product by agarose gel electrophoresis, and purify using the QIAquick Gel Extraction Kit.
6. Set up a PCR with the primer set T7B and T3Te (PCR2, Fig. 2a) following the conditions mentioned in **step 5**, but use only 12–16 cycles at the annealing temperature of 50 °C.
7. The PCR product is directly used without further purification for the in vitro transcription (*see* Subheading 3.2).

3.1.2 Preparation of the Ribosome Display PCR Product by Assembly PCR

1. Amplify the gene III spacer sequence from the plasmid pAK200 [45] using the primer set geneIIIAB and T3Te (Fig. 2a).
2. Amplify the library DNA with the primer set SDA and ABrev (Fig. 2a).
3. Separate PCR products by agarose gel electrophoresis, and purify using the QIAquick Gel Extraction Kit.
4. Using equimolar amounts of both PCR products, set up the assembly PCR in a reaction volume of 50 µL not containing any primers, and then incubate for 4 min at 94 °C followed by five cycles of 30 s 94 °C, 30 s 50 °C, and 2.5 min 72 °C in a thermal cycler.
5. Add the primers SDA and T3Te, and run 12 additional cycles of 30 s 94°C, 30 s 45 °C, and 2.5 min at 72 °C (PCR1, Fig. 2).
6. Separate PCR products by agarose gel electrophoresis, and purify using the QIAquick Gel Extraction Kit.
7. Set up a PCR with the primer set T7B and T3Te (PCR2, Fig. 2a) following the conditions mentioned in Subheading

3.1.1, **step 4**, but use only 12–16 cycles at the annealing temperature of 50 °C.

8. The PCR product is directly used without further purification for the in vitro transcription (*see* Subheading 3.2).

3.2 Transcription of PCR Products (see Note 3)

1. As template for in vitro transcription, use the DNA fragment containing all elements important for ribosome display that was obtained from the PCR with the outer primers T7B and T3Te (*see* Subheading 3.1.1, **step 7**, or *see* Subheading 3.1.2, **step 8**).
2. For in vitro transcription, set up the following reaction on ice:
   (a) 20.0 μL 5× T7 polymerase buffer.
   (b) 14.0 μL NTPs (final concentration 7 mM each).
   (c) 4.0 μL T7 RNA polymerase (20 U/μL).
   (d) 2.0 μL RNasin (40 U/μL).
   (e) 22.5 μL PCR product without further purification (*see* **Note 4**).
   (f) Add to 100 μL with H_2O.
3. Incubate the transcription for 2–3 h at 37 °C (*see* **Note 5**).

3.3 Clean-Up 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 6**):

3.3.1 Purification Protocol Using 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, and 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 Purification Protocol Using Gel Filtration

1. For purification of the RNA, small gel filtration columns (e.g., illustra MicroSpin™G50 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. Aliquot RNA and immediately flash-freeze in liquid nitrogen. Store at −80 °C.
6. Determine the RNA concentration of a 1:100 dilution by $OD_{260\ nm}$. Normally, 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:
   (a) 2.0 μL 200 mM methionine
   (b) 41 μL premixZ with optimized composition (for preparation of the premixZ, *see* Subheading 3.6)
   (c) 0.625 μL PDI (*see* Subheading 2.4)
   (d) x μL in vitro transcribed RNA (total 10 μg; *see* **Note 7**)
   (e) 50 μL S30 extract and add to 110 μL with H_2O (for preparation of the S30 extract, *see* Subheading 3.5).
2. Mix carefully by pipetting up and down, and incubate the reaction at 37 °C for 6–15 min, the time found optimal for scFv fragments (*see* **Note 8**).
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 3.6).

3.5 Preparation of S30 Extract [5, 57–60]

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 shake flask, and grow until $OD_{600\ nm}$ of 1.0–1.2 at 37 °C while shaking. This procedure can be scaled up to your

needs, and a 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 3500 × *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 stirbar.
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), and centrifuge at 4000 × *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 cell by one single passage through a French press applying 1000 psi or an EmulsiFlex at ~17,000 psi.
9. Centrifuge cells at 45,000 × *g* (corresponds to 20,000 rpm with a SS-34 rotor in a Sorvall centrifuge) at 4 °C for 30 min. Transfer the 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 will yield 8–10 mL of S30 extract), and slowly shake at 25 °C for 1 h. During this time all endogenous mRNA will be translated, and cellular nucleases will degrade mRNA and DNA [61].
11. Dialyze the S30 extract against a 50-fold volume of S30 buffer at 4 °C three times for 4 h.
12. Centrifuge S30 extract at 4000 × *g* in a table top centrifuge at 4 °C for 10 min. 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.

3.6 Selection (See Note 9)

3.6.1 Target Protein Preparation

Express and purify the target by methods of your choice, but the target for selection must be of excellent purity and structural homogeneity. To immobilize the target for capturing the ternary complexes, it is recommended to biotinylate the target. This is the method of immobilization found by far to be most robust 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 non-biotinylated 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 10**):

1. Fuse the target to an AviTag, and biotinylate it in vivo or in vitro using the *E. coli* biotin ligase BirA [50] following the guidelines posted on the Avidity webpage (www.avidity.com).
2. Alternatively, biotinylate surface lysine amino acid residues using NHS-biotin reagents from Pierce following the manufacturer's instructions.

3.6.2 Selection in Plates

1. Coat wells of a 96-well Maxisorp plate with 100 µL of a 66 nM neutravidin or streptavidin solution in PBS, and close with an adhesive plate sealer (*see* **Notes 11** and **12**). 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 PBS.
2. Block the wells with 300 µL 4% milk powder in PBS (or 0.5% BSA in PBS) 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 100–500 nM (*see* **Note 13**) in PBST/0.1% BSA and PBST/0.1% BSA without target 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 PBST 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 100 µL of the stopped in vitro translation, and supplement to a final concentration of 2% milk powder in PBST. 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 will dissociate and subsequently be 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 added *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 mixing, the RNA is stable in the lysis buffer and can be processed at room temperature until elution from the column (*see* Subheading 3.7).

3.6.3 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 to 40 µL of streptavidin-coated magnetic beads that were washed two times with 500 µL PBS and blocked with 500 µL PBST/4% milk powder (biotin-depleted; or 0.5% BSA) for 1 h in a 2 mL tube as preselection step (*see* **Note 12**). Rotate head over end at 4 °C for 1 h.
2. Transfer the supernatant to a blocked 2 mL tube, and add to 100–500 nM of biotinylated target (omit target in the control reaction), and incubate rotating at 4 °C for 1 h (*see* **Note 14**).
3. Transfer the supernatant to a blocked tube containing 40 µL of blocked streptavidin-coated magnetic beads, and capture the ternary complexes rotating at 4 °C for 30 min. Wash with 500–1000 µL ice-cold WBT containing 0.1% BSA as indicated above (*see* Subheading 3.6.2, **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 3.6.2, **step 5**).

3.7 Recovery of Eluted RNA

1. Apply the lysis buffer/eluate mixture from Subheading 3.6.2, **step 5**, on the column of the High Pure RNA isolation kit (*see* **Note 15**). Centrifuge at 8000 × *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 16**). Add 500 µL wash buffer 1 and centrifuge at 8000 × *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 8000 × *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 7** and **17**).

3.8 Reverse Transcription (RT) of mRNA

1. Transfer two times 12.5 µL of eluted RNA to fresh 1.5 mL tubes (*see* **Note 18**).
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:
   (a) 0.25 µL T3Te primer (final concentration 1.25 µM).

(b) 0.5 μL dNTPs (final concentration 125 μM of each nucleotide).

(c) 0.5 μL RNasin (40 U/μL).

(d) 0.5 μL AffinityScript™ Multiple Temperature Reverse Transcriptase.

(e) (50 U/μL).

(f) 2.0 μL 10× AffinityScript™ buffer.

(g) 2.0 μL DTT (final concentration 10 mM).

(h) 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 7.5 μL as template for PCR using the inner primers SDA and T3Te.
7. Freeze the rest of the cDNA in liquid nitrogen and store at −20 °C.

3.9 Amplification of cDNA Coding for scFv Fragments (PCR1)

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:
   (a) 7.5 μL cDNA from Subheading 3.8, **step 7.**
   (b) 5.0 μL 10× Taq buffer.
   (c) 1.55 μL 50 mM $MgCl_2$.
   (d) 2.0 μL dNTPs (final concentration 200 μM of each nucleotide).
   (e) 2.5 μL DMSO (final concentration 5%).
   (f) 0.5 μL SDA primer (final concentration 1 μM).
   (g) 0.5 μL T3Te primer (final concentration 1 μM).
   (h) 0.5 μL Taq DNA polymerase (5 U/μL).
   (i) Add to 50 μL with H_2O (*see* **Note 19**).
2. Perform a hot start PCR to reduce unspecific amplification. Use the following cycling parameters: 4 min at 94 °C; 20 cycles, 30 s at 94 °C, 30 s at 50 °C, 2.5 min at 72 °C; final extension 10 min at 72 °C (*see* **Note 20**).
3. Verify the product on an agarose gel (*see* **Note 21**).

3.10 Incorporation of Promoter Elements and 5′ RNA-Stabilizing Stem Loop (PCR2)

1. Purify PCR products from Subheading 3.9, **step 3**, by excision of the appropriate bands from an agarose gel, and purify using the QIAquick Gel Extraction Kit.
2. Using the composition mentioned in Subheading 3.9, **step 1**, set up a PCR reaction using the outer primer set T7B and T3Te and the purified template from PCR1 or perform an error-prone PCR to increase diversity (*see* Subheading 3.11.1).
3. Perform a hot start PCR to reduce unspecific amplification. Use the following cycling parameters: 4 min at 94 °C; 10–-15 cycles, 30 s at 94 °C, 30 s at 50 °C, 2.5 min at 72 °C; final extension 10 min at 72 °C.
4. Verify the product on an agarose gel (*see* **Note 21**).
5. Use the PCR product without further purification for the in vitro transcription (*see* Subheading 3.2) or the purified product for DNase I shuffling and assembly PCR (*see* Subheading 3.11.2).

3.11 Affinity Maturation (See Note 22)

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, 10, 18, 62, 63], since typically the association rate constants (on-rate) of protein-protein interactions fall into a very similar range and thus typically cannot be improved beyond this, because of the mechanism of the association process [16, 64]. 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 (typically in the third selection round). In this off-rate selection step, an excess of non-biotinylated target is added after the binding reaction to the biotinylated target has already been equilibrated for >1 h. Any fast dissociating binder will be immediately occupied by non-biotinylated target and thereby prevented from being captured with biotinylated target on streptavidin or neutravidin. Conversely, any high-affinity binder with a slow off-rate will retain 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 (times, concentrations, and error rates) to choose have been discussed elsewhere based on extensive calculations [16] (*see* **Note 23**). As a general guideline, we recommend to perform the affinity maturation over several selection "blocks" (usually three blocks seem sufficient), each block containing a round of randomization (*see* Subheading 3.11.1 or 3.11.2), a round of off-rate selection (*see* Subheading 3.11.3), and a low stringency round (*see* Subheading 3.6) for recovery of rare tight binders from a high background of inactive library members (carrying deleterious mutations) (*see* **Note 24**). For the off-rate

selections in block 1, we recommend to start with a modest stringency that can be increased in blocks 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, and later use a 1000- to 10,000-fold excess of competitor. Then proceed with washing and elution of the bound ternary complexes as above.

3.11.1 Introduction of Additional Diversity Applying Error-Prone PCR

1. Set up PCR reactions on template DNA from Subheading 3.9, **step 2**, introducing different mutational rates using various concentrations of the nucleotide analogs dPTP and 8-oxo-dGTP in the range of 1–20 μM (*see* **Note 25**):
   (a) 1 μL PCR1 product (10 ng/μL).
   (b) 4 μL dNTPs each (final concentration 250 μM).
   (c) 1–20 μM dPTP and 8-oxo-dGTP, each.
   (d) 0.5 μL T7B primer (final concentration 1 μM).
   (e) 0.5 μL T3Te primer (final concentration 1 μM).
   (f) 5 μL 10× polymerase buffer.
   (g) 3 μL $MgCl_2$ (final concentration 1.5 mM).
   (h) 0.5 μL Taq DNA polymerase
   In a 50 μL reaction:
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**).

3.11.2 Introduction of Additional Diversity Applying DNase I Shuffling and Assembly PCR [18]

1. Use 5 μg of purified PCR product (*see* Subheading 3.10, **step 5**) in a reaction volume of 100 μL. Add 10 μL of 10× DNase I buffer and 1 μL DNase I (0.15 U/μL; Roche), and incubate 5 min at room temperature.
2. Add 2.5 μL of DNA loading buffer containing EDTA to 5 μL of the reaction, and freeze the rest immediately in liquid nitrogen.
3. Analyze sample on a 1.5% agarose gel (*see* **Notes 26** and **27**).
4. Purify the 50–100 bp product of the DNase I digest using QIAEX II.
5. Set up an amplification reaction not containing any primers:
   (a) 5–15 μL purified fragment
   (b) 2 μL 10× PCR reaction buffer

(c) 0.88 µL $MgCl_2$ (50 mM)
(d) 0.25 µL dNTPs
(e) 0.8 µL Triton X-100
(f) 0.5 µL Taq DNA polymerase
(g) Add to 20 µL with H_2O.

6. Run the following program in a thermal cycler: 4 min at 94 °C; 20 cycles, 30 s at 94 °C, 30 s at 45 °C, 2.5 min at 72 °C; final extension 10 min at 72 °C.
7. Verify the product on an agarose gel, and purify the product of the expected size (*see* **Note 25**).

3.11.3 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 (preferentially in solution, but in principle this can also be done with target immobilized on plates) before adding non-biotinylated target in large excess as competitor. Considerations for selection conditions have been published elsewhere [16].

1. For pre-panning to remove all "sticky" ribosomal complexes, e.g., containing misfolded scFvs after randomization, add two times 500 µL diluted and stopped translation mix (*from* Subheading 3.4, **step 4**) to 20–50 µL of milk (4%) or BSA (0.5%)-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 milk- or BSA-blocked 2.0 mL tube. Add biotinylated target in the range of 0.1–10 nM to the selection reaction (*see* **Note 22**), and buffer only to the tube containing the control.
4. Allow for equilibration of the scFv-target complexes at 4 °C with head over end rotation for 1–14 h.
5. For competition of the complexed scFvs, add a large excess of non-biotinylated target. The ratio will vary dependent on the expected affinity of the binders in the pool ([16], *see* **Note 22**).
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 milk or BSA in a blocked 2.0 mL tube, and capture the scFv-target complexes remaining on the biotinylated target during 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 3.6.2, **step 4**). Separate captured scFv-target complexes using a magnetic separator between each washing step.
9. Proceed with the elution and purification of RNA as described (*see* Subheading 3.7).

3.12 Initial Analysis of Pools After Each Selection Round for Binding

To test for enrichment of specific binders, either PCRs from target and nontarget wells can be compared (Subheading 3.12.1) or the pools can be translated and analyzed by RIA (Subheading 3.12.2).

3.12.1 PCR of Enriched Pools

An aliquot of the RT-PCRs from the selection on target and on the control not containing target are compared. For this purpose an aliquot (typically about 5 µL) of the PCR after Subheading 3.9, **step 3**, from the wells with and without target are loaded on an agarose gel. From the second or third round onward, the PCR band from the target should be stronger.

3.12.2 RIA (Radioimmunoassay) of the Translated Enriched mRNA Pools

To test for enrichment of specific binders, the mRNA pool after each round of ribosome display can be transcribed with the outer primer set T7B and T3Te (*see* Subheading 3.10) and translated in vitro (*see* Subheading 3.4) using [^{35}S]Methionine for detection. The resulting ternary complexes are then probed in a RIA format for binding (identical to an ELISA setup).

1. Coat the microtiter plate wells overnight at 4 °C with 100 µL neutravidin in PBS.
2. Wash the plate two times with 300 µL PBS.
3. Block the microtiter plate wells with 4% milk in PBS for 1 h at room temperature.
4. Add 100 µL of biotinylated target in PBST (or just PBST to control wells), and incubate with gentle shaking for 30 min at 25 °C.
5. Wash three times with 300 µL PBST.
6. Set up an in vitro transcription reaction using 1 µg DNA (either an expression plasmid, e.g., pTFT74 [43] (Fig. 2b) with the gene or pool encoding the scFv or a PCR product containing the T7 promoter) as described (*see* Subheading 3.4) with the following modifications: set up only a 55 µL reaction, and do not add cold methionine but 2 µL [^{35}S]Methionine (0.3 µM, 50 µCi/mL final concentration). Translate for 30 min at 37 °C.
7. Add 220 µL PBST and centrifuge for 5 min at 14,000 × *g*.
8. Transfer the supernatant to a fresh 1.5 mL reaction tube (~250 µL), and add 250 µL of 4% milk in PBST containing

either no ligand or, for competition studies, different concentrations of non-biotinylated target.

9. Incubate for 1 h at room temperature, before adding 100 µL of this reaction mixtures to the microtiter well.
10. Incubate for 30 min at room temperature.
11. Wash five times with 300 µL PBST, and elute with 100 µL 4% SDS in PBS or 0.1 M triethylamine after an incubation time of 10 min.
12. Add eluate to 5 mL scintillation fluid, and quantify the radioactivity in a scintillation counter.

3.13 ELISA of Single scFvs

1. Usually after the third round of ribosome display, the PCR products are cloned into a vector, and *E. coli* are transformed, such that the binding specificity of single clones can be evaluated by ELISA. There are three principal strategies for achieving this. The primers chosen for the last PCR amplification should commensurate with the destination vector:
   (a) Since antibody scFv fragments carry disulfide bonds, which are required for correct folding, it is best to clone the scFv genes into a secretion vector behind a bacterial signal sequence. The vector pAK400 [52, 53] allows directional cloning via two *Sfi*I sites with different overhangs (Fig. 3) and leads to strong expression. This strategy should normally be chosen first.
   (b) The genes can alternatively be cloned into the vector pTFT74 [43] via *Nco*I and *Hin*dIII (Figs. 2b and 3). This vector does not contain a signal sequence and will express the genes under control of the T7 promoter. Thus, after plasmid minipreps, the antibody scFv gene can be translated in vitro in the presence of disulfide isomerase [19]. This strategy will of course yield much less protein than the direct expression from *E. coli* but can be very valuable in troubleshooting, as the in vitro folding conditions will be very similar as in the ribosome selection itself.
   (c) Finally, using the same vector, pTFT74 [43] (Fig. 2b), the protein can be produced from *E. coli*, albeit as inclusion bodies which have to be refolded [17, 43]. This is not practical for a very large number of samples but can sometimes provide large amounts of a particular scFv fragment very rapidly.
   (d) We cannot give a full account of antibody expression here. The different expression strategies have been compared [44, 53, 65], and general protocols for the specific procedures are found in the references above. Since the

transformation of *E. coli* with subsequent cell lysis for ELISA is very straightforward and is described in, e.g., [44, 53, 65], we concentrate here on the in vitro translation of the antibody.

2. Plasmids of single clones are isolated and used for in vitro transcription (*see* Subheading 3.2), RNA purification (*see* Subheading 3.3), and in vitro translation (*see* Subheading 3.4).
3. Dilute the reaction mixture fourfold with PBST, and centrifuge at 14,000 × *g* for 10 min at 4 °C.
4. Transfer supernatant to fresh 1.5 mL reaction tube, and add an equal volume of 4% milk in PBST. For a competition ELISA, add varying amounts of non-biotinylated target and preincubate for 1 h at room temperature.
5. Add 100 μL to target-coated wells or control wells prepared as described (*see* Subheading 3.11, **steps 1–5**), and incubate 30–60 min at room temperature.
6. Wash three times with 300 μL PBST.
7. Add 100 μL the FLAG tag antibody in a 1:1000 dilution in 1% milk in PBST. Incubate 1 h at room temperature, and wash three times with 300 μL PBST.
8. Add 100 μL of a polyclonal anti-mouse alkaline phosphatase conjugate in a dilution of 1:20,000 in PBST. Incubate 1 h at room temperature, and wash three times with 300 μL PBST.
9. Detection is performed with 100 μL pNPP substrate and reading the OD at 405 nm.

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 ultra-pure water. Alternatively, you can use 0.1% DEPC (diethylpyrocarbonate) which reacts with histidine residues but also other nucleophilic groups and therefore inactivates RNases but for the same reason cannot be used, e.g., for Tris-buffers. Chemicals 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. Use the homemade RNA polymerase buffer (*see* Subheading 2.2) 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.

3. In round one ensure that the number of mRNA molecules produced in transcription actually exceeds the library size (the initial number of different DNA molecules). Be aware that no more members than ribosomes present in the translation reaction can be displayed. Nonetheless, under standard conditions as described here, ribosomes will normally 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 [6].
4. The PCR products can be used without additional purification. We highly recommend to use non-purified PCR product at this step, since purified PCR product generally yields a greatly reduced amount of mRNA.
5. Optionally, the transcribed RNA can be analyzed on a denaturing formaldehyde agarose gel following standard procedures [66]. The mRNA product should give a sharp band. A smear or absence of 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 4**), and do use the homemade RNA polymerase buffer (*see* Subheading 2.2) for better transcription yield. If the products are not of the expected size, optimize the PCR conditions depending on your template and primers.
6. 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 include the final concentration that is usually obtained (using the purification by 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 gel filtration (*see* Subheading 3.3.2) can be performed in 10 min).
7. Always freeze RNA immediately after use; only thaw when needed, to avoid degradation.
8. The incubation time and temperature must be optimized for each type of library based on different constructs.
9. 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. Take into account the high diversity present 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. 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 3.11), introduce additional random mutations using error-prone PCR (*see* Subheading 3.11.1) or DNase I shuffling (*see* Subheading 3.11.1), and increase stringency by applying off-rate selections (*see* Subheading 3.11.3, *see* **Note 22**). Even when not using an additional mutagenic PCR step, it is highly advantageous to include an off-rate selection step in the panning rounds. Considerations of the choice of parameters for selection for high-affinity are given elsewhere [16].

10. Using the AviTag has the advantage that all biotinylated proteins are labeled uniformly and remote from epitopes which might interfere with their later use and that they are labeled only once, leading to a more homogenous target preparation. Make sure 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. Non-biotinylated target can be removed, as it will flow through a monomeric avidin column, from which the biotinylated protein can be eluted, following the manufacturer's instructions (Pierce).
11. 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 1, it is recommended to use a larger surface, e.g., four wells with immobilized target or an immunotube (Nunc).
12. To remove unspecifically binding ribosomal complexes, it is recommended to use a preselection on milk- or BSA-blocked wells coated only with neutravidin or streptavidin but omitting

the target protein, except for round 1, where this "pre-panning" should not be performed. For pre-panning, the preparation of additional wells and incubation of the ternary complexes from the in vitro transcription for 30–60 min are necessary before transferring the solution to the target-coated or control wells.

13. The amount of target can be reduced in later rounds to 1–20 nM.
14. 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 to achieve a high ratio. At still lower target concentrations, the unspecific binding might prevail over target binding, however, and thus specificity of binding must be carefully controlled.
15. *Optional*: as a positive control, also purify 2 μL of the input RNA from the in vitro transcription (*see* Subheading 3.2) after dilution in 200 μL EB.
16. This step is highly recommended to avoid amplification of nonselected template DNA that has been carried over through all steps of the selection procedure. 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.
17. The RNA should be 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.
18. Use one sample without addition of reverse transcriptase as control. The result of the following PCR will be a measure for the quality of the selection regarding DNA carry-over from the input DNA and putative over-cycling (*see* **Notes 16** and **20**). There should not be any band without reverse transcriptase.
19. 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 carry-over of DNA of unwanted unselected clones. To minimize expenses it is recommended to store aliquots of all the reagents before starting selection.
20. Depending on the round of selection, more or fewer cycles could be advantageous. In the first round, the cycle number can be increased, since only a few clones will have the desired properties. After more rounds of selection, specific binders are

being enriched; therefore, the output of eluted RNA molecules increases, and the cycle number can be decreased. 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.

21. A sharp band of the product of expected size should be observed. If the quality and amount (<10 ng/μL) of the PCR product was not satisfactory, repeat the PCR using several parallel reactions, or run more cycles.
22. Perform one cycle of non-stringent 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 will generate many nonfunctional molecules. First, *all* functional molecules should be recovered by a non-stringent selection, and then from this pool of functional (randomized) molecules, the best ones should be recovered. For this purpose perform a stringent round using off-rate selection. After this stringent round, the number of remaining functional molecules, most of them of very high-affinity, can be very low, such that background binding becomes an issue. Thus, the nonselective round will simply amplify these functional, high-affinity molecules.
23. Here are some general considerations [16]: subsequent selection rounds with modest selection pressure ("recovery rounds") are recommended to follow after high-stringency selection rounds, because this will lead 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 non-biotinylated/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 will eliminate 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 non-biotinylated/biotinylated target ratio is used (e.g., a 1000- to 10,000-fold access of competitor).
24. These two rounds should be followed again by a non-stringent round without any additional selection pressure, simply to amplify the rare molecules. Perform this cycle of error-prone PCR, off-rate selection, and non-stringent 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 non-stringent selections use 100 nM biotinylated target.

25. Add different concentrations of the nucleotide analogs, for example, 2, 5, and 10 μM, and mix products in equimolar amounts for the in vitro transcription. Up to 20 μM can be used, but the amount of product is greatly reduced at this concentration. 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.
26. A digest with DNase I should result in a product of DNA fragments of the length of 50–100 bp. If the product is of larger size, add another 1 μL of DNase I, and repeat **steps 1–3** (for handling of DNase I, *see* **Note 16**). Once the optimal length has been obtained, separate the rest of the product over a 1.5% agarose gel, and purify with QIAEX II.
27. The band will be diffuse but will generate a specific product in the second PCR with an outer primer set (e.g., SDA and ABrev; Table 1).

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