

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 and biophysical parameters. It is performed completely in vitro and does not involve living cells at any step (Hanes and Plückthun 1997). The 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 (Smith 1985; Winter et al. 1994), 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 to the transformation efficiency, which is usually about 10^9 to 10^{10} clones per microgram of DNA in *Escherichia coli* (Dower and Cwirla 1992) and $\sim 10^7$ per microgram of DNA in yeast.

Second, an evolution process occurs during the selection procedure. Low-fidelity DNA polymerases that introduce mutations are usually used during the many amplification steps that are part of the ribosome display protocol. In most cases, the mutations are detrimental to the interaction in question. However, in some cases, a mutation is

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randomly introduced that improves the stability or affinity of the displayed protein, leading to a selective advantage of this molecule 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. If evolution of the selected clones is not desired, high-fidelity 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 the use of 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 is retransformed into the host organism. Although an alternative would be to use *E. coli* mutator strains (Irving et al. 1996; Low et al. 1996), in this case, the error rate cannot be controlled as well as with 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 example, a library of single-chain fragments of an antibody (scFv), is transcribed in vitro; the purified mRNA is used for in vitro translation. Of note, the DNA lacks a translation stop codon, but instead codes for a carboxy-terminal spacer at the end of the protein library coding sequence. 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 dur-

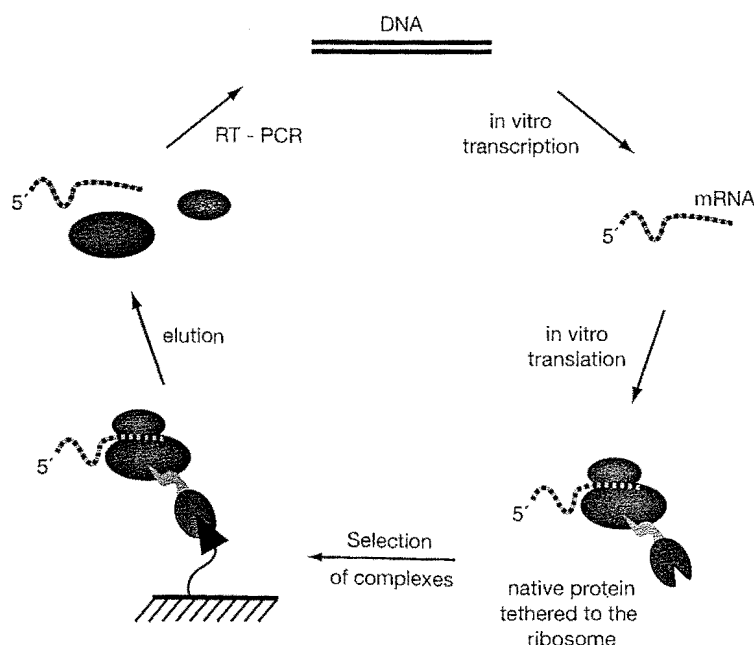


FIGURE 1. Principle of ribosome display. A DNA library encoding open reading frames lacking a stop codon at the 3' end is transcribed in vitro. The mRNA is purified and used for in vitro translation. The ribosome stalls at the end of the mRNA (or earlier). Due to the absence of a stop codon, the encoded protein is not released and can fold correctly on the ribosome. The carboxy-terminal spacer peptide is in the ribosomal tunnel, and its own carboxyl terminus is connected to the peptidyl-tRNA. The mRNA-ribosome-protein ternary complexes are used for affinity selection by an immobilized target or ligand. After washing away unbound complexes, 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.

ing in vitro translation, giving rise to a ternary complex of mRNA, ribosome, and encoded protein (Fig. 1). The presence of a carboxy-terminal spacer allows the protein to fold correctly on the ribosome, outside the ribosomal tunnel. The ternary ribosomal complexes, stabilized by high concentrations of magnesium ions and low temperature, are then subjected to selection by an immobilized ligand. The nonbinding complexes are washed away, and mRNA of the selected complexes is eluted by dissociation of the ribosomal complexes with EDTA. The mRNA is purified, reverse-transcribed, and amplified by PCR. During amplification, appropriate primers reintroduce the T7 promoter and the Shine-Dalgarno sequence. The resulting pooled DNA can be used directly for the next selection cycle, or it can be cloned into a vector for sequencing and large-scale expression of the selected binders. For antibody scFv fragments, the enrichment of a binder after one round of ribosome display is typically 100- to 1000-fold (Hanes and Plückthun 1997), although higher enrichment rates have been found with repeat proteins (see below).

Applications of Ribosome Display

Ribosome display was first established for the selection of peptide ligands binding to a protein target (Mattheakis et al. 1994; Gersuk et al. 1997). In the application of peptides, it has since been successfully applied to select streptavidin-binding peptides with up to 4 nM affinity (Lamla and Erdmann 2003) and to identify the main antigenic polypeptides of *Staphylococcus aureus* using a cDNA library (Weichhart et al. 2003).

The method was developed in our laboratory for the display of whole functional proteins that are correctly folded while still bound to the ribosome (Hanes and Plückthun 1997) and has since found many applications. In a model system using a mix of two distinct scFv fragments, 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-fold per cycle (Hanes and Plückthun 1997). Ribosome display was applied to the selection and simultaneous evolution of a scFv fragment binding with 40 pM affinity to a GCN4 mutant peptide, using a library prepared from the spleen of immunized mice (Hanes et al. 1998). This scFv fragment was subsequently evolved to 5 pM affinity (Zahnd et al. 2004). Subsequently, it was demonstrated that it is possible to select antibodies directly from a naive library. Starting with 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, thereby improving the affinity of the antibodies up to 40-fold (to 80 pM) compared to the affinity of antibodies initially present in the library. A selection for antibodies binding to an unusual DNA structure, guanine quadruplex DNA, demonstrated that antibodies with very high specificity for DNA can be generated by ribosome display. The selected anti-guanine quadruplex antibodies were used for in situ immunofluorescence experiments using macronuclei of the ciliate *Stylonychia lemnae*, providing the first evidence for the existence of guanine quadruplex DNA in vivo (Schaffitzel et al. 2001). Ribosome display selection was also applied to identify antibodies binding a herbicide from a non-immunized llama antibody library against a herbicide. However, these single-domain antibodies, isolated after six selection cycles, had affinities of only 3 μ M and 256 μ M (Yau et al. 2003). Antibodies against progesterone (He and Taussig 1997; He et al. 1999) and against hepatitis B virus DNA polymerase (Lee et al. 2004) have been selected using ribosome display, but their affinities were not reported.

Combinatorial libraries prepared from other (non-antibody) protein scaffolds can be subjected to ribosome display as well. One novel strategy was based on libraries of repeat proteins, consisting of repeating structural units that together form a contiguous target-binding surface. Natural repeat proteins such as ankyrins or leucine-rich repeat proteins were used for the design of such modular libraries (Binz et al. 2003; Stumpp et al. 2003). In contrast to scFvs, ankyrin repeat proteins can be expressed in soluble form in the cytoplasm with very good yields, and often have favorable biophysical properties. To date, ankyrin repeat libraries have been screened for binding to maltose-binding protein and two protein kinases (Binz et al. 2004), yielding ankyrins with low nanomolar affinity. Repeat proteins permit novel molecular evolution strategies, such as module insertions and module shuffling, that optimize the size and sequence of the interaction surface.

Ribosome display can also be used as a mere *in vitro* evolution method, starting from a single protein. In fact, by combination of ribosome display with error-prone PCR and DNA shuffling, and selection for decreased off-rates, the affinity of a fluorescein binding scFv was improved 30-fold to 100 pM (Jermutus et al. 2001). The same affinity maturation strategy was applied to further improve the scFv binding to a GCN4 mutant peptide (Hanes et al. 1998) from 40 pM to 5 pM (Zahnd et al. 2004). To our knowledge, this is the tightest peptide binder reported to date, demonstrating that very tight binders can be further evolved by ribosome display.

Ribosome display can be used to improve molecular properties other than affinity. For example, the stability of a scFv fragment of an antibody was enhanced by addition of increasing amounts of dithiothreitol (DTT) during *in vitro* translation (Jermutus et al. 2001). This strategy allowed selection of an antibody that was able to fold in the presence of 10 mM DTT, corresponding to the reducing environment of the cytoplasm that usually prevents formation of disulfide bridges. This approach could contribute to a general and rapid method for the generation of “intrabodies” (antibodies that can be used in the cytoplasm). Finally, libraries can be enriched for soluble, folded proteins by addition of proteases, and by passing the ribosomal complexes over a hydrophobic interaction column, separating hydrophobic and hydrophilic nascent chains (Matsuura and Plückthun 2003).

Ribosome-display selection in the presence of a “suicide substrate” inhibitor (a substrate that binds irreversibly to the enzyme that acts on it), or a substrate analog, led to the enrichment of active β -lactamase and active dihydrofolate reductase mutants. This indicated that ribosome display can also be used for enzyme selection and evolution (Amstutz et al. 2002; Takahashi et al. 2002).

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

The Construct Used for Ribosome Display

Several features of the ribosome-display construct (Fig. 2) are important for efficient ribosome display of proteins. At the DNA level, the T7 promoter allows efficient *in vitro* transcription. At the mRNA level, the ribosome-binding site (Shine-Dalgarno sequence, SDA) is followed by the sequence encoding the protein (library) to be displayed, then followed by a carboxy-terminal spacer sequence, fused in-frame to the protein. The 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 211–299 (Swissprot:P03662). Alternatively, spacers derived from the *E. coli* genes *tolA* (amino acids 118–214; Swissprot:P19934) or *tonB* (amino acids 62–229; Swissprot:P02929) can be used. The open reading frame extends to the very end of the DNA used as the template for transcription, with no stop codon. A stop codon would lead to the release of the protein and dissociation of the ribosomal complex.

At both ends of the mRNA, the ribosome-display construct includes 5' and 3' stem loops, known to stabilize mRNA against RNases *in vivo* as well as *in vitro* (Hajnsdorf et al. 1996). At least 5 of 20 *E. coli* RNases have been shown to contribute to mRNA degradation (Hajnsdorf et al. 1996), and it is likely that all are present in the S30 extract. The efficiency of ribosome display increased ~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).

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 (Protocol 1), without loss of diversity of the library due to transformation steps. As an example, we describe the generation of a construct starting from a scFv library. As a carboxy-terminal spacer, we use the carboxy-terminal domain of the gene III protein, whose coding DNA can be prepared by digestion of the vector pAK200 (Krebber et al. 1997) with *SfiI* and *HindIII*.

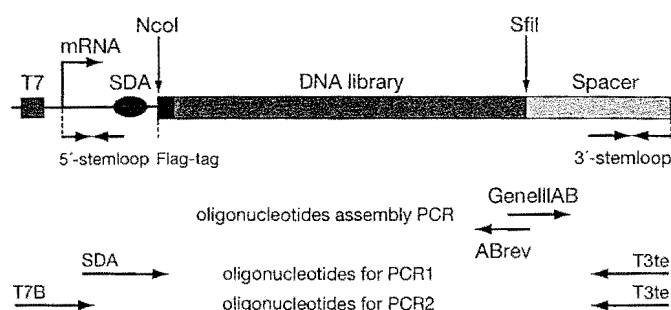


FIGURE 2. Construct used for ribosome display. A T7 promoter and a ribosome-binding site (Shine-Dalgarno sequence, SDA) are necessary for in vitro transcription and translation. The coding sequence starts with a FLAG-tag, followed by the DNA library and a spacer at the carboxyl terminus. The stop codon has been removed from the coding sequence. At the mRNA level, the construct is protected against ribonucleases by 5' and 3' stem loops. Information about the oligonucleotides used in PCR is given in the text and in Table 1. The restriction sites used in assembly of the construct are indicated.

The necessary steps to generate an scFv library from either natural sources or synthetically have been described previously (Krebber et al. 1997; Knappik et al. 2000). The DNA library must encode nonrandomized regions at the amino- and carboxy-terminal ends to permit PCR amplification. In our

TABLE 1. Oligonucleotides Used in Ribosome Display

Primer	Sequence	Remarks
T3te	5'- <u>GGCCCA</u> CCCGTGAAGGTGAGCCTCAGTAGCGACAG-3'	Introduces the 3' stem loop (stem is underlined) derived from the translated early terminator of phage T3; anneals to the gene III spacer
SDA	5'- <u>AGACCACA</u> ACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTA AGAAGGAGATATATCCATGGACTACAAAGA-3'	Introduces the ribosome-binding site (bold) and part of the mRNA 5' stem loop (underlined, see also T7B primer); used for the first PCR amplification step (PCR1); anneals to the first 11 bases of the FLAG tag sequence. The <i>NcoI</i> restriction site for cloning is shown in italics.
T7B	5'-ATACGAAATTAATACGACTCACTATAGGGAGACCACAACGG-3'	Introduces the T7 promoter (bold) and the mRNA 5' stem loop (underlined, see also SDA primer); used for the second PCR amplification step (PCR2), the transcription start is shown in italics. 12 bases of the 3' end overlap with the SDA primer.
tolAfw	5'-TATAT <u>GGCCTCGGGGGCCGAATTC</u> CAGAAGCAAGCTGAAG-3'	Forward primer to amplify the <i>E. coli</i> gene <i>tolA</i> with <i>SfiI</i> and <i>EcoRI</i> restriction sites (underlined)
tolArev	5'-GGACTGAAGCTTATCACGGTTTGAAGTCCAATGGCG-3'	Reverse primer to amplify <i>tolA</i> spacer
tonBfw	5'-TATAT <u>GGCCTCGGGGGCCGAATTC</u> AGCCGCCACCGGAG-3'	Forward primer to amplify the <i>E. coli</i> gene <i>tonB</i> with <i>SfiI</i> and <i>EcoRI</i> restriction sites (underlined)
tonBtr	5'- <u>CCGCAC</u> ACCAGTAAGGTGTGCGGTCAGGATATTCACCACAATCCC-3'	Reverse primer to amplify <i>tonB</i> spacer and to introduce a 3' stem loop (underlined)
ABrev	library dependent	Reverse primer that encodes the 3'-end of the DNA library for assembly PCR (Fig. 3)
GenelIIB	library dependent	Forward primer anneals to the 5' end of the spacer gene III and contains a 5' overhang encoding the 3' end of the DNA library for assembly PCR (Fig. 3)
anti-ssrA	5'-TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGGGACTA-3'	Inhibits the 10Sa-RNA peptide tagging system
anti-SDA	5'-TCTTTGTAGTCCATGGATATATCTCCTTCTTAAAGTTAAACAAAATTA TTTCTAGAGGGA-3'	Used for northern blotting

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 (SDA primer) and a primer that introduces an *Sfi*I restriction site at the carboxyl terminus before the stop codon of the scFv (library) (primer ABrev), if the library does not contain these restriction sites. After *Sfi*I digestion of the library or the derived PCR product, the fragment encoding the library is purified by preparative gel electrophoresis. The DNA encoding the gene III spacer (produced by digestion of the pAK200 plasmid with *Sfi*I and *Hind*III) is purified by preparative gel electrophoresis and added in at least 2-fold excess to the ligation reaction with the DNA library fragment. The ligation reaction is amplified directly by PCR (Fig. 2, PCR 1) using the primers SDA and T3te (Table 1), and the product is purified by preparative gel electrophoresis or with a PCR purification kit to remove the oligonucleotides. In a second PCR (Fig. 2, PCR 2), the primers T7B and T3te (Table 1) are used to introduce the T7 promoter (primers T7B and SDA overlap).

Alternatively, assembly PCR can be used for the generation of the ribosome-display construct (Fig. 3). Assembly PCR is relatively fast and easy; however, because the ligation method (above) is more efficient, 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 amplified from the plasmid pAK200 (Krebber et al. 1997) using the primers Gene-IIIAB (Fig. 2; containing a 5' overlap with the 3' end of the DNA library) and T3te. In parallel, the scFv library is amplified with the primers SDA and ABrev. Both PCR products are purified by gel electrophoresis, and assembly PCR is carried out with equimolar amounts of DNA of the scFv library PCR product and gene III spacer PCR product. No primers are used 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 (Fig. 2, PCR 1). In a third PCR, the ribosome display construct is amplified with T7B and T3te to introduce the T7 promoter (Fig. 2, PCR 2).

As an alternative to gene III spacers, *tolA* and *tonB* spacers can be used. The *tolA* and *tonB* spacers can be directly amplified from bacteria. For this purpose, a small amount of a bacterial colony (any *E. coli* strain, we normally use SB536 [Bass et al. 1996]) is picked with a toothpick and transferred into a standard PCR mix containing primers *tolA*fwd/*tolA*rev, or *tonB*fwd/*tonB*totrev, respectively (Table 1). After 30 cycles of PCR, a single sharp band appears that can be gel-purified, digested, and ligated to the library.

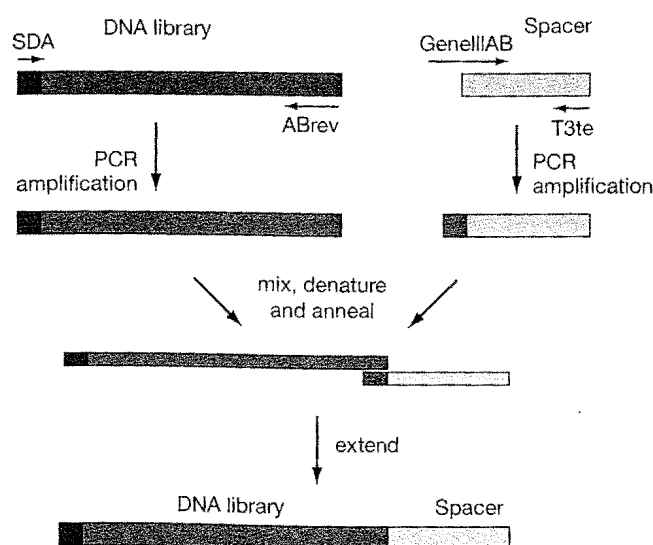


FIGURE 3. Generation of the ribosome-display construct by assembly PCR. The DNA library is amplified with appropriate primers, e.g., ABrev and SDA. The spacer is PCR-amplified using a forward primer that overlaps 5' with the 3' end of the DNA library (e.g., Gene IIIAB and an appropriate reverse primer, e.g., T3te). In the subsequent assembly PCR, no further primer is added, such that the 5' end of the spacer can anneal to the 3' end of the DNA library. After the subsequent elongation step, the spacer is fused 3' to the DNA library.

The quality of the final PCR product is crucial for ribosome display; a sample should be run on an analytical agarose gel to ensure that the PCR product contains one single strong DNA band of the expected size with no smears or by-products, either of which 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, followed by 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 (Protocol 2), ~0.1 mg of mRNA is obtained after 2–3 hr in a 200-μl transcription reaction with 1–2 μg of PCR product as a template. The transcribed mRNA is 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 1966) is carried out following a modified protocol (Protocol 3), 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 in vitro translation for ribosome display (Protocol 4) must be optimized according to Pratt (1984) for the concentration of Mg²⁺ and K⁺ ions, the amount of S30 extract used, and the translation time (Hanes et al. 1999). Protein synthesis follows a saturation curve reaching a plateau after ~30 min (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 ribosome display is usually between 6 and 10 min 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. Note 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 the 10Sa-RNA, which contributes to a surveillance mechanism responsible for the release and degradation of proteins derived from mRNA lacking a stop codon (Keiler et al. 1996). The antisense oligonucleotide anti-ssrA (Table 1), added to the in vitro translation reaction, binds to and inactivates the mRNA moiety of the 10Sa-RNA, thereby inhibiting degradation of the displayed protein.

Translation of proteins containing disulfide bridges requires an oxidizing environment. Thus, DTT is omitted from the translation reaction (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). If proteins or peptides without disulfide bridges are displayed, coupled transcription and translation can be performed as described by Pratt (1984).

Affinity Selection

The stopped, diluted translation mixture is used directly for selection experiments (Protocol 5). The affinity selection should be performed on ice, in the presence of 50 mM Mg²⁺; under these conditions the ribosomal complexes are stable for at least 10 days (Jermutus et al. 2001).

If the presence of the *E. coli* enzymes or other components of the translation mix is problematic in a particular experiment (e.g., the selection for an enzyme or a catalytic antibody), ribosomal complexes can be purified by ultracentrifugation with a sucrose cushion (Mattheakis et al. 1996). When the

stopped translation mix is resolved by 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, an alternative method for complex separation (Amstutz et al. 2002), is used to separate ribosomal complexes efficiently from free protein and small-molecular-weight compounds (Protocol 8). The fractionation range of the chosen beads should be from 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 that contains the ribosomal complexes and no free protein can be established in a simple experiment (as described in the protocol).

For affinity selection, the target protein can be immobilized on a surface; however, a protein target may be partially denatured on the surface due to hydrophobic interactions with a plastic surface, in which case the selected antibodies may not recognize the protein target. We also found that the background (unspecific binding) is lower when selection is performed in solution. For selection in solution, the target ligand must be biotin-labeled, and the ribosomal complexes binding to the ligand are captured by streptavidin-coated magnetic beads. An alternative is to immobilize the biotinylated protein indirectly to streptavidin immobilized on microtiter dishes. The ligand should contain a 30 Å linker to the biotin moiety, so that the ligand is accessible to the protein displayed on the ribosome and not hidden in the very deep streptavidin-binding pocket. To prevent the selection of streptavidin-binding proteins, streptavidin-coated magnetic beads and avidin-agarose should be used alternately for capture, after each round of ribosome display.

Selection is performed in the presence of sterilized, debiotinylated, skimmed low-fat milk (1–2%) or 0.5% BSA, and 0.25% (w/v) heparin to prevent nonspecific binding of ribosomal complexes to surfaces (heparin also 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 Mg^{2+} required for stabilization of the ribosomal complexes. Recovery of the mRNA by dissociation of the complexes has the advantage that the protein–ligand interaction is not disrupted, and thus high-affinity binders elute as efficiently as low-affinity binders.

After dissociation of the ribosomal complexes, mRNA is isolated, reverse-transcribed (Protocol 6) using the primer T3te (Fig. 2, Table 1), and PCR-amplified with the primers SDA (Table 1) and T3te (Fig. 2). The product of the first PCR is purified by preparative agarose gel electrophoresis and used as template for the second PCR with the primers T7B (Table 1) and T3te (Fig. 2). The resulting PCR product can be directly used for the next cycle of ribosome display, or cloned into an expression vector via *NcoI* and *SfiI*.

The enrichment of specific binders can be assessed by performing selection on a nonspecific surface; i.e., without adding antigen in the case of scFv (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 ligand:receptor 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 off-rate selection, the ribosomal complexes formed after translation are first equilibrated with biotin-labeled antigen target or ligand. The concentration of the labeled antigen is chosen in excess such that all complexes carrying a binding moiety are bound. After equilibration, a very large excess of competitive non-biotinylated antigen is added. Every complex that dissociates from its biotinylated antigen will be captured by the competitive antigen and therefore not captured by the streptavidin-coated magnetic beads. There is an approximate correlation between the incubation time of the complexes with competitive antigen and the mean affinity of the complexes still bound to the labeled antigen, provided a large excess of unlabeled competitor is used. In principle, small amounts of antigen could be used, but in practice, this strategy does not appear to be as successful for reasons discussed elsewhere (Plückthun et al. 2000).

This approach can be performed not only for affinity selection, but also for many other physico-chemical properties of the proteins to be selected. If the target protein (e.g., a scFv fragment) contains a disulfide bond required 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 proteases, organic solvents, detergents, or any other conditions are conceivable, provided that the mRNA–ribosome–protein complex can be kept stable.

From Selection to Evolution

Ribosome display was first established as a very efficient method for in vitro selection of antibody scFv fragments (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 original protocol, involving up to 100 cycles of PCR over all rounds, does introduce a number of mutations, especially when non-proofreading *Taq* polymerase is used (Hanes et al. 1999). This effect can be enhanced by orders of magnitude with randomization steps such as error-prone PCR and DNase I shuffling (Protocol 7) (Cadwell and Joyce 1992; Stemmer 1994; Zacco et al. 1996), which are introduced after 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 Mn^{2+} to the reaction, which decreases the fidelity of most polymerases, or by incorporation of dNTP analogs such as 8-oxo-guanosine or dPTP (Protocol 7) (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 error rate is dependent on the concentration of both manganese and dNTP analogs and thus can be controlled. It is worth mentioning that the number of mutations introduced is dependent not only 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 for recombining mutations from former selection rounds when the library consists of homologous genes (Fig. 4) (Stemmer 1994). After reverse transcription of the mRNA pool obtained by affinity selection, the DNA pool is PCR-amplified until a

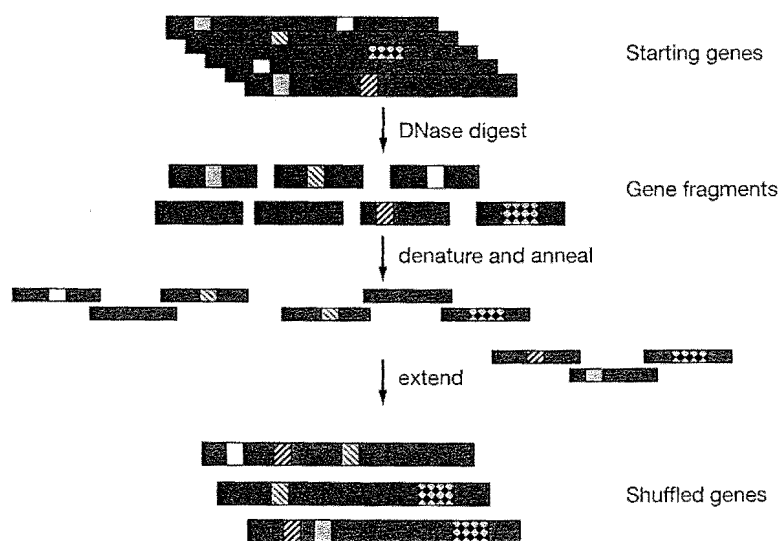


FIGURE 4. Principle of DNA shuffling. The library genes are fragmented by DNase I and then reassembled by a reaction in which homologous fragments act as primers for each other. Thus, mutations on different genes can be recombined in vitro.

band appears on the analytical agarose gel (between 15 and 30 PCR cycles). The DNA is then gel-purified and digested with DNase I until the mean fragment length is between 50 bp and 150 bp. After a given incubation time, a small sample can be analyzed on an agarose gel for the degree of digestion, to ensure that the fragments are not too small, which would make the assembly difficult. The gel-purified 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.

Radio-immunoassay Analysis of the DNA Pool and of Single Clones

Radio immunoassays (RIA) are performed to test for the presence of specific binders in the pool (Protocol 9). In RIA, the *in vitro* translation is performed with radioactive [³⁵S]methionine, and thus the binding of radioactive protein to the immobilized ligand can be quantified. The translation time is 30 min, which is when the protein synthesis reaches a plateau (Ryabova et al. 1997). After the translation is stopped, the translation mix is transferred to microtiter wells with immobilized ligand.

As a background control, the binding of pool-encoded proteins to a nonspecific surface (i.e., a milk-coated surface, or a neutravidin-coated surface) should be tested. The RIA is also 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 inhibited in 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 into 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 expression vector carries a stop codon downstream from the *Sfi*I site. 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, to allow the binders to be purified and further analyzed by enzyme-linked immunosorbent assay (ELISA), and thereby 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 a nonspecific surface. As noted above, the *in vitro* translation should be optimized for the concentration of Mn²⁺ and K⁺, the amount of extract used, and the translation time (Pratt 1984); parameters that should 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 *in vitro* translation of an enzyme, such as β -lactamase or firefly luciferase, and to measure protein synthesis with a simple activity assay. We recommend optimizing the Mg²⁺ concentration first, and then using the optimal Mg²⁺ concentration in all further experiments.

To determine the optimal translation time, when maximal amounts of functional ribosomal complexes are present, *in vitro* translation and affinity selection should 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 by addition of a high concentration of Mg²⁺ and by cooling, and subjected to affinity selection by the cognate ligand immobilized on a surface. After affinity selection, the eluted mRNA can be quantified by RT-PCR with primers SDA and T3te or by northern blot. Northern blotting is more sensitive in detecting small differences in the mRNA amount. In northern blot hybridization, the isolated mRNA is monitored and quantified with a digoxigenin-labeled probe (using DIG oligonucleotide tailing kit, Roche Diagnostics) that specifically anneals to the mRNA construct (Anti-SDA, Table 1; Protocol 10).

RIBOSOME DISPLAY—GETTING STARTED

Establishing ribosome display in your lab may require some optimization. Here, we list some critical issues that can cause problems, according to our experience.

- A key point is the *handling of RNA*. It is important to wear gloves during all manipulations. After putting on gloves, avoid touching any surfaces that might be contaminated with nucleases.
- All glassware and spatulas should be baked at 180°C for at least 4 hr. Otherwise, glass- and plasticware should be treated with RNase-inactivating reagents. Plastic tubes and tips should be sterile (from a fresh bag). Water should either be double-distilled or diethylpyrocarbonate-treated (0.1% DEPC) for 2 hr at 37°C and then autoclaved.
- Designate a special working area for RNA work only. Wipe the bench with 100% ethanol prior to use to remove microorganisms.
- Buy RNase-free reagents and separate these reagents from general-use reagents. All solutions and buffers should be DEPC-treated or generated with DEPC-treated water (except those containing primary amines, e.g., Tris).
- Prepare stock solutions and aliquot them. In case you have an RNase contamination, you can throw away aliquots and take a new aliquot.
- RNA samples should be quickly frozen in liquid nitrogen before storage at –20°C.
- A ribosome display *positive control* is necessary for optimization (see above). For example, an antibody with high affinity for its antigen (nanomolar dissociation constant) can be used to establish ribosome display with scFvs. Of course, any other protein with high affinity for its ligand can be used. Optimally, the test protein should be similar in size and topology to the protein library to be displayed. The time of translation can be thereby optimized to obtain the maximal amount of mRNA–ribosome–protein complexes (see Protocol 10).
- After each round of ribosome-display selection, the *RT-PCR* product should be a clearly defined single band. Do not proceed with smeary PCR products; the quality of the library will decrease further in subsequent rounds, possibly leading to a complete smear and inability to select molecules. Instead, repeat the respective ribosome-display selection round. In case you are not able to improve the quality of the PCR product, designing new primers may help.

Protocol 1

Preparation of the Ribosome-Display Construct by Ligation

Efficient ribosome display of proteins is influenced by several factors, including the presence of a T7 promoter at the DNA level, and the ribosome-binding site and the protein-coding sequence at the RNA level. Figure 2 provides a diagram of the construct elements. This protocol describes two alternative procedures (ligation or assembly PCR) for the preparation of the basic DNA template to be used for subsequent screening.

MATERIALS

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

Buffers, Solutions, and Reagents

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

Biological Reagents

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

Thermocycler

METHOD

I. Preparation of the Ribosome-Display Construct 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 and the QIAquick gel extraction kit, according to the manufacturer's instructions.
2. Digest the plasmid encoding the library with *SfiI* and *NcoI* and purify the products by agarose gel electrophoresis.
3. Combine 150 ng of the DNA fragment encoding the DNA library with a threefold excess of gene III spacer and 10 units of T4 DNA ligase. Incubate the ligation reaction overnight at 16°C.
4. Set up a 50- μ l reaction with the primers SDA and T3te (PCR1, Fig. 2), using 5–10 μ l of the ligation mix as PCR template. Amplify by PCR according to the following program:

Number of cycles	Denaturation	Annealing	Polymerization
1	94°C for 4 min		
5	94°C for 30 sec	37°C for 30 sec	72°C for 2.5 min
15–20	94°C for 30 sec	50°C for 30 sec	72°C for 2.5 min
Final			72°C for 10 min

- Purify the PCR product by agarose gel extraction with the QIAquick gel extraction kit. Set up a second amplification reaction using the purified product as template with the primers T7B and T3te (PCR2, Fig. 2). Amplify by PCR according to the program given in step 4; note that 12–16 cycles usually are performed in place of the 15–20 cycles.

II. Preparation of the Ribosome-Display Construct by Assembly PCR

- Amplify the carboxy-terminal domain of the gene III protein from the pAK200 vector by PCR with the primers Gene-IIIAB and T3te, and amplify the DNA library by PCR with the primers SDA and ABrev.
- Purify the PCR products by agarose gel extraction, and set up a reaction using equimolar amounts of each DNA product for the assembly PCR. Carry out amplification of these DNAs (with no primers) according to the following program:

Number of cycles	Denaturation	Annealing	Polymerization
1	94°C for 4 min		
5	94°C for 30 sec	50°C for 30 sec	72°C for 2.5 min

- Stop the program, add the primers SDA and T3te to the reaction, and resume the program as follows (PCR1, Fig. 2):

Number of cycles	Denaturation	Annealing	Polymerization
12	94°C for 30 sec	45°C for 30 sec	72°C for 2.5 min

- Purify the PCR product of the appropriate size by agarose gel extraction with the QIAquick gel extraction kit, and perform the last PCR introducing the T7 promoter with the primers T7B and T3te (see Protocol 6; PCR2, Fig. 2).

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 and ethanol precipitation.

MATERIALS

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

Buffers, Solutions, and Reagents

Ethanol <!.>
Guanidinium isothiocyanate (1 M) <!.>
LiCl (6 M) <!.> (Fluka)
Gel-loading buffer, 5x
 50% glycerol
 200 mM Tris base <!.>
 100 mM acetic acid <!.>
 5 mM EDTA
 a spatula tip of bromophenol blue <!.>
NTP solution: ATP, CTP, GTP, TTP; each at 50 mM (Sigma)
RNA denaturation buffer
 formamide <!.> 10 µl
 formaldehyde <!.> 3.5 µl
 10x MOPS buffer (pH 7.0) <!.> 2 µl
Sodium acetate, 3 M (pH 5.2) <!.>
T7 RNA polymerase buffer, 5x
 1 M HEPES-KOH (pH 7.6) <!.>
 150 mM magnesium acetate <!.>
 10 mM spermidine <!.>
 0.2 M DTT <!.>
TBE buffer (Sambrook and Russell [2001])

Biological Reagents

RNasin (40 units/µl, Promega)
T7 RNA polymerase (50 units/µl) (New England BioLabs)

Special Equipment

Tabletop refrigerated microcentrifuge

METHOD

1. To prepare the in vitro transcription reaction, thaw and mix the following reagents in a microcentrifuge tube on ice:

5 × T7 RNA polymerase buffer	40 µl
NTPs (50 mM each)	28 µl
T7 RNA Polymerase (50 units/µl)	8 µl
RNasin	4 µl
RNase-free water	75 µl
PCR-template (Protocol 1) (0.5–1 µg DNA)	45 µl
Total volume	200 µl

Incubate the reaction 2–3 hr at 37–38°C.

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.

2. To prepare a mix for mRNA purification, combine the following on ice:

in vitro transcription reaction mix (step 1)	200 µl
RNase-free water	200 µl
6 M LiCl	400 µl
Total volume	800 µl

Allow the reaction to sit on ice for 30 min.

3. Centrifuge the purification mix at 14,000g for 20–30 min at 4°C, discard the supernatant, and wash the pellet once with 500 µl of 70% ethanol.
4. Dry the pellet, resuspend it in 200 µl of RNase-free water and centrifuge at 14,000g for 5 min at 4°C. Remove the supernatant to a fresh tube.
5. Combine 180 µl of the supernatant, 18 µl of 3 M sodium acetate, and 500 µl of ethanol (97%). Incubate 30 min on ice.
6. Recover the RNA by centrifuging at 14,000g for 20–30 min at 4°C.
7. Wash the pellet with 500 µl of 70% ethanol, allow it to air-dry, and resuspend it in 40 µl of RNase-free water.
8. Determine the concentration of mRNA as follows:
 - a. Measure the OD at 260 nm (an OD_{260nm} of 1 corresponds to 40 µg/ml).
 - b. Run an analytical agarose gel (1.5%): Add 10 µl of RNA denaturation buffer to 1 µg of mRNA, and incubate for 10 min at 70°C. Chill the samples on ice, mix with 2 µl of gel loading buffer and separate by electrophoresis through a 1.5% agarose gel in TBE buffer in the presence of 20 mM guanidinium isothiocyanate.

Protocol 3

Preparation of *E. coli* S30 Cell Extract

This method describes the preparation of S30 cell lysates, the source of ribosomes for subsequent in vitro translation, 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, Solutions, and Reagents

Incomplete rich medium:

potassium dihydrogen phosphate (KH_2PO_4)<!.>	5.6 g
dipotassium hydrogen phosphate (K_2HPO_4)<!.>	28.9 g
yeast extract	1 g
thiamine	15 mg
water	to 1 liter

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

Preincubation mix<!.> (10 ml total volume)

2 M Tris-acetate<!.> (pH 7.5 at 4°C)	3.75 ml
3 M magnesium acetate<!.>	71 μl
amino acid mix, each of 20 amino acids at 10 mM (Sigma)	75 μl
0.2 M ATP	0.3 ml
phosphoenolpyruvate (Sigma)	0.2 g
pyruvate kinase (P-1506 Sigma)	50 units
water	to 10 ml

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 Reagents

E. coli strain MRE600 (Wade and Robinson 1966), lacking the major RNase activity of *E. coli*

Special Equipment

Baffled flask, 5-liter
Dialysis tubing with a cutoff of 6,000–8,000 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 OD_{550nm} of 1.0 (during early exponential growth phase) by centrifugation at 3500g for 15 min at 4°C.
4. Discard the supernatant and wash the pellet three times with 50 ml of ice-cold S30 buffer per liter culture. The cell pellet can be frozen at –80°C or in liquid nitrogen and stored for a maximum of 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 min at 4°C.
9. Transfer the supernatant to a clean centrifuge tube and centrifuge again at 30,000g for 30 min at 4°C.
10. Transfer the supernatant from the second centrifugation 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 hr 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 a 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 hr.
12. Centrifuge the cell extract at 4000g for 10 min at 4°C. Freeze the supernatant in aliquots of 100–500 µl in liquid nitrogen, and 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. 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, Solutions, and Reagents

Anti-ssrA oligonucleotide, 200 μ M (Table 1)
Methionine in water, 200 mM
Magnesium acetate<!.>, 100 mM
Potassium glutamate, 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 (all Sigma)
Washing buffer (WBTH)
 50 mM Tris-acetate<!.> (pH 7.5 at 4°C)
 150 mM NaCl
 50 mM magnesium acetate<!.>
 0.1% Tween-20
 2.5 mg/ml heparin (Sigma)
 RNase-free water (DEPC-treated)

Biological Reagents

E. coli S30 cell extract (from Protocol 3)
Library mRNA, 1 μ g/ μ l (from Protocol 2)
Protein disulfide isomerase (bovine PDI) (Sigma P-3818) (22 μ M in water)

Special Equipment

Tabletop refrigerated microcentrifuge

METHOD

1. Chill all solutions to 4°C and mix the components of the translation reaction on ice in the indicated order:

RNase-free water	14.3 μ l
Potassium glutamate (2 M)*	11 μ l
Magnesium acetate (0.1 M)*	7.6 μ l
Methionine (200 mM)	1.1 μ l
Anti-ssrA oligonucleotide (200 μ M)	2 μ l
PremixZ ice-cold, thaw on ice and vortex before pipetting	22 μ l
<i>E. coli</i> S30 extract (from Protocol 3)	40 μ l
PDI (if disulfides are present in the target protein library)	2 μ l
library mRNA from Protocol 2 (10 μ g), thaw just before use and freeze the remainder immediately.	10 μ l
Total volume	110 μ l

* To optimize translation conditions, test the following conditions for each new batch of S-30 extract (concentrations indicate final reaction concentrations): first 7–15 mM magnesium acetate, 180–220 mM potassium glutamate, and 20–50 μ l of S30 extract for a 110- μ l reaction as described by Pratt (1984). Finally, translation time should be optimized to obtain the maximal amount of mRNA–ribosome–protein complexes (see Protocol 10, Northern Blot).

2. Incubate the translation reaction for the optimized translation time (usually 6–15 min) at 37°C.
3. Stop the translation reaction with 400 μ l of ice-cold WBTH, vortex briefly and gently, and place on ice.
4. Centrifuge the translation mix at 14,000g for 5 min at 4°C and transfer to a fresh ice-cold tube.
5. Optionally, the purification described in Protocol 8 can then be used if difficulties arose performing Protocol 5.

Protocol 5

Affinity Selection

This procedure details two alternatives for the affinity selection of ribosomal complexes, using either selection on a target previously immobilized to a surface or a solution-base method. An additional two methods provide variations that enable the selection of proteins with lengthened off-rate or increased stability forms of proteins. Generally, selection in solution is preferred, as protein ligands could become denatured when immobilized on a surface. Note that native protein can often be immobilized if it is biotinylated and bound to immobilized streptavidin (as discussed in the introduction). After two or more ribosome-display selection rounds, a control should be included: With half of the translation reaction mix, the same affinity selection should be carried out without addition of ligand (background control). In this control, ribosomal complexes specific to the ligand should be washed away, and thus the PCR band after the selection round should be much fainter than that obtained after selection in presence of ligand.

MATERIALS

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

Buffers, Solutions, and Reagents

Elution buffer EB-20

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 skimmed milk (12% in water or in WBT; autoclave in a 50-ml plastic tube)

PBS buffer, pH 7.4

137 mM NaCl

2.7 mM KCl<!.>

10 mM Na₂HPO₄ <!.>

1.8 mM KH₂PO₄ <!.>

Washing buffer (WB), 10x

0.5 M Tris-acetate<!.> (pH 7.5 at 4°C)

1.5 M NaCl

0.5 mM magnesium acetate

Washing buffer with Tween (WBT), 1x

50 mM Tris-acetate<!.> (pH 7.5 at 4°C)

150 mM NaCl

50 mM magnesium acetate<!.>

0.1% Tween-20

Biological Reagents

Avidin immobilized on agarose beads (Sigma)

Biotinylated ligand and free ligand

The ligand can be biotinylated chemically using a biotin-LC-NHS ester (Pierce) or enzymatically with a tag that can be biotinylated by the *E. coli* enzyme BirA (Cull and Schatz 2000). This is also available in kit form (Avidity).

Streptavidin-coated magnetic particles (Roche Diagnostics)

Special Equipment

Magnet (Dyna)
 Microtiter plate strips or plates (Nunc)
 Panning tubes, 5 ml (Nunc)
 Rocking table or shaker

METHOD

I. Selection Using an Immobilized Target Protein

It is important that the buffers, the microtiter plates, and the 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 per well in 100 µl of PBS.
2. Wash the coated strips or plates with PBS and block for 1 hr with 4% milk powder in PBS. As a control surface, block several non-coated microtiter wells with milk.
3. Wash the wells three times with PBS and twice with WBT.
4. Fill the wells with 250 µl of ice-cold WBT and put on ice.
5. For affinity selection, remove the WBT from the ice-cold microtiter wells.
6. Supplement the 510 µl of in vitro translation mix (from Protocol 4) with 100 µl of 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 (i.e., up to 3 wells total).
7. Gently shake the microtiter plates for 1 hr in the cold room. Wash five times with WBT.
8. Aspirate WBT. To elute the mRNA, add 200 µl of ice-cold elution buffer EB for 5 min on ice and shake it gently. Transfer eluted mRNA to a new tube.

The eluted mRNA must be immediately purified (see Protocol 6).

II. Selection in Solution

1. Block 5-ml panning tubes with 4% (w/v) milk in PBS for 1 hr by end-over-end rotation.
2. Wash the 5-ml panning tubes three times with PBS, three times with WBT, and finally fill with WBT.
3. Remove any biotin from the sterilized skimmed low-fat 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 (1-mg of particles) for 1 hr at room temperature. Remove the streptavidin-coated beads with a magnet and discard them. Transfer the milk in a new tube and store it on ice.
4. Wash 100 µl of streptavidin-coated magnetic beads four times with ice-cold WBT and resuspend them in 100 µl of ice-cold WBT.
5. Empty the panning tubes from step 2 and add 60 µl of sterilized, biotin-depleted skimmed low-fat milk.
6. Add the in vitro translation mix (510 µl, from Protocol 4) and 10 pmole of biotinylated ligand in WBT to the panning tubes. Seal the immunotubes, place into a larger tube, e.g., a 250-ml centrifuge tube that is filled with ice, and rotate end-over-end for 1 hr in the cold room.

7. For capture, supplement 100 μ l of streptavidin-coated magnetic beads and rotate end-over-end on ice for 15 min in the cold room.
8. Wash the magnetic beads five times with WBT and bind them to the side of the tube with a magnet.
9. To elute the mRNA, add 200 μ l of ice-cold elution buffer EB for 5 min on ice and shake it gently.
The eluted mRNA must be immediately purified (see Protocol 6). It should be noted that the capacity of the streptavidin-coated magnetic beads is dependent on the size of the biotinylated ligand. It is important to ensure that all biotinylated ligand can be captured by the streptavidin-coated magnetic beads; therefore, not more than 10 pmole 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. Streptavidin-coated magnetic beads and avidin-agarose should be used alternately in order to avoid the selection for streptavidin-binding proteins.

III. Off-rate Selection

1. Perform steps 1–3 and 5 of Protocol 5, Part II above, to prepare the panning tubes.
2. Split the translation mix. Add biotinylated ligand to both 255 μ l of the translation reactions (ligand concentration 1–100 nM; the better the binder is, the less antigen is needed).
3. Equilibrate for 2 hr to overnight.
4. Add competing non-biotinylated ligand to one of the translation reactions (1000-fold excess over the biotinylated ligand).
5. Incubate for off-rate selection time (2 hr–15 days; start with 2 hr), depending on the expected off-rate of the best binders.
6. Recover the ribosomal complexes (steps 7–9, Protocol 5, Part II).

IV. Stability Selection of Disulfide-containing Proteins

1. Perform the selection as described in Protocol 5, Part II, but during in vitro translation (Protocol 4) and during the affinity selection (Protocol 5, Part II), add defined amounts of DTT (normally ranging from 0.5 to 10 mM; start with 0.5 mM and increase the DTT concentration in subsequent ribosome-display rounds).
2. Add DTT also for the analysis of the selected pools and single binders by RIA (see Protocol 9).

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, as this depends on the amount of mRNA eluted after affinity selection. Therefore, it is useful first to perform few PCR cycles (15–20), then to check the product on an analytical agarose gel. If necessary, more PCR cycles can be performed. Typically, the more binders present in the library, the fewer PCR cycles are needed. Overamplifying the PCR product is not recommended, because this results in a smeary band on an agarose gel and, after *in vitro* transcription, in poor-quality mRNA.

MATERIALS

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

Buffers, Solutions, and Reagents

dNTP solution, each at 20 mM (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 Reagents

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

Special Equipment

Thermocycler

METHOD

1. Purify the mRNA (from Protocol 5), using the High Pure RNA Isolation Kit according to the manufacturer's instructions.
2. Elute the purified mRNA in 35 μ l of RNase-free water and immediately denature at 70°C for 10 min. Chill the mRNA samples on ice for 1–2 min after denaturation.
3. For reverse transcription (RT), prepare a premix on ice:

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

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

4. Incubate the reaction mixture for 1 hr at 50°C.

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

5. Set up the PCR by combining the following components on ice:

T3te primer (100 μ M)	0.125 μ l
SDA primer (100 μ M)	0.125 μ l
dNTP (20 mM each)	0.5 μ l
<i>Taq</i> polymerase (5 units/ μ l, GIBCO)	0.25 μ l
10x PCR-reaction buffer (GIBCO)	5 μ l
DMSO	2.5 μ l
MgCl ₂ (50 mM)	1.55 μ l
water	32.45 μ l
DNA template, directly from reverse transcription	7.5 μ l
Total volume	50 μ l

6. Carry out amplification according to the following program:

Number of cycles	Denaturation	Annealing	Polymerization
1	94°C for 4 min		
20	94°C for 30 sec	50°C for 30 sec	72°C for 2.5 min
Final		72°C for 10 min	

7. Purify the PCR product by agarose gel electrophoresis and gel extraction using the QIAquick gel extraction kit.

8. Set up a second amplification reaction with the reaction components given in step 5, but using the purified product as template with the primers T7B and T3te.

9. Carry out amplification according to the following program:

Number of cycles	Denaturation	Annealing	Polymerization
1	94°C for 4 min		
10–15	94°C for 30 sec	60°C for 30 sec	72°C for 2.5 min
Final			72°C for 10 min

Protocol 7

Evolution: Introducing Additional Diversity

The two PCR methods presented here may be 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 (Table 1)

QIAex-II gel extraction kit (QIAGEN)

Biological Reagents

DNase I (Roche Diagnostics)

DNase I buffer 10x

10 mM $MgCl_2$ <!.>

10 mM $CaCl_2$ <!.>

500 mM Tris-HCl <!.> (pH 7.6)

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

Special Equipment

Thermocycler

METHOD

1. Error-prone PCR

1. Set up a standard PCR to reverse-transcribe the mRNA as described in Protocol 6, step 5, but include dNTP analogs with the dNTP mix in the reaction mixture.
2. Carry out amplification according to the program described in Protocol 6, step 6.
The final mutation rate can be varied by adjusting both the number of PCR cycles and the concentration of dNTP analogs. With concentrations up to 85 μ M 8-oxo-dGTP and 85 μ M dPTP, and after 25 cycles of PCR, a mutation rate of 6×10^{-2} bp⁻¹ can be obtained.
3. Perform steps 7–9 of Protocol 6.

II. DNase I Shuffling and Assembly PCR

1. Remove about 5 μg of purified PCR product (from Protocol 6, step 7) and prepare the following mix:

10x DNase I buffer	10 μl
PCR product in water	5 μg
Water	to 100 μl

2. Add 1- μl of DNase I (0.15 units/ml) and bring the tube to room temperature.
3. Incubate the reaction for 5 min at room temperature.
4. Remove a 5- μl aliquot from the reaction and add 2.5 μl of standard DNA loading buffer (containing EDTA). Freeze the remaining reaction immediately in liquid nitrogen.
5. Analyze the sample by electrophoresis through 1.5% agarose. 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 a band of ~50–150 bp is not observed, bring the reaction to room temperature, add another 1 μl of DNase I, and repeat steps 3, 4, and 5 until the expected size range of fragment is seen.
6. Set up an amplification reaction by combining the following reagents. As template, add 5–15 μl of purified fragments from step 5 (try varying the concentration of template). Do not include primers in this reaction.

dNTP	0.25 μl
MgCl_2 (50 mM)	0.88 μl
10x PCR reaction buffer	2 μl
Triton X-100	0.8 μl
<i>Taq</i> polymerase	0.5 μl

Add water to a total volume of 20 μl .

7. Carry out amplification according to the following program:

Number of cycles	Denaturation	Annealing	Polymerization
1	4 min at 94°C		
10	30 sec at 94°C	30 sec at 45°C	2.5 min at 72°C
Final			10 min at 72°C

8. Purify the product by agarose gel electrophoresis and extract the band of the desired size from the gel.

The band will be diffuse, but will regain its sharpness after a second PCR with specific outside primers (e.g., SDA and ABrev; Table 1, Fig. 2).

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, the free nascent polypeptide chain, or small molecules in the translation mix is interfering with the selection (see Protocol 5).

MATERIALS

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

Buffers, Solutions, and Reagents

Washing buffer (WB), see Protocol 5

Washing buffer with Tween (WBT), see Protocol 5

Special Equipment

CL-4B Sepharose (Pharmacia)

1-ml gel filtration columns (QIAGEN)

Equipment and reagents for in vitro translation as described in Protocol 4

METHOD

1. Perform an in vitro translation reaction and stop it as described in Protocol 4.
2. Prepare a gel filtration column (QIAGEN) with 1 ml of CL-4B Sepharose (Pharmacia) and equilibrate with ice-cold WB, Protocol 5.
3. Apply 250 μ l of stopped translation mix (Protocol 4) to the column.
4. Add 200 μ l of ice-cold WBT (Protocol 5) 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 (by addition of WBTH; Protocol 4) and, in a parallel experiment, such that the ribosomal complexes dissociate due to lacking stabilization by Mg^{2+} (by addition of WBTH without Mg^{2+}), and therefore the polypeptide is released. 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

Radio Immunoassay

This protocol determines whether a selected pool contains specific binders to a known ligand. Note that the following control experiments should be performed: a background control (e.g., without immobilized ligand) and a control in which free ligand is added as a competitor and therefore interferes with binding to the immobilized ligand.

MATERIALS

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

Buffers, Solutions, and Reagents

PBS buffer 1x

10 mM Na₂HPO₄ (pH 7.4)

140 mM NaCl

15 mM KCl

PBST buffer 1x: PBS with 0.5% (v/v) Tween-20

[³⁵S]Methionine (10 mCi/ml, 1175 Ci/mmol; New England Nuclear)

SDS (4% [w/v]) in PBS

Biological Reagents

Neutravidin (Pierce)

Neutravidin is a deglycosylated form of avidin, whose isoelectric point has been brought to neutral by chemical modification, resulting in lower nonspecific binding properties.

Special Equipment

Liquid scintillation cocktail OptiPhase2 (Wallac)

Scintillation counter

Equipment and reagents for in vitro translation as described in Protocol 4

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, alternatively, with neutravidin (100 µl per well, 4 µg/ml in PBS).
2. Wash the plate three times with PBS. In case of neutravidin coating in step 1, add 50 pmole of biotinylated ligand in 100 µl of PBS and incubate for 30 min at 25°C.
3. After washing with PBST, block the microtiter plate wells with 4% skimmed low-fat milk in PBS for 1 hr.
4. Carry out an in vitro translation using the RNA of the pool or of single clones as a template, as described in Protocol 4 (10 µg of mRNA per 110 µl reaction volume) with the following modifi-

cations: Carry out the *in vitro* translation for 30 min at 37°C. Add 2 μ l of [³⁵S]methionine (0.3 μ M, 50 μ Ci/ml final), but no cold methionine per 110 μ l translation reaction.

5. Dilute the reaction mixture with 440 μ l of PBST after translation and centrifuge for 5 min at 14,000g.
6. Dilute the supernatant with 440 μ l of 4% milk in PBST containing either no ligand or, for competition studies, different concentrations of free unbiotinylated ligand ($0.5\times K_D$ to $10\times K_D$). Preincubate for 1 hr at room temperature, before applying to the microtiter well.
7. Add 100 μ l of the radioactive reaction mix into the microtiter well and allow the binding reaction with the immobilized ligand to take place for 30 min at room temperature, while shaking gently.
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 for optimization of ribosome display to obtain the maximal amount of mRNA–ribosome–protein complexes. It should also be used to establish the optimal time of in vitro translation for a new ribosome-display construct or a new affinity selection scheme.

MATERIALS

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

Buffers, Solutions, and Reagents

Chemiluminescent substrate CSPD (disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)-tri-cyclo-[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate) (Roche Diagnostics)
DIG (digoxigenin) Oligonucleotide Tailing Kit (Roche Diagnostics)
DIG DNA labeling and detection kit
Elution buffer EB-5
50 mM Tris-acetate<!.> (pH 7.5)
150 mM NaCl
5 mM EDTA
50 µg/ml *S. cerevisiae* RNA (Sigma)
Oligonucleotide Anti-SDA (see Table 1)
Gel loading buffer (DNA) 5x
200 mM Tris base<!.>
100 mM acetic acid<!.>
5 mM EDTA
50% glycerol
1% SDS<!.>
a spatula tip of bromophenol blue
Guanidinium isothiocyanate (1 M)
Ethanol, 99%, ice-cold<!.>

Special Equipment

Apparatus for agarose gel electrophoresis
Turboblotter with Nytran Nylon membrane (Schleicher & Schuell)
X-ray films and a developing machine
Equipment and reagents for in vitro translation (Protocol 4) and affinity selection (Protocol 5)

METHOD

1. To optimize the translation time (to maximize the amount of mRNA–ribosome–protein complexes), carry out in vitro translations and affinity selections with a single model mRNA construct under similar conditions as described in Protocol 4. Test translation times from 6 to 12 min.

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

2. After in vitro translation for 5, 7, 9, and 11 min, carry out an affinity selection as described in Protocol 5, Part I, with one modification: After washing, elute the mRNA with 200 μ l of elution buffer EB-5.
3. Precipitate the mRNA immediately by addition of 600 μ l of ice-cold ethanol (99%) and incubate the samples for 30 min on ice.
4. Recover the mRNA by centrifugation at 14,000g for 30 min at 4°C.
5. Remove the supernatants and dry the RNA pellets for 10–15 min at room temperature.
6. Dissolve the RNA pellet in 10 μ l of RNA denaturation buffer (Protocol 2) on ice and incubate for 10 min 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 2 μ l of gel loading buffer, and separate by electrophoresis through a 1.5% agarose gel in the presence of TBE (Protocol 2) and 20 mM guanidinium isothiocyanate.
8. Blot the mRNA samples to Nytran Nylon membrane using a Turboblotter according to the manufacturer's recommendations.
9. Carry out hybridization for at least 4 hr 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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