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In Vitro Selection and Evolution of Protein–Ligand Interactions by Ribosome Display

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INTRODUCTION

Ribosome display is a method for selecting and further evolving functional proteins for their properties of interaction. It is performed completely *in vitro* and does not involve living cells at any step (Hanes and Plückthun 1997). This method has two main advantages over most other selection methods. First, the screening of very large libraries with up to 10^{14} members is possible. In contrast, other selection methods such as phage display (Winter et al. 1994; Smith 1985), the yeast two-hybrid system (Fields and Song 1989; Chien et al. 1991), or cell-surface display methods (Georgiou et al. 1993; Boder and Wittrup 1997) all necessarily involve transformation steps in which the original library is introduced into cells. This step limits the size of the library by the transformation efficiency, which is about 10^9 to 10^{10} per microgram of DNA in *E. coli* (Dower and Cwirla 1992) and about $\sim 10^7$ per microgram of DNA in yeast. Second, there is a built-in evolution during the ribosome display selection procedure. During the many PCR amplification steps that are part of the ribosome display protocol, low-fidelity DNA polymerases that introduce mutations are usually used. In most cases, the mutations are detrimental. However, in some cases, a beneficial mutation is introduced randomly that improves the stability or affinity of the displayed protein, leading to a selection advantage of this clone in the subsequent ribosome display cycle. Thus, the sequence space sampled is not limited by the initial size of the library applied to ribosome display, and diversification can be easily introduced during subsequent selection rounds if low-fidelity polymerases are used. If evolution of the selected clones is not desired, proofreading polymerases can be used, thereby virtually maintaining the original repertoire of the library (He et al. 1999). On the other hand, if *in vitro* evolution of a protein is desired, the diversification can be additionally enhanced by error-prone PCR (Cadwell and Joyce 1992; Zacco et al. 1996) and DNA shuffling (Stemmer 1994).

In comparison, all selection methods that use cells to express proteins, whether in the cytoplasm, on the cell surface, or on phages secreted from the cells, require a switch between *in vitro* diversification and *in vivo* selection in every selection round. After every *in vitro* diversification step, the newly generated library has to be religated and retransformed into the host organism, which is a rather time-consuming and laborious procedure. An alternative would be to use *E. coli* mutator strains (Irving et al. 1996; Low et al. 1996), but the error rate cannot be as well controlled as by *in vitro* methods.

The Principle of Ribosome Display

A prerequisite of protein selection is the coupling of genotype (RNA, DNA) and phenotype (protein). In ribosome display, this link is accomplished during *in vitro* translation by the ribosomal complexes, consisting of messenger RNA (mRNA), the ribosome, and the nascent polypeptide, which can fold correctly while still attached to the ribosome (Fig. 1) (Hanes and Plückthun 1997).

The DNA library coding for particular proteins of interest (for instance, a library of single-chain fragments of an antibody [scFv]) is transcribed *in vitro*. The mRNA is purified and used for *in vitro* translation. Because the stop codon has been removed from the protein encoding sequences in the DNA library, the ribosome stalls at the 3' end of the mRNA during *in vitro* translation, giving rise to a ternary complex of mRNA, ribosome, and encoded protein (Fig. 1). The protein can fold correctly on the ribosome, because a carboxy-terminal spacer had been genetically fused to it, thus allowing the protein of interest to fold outside of the ribosomal tunnel. The ribosomal complexes are stabilized by high concentrations of magnesium ions and low temperature. After *in vitro* translation, the ribosomal complexes are used directly for selection either on a ligand immobilized on a surface or in solution, with the bound ribosomal complexes subsequently being captured by streptavidin-coated beads. In several washing steps, the nonbinding complexes are removed and the mRNA of the selected complexes can be eluted by dissociation of

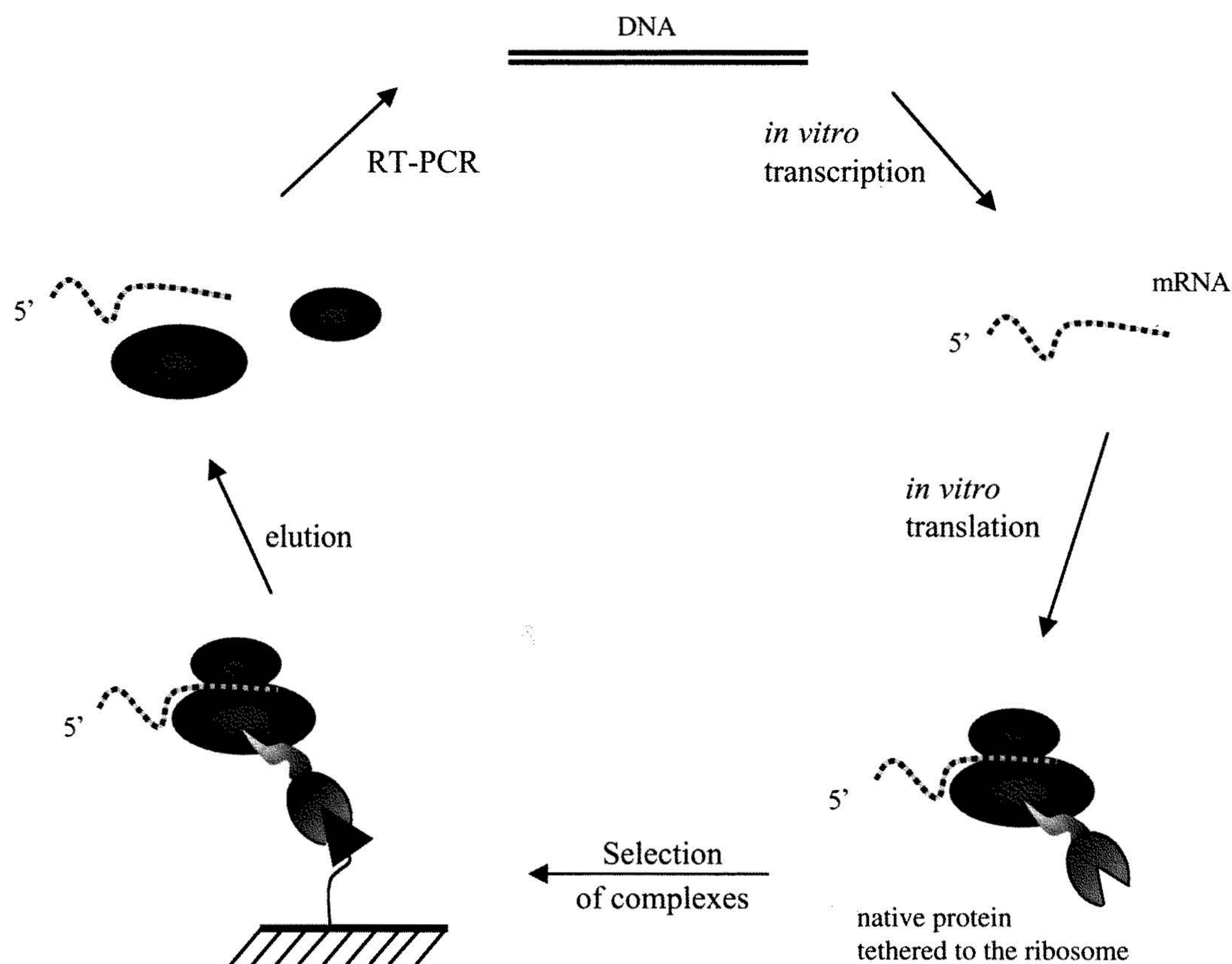


FIGURE 1. Principle of ribosome display. A DNA library encoding open reading frames lacking stop codons is transcribed *in vitro*. The mRNA is purified and used for *in vitro* translation. The ribosome stalls at the end of the mRNA. The encoded protein is not released and can fold correctly on the ribosome. The mRNA–ribosome–protein complexes are used for affinity selection on an immobilized target. After washing, the bound ribosomal complexes are dissociated. The mRNA is purified and used for reverse transcription and PCR amplification. The PCR product can be used directly for the next ribosome display selection cycle.

the ribosomal complexes with EDTA. The mRNA is purified, reverse-transcribed, and amplified by PCR. During the following PCR amplification, appropriate primers reintroduce the T7 promoter and the Shine-Dalgarno sequence. The resulting pooled DNA can either be used directly for the next selection cycle or for radioimmunoassays (RIAs), or it can be cloned in a vector for sequencing and large-scale expression of the selected binders. The enrichment of a binder after one round of ribosome display is typically 100- to 1000-fold (Hanes and Plückthun 1997).

Applications of Ribosome Display

Ribosome display was first established for the selection of peptide ligands against a protein target (Mattheakis et al. 1994; Gersuk et al. 1997). The method was further developed in our laboratory for the display of whole functional proteins that have to be correctly folded while still bound to the ribosome (Hanes and Plückthun 1997). In a model system using two distinct scFv fragments of an antibody, a 10^9 -fold enrichment of a specific scFv over the nonspecific scFv was achieved by five selection cycles of ribosome display, with an average enrichment of 100 per cycle (Hanes and Plückthun 1997). Second, ribosome display was applied to the selection and simultaneous evolution of a scFv fragment binding with 40 pM affinity to a Gcn4p mutant peptide, using a library prepared from the spleen of immunized mice (Hanes et al. 1998).

Subsequently, it was demonstrated that it is possible to generate antibodies of novel specificity *de novo*. Starting from the human combinatorial antibody library HuCAL (Knappik et al. 2000), picomolar affinity binders to insulin were selected and evolved during ribosome display selection (Hanes et al. 2000). All antibodies selected had accumulated many mutations during the PCR amplification cycles included in the ribosome display protocol, and the affinity of the antibodies improved up to 40-fold compared to the antibodies initially present in the library. In a selection against an unusual DNA structure, namely the guanine quadruplex DNA, it was demonstrated that antibodies with high specificity could be generated by ribosome display (Schaffitzel et al. 2001). The selected anti-guanine quadruplex antibodies were applied *in vivo* in macronuclei of the ciliate *Stylonychia lemnae* and provided the first evidence for the existence of guanine quadruplex DNA in a physiological system (Schaffitzel et al. 2001).

Ribosome display can also be used as an *in vitro* evolution method starting from a single protein. In fact, by combination of ribosome display with error-prone PCR and DNA shuffling, the affinity of a scFv for its antigen was improved 30-fold, to picomolar affinity (Jermutus et al. 2001). Furthermore, ribosome display was used to improve the stability of a scFv fragment by addition of increasing amounts of dithiothreitol (DTT) during sequential rounds of *in vitro* translation (Jermutus et al. 2001). Using this strategy, an antibody was selected that maintained proper folding in the presence of 10 mM DTT, which is comparable to the reducing environment of the cytoplasm in which disulfide bridges usually cannot be formed. These examples suggest that ribosomal display could constitute a general and rapid method for the generation of intrabodies (antibodies that can be used in the cytoplasm). Finally, selection in the presence of a “suicide substrate” inhibitor, an active β -lactamase, was enriched over an inactive mutant with ribosome display, indicating that ribosome display can also be used for enzyme selection (P. Amstutz et al., unpubl.).

In the following protocols, we describe ribosome display selection using an *E. coli* S30 extract for *in vitro* translation. It should be mentioned, however, that eukaryotic *in vitro* translation systems such as the rabbit reticulocyte lysate and the wheat germ lysate can also be used in ribosome display (Gersuk et al. 1997; He and Taussig 1997; Hanes et al. 1999; He et al. 1999).

OUTLINE OF PROCEDURE

The Construct Used for Ribosome Display

Several features of the ribosome display construct (Fig. 2) are important for efficient ribosome display of proteins. On the DNA level, the construct needs a T7 promoter for strong *in vitro* transcription to generate mRNA. On the mRNA level, the construct contains the ribosome-binding site, followed by the sequence encoding the protein (library) to display. The sequence for the protein library is followed by a spacer sequence fused in-frame to the protein. The carboxy-terminal spacer tethers the nascent protein to the ribosome, and it keeps the structured part of the protein outside the ribosomal tunnel, allowing folding and interaction of the protein with ligands. One successfully applied spacer sequence is derived from gene III of filamentous phage M13, covering amino acids 130–204 (SwissProt P03622). Alternatively, spacers derived from *E. coli* genes, namely *tolA* or *tonB*, can be used as spacer. For *tolA*, the amino acids 130–204 were used (SwissProt JV0057), and for *tonB*, amino acids 62–289 (SwissProt PO2929). The open reading frame extends to the very end of the DNA used as the template for transcription, and there is no stop codon present. The presence of a stop codon would lead to the release of the protein, and thereby the ribosomal complexes would dissociate.

At both ends of the mRNA, the ribosome display construct should include stem-loops. 5′ and 3′ stem-loops are known to stabilize mRNA against RNases *in vivo* as well as *in vitro* (Hajnsdorf et al. 1996). The presence of stem-loops is important, especially in the *E. coli* ribosome display system, because at least five of 20 *E. coli* RNases have been shown to contribute to mRNA degrada-

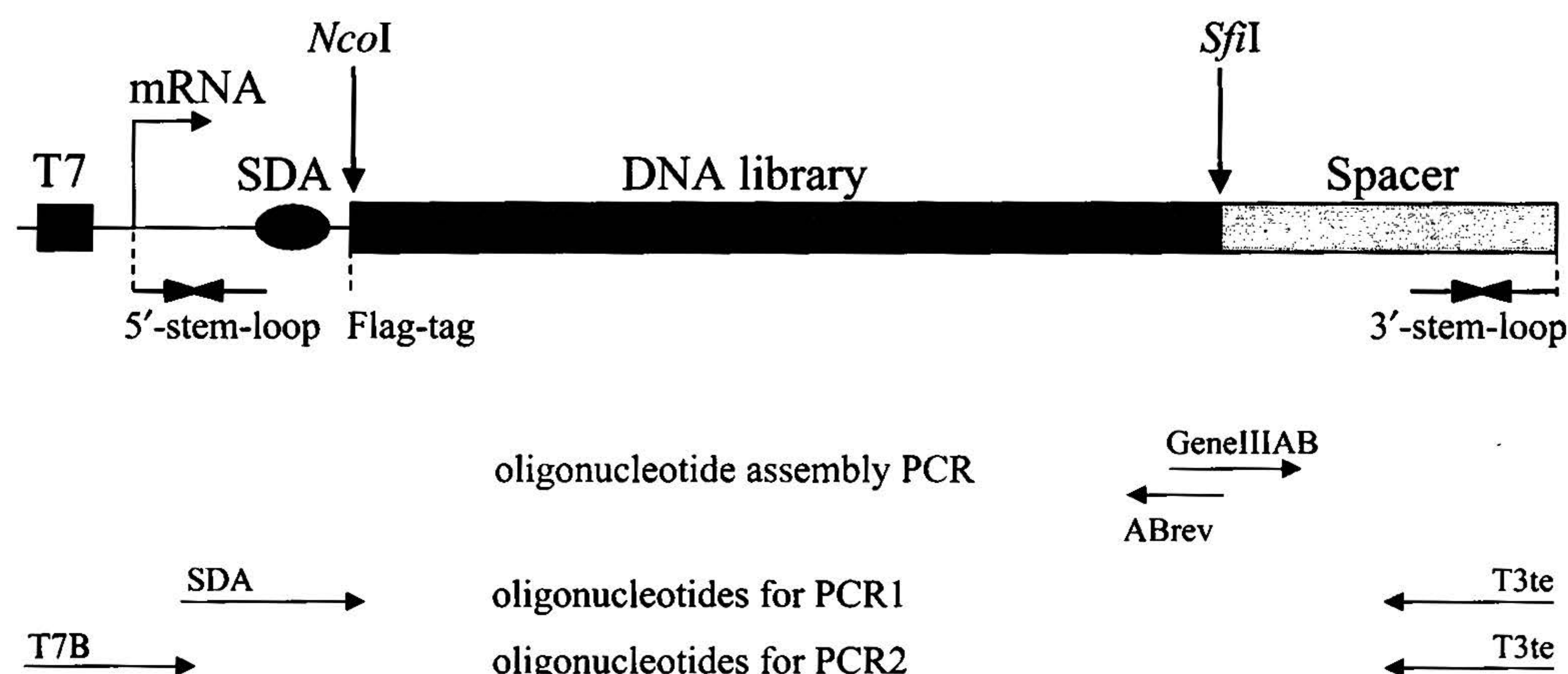


FIGURE 2. Construct used for ribosome display. A T7 promoter and a ribosome binding site (SDA) are necessary for in vitro transcription and translation. The coding sequence starts with a Flag-tag, the DNA library, and a spacer at the carboxyl terminus. The stop codon has been removed from the coding sequence. On the mRNA level, the construct is protected against ribonucleases by a 5' and a 3' stem-loop. Information about the oligonucleotides used in PCR is given in the text. The restriction sites used in assembly of the construct are indicated.

tion (Hajnsdorf et al. 1996), and they are probably all present in the S30 extract used for in vitro translation. The efficiency of ribosome display increased approximately 15-fold (Hanes and Plückthun 1997) when the 5' stem-loop derived from gene 10 of phage T7 (Table 1) and the 3' stem-loop derived from the early terminator of phage T3, slightly modified to give an open reading frame (Table 1), were introduced into the ribosome display construct (Hanes and Plückthun 1997).

TABLE 1. Oligonucleotides Used in Ribosome Display

Primer	Sequence	Remarks
T3te	5'-GGCCCACCCGTGAAGGTGAGCCTCAGTAG CGACAG-3'	introduces the 3'-stem-loop derived from the translated early terminator of phage T3; anneals to the gene III spacer
SDA	5'-AGACCACAACGGTTTCCCTCTAGAAATA ATTTTGTTTAACTTT AAGAAGGAGATATATCCATGGACTACAAAGA-3'	introduces the ribosome-binding site (bold); used for the first PCR amplification step; underlined is the <i>NcoI</i> restriction site for cloning
T7B	5'-ATACGAAATTAATACGACTCACTATAGGG AGACCACAACGG-3'	introduces the T7 promoter (bold) and the 5'-stem-loop; used for the second PCR amplification step, the transcription start is underlined.
GeneIIIAB	5'-GAGGGCGGCGGTTCTGGTT-3'	contains a 5'-overhang encoding the 3'-end of the DNA library for assembly PCR
tolAfwd	5'-TATATGGCCTCGGGGGCCGAATTCCAGA AGCAAGCTGAAG-3'	forward primer to amplify the <i>E. coli</i> gene <i>tolA</i> with <i>EcoRI</i> and <i>SfiI</i> restriction sites
tolArev	5'-GGACTGAAGCTTATCACGGTTTGAAGT CCAATGGCG-3'	reverse primer to amplify <i>tolA</i> spacer
tonBfwd	5'-TATATGGCCTCGGGGGCCGAATTCAGCCG CCACCGGAG-3'	forward primer to amplify the <i>E. coli</i> gene <i>tonB</i> with <i>EcoRI</i> and <i>SfiI</i> restriction sites
tonBtotrev	5'-CCGCACACCAGTAAGGTGTGCGGTCAGGA TATTCACCACAATCCC-3'	reverse primer to amplify <i>tonB</i> spacer and to introduce a 3'-stem-loop
ABrev	library dependent	reverse primer that encodes the 3'-end of the DNA library
anti-ssrA	5'-TTAAGCTGCTAAAGCGTAGTTTTTCGTCGT TTGCGACTA-3'	inhibits the 10S-RNA peptide tagging system
anti-SDA	5'-TCTTTGTAGTCCATGGATATATCTCCTTCTT AAAGTTAAACAAAATTATTTCTAGAGGGA-3'	used for northern blotting

Generation of the Ribosome Display Construct

The ribosome display construct can be easily and rapidly prepared completely in vitro by ligation or by assembly PCR, with the advantage of not losing any diversity of the library in transformation steps. As an example, we describe the generation of a construct starting from a scFv library. As a spacer, we use the carboxy-terminal domain of the gene III protein, which can be prepared by digestion of the vector pAK200 (Krebber et al. 1997) with *Sfi*I and *Hind*III.

The necessary steps to generate a scFv library either from natural sources or synthetically are described elsewhere (Krebber et al. 1997; Knappik et al. 2000). The DNA library needs to encode constant regions at the amino- and carboxy-terminal ends to permit PCR amplification. In our construct, the coding sequence starts with a FLAG-tag used for detection of protein expression. The library is prepared and amplified by PCR using a primer that introduces an *Nco*I site at the amino terminus (e.g., SDA primer) and a primer that introduces an *Sfi*I restriction site at the carboxyl terminus before the stop codon of the scFv library if the library does not contain the restriction site already. After *Sfi*I digestion, the fragment encoding the library is purified by preparative gel electrophoresis. The DNA encoding the gene III spacer, which must be cut beforehand with *Sfi*I, is also purified by preparative gel electrophoresis and added in at least twofold excess to the ligation reaction with the DNA library fragment. The ligation reaction is amplified directly by PCR using the primers SDA and T3te (Table 1). The PCR product is purified by preparative gel electrophoresis or by using a PCR purification kit to remove the oligonucleotides. In a second PCR, the primers T7B and T3te (Table 1) are used.

Alternatively, assembly PCR can be used for the generation of the ribosome display construct. The advantage of assembly PCR over the ligation protocol is that handling is relatively fast and easy. Because the ligation is more efficient, however, assembly PCR is not recommended for the cloning of very complex libraries into the ribosome display format. For assembly PCR using the gene III spacer, the spacer encoding the carboxy-terminal domain of the gene III protein is PCR-amplified from the plasmid pAK200 (Krebber et al. 1997) using the primers Gene-IIIAB (containing a 5' overhang encoding the 3' end of the DNA library) and T3te. In parallel, the scFv library is PCR-amplified using the primers SDA and ABrev. Both PCR products are purified by gel electrophoresis. The assembly PCR is carried out by adding equimolar amounts of DNA of the scFv library and gene III spacer to the PCR without using any primers in the first few cycles, to obtain an assembled library–gene III fusion product. In the last 10–15 cycles, the primers SDA and T3te are added to the assembly PCR to amplify the desired ribosome display construct.

The *tolA* and *tonB* spacers can be directly amplified from any *E. coli* colony. For this purpose, a small amount of a colony (any *E. coli* strain; we normally use SB536; Bass et al. 1996) is picked with a toothpick and then transferred into a standard PCR mix containing primers *tolA*fwd/*tolA*rev or *tonB*fwd/*tonB*totrev, respectively. After 30 cycles of PCR, a sharp band appears that can be gel-purified. This DNA can be directly used for assembly PCR. Of course, these spacers can be amplified or digested from a plasmid containing the cloned spacers.

The quality of the PCR product is crucial for ribosome display. Thus, it should be checked on an analytical agarose gel that the PCR product contains one single strong DNA band of the expected size and no smears or by-products, which both would reduce the efficiency of ribosome display. The DNA concentration should be 20 ng/μl or more.

In Vitro Transcription

The PCR product (with the T7 promoter, the ribosome-binding site, the DNA library with the spacer fused in-frame) can be directly used for in vitro transcription using T7 RNA polymerase (Pokrovskaya and Gurevich 1994). With the given protocol, ~0.1 mg of mRNA is obtained after 2–3 hours in a 200-μl transcription reaction with 1–2 μg of PCR product as a template. The transcribed mRNA can be purified by LiCl precipitation and a subsequent ethanol precipitation.

In Vitro Translation Using *E. coli* S30 Cell Extract

For *E. coli* in vitro translation, the preparation of S30 extracts from *E. coli* MRE600 cells (Wade and Robinson 1996) is carried out following a modified protocol, based on the procedure described by Chen and Zubay (1983) and Pratt (1984). In particular, the reducing agents DTT and β -mercaptoethanol are omitted from all buffers for the display of proteins containing disulfide bridges. The *E. coli* system used for ribosome display needs to be optimized according to Pratt (1984) with respect to the concentration of Mg^{++} and K^+ ions, the amount of S30 cell extract used, and the translation time (Hanes et al. 1999). Protein synthesis follows a saturation curve reaching a plateau after ~30 minutes (Ryabova et al. 1997). At the same time, mRNA is continuously degraded. Thus, an optimal time exists, at which the concentration of intact mRNA–ribosome–protein complexes that can be used for selection is at a maximum. This optimal time for in vitro translation to be used for *E. coli* ribosome display is usually between 6 and 10 minutes after translation starts, but has to be optimized for each S30 batch. Although most proteins generally fold more efficiently at lower temperatures in vitro, we found that, at least for scFv fragments of antibodies, more ribosomal complexes containing functional protein were obtained when the reaction was carried out at 37°C, which may be attributed to the chaperone activity in the *E. coli* extract. It should be mentioned here that the synthesis of large proteins with a molecular weight >70,000 is not very efficient due to premature termination of translation (Ramachandiran et al. 2000).

An important prerequisite for efficient ribosome display is the elimination of 10Sa-RNA, a surveillance mechanism that is responsible for the release and degradation of proteins derived from mRNA lacking a stop codon (Keiler et al. 1996). To inhibit this degradation mechanism, an antisense oligonucleotide named anti-ssrA (Table 1) is added to the in vitro translation reaction, binding to the mRNA moiety of the 10Sa-RNA.

For the translation of proteins containing disulfide bridges, it is important to have an oxidizing environment. Thus, no DTT should be present in the translation reaction to allow formation of the disulfide bridges (Ryabova et al. 1997). During in vitro transcription, however, the presence of reducing agents is necessary for the stability of T7 RNA polymerase. Therefore, in vitro transcription and cell-free translation are carried out in two separate steps, requiring the isolation and purification of the mRNA. Protein disulfide isomerase (PDI), a eukaryotic chaperone that catalyzes the formation of disulfide bonds (Freedman et al. 1995), improves the efficiency of ribosome display of antibody fragments threefold (Hanes and Plückthun 1997).

Affinity Selection

The stopped diluted translation mixture can be used directly for selection experiments. The affinity selection should be performed on ice in the presence of 50 mM magnesium ions; under these conditions the ribosomal complexes are stable for at least 10 days (Jermutus et al. 2001).

If the presence of the *E. coli* proteins or other components of the translation mix is problematic in a particular experiment, ribosomal complexes can be purified by ultracentrifugation with a sucrose cushion (Mattheakis et al. 1996). When the stopped translation mix is applied to ultracentrifugation, the ribosomal complex will form a pellet and can be separated from free proteins and low-molecular-weight compounds, which stay in the supernatant. Gel filtration is a more gentle method for complex separation (P. Amstutz, unpubl.). Gel filtration columns separate ribosomal complexes efficiently from free protein and small-molecular-weight compounds. The fractionation range of the beads should be chosen from a range of 5×10^4 to 2×10^7 D (CL-4B Sepharose, Pharmacia) and the bed volume for this fractionation range should be four times the sample volume. The elution fraction containing the ribosomal complexes but no free protein can be established in a simple experiment (see below).

For affinity selection, the target protein can be immobilized on a surface. This procedure entails the risk that a protein target may be partially denatured on the surface due to hydropho-

bic interactions with the plastic surface, in which case the selected antibodies may not recognize the protein target in its native conformation. We found that the background (unspecific binding) is lower when selection is performed in solution. For selection in solution, the ligand has to be biotin-labeled, and the ribosomal complexes binding to the ligand are captured by streptavidin-coated magnetic beads. The ligand should contain a 30 Å linker to the biotin moiety, such that the ligand is still accessible to the ribosomally displayed protein and not hidden in the streptavidin-binding pocket. To prevent the selection of streptavidin binders, alternation between streptavidin-coated magnetic beads and avidin-agarose for capture after each round of ribosome display is recommended.

Selection is performed in the presence of sterilized, debiotinylated skimmed low-fat milk (1–2%) and 0.25% (w/v) heparin to prevent nonspecific binding of ribosomal complexes to surfaces and to decrease the background signal. In addition, heparin inhibits nucleases. After several washing steps, non- or weakly bound ribosomal complexes are mostly eliminated. The mRNA of bound ribosomal complexes is recovered by dissociation of the complexes with EDTA-containing buffer, which chelates magnesium ions that are required for stabilization of the ribosomal complexes. The recovery of the mRNA by dissociation of the complexes has the advantage that the protein–ligand interaction does not have to be disrupted, and thus high-affinity binders elute as efficiently as low-affinity binders.

After dissociation of the ribosomal complexes, mRNA is isolated, reverse-transcribed using the primer T3te (Table 1), and PCR-amplified with the primers SDA (Table 1) and T3te. The product of the first PCR is purified by preparative agarose gel electrophoresis and used as the template for the second PCR amplification with the primers T7B (Table 1) and T3te. The resulting PCR product can be directly used for the next ribosome display cycle, for radioimmunoassay (RIA), or cloned in an expression vector via *NcoI* and *SfiI*.

Whether or not there is an enrichment of specific binders can be controlled by performing a selection round on a nonspecific surface, i.e., without adding antigen (background control). If the DNA pool is enriched for specific binders, the PCR signal after ribosome display selection should be higher when antigen is present during selection.

Tailoring Molecules by Adapting the Selection Pressure

Affinity is essentially determined by the off rate, as on rates fall into a relatively narrow window (Schwesinger et al. 2000, and references therein). To select for very high affinities, off-rate selection has been applied successfully (Hawkins et al. 1992; Yang et al. 1995; Boder and Wittrup 1997; Chen et al. 1999; Jermutus et al. 2001). In principle, one could also use very small amounts of antigen, but in practice, this strategy does not appear to be successful for reasons discussed elsewhere (Plückthun et al. 2000). In off-rate selection, the ribosomal complexes formed after translation are first equilibrated with biotin-labeled antigen. The concentration of the labeled antigen is chosen such that basically all complexes carrying a binding moiety are bound. After equilibration, a large excess of competitive nonlabeled antigen is added. Every complex that dissociates from its labeled antigen will be captured by the competitive antigen and therefore not be captured by the streptavidin-coated magnetic beads. There is a simple correlation between the incubation time of the complexes with competitive antigen and the mean affinity of the complexes still being bound to the labeled antigen.

This type of selection can be performed not only for affinity, but also for many other physicochemical properties of the library. If the target protein, e.g., a scFv fragment, contains a disulfide bond needed for activity, a selection can be carried out for stability under reducing conditions (Jermutus et al. 2001). Several rounds of ribosome display with increasing concentrations of DTT present during the panning procedure lead to an increase in stability of the evolved molecules. Following this principle, selections for stability in the presence of organic solvents, detergents, or any other conditions are conceivable, provided that the ribosomal complex can be kept stable.

From Selection to Evolution

Ribosome display was first established as a very efficient method for in vitro selection (Hanes and Plückthun 1997) with enrichment factors up to 10^9 over five rounds. The complete in vitro nature of the method makes it very convenient to perform further randomization steps between different rounds of selection. The intrinsic diversification present in the original protocol, involving up to 100 cycles of PCR over all rounds, does introduce a number of mutations, especially when using nonproofreading *Taq* polymerase (Hanes et al. 1999). This effect can be enhanced by orders of magnitude by error-prone PCR and DNase I shuffling (Cadwell and Joyce 1992; Stemmer 1994; Zacco et al. 1996). These randomization steps are introduced after the reverse transcription (RT)-PCR.

The sequence randomization induced during error-prone PCR amplification is a well-established way to introduce mutations into a gene. Induction of errors is achieved either by addition of manganese to the PCR, which decreases the fidelity of most polymerases, or by the incorporation of dNTP analogs such as 8-oxo-guanosine or dPTP (Zacco et al. 1996). This leads to a mismatch base-pairing in the second-strand synthesis and, consequently, to a mutation at this position. The degree of randomization is dependent on the concentration of both manganese and dNTP-analogs and can thereby be controlled. It is worth mentioning that the number of mutations introduced is not only dependent on the polymerases used, or the concentration of additives during PCR amplification, but also on the number of PCR amplification steps.

DNase I shuffling is a highly efficient tool to introduce mutations into a gene and to recombine mutations from former selection rounds (Stemmer 1994). Because this uncouples mutations, which would otherwise be physically linked, and recombines them randomly, it is normally thought to ensure that not too many deleterious mutations are accumulated (Stemmer 1994).

After reverse transcription of the mRNA pool obtained after affinity selection, the DNA pool is PCR-amplified until a strong band appears on the analytical agarose gel. The DNA is then gel-purified and digested with DNase I until the mean fragment length is between 50 bp and 150 bp. It is worth taking a small sample after a given incubation time, shock-freezing the rest of the sample, and analyzing the degree of digestion on an agarose gel. This monitoring ensures that the fragments are not getting too small, which is undesirable and makes the assembly difficult. The digested fragments are purified over an agarose gel and isolated using a small fragment recovery kit. The fragments are then reassembled to a full-length construct using a special PCR containing Tween-20, or similar detergent, but no primers. The full-length band is isolated from an agarose gel and amplified with normal PCR to the ribosome display construct. A combination of both methods—error-prone PCR and DNase I shuffling—ensures a high degree of randomization, while decreasing the risk that the effects of beneficial mutations are hidden by deleterious ones.

RIA Analysis of the DNA Pool and of Single Clones

RIAs are performed to test for the presence of specific binders in a pool. In RIA, the in vitro translation is performed with radioactive [^{35}S]methionine, and thus the binding of radioactive protein to the immobilized ligand can be quantified. The translation time is generally about 30 minutes, which is when the protein synthesis reaches a plateau (Ryabova et al. 1997). After the translation is stopped, free ligand can be added for inhibition tests, or the translation mix is directly transferred to microtiter wells with immobilized ligand.

Several control experiments should be performed: The binding of pool-encoded proteins to a nonspecific surface (i.e., a milk-coated surface, or a neutravidin-coated surface) should be tested. Also, the RIA should be performed both in the presence and absence of free ligand as a competitor. If specific binders are present, the RIA signal should be higher on the specific surface compared to the control surface, and, particularly important, the binding should be inhibitable by the presence of free ligand that acts as a competitor.

If this is the case, the pool is clearly enriched for binders and the DNA encoding the pool is cloned in an expression vector via the *Sfi*I and *Nco*I restriction sites for identification of individual binders and for further characterization (Fig. 2; Table 1). The plasmid DNA of single clones is transcribed in vitro according to the protocol, and the mRNA of the single clones is used for RIA analysis to identify specific binders. The enriched DNA pool can also be cloned into a plasmid that adds a peptide detection tag to the protein. This allows the binders to be purified and further analyzed by enzyme-linked immunosorbent assay (ELISA), avoiding the use of radioactivity in RIA.

Optimization of Ribosome Display

Ribosome display should first be established and optimized with a defined, well-known model system. The model protein, e.g., an antibody scFv fragment, should clearly bind to its ligand in ribosome display and thus give an enrichment on a surface coated with its ligand compared to an unspecific surface. As mentioned before, the in vitro translation should be optimized for the concentration of magnesium and potassium ions, the amount of extract used, and the translation time (Pratt 1984). These parameters need to be optimized for each new preparation of *E. coli* S30 cell extract. To establish the optimal ion concentrations, it is most convenient to carry out an in vitro translation of an enzyme, such as β -lactamase or firefly luciferase, with a simple activity assay. This way, protein production under different conditions can be monitored easily. It is recommended that Mg^{++} concentration be optimized first, and then that concentration be used in all other experiments.

To determine the optimal translation time, where maximal amounts of functional ribosomal complexes are present, in vitro translation and affinity selection have to be performed using a ribosome display construct encoding a model protein, e.g., an antibody. After various incubation times at 37°C, aliquots of the in vitro translation reaction are stopped. All of the aliquots are used for affinity selection with the cognate ligand immobilized on a surface. After affinity selection, the eluted mRNA can be used either for RT-PCR or for a northern blot. Northern blotting is more sensitive in detecting small differences in the mRNA amount. In northern blot hybridization, the isolated mRNA can be monitored and quantified with a digoxigenin-labeled probe that specifically anneals to the mRNA construct (Anti-SDA, Table 1). Figure 3 shows the relationship of all of the steps in performing ribosomal display.

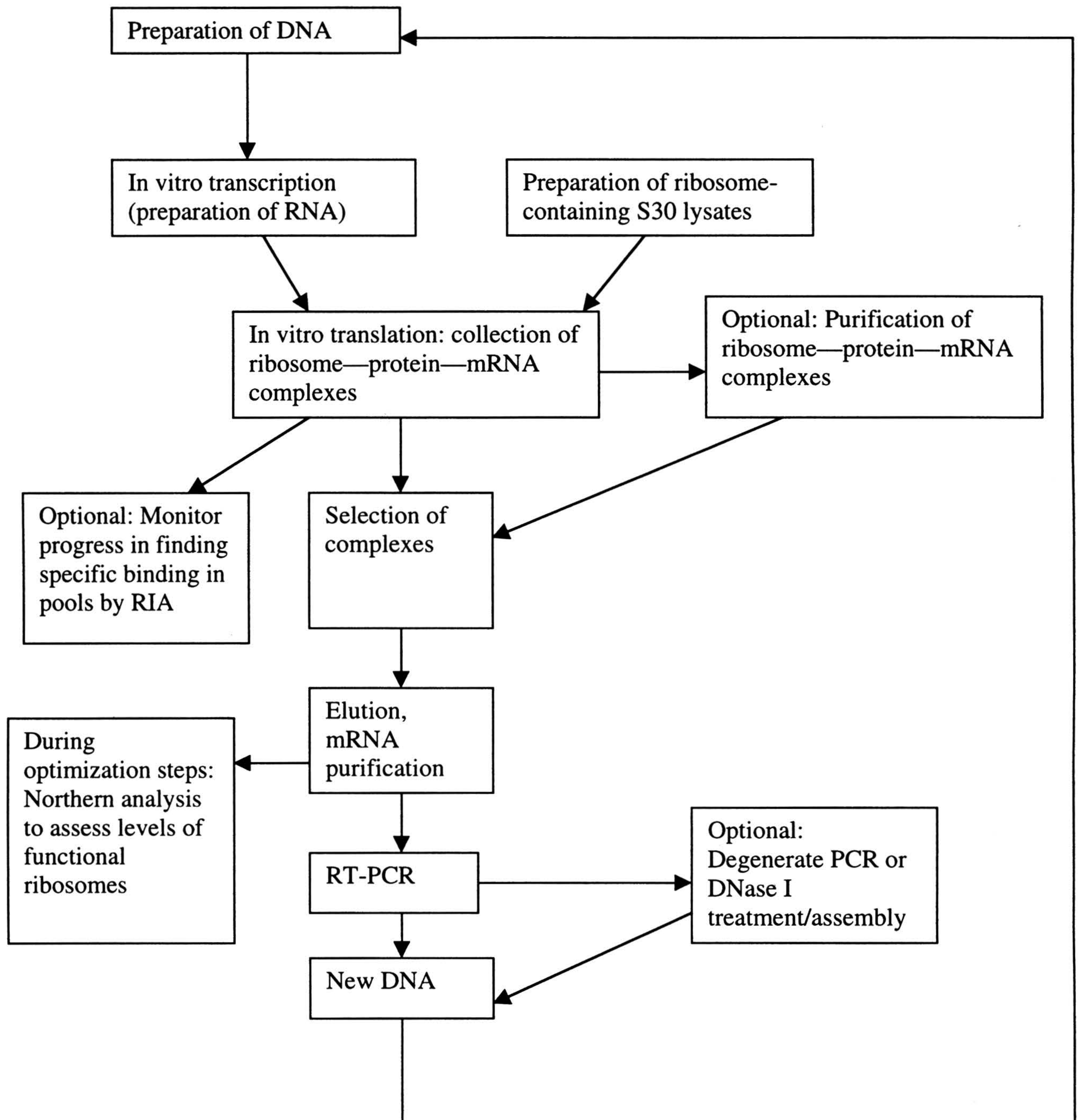


FIGURE 3. Flowchart of ribosomal display steps.

Protocol 1

Preparation of the Ribosome Display Construct by Ligation

Efficient ribosomal display of proteins is influenced by several factors, including the presence of a T7 promoter on the DNA level and the ribosome-binding site and the sequence encoding the protein on the RNA level. Figure 2 provides a diagram of the construct elements. This protocol describes the preparation of the basic DNA template for subsequent screening using either ligation or assembly PCR.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers, Solutions, and Reagents

dNTPs, 20 mM each (Roche Diagnostics)
Dimethylsulfoxide (DMSO) (Fluka)<!.>
Oligonucleotides SDA, T7B, T3te, Gene-IIIAB, ABrev (see Table 1)
QIAquick gel extraction kit (Qiagen)
Tris-HCl (10 mM, pH 8.5)

Biological molecules

SfiI/NcoI fragment of protein encoding DNA library, e.g., scFv antibody library (150 ng, $\sim 2 \times 10^{11}$ molecules)
SfiI/HindIII fragment of the gene III spacer
T4 DNA ligase (Roche Diagnostics)
Taq DNA polymerase (GIBCO BRL)

Special Equipment

Apparatus for agarose gel electrophoresis
Thermocycler

METHOD

Preparation by Ligation

1. Digest the vector pAK200 (Krebber et al. 1997) with *HindIII* and *SfiI* and purify the resulting 481-bp fragment encoding the gene III spacer by agarose gel electrophoresis using the QIAquick gel extraction kit.
2. Digest the plasmid encoding the library with *SfiI* and *NcoI* and purify it by agarose gel electrophoresis.

3. Ligate 150 ng of DNA fragment encoding the DNA library to a threefold excess of gene III spacer overnight at 16°C using 10 units of T4 DNA ligase.
4. Perform PCR amplification as described in step 5 of mRNA Purification and RT-PCR protocol (Protocol 6, p. 557) using the primers SDA and T3te. Use 5–10 µl of the ligation mix as PCR template for a 50-µl PCR. The PCR program is 4 minutes at 94°C, then 5 cycles of 30 seconds at 94°C, 30 seconds at 37°C, 2.5 minutes at 72°C, followed by 15 to 20 cycles with the same settings except with 50°C annealing temperature instead of 37°C, and finished by 10 minutes at 72°C.
5. Purify the PCR product by agarose gel extraction, and use it for the second PCR with the primers T7B and T3te (see step 7 in mRNA Purification and RT-PCR, Protocol 6, p. 557). Usually, 12–16 cycles are performed.

Preparation of the Ribosome Display Construct by Assembly PCR

1. PCR-amplify the carboxy-terminal domain of the gene III protein from the vector pAK200 with the primers Gene-IIIAB and T3te, and the DNA library with the primers SDA and ABrev.
2. Purify the PCR products by agarose gel extraction, and use equimolar amounts of each DNA for the assembly PCR. Perform the assembly PCR as follows: 4 minutes at 94°C, then 5 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 2.5 minutes at 72°C, followed by adding the primers SDA and T3te and performing an additional 12 cycles of 30 seconds at 94°C, 30 seconds at 45°C, and 2.5 minutes at 72°C.
3. Purify the PCR product of the appropriate size by agarose gel extraction, and perform the last PCR introducing the T7 promoter with the primers T7B and T3te (see step 7 in mRNA Purification and RT-PCR, Protocol 6, p. 557).

Protocol 2

In Vitro Transcription and mRNA Purification

The DNA template from Protocol 1 is used to prepare the mRNA template by in vitro transcription. The transcribed mRNA is then purified by LiCl precipitation and ethanol precipitation.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers and Solutions

Ethanol <!.>
Guanidinium isothiocyanate (1 M) <!.>
Lithium chloride (6 M) (LiCl) (Fluka)<!.>
5X Loading buffer
 50% glycerol
 200 mM Tris-HCl (pH 8)
 100 mM acetic acid <!.>
 5 mM EDTA
 bromophenol blue <!.>
NTPs: ATP, CTP, GTP, TTP (50 mM each) (Sigma)
RNA denaturation buffer
 10 µl of formamide<!.>
 3.5 µl of formaldehyde <!.>
 2 µl of 10X MOPS buffer (0.2 M MOPS [pH 7.0], 80 mM sodium acetate, 10 mM EDTA)
Sodium acetate (3 M, pH 5.2)
5X T7 RNA polymerase buffer
 1 M HEPES-KOH <!.> (pH 7.6)
 150 mM magnesium acetate
 10 mM spermidine
 0.2 mM DTT <!.>
TBE buffer
 90 mM Tris-HCl
 90 mM boric acid
 10 mM EDTA

Biological Molecules

RNasin (40 units/µl, Promega, Madison, Wisconsin)
T7 RNA Polymerase (New England Biolabs, Beverly, Massachusetts)

Special Equipment

Apparatus for gel electrophoresis
Heating block
Tabletop refrigerated microcentrifuge

METHOD

1. For the in vitro transcription reaction, thaw and mix the following reagents on ice:

40 μ l	5 $\times$ T7 RNA polymerase buffer
28 μ l	NTPs (50 mM each)
8 μ l	T7 RNA polymerase (50 units/ μ l)
4 μ l	RNasin (40 units/ μ l)
75 μ l	RNase-free H ₂ O
45 μ l	PCR-template (from 1) (0.5–1 μ g of DNA)
200 μ l	

Incubate for 2–3 hours at 37–38°C.

2. For mRNA purification, combine:

200 μ l	in vitro transcription mixture
200 μ l	RNase-free H ₂ O
400 μ l	6 M LiCl
800 μ l	

Keep on ice for 30 minutes.

3. Centrifuge for 20–30 minutes at 14,000 rpm (4°C), discard the supernatant, and wash the pellet once with 500 μ l of 70% ethanol.
4. Dry the pellet, resuspend it in 200 μ l of RNase-free H₂O, and centrifuge at 14,000 rpm for 5 minutes at 4°C.
5. Mix 180 μ l of the supernatant, 18 μ l of 3 M sodium acetate, and 500 μ l of ethanol (97%) and incubate for 30 minutes on ice.
6. Centrifuge at 14,000 rpm for 20–30 minutes at 4°C.
7. Wash the pellet with 500 μ l of 70% ethanol, air-dry, and resuspend it in 40 μ l of RNase-free H₂O.
8. Determine the concentration of mRNA as follows:
 - a. Measure the OD at 260 nm (an OD₂₆₀ of 1 corresponds to 40 μ g/ml).
 - b. Run an analytical agarose gel (1.5%): Add 10 μ l of RNA denaturation buffer to 1 μ g of mRNA on ice and incubate for 10 minutes at 70°C. Chill the samples on ice, mix with 2 μ l of gel loading buffer and separate by 1.5% agarose gel electrophoresis in TBE buffer and in the presence of 1 M guanidinium isothiocyanate.

The scale of the transcription reaction can be adjusted. Care should be taken, however, that the diversity of the DNA library is conserved. Thus, the number of molecules of DNA template used should be several times higher than the diversity of the library.

Protocol 3

Preparation of *E. coli* S30 Cell Extract

Described here is the preparation of S30 cell lysates, the source of ribosomes for subsequent in vitro translation. This protocol is based on the procedures described by Chen and Zubay (1983) and Pratt (1984).

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers and Solutions

Incomplete rich medium

5.6 g of potassium dihydrogen phosphate (KH_2PO_4)<!.>

28.9 g of potassium hydrogen phosphate (K_2HPO_4)<!.>

10 g of yeast extract

15 mg of thiamine per 1 liter of culture medium

Autoclave the medium first and then add 25 ml of 40% (w/v) glucose, sterile-filtered.

Preincubation mix (10 ml total volume):

3.75 ml of 2 M Tris-acetate (pH 7.5 at 4°C)

71 μl of 3 M magnesium acetate

75 μl of amino acid mix (10 mM of each of the 20 amino acids; Sigma)

0.3 ml of 0.2 M ATP

0.2 g of phosphoenolpyruvate (Sigma)

50 units of pyruvate kinase (Sigma P 1506)

The preincubation mix must be prepared fresh immediately before use.

S30 buffer

10 mM Tris-acetate (pH 7.5 at 4°C)

14 mM magnesium acetate

60 mM potassium acetate

Store at 4°C or chill the buffer solution before use.

Biological Molecules

Escherichia coli strain MRE600 (Wade and Robinson 1966)

Special Equipment

Baffled flask (5-liter)

Dialysis tubing with a cutoff of 6000–8000 D

French press

Refrigerated centrifuge (30,000g)

Shaker for bacterial culture at 25°C and 37°C

METHOD

1. Grow a 100-ml starter culture of *E. coli* MRE600 overnight at 37°C in incomplete rich medium with shaking.
2. The next day, inoculate 1 liter of incomplete rich medium in a 5-liter baffled shaker flask with 10 ml of the overnight culture and grow at 37°C.
3. Harvest the cells at OD₅₅₀ of 1.0 (corresponding to the early exponential growth phase) by centrifugation at 3,500g for 15 minutes at 4°C.
4. Discard the supernatant and wash the pellet three times with 50 ml of ice-cold S30 buffer per liter of culture.
The cell pellet can be frozen at -80°C or in liquid nitrogen and stored for up to 2 days.
5. Thaw the cell pellet on ice and wash it once again with S30 buffer.
6. Weigh the cell pellet and resuspend it in ice-cold S30 buffer at a ratio of 1.27 ml of buffer per gram of wet cells.
7. Lyse the cells by one passage through a French press using a chilled French press cell at 6000 psi.
More than one passage of the cell suspension results in decreased translation activity of the cell extract.
8. Centrifuge the lysed cells immediately at 30,000g for 30 minutes at 4°C.
9. Transfer the supernatant to a clean centrifuge tube and centrifuge again at 30,000g at 4°C for 30 minutes.
10. Transfer the supernatant of the second centrifugation again to a clean flask and add 1 ml of preincubation mix for each 6.5 ml of S30 extract. Shake this solution slowly for 1 hour at 25°C (no foaming should occur).
During this time, all translation of endogenous mRNA will be finished and the endogenous mRNA and DNA will be degraded by nucleases present in the cell extract.
11. Transfer the S30 cell extract to dialysis tubing and dialyze in the cold room three times against a 50-fold volume of chilled S30 buffer. Replace each dialysis solution after 1 hour.
12. Centrifuge the cell extract at 4000g for 10 minutes at 4°C and freeze the supernatant in aliquots of 100–500 µl in liquid nitrogen. Store it at -80°C.
The extract can be stored for months without losing activity. It can even be frozen a second time after thawing. However, if the extract is thawed more than twice, it starts losing activity.

Protocol 4

In Vitro Translation

The actual process of in vitro translation to prepare mRNA- and protein-tethered ribosomes is described in this protocol.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers and Solutions

Anti-ssrA oligonucleotide (200 μ M) (see Table 1)
Methionine in H₂O (200 mM)
Magnesium acetate in H₂O (100 mM)
Potassium glutamate in H₂O (2 M)
PremixZ
250 mM Tris-acetate (pH 7.5 at 4°C)
1.75 mM of each amino acid except methionine
10 mM ATP
2.5 mM GTP
5 mM cAMP
150 mM acetylphosphate
2.5 mg/ml *E. coli* tRNA
0.1 mg/ml folinic acid
7.5% PEG 8000 <!.>
Washing buffer WBTH
50 mM Tris-acetate (pH 7.5)
150 mM NaCl
50 mM magnesium acetate
0.1% Tween-20
2.5 mg/ml heparin (Sigma)

Biological Molecules

E. coli S30 cell extract (from Preparation of *E. coli* S30 Cell Extract protocol, p. 550)
Library mRNA (1 μ g/ μ l) (from In Vitro Transcription and mRNA Purification protocol, p. 548)
Protein disulfide isomerase (22 μ M) (bovine PDI) (Sigma) in H₂O

Special Equipment

Heating block
Tabletop refrigerated microcentrifuge

METHOD

1. Chill all solutions and mix the translation reaction on ice in the indicated order:

14.3	μl	RNase-free H ₂ O
11	μl	Potassium glutamate (2 M) (see note below)
7.6	μl	Magnesium acetate (0.1 M) (see note below)
1.1	μl	Methionine (200 mM)
2	μl	Anti-ssrA oligonucleotide
22	μl	PremixZ ice-cold (thaw on ice and vortex before pipetting)
40	μl	<i>E. coli</i> S30 extract
2	μl	PDI (if disulfides are present in the target protein library)
10	μl	Library mRNA (10 μg), thaw just before use and freeze the
—————		remainder immediately
110	μl	

To optimize translation conditions, test the following conditions: for each new batch of S30, extract 11–15 mM magnesium acetate, 180–220 mM potassium glutamate, and 20–50 μl of S30 extract for a 110-μl reaction.

2. Incubate the translation reaction at 37°C for the optimized translation time (usually 6–15 minutes).
3. Stop the translation with 440 μl of ice-cold WBTH, vortex briefly, and gently place on ice.
4. Centrifuge the translation mix at 14,000g for 5 minutes at 4°C and transfer to a new ice-cold tube.

Protocol 5

Affinity Selection

This procedure details the affinity selection of ribosomal complexes using either panning or a solution-based method. Variations that enable the selection of proteins with lengthened off-rate or increased stability forms of proteins are also noted.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers and Solutions

Elution buffer (EB)
50 mM Tris-acetate (pH 7.5 at 4°C)
150 mM NaCl
20 mM EDTA
50 µg/ml *S. cerevisiae* RNA (Sigma)
Low-fat skim milk (sterilized) (12% in H₂O)
PBS buffer
137 mM NaCl
2.7 mM KCl <!.>
10 mM Na₂HPO₄ <!.>
1.8 mM KH₂PO₄ <!.>
10x Washing buffer (WB)
0.5 M Tris-acetate (pH 7.5 at 4°C)
1.5 M NaCl
0.5 mM magnesium acetate
1x Washing buffer (WBT)
50 mM Tris-acetate (pH 7.5 at 4°C)
150 mM NaCl
50 mM magnesium acetate
0.1% Tween-20

Biological Molecules

Avidin immobilized on agarose beads (Sigma)
Biotinylated ligand and free ligand
Streptavidin magnetic particles (Roche Diagnostics)

Special Equipment

Magnet
Microtiter plate strips or plates (Nunc)
Panning tubes (Nunc)
Rocking table or shaker

METHOD

Selection Using an Immobilized Target Protein

It is important that the buffers, microtiter plates, and pipette tips are temperature-equilibrated in the cold room before use.

1. Coat the microtiter wells overnight at 4°C with 400 ng of ligand in 100 µl of PBS.
2. Wash the coated microtiter plates or strips with PBS and block for 1 hour with 4% milk powder in PBS. As a control surface, block several noncoated microtiter wells with milk.
3. Wash the wells three times with PBS and twice with washing buffer WBT.
4. Fill the wells with 250 µl of ice-cold washing buffer WBT and put on ice.
5. For affinity selection, remove the washing buffer WBT from the ice-cold microtiter wells.
6. Supplement the *in vitro* translation mix (from the *In Vitro* Translation protocol, p. 552) with ice-cold sterilized milk in WBT to a final concentration of 2% (w/v) milk and transfer 200 µl of translation mix in each ligand-coated and milk-blocked microtiter well.
7. Gently shake the microtiter plates for 1 hour in the cold room. Pour off the translation reaction and then wash five times with WBT.
8. For elution, add 200 µl of ice-cold elution buffer EB for 5 minutes on ice and shake it gently. The eluted mRNA must be immediately purified (see step 1 in mRNA Purification and RT-PCR protocol, p. 557).

Alternative Protocol: Selection in Solution

1. Block 5-ml panning tubes with 4% (w/v) milk in PBS for 1 hour by end-over-end rotation.
2. Wash the 5-ml panning tubes three times with PBS and three times with washing buffer WBT, and then finally fill with WBT.
3. Remove biotin from the sterilized low-fat skim milk before use by end-over-end rotation of 1 ml of sterilized 12% (w/v) milk powder with 100 µl of streptavidin-coated magnetic beads for 1 hour at room temperature. Remove the streptavidin-coated beads with a magnet and discard them; transfer the milk in a new tube. Store it on ice.
4. Wash 100 µl of streptavidin-coated magnetic beads four times with ice-cold washing buffer WBT and resuspend the beads in their original volume in ice-cold WBT.
5. Empty the immunotubes and add 60 µl of sterilized, biotin-depleted low-fat skim milk to 1–2% (w/v) final concentration.
6. Add the *in vitro* translation mix (from mRNA Purification and RT-PCR protocol, p. 557) and 10 pmoles of biotinylated ligand to the panning tubes. Seal the immunotubes, put them into a larger tube (e.g., a 250-ml centrifuge tube that is filled with ice), and rotate end-over-end for 1 hour in the cold room.
7. For capture, add 100 µl of streptavidin-coated magnetic beads to the tubes and rotate the tube end-over-end on ice for 15 minutes in the cold room.
8. Pour off the translation reaction, then wash the magnetic beads five times with WBT, and bind them to the side of the tube with a magnet.

9. For elution, add 200 μ l of ice-cold elution buffer EB for 5 minutes on ice and shake it gently. The eluted mRNA must be immediately purified (see mRNA Purification and RT-PCR, Protocol 6, p. 557).

The capacity of the streptavidin-coated magnetic beads is dependent on the size of the biotinylated ligand. Because it is important to ensure that all biotinylated ligand can be captured by the streptavidin-coated magnetic beads, not more than 10 pmoles of biotinylated ligand should be used per 100 μ l of streptavidin-coated magnetic beads, although this amount of beads can bind 100 times more free biotin. Alternating with streptavidin-coated magnetic beads, avidin-agarose should be used to avoid the selection for streptavidin-binding proteins.

Off-rate Selection

1. Perform steps 1–3 of the above protocol, Selection in Solution, to prepare the panning tubes.
2. Split the translation mix. Add to one reaction biotinylated antigen (concentration 1–100 nM; the better the binder is, the less antigen is needed).
3. Equilibrate for 2 hours to overnight.
4. Add competing antigen (1000-fold excess over the biotinylated antigen).
5. Incubate for off-rate selection time (2 hours to 15 days), depending on the expected off-rate of the best binders.
6. Wash the streptavidin-coated magnetic beads (see step 4 in the above protocol, Selection in Solution) and recover the ribosomal complexes (steps 7 and 8 in the above protocol, Selection in Solution).

Stability Selection of Disulfide Containing Proteins

1. Perform the selection as described in the Selection in Solution protocol, but during in vitro translation (step 1, In Vitro Translation protocol, p. 552) and during the affinity selection (step 6 in Selection in Solution protocol, above), add defined amounts of DTT (normally ranging from 0.5 to 10 mM).
2. Add DTT also for the analysis of the selected pools and single binders by RIA (see Radioimmunoassay, Protocol 9, p. 562).

Protocol 6

mRNA Purification and RT-PCR

The purpose of this protocol is the purification and subsequent use for RT-PCR of mRNA from the selected ribosomal complexes. It is difficult to predict the number of PCR cycles necessary to recover the genetic information, because this depends on the amount of mRNA eluted after affinity selection. Therefore, it is useful first to perform a few PCR cycles (15–20) and then check the PCR product on an analytical agarose gel. If necessary, add more PCR cycles. Usually the fewer cycles of PCR that are needed, the more binders are present in the library. However, overamplifying the PCR product is not recommended because this results in a smeary band on an agarose gel and subsequently, after in vitro transcription, in poor-quality RNA.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers, Solutions, and Reagents

dNTPs (20 mM each; Eurogentec)
Dimethylsulfoxide (DMSO) (Fluka) <!.>
Dithiothreitol (DTT) (0.1 M) <!.>
High Pure RNA Isolation Kit (Roche Diagnostics)
MgCl₂ (50 mM) <!.>
Oligonucleotide primers T3te, SDA, and T7B (see Table 1)
QIAquick gel extraction kit (Qiagen)
Tris-HCl (10 mM, pH 8.5)

Biological Molecules

Superscript reverse transcriptase
5X Superscript first-strand synthesis buffer (GIBCO BRL)
RNasin (Promega)
Taq DNA polymerase, 10X PCR buffer (GIBCO BRL)

Special Equipment

Apparatus for agarose gel electrophoresis
Heating block
Thermocycler

METHOD

1. Isolate the mRNA (from Affinity Selection protocol, p. 554) using the High Pure RNA isolation kit according to the manufacturer's instructions.

2. Elute the purified RNA in 35 μl of RNase-free H_2O and immediately denature for 10 minutes at 70°C for reverse transcription. Chill the mRNA samples for 1–2 minutes on ice after denaturation.

3. For reverse transcription, prepare a premix on ice as follows:

0.25	μl	T3te primer (100 μM)
0.5	μl	dNTP (20 mM each)
0.5	μl	RNasin (40 units/ μl , Promega)
0.5	μl	Superscript reverse transcriptase (GIBCO, 200 units/ μl)
4	μl	5x Superscript first-strand synthesis buffer (GIBCO)
2	μl	DTT (0.1 M)
7.75	μl	

Add 12.25 μl of denatured mRNA to this premix, mix, and centrifuge briefly at 4°C .

4. Incubate for 1 hour at 50°C .

In addition to the RT-PCR sample, a negative control should be performed without template to test the buffers and primers for contamination.

5. Set up the PCR on ice:

0.125	μl	T3te primer (100 μM)
0.125	μl	SDA primer (100 μM)
0.5	μl	dNTP (20 mM each)
0.25	μl	<i>Taq</i> Polymerase (5 units/ μl , GIBCO)
5	μl	10x PCR buffer (GIBCO)
2.5	μl	DMSO
1.55	μl	MgCl_2 (50 mM)
32.45	μl	H_2O
7.5	μl	DNA template, directly from reverse transcription
50	μl	

The PCR is carried out for 4 minutes at 94°C , followed by 20 cycles of 30 seconds at 94°C , 30 seconds at 50°C , 2.5 minutes at 72°C , and is finished by 10 minutes at 72°C .

6. Purify the PCR product by agarose gel electrophoresis.
7. Use the purified PCR product for the second PCR amplification using the same reaction setup (as above in step 5) and the primers T3te and T7B. After initial denaturation for 4 minutes at 94°C , 10–15 PCR cycles are performed with 30 seconds at 94°C , 30 seconds at 60°C , 2.5 minutes at 72°C , and the reaction is finished by 10 minutes at 72°C .

Protocol 7

Evolution: Introducing Additional Diversity

These PCR methods are used to introduce mutations and to recombine mutations from previous selection rounds. Degenerate PCR and DNase I digestion/reassembly methods can be incorporated into the selection procedure at the RT-PCR step to increase diversity for subsequent rounds of selection.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers, Solutions, and Reagents

dNTP-analogs

2 mM 6(2-deoxy- β -D-ribofuranosyl)-3,4-dihydro-8H-pyrimido(4,5-c)(1,2)-oxazin-7-one triphosphate (dPTP, Nucleix Plus, Amersham)

2 mM 8-oxo-2'-deoxyguanosine triphosphate (8-oxo-dGTP, Nucleix Plus, Amersham)

Triton X-100 (Fluka)

Oligonucleotide primers SDA, T7B, ABrev (see Table 1)

QIAex-II gel extraction kit (Qiagen)

Biological Molecules

DNase I (Roche Diagnostics)

10x DNase I buffer

10 mM MgCl₂ <!.>

10 mM CaCl₂ <!.>

500 mM Tris-HCl (pH 7.6)

Taq DNA polymerase, 10x PCR buffer (GIBCO BRL)

Special Equipment

Apparatus for agarose gel electrophoresis

Heating block

Thermocycler

METHOD

Error-prone PCR

Perform a standard PCR (see step 5 in the mRNA Purification and RT-PCR protocol, p. 557), but add dNTP analogs to the reaction mixture. The final mutation rate can be varied both with the

number of PCR cycles and with the concentration of dNTP analogs. With up to 85 μM 8-oxo-dGTP and 85 μM dPTP, a mutation rate of $6 \times 10^{-2} \text{ bp}^{-1}$ can be obtained after 25 cycles of PCR.

DNase I Shuffling and Assembly PCR

1. Take about 5 μg of purified PCR product (from step 5 in the mRNA Purification and RT-PCR protocol, p. 557) and dissolve it in the following mix:

10x DNase I buffer	10 μl
PCR product in H_2O	5 μg
H_2O	up to 100 μl

2. Add 1 μl of DNase I (0.15 unit/ml) and bring the tube to room temperature.
3. Incubate for 5 minutes at room temperature.
4. Take an aliquot of 5 μl and add 2.5 μl of standard DNA loading buffer (containing EDTA). Freeze the remaining reaction immediately in liquid nitrogen.
5. Analyze the sample on a 1.5% agarose gel. The original DNA band should have been shifted to a broad band at 50–100 bp. If this is observed, purify the remaining 95 μl on a preparative agarose gel (extract from gel with Qiaex-II, Qiagen). If this is not observed, bring the reaction again to room temperature and add another 1 μl of DNase I. Repeat until the expected size range of fragment is found.
6. Mix the following PCR, containing no primers, and add 5–15 μl of the purified fragments from step 5 (try different concentrations of the template).

dNTP	0.25 μl
MgCl_2 (50 mM)	0.88 μl
10x PCR reaction buffer (see step 5, mRNA Purification and RT-PCR protocol, p. 557)	2 μl
Triton X-100	0.8 μl
<i>Taq</i> polymerase	0.5 μl

Add H_2O to a total volume of 20 μl .

The PCR conditions are 4 minutes at 94°C, followed by 10 cycles of 30 seconds at 94°C, 30 seconds at 40°C, 2.5 minutes at 72°C, then followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 45°C, 2.5 minutes at 72°C, and the reaction is finished by 10 minutes at 72°C.

7. Extract the band of the desired size from the agarose gel. The band will be diffuse, but it will regain its sharpness after a second PCR with specific “outside” primers (e.g., SDA and ABrev).

Protocol 8

Separation of Ribosomal Complexes from Free Protein and Small-molecular-weight Compounds

This is a simple optional method for purifying ribosomal complexes prior to use in affinity selection, if the presence of *E. coli* proteins in the translation mix is problematic (see Protocol 5, p. 554).

MATERIALS

Special Equipment

CL-4B Sepharose (Pharmacia)
Gel filtration columns (1-ml) (Qiagen)

METHOD

1. Perform an in vitro translation reaction and stop it as described above (In Vitro Translation, Protocol 4, p. 552).
2. Prepare a gel filtration column (Qiagen) with 1 ml of CL-4B Sepharose (Pharmacia) and equilibrate it with ice-cold washing buffer, WB.
3. Apply 250 μ l of the stopped translation mix to the column.
4. Add 200 μ l of ice-cold WBT and discard the flowthrough.
5. Add another 300 μ l of ice-cold WB and collect the flowthrough, containing the ribosomal complexes, which can be used for selection or other experiments.

The fraction containing only the ribosomal complexes and no released protein can be determined in a simple experiment. Translation of an enzyme-ribosome display-construct is stopped such that stable complexes form (WB) and, in a parallel experiment, such that no complexes form (WB without Mg^{++}). Both samples are applied to gel filtration columns, and the elution profile of the enzyme is monitored by activity measurements. By comparing the two elution profiles, the fraction containing only protein in the ribosomal complex is determined.

Protocol 9

Radioimmunoassay (RIA)

This protocol determines whether a selected pool contains specific binders to a ligand. Please note that several control experiments should be performed. For a discussion of these experiments, see page 543.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Use all of the material used for in vitro translation (see In Vitro Translation, Protocol 5, p. 552) and in addition:

Buffers and Solutions

1x PBS buffer
10 mM Na₂HPO₄ (pH 7.4) <!.>
140 mM NaCl
15 mM KCl <!.>
1x PBST buffer
PBS with 0.5% (v/v) Tween-20
[³⁵S]Methionine (10 mCi/ml, 1175 Ci/mmol; New England Nuclear) <!.>
SDS (4%) in PBS

Special Equipment

Liquid scintillation cocktail "OptiPhase2" (Wallac, Finland)
Scintillation counter

METHOD

1. Coat the microtiter plate wells overnight at 4°C either directly with the ligand (for proteins typically 100 µl of 0.2 µM) or with neutravidin (100 µl per well, 4 µg/ml in PBS).
2. Wash the plate three times with PBS. In case of neutravidin coating, add 50 pmole of biotinylated ligand in 100 µl of PBS and incubate for 30 minutes at 25°C.
3. After washing with PBST, block the microtiter plate wells with 4% skimmed low-fat milk in PBS.
4. Carry out an in vitro translation using the RNA of the pool or of single clones as a template as described in step 1 of In Vitro Translation protocol (p. 552) (10 µg of mRNA per 110-µl reaction volume) with the following modifications: Carry out the in vitro translation for 30 minutes at 37°C. Add 2 µl of [³⁵S]methionine (0.3 µM, 50 µCi/ml final), but no cold methionine.

5. Dilute the reaction mixture fourfold with PBST after translation and centrifuge the mixture at 14,000g for 5 minutes.
6. Dilute the supernatant with the same volume of 4% milk in PBST containing either no ligand or, for inhibition studies, different concentrations of free ligand, and preincubate for 1 hour at room temperature before applying to the microtiter well.
7. Add 100 μ l of the radioactive reaction mix into the microtiter well and let the binding reaction with the immobilized ligand take place for 30 minutes at room temperature, with gentle shaking.
8. Wash five times with PBST and elute with 4% SDS in PBS.
9. Prepare 5 ml of scintillation fluid, add the eluted fraction, and quantify the radioactivity in a scintillation counter.

Protocol 10

Northern Blot

Northern blotting is a sensitive method for detecting small differences in the mRNA amount. This procedure can be used during optimization of ribosome display to characterize a new preparation of S30 cell extract.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Use all of the material used for in vitro translation (In Vitro Translation, Protocol 4, p. 552) and affinity selection (Affinity Selection, Protocol 5, p. 554) and in addition:

Buffers, Solutions, and Reagents

Chemiluminescent substrate CSPD (disodium 3-[4-methoxyspiro]1,2-dioxetane-3,2'
[5'-chloro]-tricyclo-[3.3.1.1^{3,7}]decan[-4-yl]phenyl phosphate; Roche Diagnostics)

DIG Oligonucleotide Tailing Kit (Roche Diagnostics)

Elution buffer EB5

50 mM Tris-acetate (pH 7.5)

150 mM NaCl

5 mM EDTA

50 µg/ml *S. cerevisiae* RNA (Sigma)

Guanidinium isothiocyanate (20 mM) <!.>

Oligonucleotide Anti-SDA (see Table 1)

TBE

89 mM Tris

89 mM boric acid

2 mM EDTA in H₂O

Special Equipment

Apparatus for agarose gel electrophoresis

Turboblotter with Nytran nylon membrane (Schleicher & Schuell)

X-ray films and a developing machine

METHOD

1. To optimize translation time, carry out in vitro translations and affinity selections with a single model mRNA construct under similar conditions as described in step 1 of the In Vitro Translation protocol. Test reaction translation times from 6 to 12 minutes.

Longer translation times may be necessary if a longer library mRNA is used for ribosome display.

2. After in vitro translation for 5, 7, 9, and 11 minutes, carry out an affinity selection as described in Affinity Selection, Protocol 5 (p. 554) with one modification: After washing, elute mRNA with 200 μ l of elution buffer EB5.
3. Precipitate the RNA immediately by addition of 600 μ l of ice-cold ethanol and incubate the samples for 30 minutes on ice.
4. Centrifuge at 14,000g for 30 minutes at 4°C.
5. Remove the supernatants and dry the RNA pellets for 10–15 minutes at room temperature.
6. Dissolve the RNA pellet in 10 μ l of RNA denaturation buffer on ice and incubate for 10 minutes at 70°C.

For an estimation of the amount of recovered mRNA, several other control samples containing between 0.2 ng and 10 ng of the original model mRNA can be prepared as standards.
7. Chill the samples on ice, mix with 1 μ l of gel loading buffer, and separate by 1.5% agarose gel electrophoresis in the presence of TBE and 20 mM guanidinium isothiocyanate.
8. Blot the RNA samples to a Nytran nylon membrane using a Turboblottter according to the manufacturer's recommendations.
9. Carry out hybridization for at least 4 hours at 60°C as described previously (Hanes and Plückthun 1997) with the oligonucleotide Anti-SDA, labeled by 3'-tailing with digoxigenin-11-dUTP/dATP using the DIG Oligonucleotide Tailing Kit.
10. Detect the hybridized oligonucleotide probe using the DIG DNA Labeling and Detection Kit with the chemiluminescent substrate CSPD and exposure to X-ray film.

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