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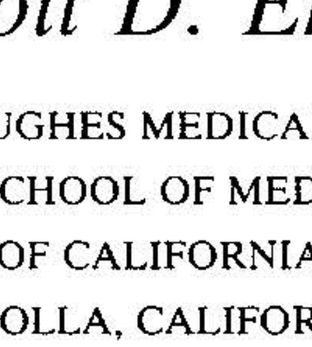

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[24] Selecting and Evolving Functional Proteins *in Vitro* by Ribosome Display

By JOZEF HANES, LUTZ JERMUTUS, and ANDREAS PLÜCKTHUN

Introduction

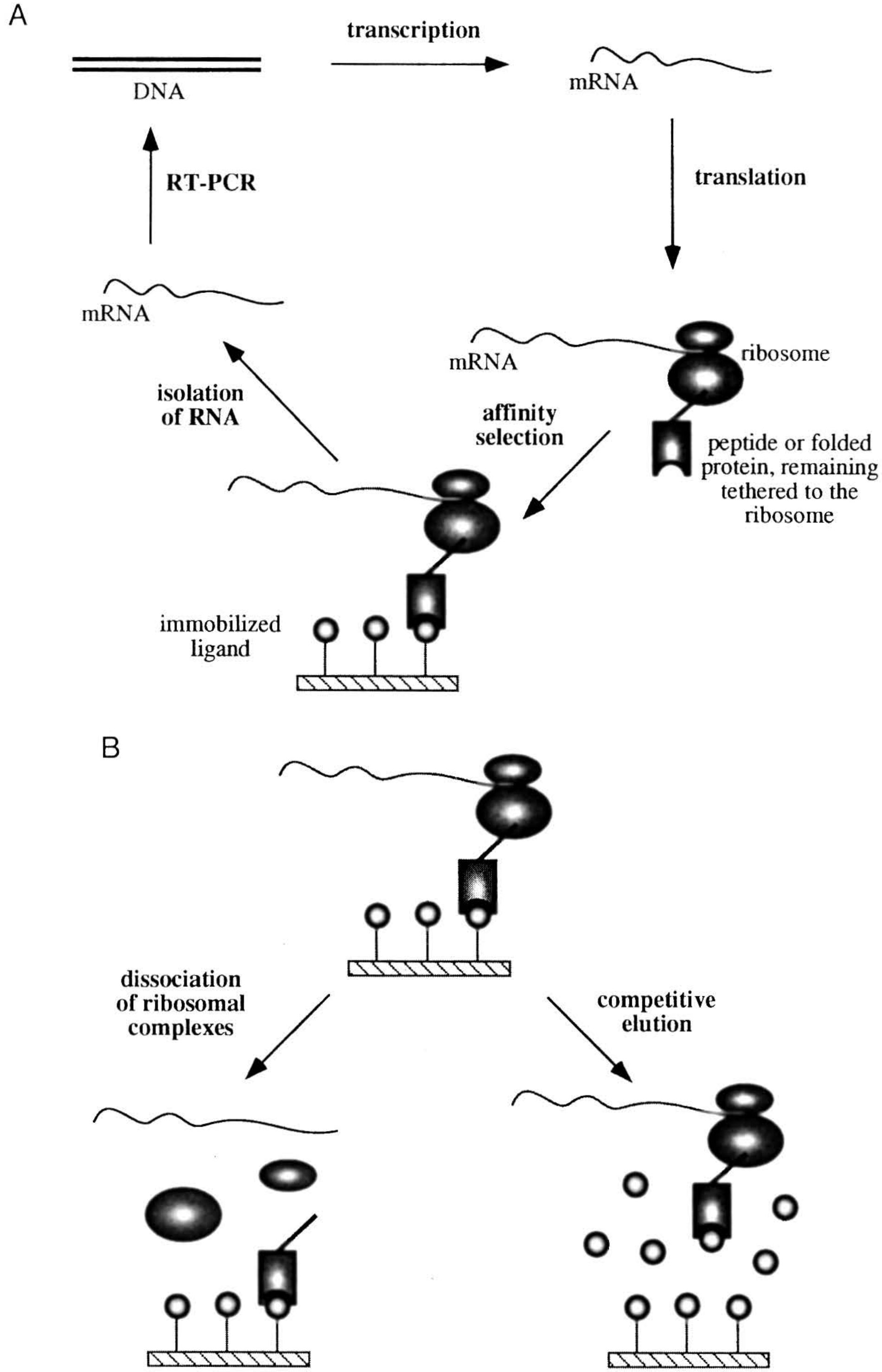
Most techniques used for the screening and selection of protein libraries are based on *in vivo* systems. They either use living cells directly, such as cell surface display,[1,2] the yeast two-hybrid system,[3] or the protein–fragment complementation assay (see [14] in this volume[4]), or they use cells indirectly for the production of phages or viruses in techniques such as phage display[5,6]

[1] E. T. Boder and K. D. Wittrup, *Nature Biotechnol.* **15,** 553 (1997).
[2] S. Stahl and M. Uhlen, *Trends Biotechnol.* **15,** 185 (1997).
[3] C. Bai and S. J. Elledge, *Methods Enzymol.* **283,** 141 (1997).
[4] S. W. Michnick, I. Remy, F. X. Campbell-Valois, A. Vallée-Belisle, and J. N. Pelletier, *Methods Enzymol.* **328,** 208 (2000).
[5] G. P. Smith and J. K. Scott, *Methods Enzymol.* **217,** 228 (1993).
[6] G. P. Smith, *Science* **228,** 1315 (1985).

or the selectively infective phage (SIP) technology.[7] However, *in vivo* systems have a number of limitations. One important restriction is that the library size is limited by the transformation efficiency,[8] a step that all of these techniques have in common. Usually, libraries of not more than 10^9 to 10^{10} independent members can be prepared in *Escherichia coli,* and yeast libraries are still smaller by several orders of magnitude because transformation is less efficient. Another limitation of *in vivo*-based selection methods becomes apparent if a diversification step must be included. It requires either repeatedly switching between *in vivo* selection and *in vitro* diversification[9–11] or the use of mutator strains.[12] The former approach is quite laborious, as it makes a new library generation and large-scale transformation necessary for each cycle of sequence diversification and selection. The latter approach may have the disadvantage that possible candidate molecules may be removed from the library by mutations generated either in the host genome or in the plasmid regions important for expression or replication.

All these limitations can be simultaneously overcome in ribosome display. Ribosome display is an *in vitro* technology for the simultaneous selection and evolution of proteins from diverse libraries (reviewed in Refs. 13 and 14). Because no transformation is necessary, large libraries can be prepared and applied for selection. Furthermore, diversification is conveniently introduced in this method, making evolutionary approaches easily accessible. Ribosome display was first applied to short peptides[15,16] and has subsequently been improved to work with folded proteins.[17–19] In this

[7] S. Spada, C. Krebber, and A. Plückthun, *Biol. Chem.* **378,** 445 (1997).
[8] W. J. Dower and S. E. Cwirla, *in* "Guide to Electroporation and Electrofusion" (D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers, eds.), p. 291. Academic Press, San Diego, California, 1992.
[9] A. C. Braisted and J. A. Wells, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5688 (1996).
[10] R. Schier, A. McCall, G. P. Adams, K. W. Marshall, H. Merritt, M. Yim, R. S. Crawford, L. M. Weiner, C. Marks, and J. D. Marks, *J. Mol. Biol.* **263,** 551 (1996).
[11] W. P. Yang, K. Green, S. Pinz-Sweeney, A. T. Briones, D. R. Burton, and C. F. Barbas III, *J. Mol. Biol.* **254,** 392 (1995).
[12] N. M. Low, P. H. Holliger, and G. Winter, *J. Mol. Biol.* **260,** 359 (1996).
[13] J. Hanes and A. Plückthun, *in* "Combinatorial Chemistry in Biology" (M. Famulok and E. L. Winnacker, eds.). *Curr. Top. Microbiol. Immunol.* **243,** 107 (1999).
[14] L. Jermutus, L. A. Ryabova, and A. Plückthun, *Curr. Opin. Biotechnol.* **9,** 534 (1998).
[15] L. C. Mattheakis, R. R. Bhatt, and W. J. Dower, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 9022 (1994).
[16] L. C. Mattheakis, J. M. Dias, and W. J. Dower, *Methods Enzymol.* **267,** 195 (1996).
[17] J. Hanes and A. Plückthun, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 4937 (1997).
[18] J. Hanes, J. Jermutus, S. Weber-Bornhauser, H. R. Bosshard, and A. Plückthun, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14130 (1998).
[19] J. Hanes, L. Jermutus, C. Schaffitzel, and A. Plückthun, *FEBS Lett.* **450,** 105 (1999).


A
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RT-PCR
translation
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chapter we describe in detail the methodology and the nature of the improvements.

The principle of ribosome display is shown in Fig. 1A. DNA, usually a polymerase chain reaction (PCR) product, which encodes a protein library in a special ribosome display cassette (discussed below), is transcribed *in vitro* to mRNA. The mRNA is purified and used for *in vitro* translation. This *in vitro* translation is performed under such conditions that ternary ribosomal complexes are formed, consisting of mRNA, ribosome, and translated polypeptide, which do not dissociate. These complexes can then be used for affinity selection. Complexes that do not encode and produce a polypeptide that specifically recognizes the target are removed by intensive washing. The mRNA of the selected ribosomal complexes, which encode a polypeptide cognate for the target, is isolated and used for reverse transcription and PCR. This amplified DNA can then be used for another round of ribosome display or for analysis. The mRNA can be isolated from bound ribosomal complexes either directly by removing Mg^{2+} with an excess of EDTA, and thus causing dissociation of all bound complexes, or by competitive elution of ribosomal complexes with free ligand, followed by RNA isolation from the eluted complexes (Fig. 1B). In this chapter we provide the information necessary to perform ribosome display for the selection and evolution of functional proteins, using either an *E. coli* or a rabbit reticulocyte translation system.

The *Escherichia coli* Ribosome Display System

Construction of a DNA Library and Its Transcription to mRNA

The ribosome display construct (Fig. 2A) contains, on the DNA level, a T7 promoter for efficient transcription to mRNA. On the RNA level,

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FIG. 1. (A) Principle of ribosome display for screening protein libraries for ligand binding. A DNA library containing all important features necessary for ribosome display (for details see text) is first transcribed to mRNA and after its purification, mRNA is translated *in vitro*. Translation is stopped by cooling on ice, and the ribosome complexes are stabilized by increasing the magnesium concentration. Ribosomal complexes are affinity selected from the translation mixture by the native, newly synthesized protein binding to immobilized ligand. Nonspecific ribosome complexes are removed by intensive washing, and mRNA is isolated from the bound ribosome complexes, reverse transcribed to cDNA, and cDNA is then amplified by PCR. This DNA is then used for the next cycle of enrichment, and a portion can be analyzed by cloning and sequencing and/or by ELISA or RIA. (B) Two methods for mRNA isolation from bound ribosomal complxes. The bound ribosomal complexes can either be dissociated by an excess of EDTA and then RNA is isolated, or they can first be eluted specifically with free ligand followed by RNA isolation.

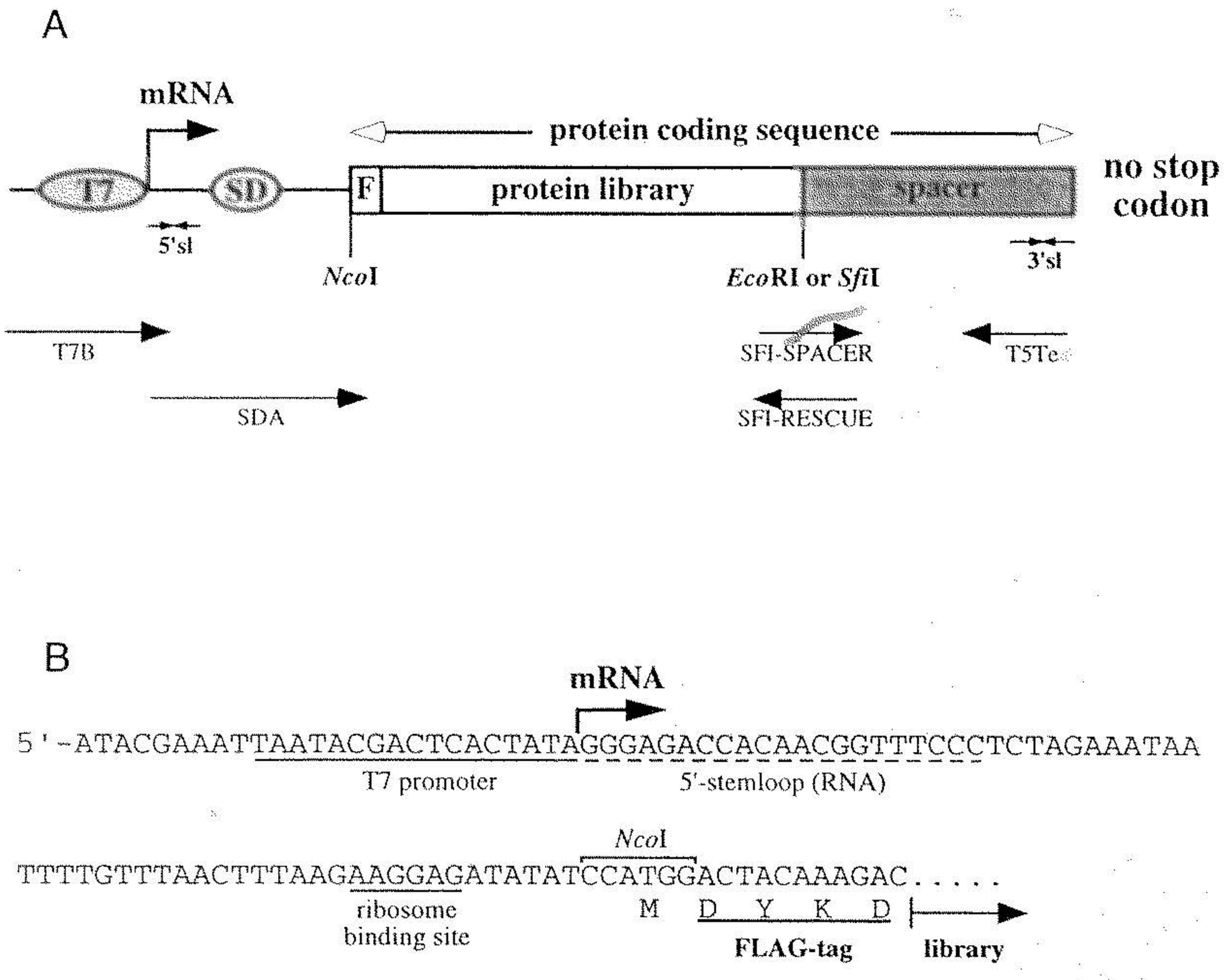

FIG. 2. (A) Schematic drawing of the DNA construct used for *E. coli* ribosome display system. *T7* denotes the T7 promoter, *SD* the ribosome-binding site, *spacer* the part of protein construct connecting the folded protein to the ribosome, *F* the FLAG tag sequence and *5′sl* and *3′sl* the stem–loops on the 5′ and 3′ end of the mRNA. The filled arrow indicates the transcriptional start and open arrows the protein coding sequence. The primers used for the PCR amplification are shown below the scheme. If the construct is prepared by ligation of the library coding sequence to the spacer, in the first PCR this ligation mixture is amplified with primers SDA and T5Te, followed by a second amplification with primers T7B and T5Te. For the preparation of the construct by assembly PCR, the library sequence and the spacer are amplified separately with primers SDA and SFI-RESCUE for library or SFI-SPACER and T5Te for spacer. Purified PCR products are then assembled by PCR without primers for several cycles after amplification with primers SDA and T5Te in the first step. The second amplification is carried out with primers T7B and T5Te. (B) DNA upstream sequence used for the *E. coli* ribosome display constructs.

the construct contains a prokaryotic ribosome binding site (Shine–Dalgarno sequence), followed by the open reading frame encoding the protein library without any stop codon, to avoid release of synthesized peptide, and its mRNA from the ribosome. The mRNA also contains 5′ and 3′ stem–loops, which are known to stabilize mRNA against RNases and therefore increase its half-life *in vivo* as well as *in vitro*.[20] In our most frequently used constructs

[20] J. G. Belasco and G. Brawerman, "Control of Messenger RNA Stability." Academic Press, San Diego, California, 1993.

the 5′-untranslated region of the mRNA, including the 5′ stem–loop and the ribosome-binding site, is derived from gene 10 of phage T7[21] (Fig. 2B), and the 3′ stem–loop is derived from the early terminator of phage T3[22] (Fig. 3) that has been slightly modified to give an open reading frame.

The protein-coding sequence comprises two portions: the N-terminal part, which encodes the polypeptide to be selected (the library), and the C-terminal part, which is constant and serves as a spacer (Fig. 2A). The spacer has two functions: (1) it tethers the synthesized protein to the ribosome by maintaining the covalent bond to the tRNA, which is bound at the P-site of the ribosome, and (2) it provides an unstructured portion at the C terminus of the protein to be folded, such that the ribosomal tunnel can cover at least 20–30 amino acid residues[23–25] of the emerging polypeptide without interfering with the folding of the main portion of the protein. We currently use two different spacers, named here spacer A and spacer B, both derived from gene III of filamentous phage M13mp19[26] (Swissprot: P03662), covering amino acids (aa) 211–318 or 249–318, respectively. The use of the longer spacer A (121 aa; Fig. 3A) results in an approximately twofold higher efficiency of ribosome display than that of spacer B (82 aa; Fig. 3B). However, it is somewhat more difficult to introduce the longer spacer by PCR amplification with oligonucleotides that anneal to the ends of the cDNA, usually resulting in lower amount and quality of the PCR products than the shorter one.

The ribosome display library can be constructed *in vitro,* without any *E. coli* transformation, either by ligating the DNA encoding the library to the spacer, or by assembly PCR. If the library is prepared by ligation, the spacer is most conveniently isolated from a restriction fragment of a phage or phagemid. To keep restriction sites convenient, either *Eco*RI/*Hin*dIII or *Sfi*I/*Hin*dIII fragments are used (Fig. 2A). Thus, spacer A can be isolated from an fd phage (f17/9),[27] which displays a single-chain Fv fragment and had been engineered to contain appropriate restriction sites in gene III, by *Eco*RI/*Hin*dIII digestion. Similarly, spacer B can be isolated from the vector pAK200[28] by cutting with *Sfi*I/*Hin*dIII. The DNA library must be

[21] F. W. Studier, A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff, *Methods Enzymol.* **185,** 60 (1990).
[22] R. Reynolds, R. M. Bermudez-Cruz, and M. J. Chamberlin, *J. Mol. Biol.* **224,** 31 (1992).
[23] W. P. Smith, P. C. Tai, and B. D. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5922 (1978).
[24] R. Jackson and T. Hunter, *Nature* (*London*) **227,** 672 (1970).
[25] L. I. Malkin and A. Rich, *J. Mol. Biol.* **26,** 329 (1967).
[26] P. M. van Wezenbeek, T. J. Hulsebos, and J. G. Schoenmakers. *Gene* **11,** 129 (1980).
[27] C. Krebber, S. Spada, D. Desplancq, and A. Plückthun. *FEBS Lett.* **377,** 227 (1995).
[28] A. Krebber, S. Bornhauser, J. Burmester, A. Honegger. J. Willuda, H. R. Bosshard, and A. Plückthun, *J. Immunol. Methods* **201,** 35 (1997).

A

*Eco*RI

library → GCTTCCGGAGAATTCCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCT
A S G E F P Q P P V N A G G G S

GGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCTGAGGGT
G G G S G G G S E G G G S E G G G S E G

GGCGGCTCTGAGGGTGGCGGTTCCGGTGGCGGCTCCGGTTCCGGTGATTTTGATTATGAA
G G S E G G G S G G G S G S G D F D Y E

AAGATGGCAAACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTACAG
K M A N A N K G A M T E N A D E N A L Q

TCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGT
S D A K G K L D S V A T D Y G A A I D G

TTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATACCGCACAC
F I G D V S G L A N G N G A T G D T A H

3'-stemloop (RNA)

CTTACTGGTGTGCGG
L T G V R

B

*Sfi*I

library → G|GCCTCGGGGGCCGAGGGCGGCGGTTCTGGTTCCGGTGATTTTGATTAT
A S G A E G G G S G S G D F D Y

GAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTA
E K M A N A N K G A M T E N A D E N A L

CAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGAT
Q S D A K G K L D S V A T D Y G A A I D

GGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATACCGCA
G F I G D V S G L A N G N G A T G D T A

3'-stemloop (RNA)

CACCTTACTGGTGTGCGG
H L T G V R

FIG. 3. DNA and deduced protein sequence of spacer A (A) and spacer B (B) used for ribosome display. The arrow indicates the 3′ end of the protein library coding sequence.

prepared in such a way (usually by PCR) that it contains *Sfi*I or *Eco*RI flanking sequences at its 3′ end and carries at least 12 nucleotides of a constant 5′-flanking sequence. The 5′-flanking sequence is necessary for the annealing of PCR primers, with which the entire DNA library is amplified to generate the full expression cassette (Fig. 2). In this way, the required upstream elements (T7 promoter, Shine–Dalgarno sequence) are introduced. For this constant sequence, either a part of the 5′-untranslated mRNA sequence can be used, or a protein tag can be added to the translated region that can later also be used for protein detection. For actually assembling the cassette (Fig. 2) by ligation, the DNA library encoding the polypeptide of interest is cut with an appropriate restriction enzyme (*Sfi*I or *Eco*RI) at the 3′ end of the polypeptide region and ligated to the selected spacer. The ligation mixture is subsequently amplified in two PCRs with two pairs of oligonucleotides (SDA and T5Te, followed by T7B and T5Te; Fig. 2A), which introduce all above-mentioned features important for ribosome display.[17]

If the whole ribosome display library construct (Fig. 2A) is prepared by assembly PCR instead of ligation, the actual protein library DNA and the constant spacer are first amplified separately. Primers are designed in such a way that the DNA library at its 3′ end and the spacer DNA at its 5′ end each contain an identical sequence of 18 nucleotides. Both PCR products are purified and used for assembly PCR (SDA and SFI-RESCUE, and SFI-SPACER and T5Te; Fig. 2A). First, during a few cycles of PCR, both fragments anneal together and are completed with *Taq* polymerase in the absence of any primers, and then this assembled template is further amplified in the presence of the "outer" primers (T7B and T5Te; Fig. 2A).

Library diversity can be increased before starting affinity selection or in between cycles of ribosome display by, e.g., DNA shuffling,[29] error-prone PCR,[30] or the staggered extension process.[31] In this case, only the DNA encoding the protein library is diversified by these PCR-based techniques, amplified with primers specific for this polypeptide cassette (e.g., SDA and SFI-RESCUE; Fig. 2A), and then either ligated to the spacer and then subjected to PCR amplification or, alternatively, directly used for assembly PCR to regenerate the whole cassette.

PCR products encompassing the whole construct with promoter, Shine–Dalgarno sequence, protein library and spacer, prepared either from liga-

[29] W. P. Stemmer, *Nature* (*London*) **370,** 389 (1994).
[30] R. C. Cadwell and G. F. Joyce, *Genome Res.* **3,** S136 (1994).
[31] H. Zhao, L. Giver, Z. Shao, J. A. Affholter, and F. H. Arnold, *Nature Biotechnol.* **16,** 258 (1998).

tion or from assembly PCR, are directly used for *in vitro* transcription with T7 RNA polymerase,[32] and RNA is purified by LiCl precipitation.

As an example, we provide here a procedure for the preparation of an antibody single-chain Fv fragment (scFv) library from immunized mice. Briefly, light (V_L) and heavy (V_H) chains are separately amplified by PCR from the natural mRNAs. After their purification they are assembled by PCR to create a DNA library *in vitro,* without *E. coli* transformation. The resulting PCR product encodes V_L linked to V_H via a flexible linker $(Gly_4Ser)_4$. This procedure, which is identical for assembling an scFv library for either phage display or ribosome display, has been described elsewhere.[28] After digestion with *Sfi*I (which flanks the scFv portion at its 3′ end), this library is used for the preparation of the ribosome display construct. The coding sequence starts with a FLAG tag (sequence MDYKD) and, of course, carries no signal sequence. To this FLAG sequence the primer SDA anneals, which is used for the PCR amplification of the whole library and that introduces the ribosome-binding site.

Materials

PCR primers:
SDA: 5′-AGA CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA TCC ATG GAC TAC AAA GA-3′; the ribosome-binding site is underlined
T7B: 5′-ATA CGA AAT TAA TAC GAC TCA CTA TAG GGA GAC CAC AAC GG-3′; the T7 promoter is underlined
T5Te: 5′-CCG CAC ACC AGT AAG GTG TGC GGT ATC ACC AGT AGC ACC-3′; the sequence creating a 3′ stem–loop at the RNA level is underlined
*Sfi*I fragment of mouse scFv DNA library (150 ng, ca. 2×10^{11} molecules)
QIAquick gel extraction kit (Qiagen, Hilden, Germany)
T4 DNA ligase (Roche Diagnostics, Mannheim, Germany)
Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD)
dNTPs, 20 m*M* each (Roche Diagnostics)
Dimethyl sulfoxide (DMSO); Fluka, (Buchs, Switzerland)
10 m*M* Tris-HCl, pH 8.5
T7 RNA polymerase (New England BioLabs, Beverly, MA)
T7 RNA polymerase buffer (5×): 1 *M* HEPES-KOH (pH 7.6), 150 m*M* magnesium acetate, 10 m*M* spermidine, 0.2 m*M* dithiothreitol (DTT)
RNasin (Promega, Madison, WI)

[32] I. D. Pokrovskaya and V. V. Gurevich, *Anal. Biochem.* **220**, 420 (1994).

LiCl, 6*M*
RNase-free water (Milli-Q-purified)

Additional Material for DNA Library Construction by Assembly Polymerase Chain Reaction

Primers:
SFI-RESCUE: 5′-GCC CTC GGC CCC CGA GGC-3′
SFI-SPACER: 5′-GCC TCG GGG GCC GAG GGC GGC GGT T-3′

Protocol for DNA Library Construction by Ligation or Assembly Polymerase Chain Reaction and Its Transcription to mRNA. The vector pAK200[28] is cut by *Sfi*I/*Hin*dIII and the resulting 481-bp fragment, encoding part of the C-terminal domain of the gene III protein, which serves as the spacer (Fig. 2A), is purified with the QIAquick gel extraction kit. The *Sfi*I fragment of an scFv library (150 ng), prepared by assembly PCR as described in Ref. 28, is ligated to a threefold molar excess of the purified 481-bp *Sfi*I/*Hin*dIII fragment (300 ng) in a 30-μl reaction overnight at 16° with 10 units (U) of T4 DNA ligase. The PCR amplification is performed in 50 μl, containing 5 μl of ligation mixture, 5 μl of 10× PCR buffer, 1.5 μl of 50 m*M* $MgCl_2$ (the ligation mixture also contains Mg^{2+}), 0.5 μl of 20 m*M* dNTPs, 2.5 μl of DMSO, 0.5 μl each of 100 μM primers SDA and T5Te, and 0.5 μl (2.5 U) of *Taq* DNA polymerase (4 min at 94°, and then 5 cycles of 30 sec at 94°, 30 sec at 37°, 2.5 min at 72°, followed by 15 to 20 similar cycles at 50 instead of 37°, and finished by 10 min at 72°). PCR products are separated by agarose gel electrophoresis, and the DNA band corresponding to the library is purified with the QIAquick gel extraction kit and eluted in 30 to 50 μl of 10 m*M* Tris-HCl, pH 8.5 or water. This DNA is subsequently used for the second PCR amplification, using the same conditions as for the first one, except that primers T7B and T5Te and 10 to 20 μl of purified PCR products are used as template for the PCR (usually 12 to 16 cycles).

To prepare a DNA library by assembly PCR instead of ligation to the 3′-tether cassette, first the vector pAK200[28] is amplified with primers SFI-SPACER and T5Te, and the *Sfi*I fragment of the mouse scFv DNA library is amplified with primers SDA and SFI-RESCUE, using the same conditions as described above. After purification of both fragments with the QIAquick gel extraction kit, the library and the spacer fragment are applied for assembly PCR. Fifteen nanograms of the library fragment and 30 ng of spacer fragment (about a threefold molar excess) are mixed and amplified by PCR, first without primers (4 min at 94°, then 5 cycles of 30 sec at 94°, 30 sec at 50°, 2.5 min at 72°), followed by amplification with primers SDA

and T5Te (12 cycles of 30 sec at 94°, 30 sec at 50°, 2.5 min at 72°, and finished by 10 min at 72°), under similar conditions as described above for the amplification from the ligation mixture. The second PCR with primers T7B and T5Te is performed as described above.

It is essential for obtaining the best ribosome display performance that the quality of the PCR product used for transcription be extremely high: the amplified sample should contain a single strong band and the DNA should be at least 10 to 20 ng/μl in concentration, without by-products or smears, when analyzed by agarose gel electrophoresis. The mRNA from a ribosome display library is prepared by *in vitro* transcription in a 150-μl reaction containing 30 μl of 5× T7 RNA polymerase buffer, 21 μl of NTPs (50 m*M* each), 3 μl (120 U) of RNasin, 6 μl (300 U) of T7 RNA polymerase, and 30 μl of the unpurified PCR products from the second PCR amplification (see above). After incubating the mixture for 2 to 3 hr at 37°, the RNA is purified as follows. The reaction is mixed with the same volume of 6 *M* LiCl, incubated on ice for 30 min, and subsequently centrifuged 20 min at 4° and 16,000*g*. The RNA pellet is washed once with 500 μl of 70% ethanol, dissolved (without prior drying) in 100 μl of RNase-free water, and precipitated a second time by adding a one-tenth volume of 1 *M* NaCl and 3 volumes of ethanol and incubating on ice for 30 min. The mixture is centrifuged as for the first precipitation, and the pellet is washed with 70% ethanol, dried, and dissolved in RNase-free water. The concentration of RNA is estimated by optical density (OD) measurement at 260 nm (1 OD_{260} = 40 μg/ml). For successful ribosome display, the mRNA quality is important. This can be controlled by agarose gel electrophoresis.[33] The sample should contain at least 90% full-length mRNA.

Preparation of S-30 Extract

In vitro translation is performed by using an S-30 *E. coli* extract, which is prepared from the *E. coli* strain MRE600 by a slightly modified procedure of Lesley,[34] based on the procedure of Chen and Zubay.[35] Our protocol for S-30 ribosome display extract preparation differs from the published method[34] only by the fact that DTT, 2-mercaptoethanol, and *p*-toluene sulfonyl fluoride are omitted in all solutions. However, if a library of proteins is to be screened that does not contain disulfide bonds, the extract can be prepared with DTT and 2-mercaptoethanol. The quality of the S-30 extract

[33] S. K. Goda and N. P. Minton, *Nucleic Acids Res.* **23,** 3357 (1995).
[34] S. A. Lesley, *Methods Mol. Biol.* **37,** 265 (1995).
[35] H. Z. Chen and G. Zubay, *Methods Enzymol.* **101,** 674 (1983).

is important for the efficiency of ribosome display and also for the *in vitro* translation itself. The cells must be harvested in early exponential growth phase, when their translational machinery is highly active. According to our experience, the cells should not be harvested at OD_{550} higher than 0.5, when 5-liter baffled flasks are used for culturing. However, cells can be harvested at a higher OD_{550} if a fermenter is used.

Material

Escherichia coli strain MRE600

Growth medium (per liter of distilled water): 5.6 g of KH_2PO_4 (anhydrous), 28.9 g of K_2HPO_4 (anhydrous), 10 g of yeast extract, 15 mg of thiamin, and 25 ml of 40% (w/v) glucose (added after separate sterilization)

S-30 buffer: 10 m*M* Tris–acetate (pH 7.5), 14 m*M* magnesium acetate, and 60 m*M* potassium acetate. The solution must be chilled on ice before use

French pressure cell (SLM-Aminco, Rochester, NY)

Dialysis tubing (6000–8000 molecular weight cutoff), prewashed in S-30 buffer

Preincubation mix (per 10 ml): 3.75 ml of 2 *M* Tris–acetate (pH 7.5), 71 μl of 3 *M* magnesium acetate, 75 μl of a solution containing the 20 amino acids (10 m*M* each), 0.3 ml of 0.2 *M* ATP, 0.2 g of phosphoenolpyruvate (trisodium salt), and 50 U of pyruvate kinase. The preincubation mix must be prepared immediately before use

Protocol for S-30 Extract Preparation. A starter culture of *E. coli* MRE600 is grown overnight at 37° in 100 ml of yeast extract containing growth medium with shaking. On the following day, 1-liter aliquots of medium in 5-liter baffled flasks are inoculated with 10 ml of the overnight culture and growth is allowed to continue at 37° up to an OD_{550} of 0.5. Then, approximately the same volume of ice is added to the *E. coli* culture, and cells are harvested by centrifugation at 3500 *g* for 15 min at 4°. The supernatant is discarded, and another 1-liter aliquot of the remaining culture can be added to the centrifuge bottles and centrifuged. After centrifugation, pellets are thoroughly resuspended in 200 ml (per liter of culture) of ice-cold S-30 buffer and centrifuged at 3500*g* for 15 min at 4°. This washing step is repeated once more, and after centrifugation, pellets are combined and weighted. At this point, pellets can be stored at −80° or immediately used for extract preparation.

The washed cells are thoroughly resuspended in ice-cold S-30 buffer at a ratio of 4 ml of buffer per gram of wet cells. The cells are lysed by a

passage through a chilled French pressure cell at 6000 psi. Repeated passing of the cell suspension through French pressure cell will result in a decrease of translational activity of the extract. The lysed cells are immediately centrifuged at 30,000*g* for 30 min at 4°. The supernatant is transferred to a clean centrifuge tube and centrifuged again at 30,000 *g* for 30 min at 4°. The supernatant of the second centrifugation is transferred again to a clean flask, and for each 6.5 ml of S-30 extract, 1 ml of preincubation mix is added, and this solution is slowly shaken for 1 hr at 25° (there should be no foaming). By this so-called "run-off" procedure the ribosomes finish translation of endogenous mRNA, and endogenous mRNA and DNA are degraded by nucleases present in the extract. This step is necessary to make the system dependent only on exogenous template. Afterward, the S-30 extract is transferred to dialysis tubing and dialyzed in the cold room three times against chilled S-30 buffer (500 ml of buffer per aliquot of extract prepared from 1 liter of culture). Each dialysis solution is replaced after 4 hr (one step can also be overnight). The extract is divided into aliquots of 100 to 500 μl and frozen in liquid nitrogen. The frozen extract can be stored for several months at −80° without loss of activity. After thawing, the extract can be frozen once more for later use. It is not recommended to thaw and freeze the extract more than twice, because it will begin losing activity.

In Vitro Translation for Ribosome Display

The *in vitro* translation for ribosome display is usually performed at 37° for a relatively short time of only about 6 to 8 min. This short time has been optimized to minimize decay of the ternary complexes.[17] Despite the general tendency of proteins to fold with higher efficiency at lower temperature *in vitro*, the yield of functional molecules from *in vitro* translation was experimentally found to be higher at 37° at least for the scFv fragments examined, which are not unusually stable molecules. This higher yield may be due to the action of molecular chaperones in the extract, and the optimal temperature is probably a complicated function of the temperature dependence of translation, folding, RNases, and perhaps proteases. The translation mixture contains the whole translational machinery present in the S-30 extract and also all low molecular weight compounds necessary for *in vitro* translation (ATP, GTP, amino acids, tRNA, the energy regeneration system, etc.). The efficiency of forming correctly folded disulfide-containing proteins such as antibody scFv fragments in the *E. coli* ribosome display system was found to be increased if eukaryotic protein disulfide isomerase (PDI), which catalyzes disulfide bond formation and

rearrangement,[36,37] was used during the reaction.[17] Furthermore, the efficiency of the *E. coli* system was found to be higher if the 10Sa-RNA (the product of the *ssrA* gene), which is responsible for tagging and releasing truncated peptides from *E. coli* ribosomes,[38–40] was eliminated. This was achieved by including an antisense oligonucleotide in the reaction mixture.[17]

To achieve the maximal efficiency of the *E. coli* ribosome display system, the *in vitro* translation must be optimized for each batch of extract regarding the concentration of magnesium and potassium ions, the concentration of the extract itself, and the translation time under ribosome display conditions (see Optimization of the *Escherichia coli* Ribosome Display System, below).

Here, we provide an example of *in vitro* translation, using optimized conditions for one particular batch of S-30 extract and using mRNA encoding an scFv library, prepared according to the protocol of the section Construction of a DNA Library and Its Transcription to mRNA, above).

Material

S-30 *E. coli* extract
Premix Z: 250 m*M* Tris–acetate (pH 7.5), a 1.75 m*M* concentration of each amino acid, except methionine, 10 m*M* ATP, 2.5 m*M* GTP, 5 m*M* cAMP, 150 m*M* acetyl phosphate, *E. coli* tRNA (2.5 mg/ml), folinic acid (0.1 mg/ml), 7.5% (w/v) polyethylene glycol (PEG) 8000 (Sigma, St. Louis, MO)
Methionine, 200 m*M*
Magnesium acetate, 100 m*M*
Potassium glutamate, 2 *M*
Anti-*ssrA* oligonucleotide, 200 μM (5′-TTA AGC TGC TAA AGC GTA GTT TTC GTC GTT TGC GAC TA -3′)
Protein disulfide isomerase (PDI), 44 μM (Sigma)
Library mRNA, 1 μg/μl
Washing buffer WBTH: 50 m*M* Tris–acetate (pH 7.5), 150 m*M* NaCl, 50 m*M* magnesium acetate, 0.1% (v/v) Tween 20, heparin (2.5 mg/ml)

Protocol for Ribosome Display in Vitro Translation. A tube containing 440 μl of washing buffer WBTH is prepared in an ice–water bath. On ice,

[36] L. A. Ryabova, D. Desplancq, A. S. Spirin, and A. Plückthun, *Nature Biotechnol.* **15,** 79 (1997).
[37] R. B. Freedman, T. R. Hirst, and M. F. Tuite, *Trends Biochem. Sci.* **19,** 331 (1994).
[38] K. C. Keiler, P. R. Waller, and R. T. Sauer, *Science* **271,** 990 (1996).
[39] Y. Komine, M. Kitabatake, T. Yokogawa, K. Nishikawa, and H. Inokuchi, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 9223 (1994).
[40] B. K. Ray and D. Apirion, *Mol. Gen. Genet.* **174,** 25 (1979).

the following ice-cold solutions are combined: 22 μl of premix Z, 1.1 μl of 200 m*M* methionine, 11 μl of 2 *M* potassium glutamate, 7.6 μl of 100 m*M* magnesium acetate, 2 μl of 200 μM anti-*ssrA* oligonucleotide, 1.5 μl of 44 μM PDI, 40 μl of S-30 *E. coli* extract, and sterile double-distilled water is added up to 100 μl. The aliquot of the library mRNA to be used should be thawed just before use, and the unused part should be immediately snap-frozen. Ice-cold library mRNA (10 μg or approximately 2×10^{13} molecules) in 10 μl of RNase-free water is added to the mixture, gently vortexed, and immediately placed in a 37° water bath. After 7 min of *in vitro* translation, the reaction mixture is immediately transferred to the chilled tube with 440 μl of WBTH in an ice–water bath, briefly and gently vortexed, and placed back in the ice–water bath.

Affinity Selection of Ribosomal Complexes

The ribosomal complexes are stabilized by both chilling the translation mixture and by increasing the Mg^{2+} concentration from about 14 m*M* during the reaction to 50 m*M*,[17] by diluting the mixture with ice-cold Mg^{2+}-containing washing buffer. The diluted translation mixture is applied for affinity selection in the presence of 2% (w/v) sterilized milk (from milk powder) and heparin (2 mg/ml),[18] which together significantly reduce nonspecific binding of ribosomal complexes to the surface of microtiter wells, streptavidin magnetic beads, or avidin immobilized on agarose beads. Milk is sterilized to eliminate its intrinsic RNase activity. Another way to reduce nonspecific binding is to apply the diluted translation mixture for a so-called "prebinding" step prior to affinity selection. For this purpose, the translation mixture is first incubated in milk-coated microtiter wells or immunotubes, where "sticky" ribosomal complexes can bind to the milk components on the surface. The supernatant of this preselected solution is then used for affinity selection, which can be carried out either with ligand immobilized on a polystyrene surface or in solution by using biotinylated ligand and subsequent capture by streptavidin magnetic beads or avidin immobilized on agarose beads. After affinity selection, either Mg^{2+} ions are complexed with an excess of EDTA, resulting in the dissociation of ribosomal complexes and elution of mRNA, or whole ribosomal complexes can be eluted with an excess of free ligand. Although EDTA elution is generally preferred as tight binders can be selected, mRNA from nonspecifically bound ribosomal complexes will also be eluted by the EDTA treatment.

For affinity selection with a ligand immobilized on a polystyrene surface, microtiter strips or plates are usually used. However, for large-scale experiments, panning tubes can also be used. If the ligand is a small molecule, e.g., a hapten or peptide, it must be coupled to the carrier protein, e.g.,

bovine serum albumin (BSA) or transferrin, or covalently coupled to activated surfaces (e.g., CovaLink NH_2; Nunc, Roskilde, Denmark).

Affinity selection in solution requires the ligand to be biotinylated, and to be captured by streptavidin magnetic particles or avidin immobilized on agarose beads. The ligand should contain a spacer arm (according to our experience at least 30 Å long) between the biotin moiety and the ligand itself, as this must be long enough to present the ligand outside of the deep streptavidin binding pocket.[41] First, the biotinylated ligand is added to the ribosomal complexes and incubated on ice with shaking for at least 1 hr, to allow the ribosomal complexes to bind to the ligand. Then, unbound biotinylated ligand and also the bound ribosomal complexes are captured by streptavidin magnetic particles, which must therefore be present in sufficient excess.

One of the main keys to success in ribosome display system is to carry out the entire ribosome display procedure, after *in vitro* translation up to the RNA isolation, on ice and to keep all material necessary for this part of the experiment (e.g., pipette tips, tubes, microtiter plates) ice-cold.

Material

Washing buffer WBT: 50 m*M* Tris–acetate (pH 7.5), 150 m*M* NaCl, 50 m*M* magnesium acetate, 0.1% (v/v) Tween 20
Washing buffer WB (10×): 0.5 *M* Tris–acetate (pH 7.5), 1.5 *M* NaCl, 0.5 m*M* magnesium acetate
PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4
Microtiter plate strips or plates (Nunc)
Panning tubes (Nunc)
Ligand
Streptavidin magnetic particles (Roche Diagnostics)
Avidin immobilized on agarose beads (Sigma)
Biotinylated ligand
Sterilized 12% (w/v) milk powder (in water)
Elution buffer EB20: 50 m*M* Tris–acetate (pH 7.5), 150 m*M* NaCl, 20 m*M* EDTA, *Saccharomyces cerevisiae* RNA (50 μg/ml; Sigma)

Protocol for Affinity Selection of Ribosomal Complexes with Ligand Immobilized on a Polystyrene Surface. Sterilized 12% (w/v) milk powder is combined with 10× washing buffer WB to give 10% (w/v) milk powder in 1× WB, and this can be stored on ice for several weeks. Microtiter strips are coated overnight at 4° with 100 μl of PBS solution, containing at least 4 mg of ligand per milliliter. The next day, the coated strips are washed

[41] W. A. Hendrickson, A. Pahler, J. L. Smith, Y. Satow, E. A. Merritt, and R. P. Phizackerley, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 2190 (1989).

with PBS and, together with the same number of noncoated strips, blocked with 4% (w/v) milk powder in PBS for 1 hr at room temperature with shaking. After washing the strips three times with PBS and two times with washing buffer, the strips are filled with 250 μl of ice-cold washing buffer, placed on ice in a cold room, and incubated for at least 20 min prior to affinity selection. This procedure is usually performed before the ribosome display *in vitro* translation is started. It is important that the strips be ice-cold, and the pipette tips temperature equilibrated in the cold room before use, otherwise it will result in a failure of ribosome display.

The washing buffer from the ice-cold strips is removed just before use, and the strips are placed on ice in an appropriate shaker in the cold room. The diluted ice-cold translation mixture (see preceding section) is centrifuged for 5 min at 4° and 16,000*g* to remove insoluble components. The supernatant is mixed with one-fifth volume of ice-cold 10% (w/v) milk powder in 1× WB, pipetted into milk-blocked microtiter strips (which do not contain ligand) for prebinding (200 μl per well), and the strips are gently shaken for 20 min on ice in the cold room. The content of the wells is then transferred to the ligand-coated and milk-blocked strips, and the strips are gently shaken for 1 hr in the cold room on ice. After five washes with ice-cold washing buffer WBT, the retained ribosomal complexes are dissociated with 200 μl of ice-cold elution buffer EB20 for 5 min on ice by gentle shaking, resulting in mRNA elution. The eluted mRNA can be frozen (e.g., in liquid nitrogen), or is immediately used for isolation.

Protocol for Affinity Selection of Ribosomal Complexes in Solution. Because the protocol for affinity selection in solution is similar to the protocol described above for affinity selection on a surface, we focus only on the differences. For the affinity selection a sufficient amount (to allow the capturing of all biotinylated ligand) of streptavidin magnetic particles is washed four times with ice-cold washing buffer WBT, then resuspended in their original volume in ice-cold WBT and aliquoted (if necessary) in ice-cold tubes. Panning tubes (5-ml volume) are blocked with 4% (w/v) milk powder for 1 hr by end-over-end rotation at room temperature, and then washed three times with PBS and three times with washing buffer WBT, filled with WBT, and placed on ice. Biotin-free milk is prepared by end-over-end rotation of 1 ml of sterilized 12% (w/v) milk powder with 100 μl of streptavidin magnetic particles for 1 hr at room temperature and then, after removing the streptavidin magnetic particles, stored on ice. The diluted and centrifuged translation mixture, containing 2% biotin-free milk, is supplemented with biotinylated ligand to the required concentration, and the mixture is transferred to the prepared panning tubes. The tubes are placed inside of an appropriate larger tube or flask filled with ice and rotated end over end for 1 hr in the cold room. In this way, affinity selection

affinity selection (with biotinylated ligand in solution) and prebinding (to the tube surface) are performed simultaneously.

After affinity selection, the solution from the panning tubes is added to streptavidin magnetic particles and rotated end over end on ice for 10 to 15 min in the cold room. Washing and elution of mRNA are performed as described in the previous section, except that a magnet is used for the capturing of streptavidin magnetic particles. The capacity of streptavidin magnetic particles for binding of biotinylated ligand is dependent on the ligand size. Because it is important to capture all ligand-bound ribosomal complexes, especially if libraries of high diversity are used for selection, we do not use more than 10 pmol of biotinylated ligand for 100 μl of streptavidin magnetic particles (the particles can bind 100 times more free biotin). To eliminate the selection of streptavidin binders from the library, we recommend switching between streptavidin magnetic particles and avidin agarose beads for the capturing of the biotinylated ligand after each or each second ribosome display cycle. For the products of the suppliers indicated above, 1 volume of avidin immobilized on agarose has the same binding capacity as 5 volumes of streptavidin magnetic particles.

mRNA Purification, Reverse Transcription, and Polymerase Chain Reaction

mRNA is isolated by using a commercial RNA isolation kit. In some cases, eluted mRNA samples may contain residual DNA. Therefore, we recommend the use of an RNA isolation kit that includes a DNase treatment of the sample. Purified RNA is subsequently used for reverse transcription and PCR, and the resulting DNA is used for another round of ribosome display, for radioimmunoassay (RIA) analysis, or for cloning and subsequent analysis of single clones.

PCR amplification is usually performed in two steps in a similar way as described in the section Construction of a DNA Library and Its Transcription to mRNA (above), by using the SDA and T5Te primers (Fig. 2A) in the first step, subsequent purification of the resulting PCR product by agarose gel electrophoresis, and amplification of the purified template with primers T7B and T5Te in the second PCR. In principle, it is possible to carry out only one PCR with primers T7B and T5Te; however, the quality of the PCR products and also of the mRNA prepared from them is quite often not sufficient to perform a ribosome display experiment for the subsequent round.

Material

Primers T5Te, SDA, and T7B (see above)
High Pure RNA isolation kit (Roche Diagnostics)

Superscript reverse transcriptase (GIBCO-BRL)
RNasin (Promega)
Taq DNA polymerase (GIBCO-BRL)
Solution of dNTPs (20 m*M* each)
DMSO (Fluka)
QIAquick gel extraction kit (Qiagen)
Tris-HCl, pH 8.5 (10 m*M*)

Protocol for mRNA Purification, Reverse Transcription, and Polymerase Chain Reaction. RNA is isolated according to the manufacturer's instructions, using the High Pure RNA isolation kit. The DNase treatment, which is carried out after loading the columns with the RNA sample, is performed for 5 to 10 min at room temperature. Purified RNA is eluted in 35 μl of RNase-free water, and immediately placed in a 70° water bath for 10 min. During the incubation the premix for the reverse transcription reaction is prepared. The following solutions are combined on ice for each reaction: 0.5 μl of T5Te primer (100 μM), 1.25 μl of each dNTP (20 m*M* each), 1.25 μl of RNasin (50 U), 10 μl of 5× first-strand synthesis buffer, 5 μl of 0.1 *M* DTT, and 1.25 μl of Superscript reverse transcriptase (250 units) (the last three components are from the Superscript reverse transcriptase kit). After the 70° incubation, the samples are chilled on ice for 1 to 2 min, centrifuged at 4°, and the whole RNA preparation of approximately 30 μl is combined with the prepared reverse transcription premix and incubated for 1 hr at 50°.

Because it is difficult to predict the number of cycles necessary for PCR amplification at this point, as this depends on the amount of isolated mRNA, a test PCR should first be performed in a 50-μl reaction, containing 5 μl of reverse transcript, 5 μl of 10× PCR buffer, 1.7 μl of 50 m*M* $MgCl_2$, 0.5 μl of dNTPs (20 m*M* each), 2.5 μl of DMSO, 0.5 μl of 100 μM of each of the primers SDA and T5Te, and 0.5 μl (2.5 U) of *Taq* DNA polymerase (4 min at 94°, followed by 20 to 30 cycles of 30 sec at 94°, 30 sec at 50°, 2.5 min at 72°, and finished by 10 min at 72°). Usually, the more ribosome display cycles have already been performed with a diverse library the more mRNA encoding a cognate product to the antigen is isolated and the fewer PCR cycles are necessary. It is better to perform the test PCR first for fewer cycles and to add further cycles, if necessary, after analysis of PCR products by agarose gel electrophoresis. For this purpose, usually no additional components need to be added even after overnight PCR. It is not recommended to overamplify DNA. If PCR products are too smeary, usually resulting from overamplification, the RNA quality will not be sufficient for successful ribosome display. The remaining reverse transcript (about 45 μl) or a portion of it is used for a large-scale PCR, under the optimal PCR conditions established from the small aliquot of reverse transcript. The whole reverse transcript should be used for amplification mainly in

cycles of the ribosome display experiment to quantitatively amplify all recovered binders from the library.

PCR products are purified by agarose gel electrophoresis and used for the second amplification, using primers T7B and T5Te as described for the second PCR in the section Construction of a DNA Library and Its Transcription to mRNA, above.

Optimization of the Escherichia coli Ribosome Display System

To achieve maximal efficiency, the *E. coli* ribosome display system should be optimized. For this purpose, a model protein is required, which gives a clear enrichment on a surface coated with a cognate ligand, compared to a control surface in a ribosome display experiment (e.g., for an scFv library, the antibody scFvhag can be used[17]). The system should be optimized for the concentration of magnesium and potassium ions, the concentration of the extract itself and the translation time for each batch of *E. coli* extract, using ribosome display conditions. The optimization of the system is performed in such a way that the mRNA ribosome display construct, encoding a model protein, is used for several ribosome display *in vitro* translation reactions, which differ either in the concentration of Mg^{2+}, potassium glutamate, or extract. After various incubation times at 37° aliquots of the reactions are stopped and applied for affinity selection. The affinity selection is carried out either with the appropriate ligand, immobilized on the surface, or in solution, using biotinylated ligand. After affinity selection either the mRNA is isolated, reverse transcribed to cDNA, amplified by PCR, and the PCR products analyzed by agarose gel electrophoresis, or the isolated mRNA is analyzed by Northern blot hybridization with a probe specific for the mRNA construct. We prefer the latter approach, because it is possible to directly quantify the amount of isolated mRNA, and this technique is more sensitive in the detection of differences among the amounts of recovered mRNA from different samples. In the optimization of the system, first the magnesium concentration is optimized, and this optimal Mg^{2+} concentration is then used for the further optimization of potassium glutamate and extract concentrations. For the optimization of the Mg^{2+} concentration it should be kept in mind that the extract itself already contains 14 m*M* Mg^{2+}.

We provide here an example for the optimization of the *E. coli* ribosome display system using a ribosome display construct, in which a model protein is fused to spacer B.

Material

High Pure RNA isolation kit (Roche Diagnostics) (optional, see protocol)

Washing buffer WBTH
Sterilized milk, 4% (w/v) milk powder in WBT
Elution buffer EB5: 50 m*M* Tris–acetate (pH 7.5), 150 m*M* NaCl, 5 m*M* EDTA, *S. cerevisiae* RNA (50 μg/ml; Sigma)
RNA denaturation buffer (prepared fresh before use): 10 μl of formamide, 3.5 μl of formaldehyde, 2 μl of 10× morpholinepropanesulfonic acid (MOPS) buffer [0.2 *M* MOPS (pH 7.0), 80 m*M* sodium diacetate, 10 m*M* EDTA]
Gel loading buffer: 50% (w/v) glycerol, 1 m*M* EDTA, 0.25% (v/v) bromphenol blue
Turboblotter with Nytran Nylon membrane (Schleicher & Schuell, Dussel, Germany)
Oligonucleotide SFI-LINK: 5′-CCT TTA AGC AGC TCA TCA AAA TCA CCG GAA CCA GAA CCG CCG CCC TCG GCC CCC GAG GCC G-3′. This oligonucleotide anneals to the *Sfi*I site and a part of the spacer B of ribosome display construct mRNA
Digoxigenin (DIG) oligonucleotide tailing kit (Roche Diagnostics)
Chemiluminescent substrate disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}]decan]4-yl)phenyl phosphate (CSPD; Roche Diagnostics)

Protocol for the Optimization of the Escherichia coli Ribosome Display System by Northern Blot Hybridization. First, the mRNA of a ribosome display construct of a model protein, containing the spacer B, is prepared as described above for the library construction. To optimize the system, *in vitro* translations and affinity selections of the ribosome display system are carried out with a single model mRNA under similar conditions as described in the section *In Vitro* Translation for Ribosome Display (above), with the following modifications. From our experience, the optimal conditions for ribosome display *in vitro* translation can vary in the following ranges: 11 to 15 m*M* magnesium acetate, 180 to 220 m*M* potassium glutamate, 30 to 50 μl of S-30 extract for a 110-μl reaction, and 6 to 9 min for the time of translation. Longer translation times might be necessary if a longer library mRNA is used for ribosome display.

In vitro translation reactions, differing in the concentration of the compound to be tested (magnesium acetate, potassium glutamate, or amount of extract), each prepared in a 110-μl volume, are incubated at 37°. After selected time points (e.g., for an antibody scFv library, after 5, 7, 9, and 11 min) aliquots of 25 μl are taken and immediately added to tubes containing 100 μl of ice-cold washing buffer WBTH (prepared in advance in a water–ice bath), gently vortexed, and stored in the ice bath until the experiment is finished. Samples are then centrifuged, 110 μl of supernatant is mixed with the same volume of 4% (w/v) sterilized milk in WBT, and two 100-

μl volumes of each sample are applied for affinity selection. After washing, mRNA is eluted with 200 μl of elution buffer EB5, and RNA is immediately precipitated by adding 600 μl of ice-cold ethanol and incubating the samples for 30 min on ice. After 30 min of centrifugation at 4° at 16,000*g*, the supernatants are removed and RNA pellets are dried at room temperature for 10 to 15 min, dissolved in 10 μl of RNA denaturation buffer on ice, and incubated for 10 min at 70°. For the estimation of the amount of recovered mRNA several other control samples containing between 0.2 and 10 ng of the original model mRNA can be prepared in a similar way. RNA samples of up to 5 μl can be directly mixed with 10 μl of RNA denaturation buffer. After the 70° incubation, samples are chilled on ice, mixed with 1 μl of gel loading buffer, and separated by 1.5% (w/v) agarose gel electrophoresis in the presence of TBE and 20 m*M* guanidinium isothiocyanate.[33] The use of elution buffer containing higher EDTA concentrations and/or RNA precipitation at lower temperatures may lead to the coprecipitation of EDTA, resulting in a failure of the Northern analysis. Alternatively, RNA may be eluted with buffer EB20 and subsequently purified by the High Pure RNA isolation kit. This procedure, however, requires an additional concentration step of the RNA samples by precipitation.

RNA samples separated by agarose gel electrophoresis are blotted to Nytran Nylon membrane, using a Turboblotter according to the manufacturer recommendations. Hybridization is carried out at least for 4 hr at 60° as described[17] with the oligonucleotide SFI-LINK, labeled by 3′ tailing with digoxigenin–11-dUTP/dATP using the DIG oligonucleotide tailing kit. The hybridized oligonucleotide probe is detected with the DIG DNA labeling and detection kit with the chemiluminescent substrate CSPD and exposure to X-ray film.

Monitoring the Enrichment of a Ribosome Display Library for Specific Binders and Analysis of Single Clones by Radioimmunoassay

After each round of ribosome display, isolated mRNA from affinity-selected ribosomal complexes is reverse transcribed to cDNA and amplified by PCR. The PCR products are used for mRNA preparation, which is used for the next round of ribosome display. This mRNA can also be used for RIA analysis of the ribosome display library to check for the presence of specific binders. Although this mRNA contains no stop codon, translation for more than 30 min in the absence of anti-*ssrA* oligonucleotide and the use of buffers without magnesium for affinity selection lead to protein release from ribosomal complexes, and a RIA can be performed. After *in vitro* translation, which is carried out in the presence of [^{35}S]methionine, samples are tested for ligand binding, with ligand immobilized on a polysty-

rene surface. The RIA is performed both in the presence and absence of free ligand to verify specific binding, as true specific binding always must be inhibitable by free ligand. After affinity selection, bound radioactive protein is eluted with sodium dodecyl sulfate (SDS) and quantified by scintillation counting. The whole procedure after *in vitro* translation can be performed at room temperature. If the analyzed proteins are not stable under such conditions, however, lower temperatures (e.g., 4°) can also be used. The elution must be performed at room temperature, because SDS precipitates at 4°.

If the translation product of the mRNA pool binds to the ligand and this binding can be clearly inhibited with free ligand, the library is already enriched for specific binders. The library-encoded PCR product, from which the mRNA was transcribed for RIA analysis, is cloned, and plasmids are isolated from single clones and used for *in vitro* transcription. After purification, these plasmid mRNAs are used for *in vitro* translation in the presence of [^{35}S]methionine and the translation mixtures are applied for RIA analysis as described above. If the enriched DNA pool is cloned into a plasmid that adds a peptide detection tag to the protein, binders can also be analyzed by enzyme-linked immunosorbent assay (ELISA).

Material

S-30 *E. coli* extract
Premix Z
[^{35}S]Methionine (10 mCi/ml, 1175 Ci/mmol; New England Nuclear, Boston, MA)
Magnesium acetate, 100 m*M*
Potassium glutamate, 2 *M*
PBST: PBS containing 0.5% (v/v) Tween 20
SDS elution buffer: 4% SDS in PBS

Protocol for Radioimmunoassay Analysis of the DNA Pool Enriched for Specific Binders or for Radioimmunoassay of Single Clones. In vitro translation is performed as described in the section *In Vitro* Translation for Ribosome Display (above) with the following modifications. In a 110-μl *in vitro* translation reaction 1.1 μl of cold methionine is replaced with 2 μl of [^{35}S]methionine, anti-*ssrA* oligonucleotide is omitted, and the translation is performed with either 10 μg of enriched library mRNA or 10 μg of mRNA, obtained by transcription from a plasmid, for 30 to 40 min at 37°. After translation, samples are diluted fourfold or more, if required, in PBST, and centrifuged for 5 min to remove any insoluble components. The ligand is diluted in 4% (w/v) milk in PBST to a twofold higher concentration than used in the inhibition reaction. This ligand solution is then mixed with an equal volume of the supernatant from the centrifuged translation mixture

applied for binding to immobilized ligand on the surface of microtiter wells. After washing five times with PBST, bound radioactive protein is eluted with SDS elution buffer for 15 min with shaking, and the eluted protein is quantified by liquid scintillation counting.

The Rabbit Reticulocyte Ribosome Display System

As has been shown,[19] the use of a rabbit reticulocyte lysate as a ribosome source does not offer any clear advantage over the *E. coli*-based display system. In a direct comparison with antibody scFv fragments, which are of course derived from eukaryotic proteins, the rabbit reticulocyte lysate system gave rise to lower amounts of functional complexes, at least for those tested, lower enrichment factors, and higher costs. However, because the lysates are commercially available and contain a lower intrinsic RNase activity, the use of this system might be perceived as being more convenient, and it is also possible that different proteins might be expressed with different efficiencies in the two translation systems.

Construction of a DNA Library and Its Transcription to mRNA

The ribosome display construct for the eukaryotic translation system differs from the construct for the *E. coli*-based system in two respects. First, because of the lower nuclease activity in the reticulocyte translation system, the 5′ and 3′ stem–loops may be omitted, although their presence has no negative influence on the efficiency of the rabbit reticulocyte ribosome display system. Second, to allow efficient translation of any sequence, the gene 10 region of phage T7 is replaced with the translational enhancer sequence of the *Xenopus laevis* β-globin gene, together with an optimal Kozak sequence,[42] the eukaryotic ribosome-binding sequence (Fig. 4). In our construct, the 5′ stem–loop is omitted, while the entire 3′ part of the construct is kept the same as in the *E. coli* ribosome display construct. Therefore, the primer SDA is replaced by the primer SDA-RRL and the primer T7B by T7RR-EN (Fig. 4A).

The preparation of the construct is carried out as described for the *E. coli*-based system. For transcription, we tested the use of a cap analog, which has been reported to enhance the efficiency of *in vitro* translation in some cases.[43,44] However, for maximal enhancement probably both a poly(A) tail and a 5′ cap may be required as the interaction between these

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[44] H. J. Song, D. R. Gallie, and R. F. Duncan, *Eur. J. Biochem.* **232,** 778 (1995).

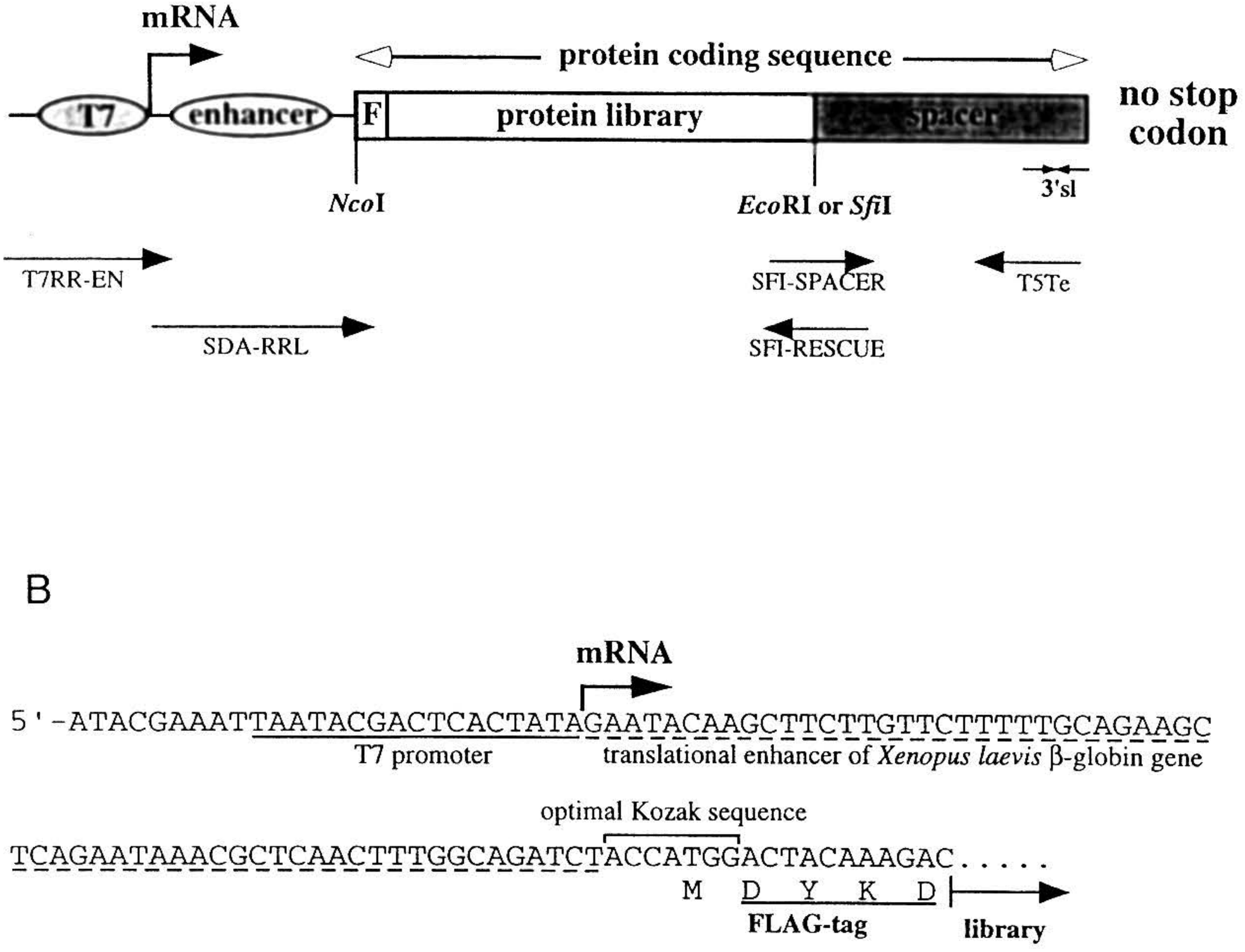

FIG. 4. (A) Schematic drawing of the DNA construct used for rabbit reticulocyte ribosome display system. *T7* denotes the T7 promoter, *enhancer* the translational enhancer of *Xenopus laevis* β-globin gene, *spacer* the part of protein construct connecting the folded protein to the ribosome, *F* the FLAG tag sequence, and *3′sl* the stem–loop at the 3′ end of the mRNA. The filled arrow indicates the transcriptional start and open arrows the protein coding sequence. The primers used for the PCR amplification are shown below the scheme. If the construct is prepared by ligation of the library coding sequence to the spacer, in the first PCR this ligation mixture is amplified with primers SDA-RRL and T5Te, followed by a second amplification with primers T7RR-EN and T5Te. For the preparation of the construct by assembly PCR, the library sequence and the spacer are amplified separately with primers SDA-RRL and SFI-RESCUE for library or SFI-SPACER and T5Te for spacer. Purified PCR products are then assembled by PCR without primers for several cycles after amplification with primers SDA-RRL and T5Te in the first step. The second amplification is carried out with primers T7RR-EN and T5Te. (B) DNA upstream sequence used for the rabbit reticulocyte ribosome display constructs.

termini has been shown to allow for efficient translation initiation.[45] In ribosome display, we could observe only a marginal effect of about a twofold improvement. However, the yield of RNA synthesis drops dramatically, if the cap analogs are used.

[45] M. Piron, P. Vende, J. Cohen, and D. Poncet, *EMBO J.* **17,** 5811 (1998).

The procedures for the construction of a DNA library and its transcription to mRNA remain unchanged relative to the *E. coli* system.

Materials

PCR primers:
SDA-RRL: 5-TTT GCA GAA GCT CAG AAT AAA CGC TCA ACT TTG GCA GAT CTA CCA TGG ACT ACA AAG A-3
T7RR-EN: 5-ATA CGA AAT TAA TAC GAC TCA CTA TAG AAT ACA AGC TTC TTG TTC TTT TTG CAG AAG CTC-3
Cap analog ^{7m}GpppG (Stratagene La Jolla, CA)

Ribosome Display In Vitro Translation and Affinity Selection

The best results in using the rabbit reticulocyte system in ribosome display were obtained with a commercial kit, in which DTT can be omitted and concentrations of Mg^{2+} and K^+ can be optimized.[19] We found that the efficiency of the rabbit reticulocyte ribosome display system is related to the amount of protein produced in the *in vitro* translation reaction. Therefore, potassium and magnesium concentrations are optimized by carrying out *in vitro* translation, which is performed for 20 min at 30° in the presence of radioactive methionine, followed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the translation products and autoradiography. We did not find it necessary to adjust the optimal conditions for each new batch from the commercial supplier. The efficiency for disulfide containing proteins, such as antibody single-chain Fv fragments, can be increased if eukaryotic protein disulfide isomerase (PDI) is used cotranslationally. The translation is stopped in a similar way as for the *E. coli* system. However, we observed that 50 m*M* Mg^{2+} present during affinity selection may occasionally lead to artifacts, when affinity selection is carried out on polystyrene surfaces.[19] Therefore, the magnesium concentration is reduced to 5 m*M* in the eukaryotic system, without significantly altering the efficiency of this system. Because the preferred anion in the rabbit reticulocyte translation system is chloride, the Tris–acetate-based buffer of the *E. coli* system is replaced with a PBS-based buffer.

Materials

Flexi rabbit reticulocyte lysate kit (Promega)
[^{35}S]Methionine
SDS–PAGE equipment
Washing buffer PBSM (10×): 1.37 *M* NaCl, 0.027 *M* KCl, 0.1 *M* Na_2HPO_4, 0.018 *M* KH_2PO_4, 0.05 *M* $MgCl_2$
Washing buffer PBSTM: PBS with 0.05% (v/v) Tween 20, 5 m*M* $MgCl_2$

Washing buffer PBSTMH: PBSTM with heparin at 2.5 mg/ml
Elution buffer PEB20: PBS with 20 m*M* EDTA

Protocol for Ribosome Display in Vitro Translation and Affinity Selection. Sterilized 12% (v/v) milk is combined with 10× PBSM to obtain 10% (v/v) milk in 1× PBSM, and this solution can be stored on ice for several weeks. In an ice–water bath, a tube containing 200 μl of PBSTMH buffer and 62.5 μl of 10% (v/v) sterilized milk in PBSM is prepared. On ice, the following ice-cold solutions are combined: 0.8 μl of 2.5 *M* KCl, 0.2 μl of 200 m*M* methionine, 1 μl of 1 m*M* each amino acid except methionine, 33 μl of Flexi rabbit reticulocyte lysate, and sterile, double-distrilled water is added up to 40 μl. The amino acid mix (1 m*M* each) without methionine is included in the translation kit, as is KCl. The library mRNA should be thawed only directly before use, and the remainder should be immediately frozen. Five micrograms (approximately 1×10^{13} molecules) of ice-cold library mRNA, either capped or uncapped, in 10 μl is added to the mixture, gently vortexed, and immediately placed in a 30° water bath. After 20 min of *in vitro* translation, the mixture is pipetted out of the reaction tube, immediately added to the tube containing buffer PBSTMH and milk, briefly and gently vortexed, and placed in the ice–water bath. We did not find it necessary to centrifuge the translation mixture prior to selection. Selection is carried out as described for the *E. coli* system, except that buffers WBT and EB20 are replaced by buffers PBSTM and PEB20, respectively. mRNA purification, reverse transcription, and PCR are performed as described for the *E. coli* system, except that primers SDA and T7B are replaced by primers SDA-RRL and T7RR-EN.