mRNA display for the selection and evolution of enzymes from *in vitro*-translated protein libraries

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The mRNA display technology enables the *in vitro* selection and directed evolution of functional proteins from libraries of more than 10[12] different mutants in a single test tube. The size of these libraries is well beyond the limit of screening technologies and of most *in vivo* and *in vitro* selection methods. The mRNA display technology has been used to select peptides and proteins that bind to a specific ligand, as well as novel enzymes. This protocol details the procedure to produce mRNA-displayed proteins (3 d) and to subject them to a selection and evolution of enzymes for bond-forming reactions (4–10 weeks). This method is demonstrated by the generation of new RNA ligase enzymes.

INTRODUCTION

Protein engineering methods

Protein engineering is a method of generating altered proteins with desirable properties and can be pursued by two general strategies. The first approach is a rational design that uses detailed knowledge of the structure and function of a protein to make useful changes to the amino acid sequence. The second approach harnesses the power of natural selection to modify the protein's properties and is known as directed evolution. Similar to natural selection, in a directed evolution strategy, first a library of mutant proteins is generated from which mutants with the desired properties are isolated. There are numerous different methods for directed evolution of proteins that have been reviewed recently[1–5]. Directed evolution can be performed *in vivo* or *in vitro*. For applications that use the evolved protein inside a cell, it is likely to be advantageous to evolve a protein *in vivo*. However, because *in vivo* methods rely on the transformation of DNA into cells, they are inherently restricted to library sizes of about 10[4]–10[9] mutants. In contrast, *in vitro* selection methods can process more complex libraries of more than 10[12] variants. This numerical advantage increases the chance of finding particularly rare mutants in a directed evolution experiment.

mRNA display

This protocol uses the mRNA display technology, which is an *in vitro* method and allows a large number of mutants to be examined in a single experiment[6,7]. mRNA-displayed proteins are constructs in which a protein is covalently attached to the mRNA that encodes it. The covalent connection of phenotype (protein) and genotype (RNA) effectively renders the protein directly amplifiable. This linkage enables successive cycles of selection, amplification and, optionally, mutagenesis to be carried out on protein libraries with sizes greater than 10[12]. This library size is much bigger than that used for most *in vivo* and *in vitro* selection methods and several orders of magnitude larger than that used in traditional screening technologies. The mRNA display procedure is carried out entirely *in vitro*, which makes it possible to select under a wide range of interesting conditions that would be incompatible with traditional *in vivo* selection methods (salts, pH, additives and so on). If the RNA stability is of concern in certain environments, the RNA can be replaced by its cDNA, as described in a variation of the mRNA display[8]. mRNA display has been used to select for binders to a specific ligand from libraries of proteins[9–12], peptides[13–15], circularized peptides[16,17] and peptides built from unnatural amino acids[18,19]. Remarkably, the mRNA display technology has even yielded *de novo* proteins that bind ATP from a library of polypeptides containing 80 random amino acid positions[20]. Methods on how to prepare mRNA-displayed proteins for the selection of binders have been described previously[21–23].

Selection of enzymes using mRNA display

Recently, the mRNA display technology was developed further to enable a selection for enzymes[24], which is the focus of this protocol. A general scheme for the direct selection of enzymes catalyzing bond-forming reactions was established. This scheme uses the formation of the reaction product as the sole selection criterion and allows the isolation of new enzymatic activities without knowledge of the reaction mechanism. Although mRNA display has only been used for bond-forming enzymes to date, this method could also be modified to select enzymes that catalyze bond-breaking reactions or other covalent modification reactions (**Box 1**).

The novel ligases previously isolated by mRNA display catalyzed multiple conversions of substrate to product (multiple turnovers), although the selection method required only a single conversion and did not exert any selective pressure for product release[24]. To directly improve on those properties, enzymes can be evolved further, e.g., with an *in vitro* compartmentalization (IVC) selection scheme[25,26]. IVC selections can directly enrich for increased substrate affinity and multiple turnover activity, but are limited to protein libraries that are several orders of magnitude smaller than those accessible by mRNA display. To create proficient novel enzymes, a combination of the two methods may be advantageous: mRNA display has the necessary library diversity to generate a novel enzymatic activity and IVC can subsequently evolve this activity to yield highly efficient enzymes.

An alternative *in vitro* selection method that can handle protein libraries of similar size as mRNA display is ribosome display[27,28]. In contrast to the covalent genotype-phenotype linkage generated during mRNA display, in ribosome display this connection is maintained by preventing the ribosome from dissociating. This is achieved by eliminating the stop codon of the mRNA before translation and maintaining the mRNA-ribosome-protein complex

BOX 1 | SELECTION FOR DIVERSE ENZYMATIC ACTIVITIES

The selection scheme for isolating enzymes that catalyze bond-forming reactions presented in **Figure 1** can be applied to a wide range of different A and B substrates. In every case, substrate A needs to be linked to the RT primer and substrate B has to carry a selectable anchor group. The attachment of substrate A to a synthetic oligonucleotide (RT primer) can be conveniently achieved by a variety of commercially available coupling chemistries (http://www.glenresearch.com/Catalog/modifiers.html#terminus). Although biotin is a uniquely practical anchor group for substrate B, any other moiety that allows the specific isolation can be used instead.

The mRNA display scheme can also potentially be used to select for enzymes that catalyze bond-breaking reactions. For this purpose, the mRNA-displayed proteins are immobilized via the substrate and then those molecules are isolated that can release themselves by cleaving the substrate. In addition, enzymes that catalyze other covalent modification reactions can be selected by first attaching the substrate to the cDNA and then isolating the active enzymes with product-specific agents such as antibodies. For a more detailed discussion, see a recent review article[42].

at a high salt concentration and low temperature. A ribosome display selection has yielded novel enzymes by using a mechanism-based reaction scheme[29]. In principle, it also appears feasible to use ribosome display to select enzymes in a manner very similar to the procedure described in this protocol for mRNA display, which was demonstrated as a proof of concept[30].

Experimental design

An overview of the experimental procedure is given in **Figure 1**. The starting point of the selection is a DNA library encoding the protein library of interest. DNA libraries can be produced by a variety of methods described previously[31–34]. One approach to prepare particularly large libraries assembles the mutant genes from oligonucleotides containing randomized codons that are synthesized chemically by automated DNA synthesis[35]. By choosing a certain minimum number of codons to be randomized, it is virtually assured that every single mutant has a unique sequence. For example, randomizing 13 residues in a protein with all 20 amino acids can theoretically yield 20^{13} ($= 8 \times 10^{16}$) possible mutants, whereas only about 10^{13} proteins are typically used in an mRNA display selection.

To facilitate the mRNA display of a given DNA library, the DNA library is first modified at both termini by PCR using suitable primers to introduce specific constant sequence regions[35] (**Fig. 2**). The DNA library is then transcribed into an RNA library. To create the covalent link between the RNA and its encoding protein, all RNA molecules are modified at the 3′ terminus with a puromycin-containing oligonucleotide by ligation or photo-crosslinking[36] before the *in vitro* translation reaction.

Puromycin is an antibiotic that mimics the aminoacyl end of tRNA and functions as translation inhibitor by entering the ribosomal A site and accepting the nascent peptide as a result of the peptidyl transferase activity of the ribosome. In the mRNA display context, the puromycin-modified mRNA template is translated by the ribosome until the modified 3′ end is reached. Puromycin enters the A site and becomes attached to the peptide chain. As puromycin is already coupled to mRNA, the mRNA becomes covalently linked to its protein product via puromycin because of the formation of a stable amide bond. The library of mRNA-displayed proteins is then separated from RNAs and proteins that have not been linked and from other components of the translation reaction by a series of purifications. An oligo(dT)cellulose column is used to purify the mRNA-displayed proteins from unfused proteins on the basis of the A_{15} sequence that is part of the puromycin-containing oligonucleotide. Thereafter, an anti-Flag affinity column is used to purify on the basis of the presence of the Flag epitope in the translated protein to discard unfused RNAs. Alternatively, other affinity tags such as a hexahistidine tag can be used either C or N terminally (**Fig. 2**). Subsequently, the mRNA is reverse transcribed (RT) into cDNA, which serves several purposes. The cDNA will later be used
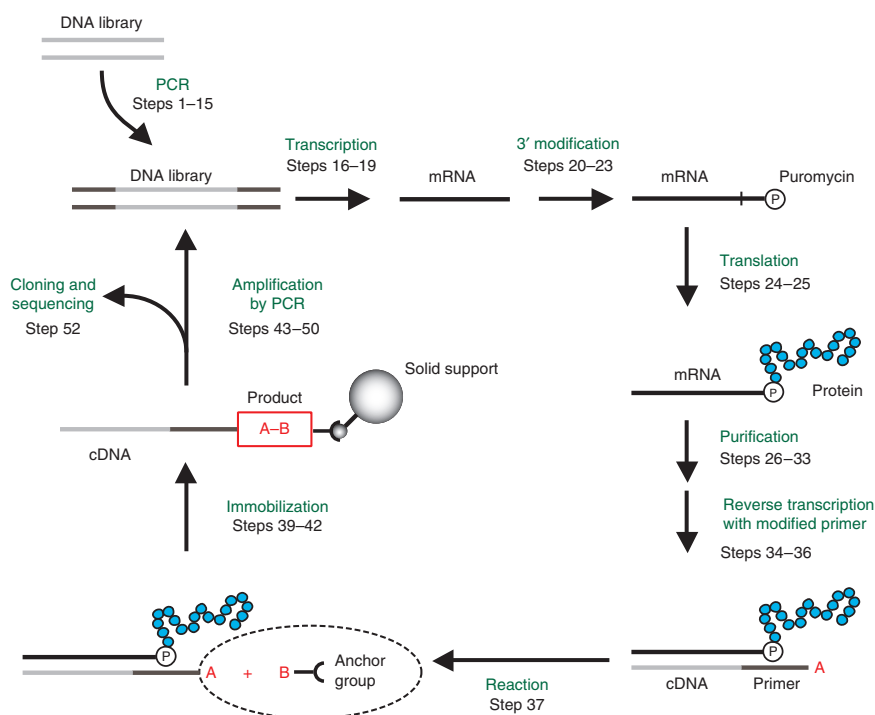
Figure 1 | Overview scheme of the selection of enzymes for bond-forming reactions by mRNA display. A DNA library is transcribed into RNA, which is subsequently translated into mRNA-displayed proteins. Substrate A is linked to the complex by reverse transcription. The enzymatically active proteins catalyze the bond formation between A and B and thereby attach the anchor group to their encoding cDNA. The cDNA is immobilized, amplified and either subjected to an additional round of enrichment or cloned and sequenced for identification.
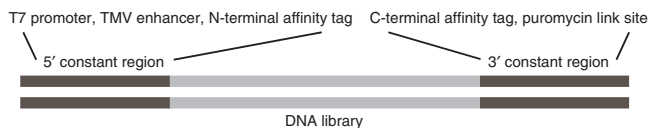
Figure 2 | DNA library design for an mRNA display selection. The DNA library encoding the protein library is flanked by constant regions. The T7 promoter and TMV translation enhancer sequences are necessary for transcription initiation and efficient translation. The puromycin link site is complementary to the puromycin-containing oligonucleotide. Affinity tags can be included on either end or on both.

Figure 3 | Selection of enzymes catalyzing a bond-forming reaction of two substrates, A and B. (**a**) Substrate A is linked to the reverse transcription primer used for cDNA synthesis and substrate B carries a selectable anchor group. Upon reaction between A and B, the anchor group is connected to the primer and, consequently, to the cDNA. (**b**) To demonstrate the general concept, this protocol describes the selection of enzymes that perform a template-dependent ligation of a 5′-triphosphate-activated RNA (PPP-substrate) to a second RNA (HO-substrate). The use of a photocleavable (PC) group allows the specific release of immobilized cDNA. The splint is a complementary RNA oligonucleotide that base pairs to both substrates.

to amplify selected mRNA-displayed proteins, and the cDNA-RNA heteroduplex also confers a greater stability of the RNA toward nucleases. In addition, the double strand prevents the RNA from folding onto itself into secondary structures that might interfere with the subsequent selection step.

The purified library of mRNA-displayed proteins can be used to select for proteins that bind to a ligand, as described above[9–20]; however, this protocol instead will focus on the selection of enzymes for a bond-forming reaction of two substrates, A and B (**Fig. 3a**). In this case, the library of mRNA-displayed proteins is reverse transcribed with a primer bearing substrate A at its 5′ end. The second substrate B is added, which carries an anchor group. Proteins that catalyze the bond formation between A and B attach the anchor group to their encoding cDNA and can be separated from inactive molecules. To demonstrate the general concept, this protocol describes the selection of enzymes that perform a template-dependent ligation of a 5′-triphosphate-activated RNA (PPP-substrate) to a second RNA with a 3′-hydroxyl group (HO-substrate, **Fig. 3b**). The PPP-substrate is ligated to a primer that is used for reverse transcription (RT) of the RNA and the HO-substrate is biotinylated. The cDNA of the catalytically active molecules is immobilized on streptavidin-coated beads via biotin, washed and then released by UV irradiation of the photocleavable (PC) linker. The selected cDNA molecules are amplified by PCR and used as input for the next round of selection or analyzed by DNA sequencing. The use of the PC linker is optional, but it will minimize the potential enrichment of proteins that bind nonspecifically to the streptavidin beads. Alternatively, the immobilized cDNA can be directly amplified by PCR. The process of selection and amplification is repeated until the library is dominated by proteins with the desired properties. The number of repetitions needed depends on the diversity of the starting library and the enrichment achieved in the selection step. To expand the *in vitro* selection to an *in vitro* evolution procedure,
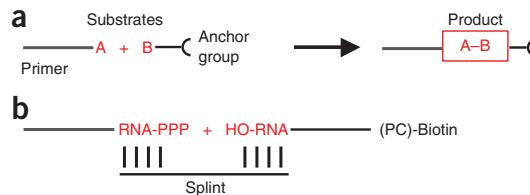
additional genetic diversity can be introduced during each round; e.g., by random mutagenesis and DNA recombination (**Box 2**).

At the beginning of a selection experiment, it may not be known whether the desired activity even exists in the given library, especially when the goal of a selection is to find a *de novo* enzyme. Therefore, if no enzymes could be isolated it is difficult to distinguish whether the selection failed because of the lack of enzymes in the library or because of problems during the selection process. To maximize the success of any selection, it is advisable to optimize all individual steps of the procedure before the actual selection is attempted using the specific library and reaction of interest. To increase the chances of finding a new enzyme, it might also be beneficial to use several different libraries in parallel if available. When a selection aimed at evolving an already existing activity fails, the problem often lies in the execution of the experiment (e.g., the chosen mutation rate might have been too high; **Box 2**).

Because of the complexity of a selection, which usually requires a number of rounds and each round comprises numerous steps, it is important to continuously scrutinize the integrity of individual steps to ensure the success of the whole protocol. To enable simple and sensitive monitoring of this process, radioactive labeling is used throughout the protocol. Furthermore, the analysis by gel electrophoresis at different points in the protocol ensures that potential issues can be detected immediately and addressed. Although, in principle, gel electrophoresis is not absolutely required for the success of the protocol, it is strongly encouraged as a control.

BOX 2 | *IN VITRO* EVOLUTION

The *in vitro* selection process can be expanded to an *in vitro* evolution procedure by introducing mutations. A wide variety of methods for the generation of mutants that is commonly used with other directed evolution methods is also compatible with the mRNA display selection. Those methods range from using error-prone PCR protocols to enabling the recombination of genes[32,43–47]. Although mutations can be introduced in any or all rounds of selection, the detrimental effects of mutagenesis need to be balanced against potential benefits, as random mutations are much more likely to be deleterious rather than beneficial. Therefore, there is a risk of obliterating the enzymatic activity of the library by choosing too high a mutation rate. If the desired activity is potentially very rare in the starting library, it might be advantageous not to introduce mutagenesis until a few rounds of selection have generated sufficient copies of the active enzymes. In the case of the RNA ligase selection, after detecting the increase in immobilized DNA for rounds 9–12 (**Figure 5**), an aliquot of DNA isolated after round 8 was mutagenized by error-prone PCR at an average mutagenic rate of 3.8% at the amino acid level and then used for the next round (round 9—mutagenic). Mutagenesis was repeated in the following two rounds and the selection was continued while the selection pressure was increased by reducing the reaction time. In this example, the *in vitro* evolution increased the enzymatic activity 100-fold[24].

MATERIALS

REAGENTS

- Anti-Flag M2-agarose affinity gel (Sigma, cat. no. A-2220)
- BSA (NEB, cat. no. B9001)
- 1-Butanol (Sigma, cat. no. B7906)
- Chloroform (Amresco, cat. no. 0757) ❗ **CAUTION** Chloroform is toxic. Wear goggles and gloves, handle carefully and work in a fume hood.
- DeepVent polymerase, ThermoPol buffer (NEB, cat. no. M0258)
- DTT (Sigma, cat. no. D9779)
- dATP, [α-^{32}P] EasyTide (PerkinElmer, cat. no. BLU512H) ❗ **CAUTION** dATP, [α-^{32}P] is radioactive. Wear goggles, gloves and a lab coat and follow all relevant local and institutional radiation safety procedures.
- dNTPs (NEB, cat. no. N0446)
- EDTA, pH 8.0 (USB, cat. no. 15694)
- Ethanol (Decon Labs, cat. no. 3916EA)
- Flag peptide (Sigma, cat. no. F3290)
- Glycine (USB, cat. no. 16407)
- HCl (Sigma, cat. no. H1758) ❗ **CAUTION** HCl is corrosive. Wear goggles, gloves and a lab coat.
- HEPES (Sigma, cat. no. H4034)
- Inorganic pyrophosphatase from *E. coli* (Sigma, I2267)
- KCl (Sigma, cat. no. P9541)
- KOAc (Sigma, cat. no. P1190)
- KOH (Sigma, cat. no. P5958) ❗ **CAUTION** KOH is corrosive. Wear goggles, gloves and a lab coat.
- 2-Mercaptoethanol (Sigma, cat. no. M7154) ❗ **CAUTION** 2-Mercaptoethanol is toxic. Wear goggles and gloves, handle carefully and work in a fume hood.
- Methionine, L-[^{35}S] (PerkinElmer, cat. no. NEG009A) ❗ **CAUTION** Methionine, L-[^{35}S] is radioactive. Wear goggles, gloves and a lab coat and follow all relevant local and institutional radiation safety procedures. ▲ **CRITICAL** Radioactive labeling was used throughout this protocol because it allows easy and highly sensitive detection. Alternatively, fluorescent labeling of mRNA-displayed proteins may potentially be used[37]. We have not explored this approach.
- MgCl$_2$ (Fluka, cat. no. 63068)
- NaCl (ISCBioExpress, cat. no. 0241)
- NaOH (Sigma, cat. no. S8045) ❗ **CAUTION** NaOH is corrosive. Wear goggles, gloves and a lab coat.
- NTPs (USB, cat. no. 77245)
- PBS (USB, cat. no. 75889)
- Phenol (equilibrated, pH 8; USB, cat. no. 75829) ❗ **CAUTION** Phenol is toxic and corrosive. Wear goggles, gloves and a lab coat, handle carefully and work in a fume hood.
- Oligo(dT)cellulose Type 7 (GE Healthcare, cat. no. 27-5543-02)
- Oligonucleotides and primers (see below for details)
- PBS, 10× (pH 7.4) (USB, cat. no. 75889)
- Potassium phosphate monobasic (Sigma, cat. no. P9791)
- Rabbit reticulocyte lysate system, nuclease treated (Promega, L4960)
- RNAsin ribonuclease inhibitor (Promega, cat. no. N2511)
- Spermidine (Sigma, cat. no. S85558) ❗ **CAUTION** Spermidine is corrosive. Wear goggles, gloves and a lab coat.
- Streptavidin agarose resin (Thermo Scientific, cat. no. 20353)
- Superscript II reverse transcriptase (Invitrogen, cat. no. 18064-014)
- T4 DNA ligase (NEB, cat. no. M0202)
- T7 RNA polymerase (NEB, cat. no. M0251)
- Taq DNA polymerase (NEB, cat. no. M0267)
- TBE (National Diagnostics, cat. no. EC-860)
- TOPO TA cloning kit for sequencing (Invitrogen, cat. no. K4500-01)
- Tris-HCl Ready Gel, SDS-PAGE precast gel (7.5%) and Tris/Glycine/SDS running buffer (Bio-Rad, cat. nos. 161-1100 and 161-0732)
- Tris(hydroxymethyl)aminomethane (USB, cat. no. 75825)
- Triton X-100 (Sigma, cat. no. T8787)
- tRNA from baker's yeast (Roche, cat. no. 10109495001)
- Urea (Bio-Rad, cat. no. 161-0731)
- Water, RNase free, from Barnstead Easypure II UV/UF (Thermo Scientific, cat. no. D8611)
- ZnCl$_2$ (Fluka, cat. no. 96468) ❗ **CAUTION** ZnCl$_2$ is corrosive. Wear goggles, gloves and a lab coat.

Forward primer

The forward primer contains the T7 polymerase promoter (underlined), tobacco mosaic virus (TMV) translational enhancer (italics), N-terminal affinity tag such as Flag (bold) and ~18 nucleotides complementary to the 5′ terminus of the specific DNA library (X)$_{18}$: 5′-<u>TCTAATACGACTCACTATA</u>*GGGACAATTACTATTTACAATTACA***ATGGACTACAAGGACGACGACGACAAG**(X)$_{18}$-3′ ▲ **CRITICAL** The (X)$_{18}$ part of this primer has to be designed according to the DNA library of your choice. ▲ **CRITICAL**: DNA oligonucleotides can be purchased from commercial vendors such as Integrated DNA Technologies (IDT) (http://www.idtdna.com/) and should be gel purified.

Reverse primer

The reverse primer contains a stop codon (italics), a sequence complementary to the puromycin oligonucleotide for crosslinking purposes (underlined), an optional C-terminal purification tag (bold) and ~18 nucleotides complementary to the 3′ terminus of the respective DNA library (X)$_{18}$: 5′-*TTA*<u>ATAGCCGGTG</u>(**optional tag**)(X)$_{18}$-3′ ▲ **CRITICAL** The (X)$_{18}$ part of this primer has to be designed according to the DNA library of your choice. ▲ **CRITICAL** DNA oligonucleotides can be purchased from commercial vendors such as IDT (http://www.idtdna.com/) and should be gel purified.

Puromycin-containing oligonucleotide

Puromycin-containing oligonucleotide sequence (see below for synthesis and reagents information): 5′-X(tagccggtg)AAAAAAAAAAAAAAAAAZZACCP-3′. Can be synthesized at the W.M. Keck Facility at Yale University with the following reagents (corresponding to the sequence) from Glen Research: X = psoralen C6, lower case letters = 2′-OMe, Z = spacer 9, P = puromycin, stretch of A's and ACC = DNA. Desalt this oligonucleotide by NAP 25 column according to manufacturer's instructions.

Splint

Splint: 5′-GAGUCUCCGCGAACGU-3′ ▲ **CRITICAL** RNA oligonucleotides can be purchased from vendors such as Dharmacon (http://www.dharmacon.com/) and can be used immediately after deprotection following manufacturer's instructions.

HO-substrate-(PC)-biotin

This RNA oligonucleotide is the ligation substrate with a 3′-hydroxyl group. 5′-Biotin-(PC)-UCACACUGUCUAACGUUCGC-3′ (Biotin-(PC) = PC biotin phosphoramidite from Glen Research) ▲ **CRITICAL** RNA oligonucleotides can be purchased from vendors such as Dharmacon (http://www.dharmacon.com/) and can be used immediately after deprotection following the manufacturer's instructions.

RT primer modified with substrate A

See REAGENT SETUP section.

Other DNA oligonucleotides

BS47: 5′-TTCTAATACGACTCACTATAGGGAGACTCTTT-3′; BS48: 5′-AAAGAGTCTCCTATAGTGAGTCGTATTAGAA-3′; BS50: 5′-pTTTT TTTTTTTTTTTTTCCCAGATCCAGACATTC-3′ ▲ **CRITICAL** This oligonucleotide contains a 5′-monophosphate to enable the ligation with T4 DNA ligase. BS56: 5′-CATATGGGAATGTCTGGATCTGGG AAAAAAAAAAAAAAAAAAAAGAGTCTCCGCGAACGTTAGACAGTG TGACTTCGTCATGCTATTCA-3′ ▲ **CRITICAL** All DNA oligonucleotides can be purchased from commercial vendors such as IDT (http://www.idtdna.com/) and should be gel purified.

EQUIPMENT

- Bottletop filter (0.22 μm; Corning, cat. no. 430513)
- Syringe filter, Acrodisc (25 mm, 0.22 μm; Pall, P/N 4192)
- Centrifugal filter device, Ultrafree-MC and CL, Durapore polyvinylidene fluoride (PVDF) (0.45 μm; Millipore, cat. no. UFC30HV00 and UFC40HV25)
- Electroelution system, Elutrap (Whatman, cat. no. 10447705)
- NAP 25 column (GE Healthcare, cat. no. 17-0852-02)
- Scintillation counter, LS 6500 (Beckman, cat. no. 5110705)
- Chromatography column, Econo-Pac (Bio-Rad, cat. no. 732-1010)
- Costar 96-well plate (Sigma, cat. no. Z712485; Corning, cat. no. 2797)
- Microtiter sealing tape (Fisher, cat. no. 9503130)
- Slide-A-Lyzer dialysis cassette, 20,000 MWCO, 0.1–0.5 ml (Pierce, cat. no. 66005)
- Autoradiography system, such as Storm 820 Imaging System & ImageQuant TL Image Analysis Software (GE Healthcare, cat. no. 28-9328-12)
- UV lamp (365 nm, 4 Watts; UVP, UVL-21, part no. 95-0018-02)

REAGENT SETUP

Buffers and solutions ▲ **CRITICAL** RNA can be degraded by ubiquitous RNase enzymes. General precautions for the work with RNA-containing samples

include the exclusive use of RNase-free water and chemicals for all buffers and solutions. Wear disposable gloves when handling tubes or equipment that comes into direct contact with RNA. Use disposable tubes and containers. Before use, filter all solutions through a 0.22-μm bottletop filter (>50 ml) or a 0.22-μm syringe filter (<50 ml). Unless noted otherwise, keep RNA-containing solutions on ice at all times and return them to −20 °C when not in use. In case of possible contamination with dust particles, filter the RNA solution through a 0.45-μm centrifugal filter.

T7 buffer (4×) 140 mM MgCl$_2$, 8 mM spermidine, 800 mM HEPES, adjusted to pH 7.5 with KOH. Store in 1-ml aliquots at −20 °C for up to 6 months.

XL buffer (10×) 1 M KCl, 10 mM spermidine, 10 mM EDTA (pH 8.0) and 200 mM HEPES, adjusted to pH 7.5 with KOH. Store in 1-ml aliquots at −20 °C for up to 6 months.

Oligo(dT)cellulose-binding buffer 1 M NaCl, 10 mM EDTA pH 8.0, 0.2% (wt/vol) Triton X-100, 20 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.0 with HCl) and 10 mM 2-mercaptoethanol. Store at 4 °C for up to 6 months. ▲ CRITICAL Solutions are prepared and stored without 2-mercaptoethanol, which is only added immediately before use because it is prone to oxidation by dissolved oxygen over time.

Oligo(dT)cellulose wash buffer 300 mM KCl, 20 mM Tris(hydroxymethyl)aminomethane adjusted to pH 8.0 with HCl, and 5 mM 2-mercaptoethanol. Store at 4 °C for up to 6 months. ▲ CRITICAL Solutions are prepared and stored without 2-mercaptoethanol, which is only added immediately before use because it is prone to oxidation by dissolved oxygen over time.

Oligo(dT)cellulose elution buffer 2 mM Tris(hydroxymethyl)aminomethane (adjusted to pH 8.0 with HCl) and 5 mM 2-mercaptoethanol. Store at 4 °C for up to 6 months. ▲ CRITICAL Solutions are prepared and stored without 2-mercaptoethanol, which is only added immediately before use because it is prone to oxidation by dissolved oxygen over time.

Flag-binding buffer (10×) 1.5 M KCl, 0.1% (wt/vol) Triton X-100, 500 mM HEPES (adjusted to pH 7.4 with KOH) and 50 mM 2-mercaptoethanol. Store at 4 °C for up to 6 months. ▲ CRITICAL Solutions are prepared and stored without 2-mercaptoethanol, which is only added immediately before use because it is prone to oxidation by dissolved oxygen over time.

Flag clean buffer 100 mM Glycine (adjusted to pH 3.5 with HCl) and 0.25% (wt/vol) Triton X-100. Store at 4 °C for up to 6 months.

Selection buffer (5×) 2 M KCl, 25 mM MgCl$_2$, 0.5 mM ZnCl$_2$, 0.05 % (wt/vol) Triton X-100, and 100 mM HEPES (adjusted to pH 7.4 with KOH). Store at 4 °C for up to 6 months.

SA-binding buffer 1 M NaCl, 5 mM EDTA, and 10 mM HEPES (adjusted to pH 7.2 with NaOH). Store at 4 °C for up to 6 months.

SA urea wash buffer 8 M urea and 0.1 M Tris(hydroxymethyl)aminomethane (adjusted to pH 7.4 with NaOH). Store at room temperature (~25 °C) for up to 6 months.

SA basic wash solution 20 mM NaOH and 1 mM EDTA. Store at 4 °C for up to 6 months.

RT primer modification with substrate A For each enzymatic activity to be selected, a specific substrate A has to be coupled to the RT primer (**Box 1**). The example reaction used to demonstrate this protocol is an RNA ligation in which substrate A is a 5′-triphosphate RNA (PPP-substrate, 5′-PPP-substrate UCUUU-3′). This particular modified RT primer is generated as follows. Prepare substrate A by *in vitro* transcription using T7 RNA polymerase and the double-stranded template formed by primers BS47 and BS48 that are complementary to each other. Purify by denaturing PAGE[38]. Ligate the PPP substrate to the RT primer BS50 by splinted ligation with T4 DNA ligase[39] using BS56 as splint. Purify by PAGE to obtain the final modified RT primer: 5′-pppGGAG ACUCUUUTTTTTTTTTTTTTTTTTTTCCCAGATCCAGACATTC-3′. The underlined region represents substrate A, which is now linked to the RT primer.

PROCEDURE
Modify DNA library by PCR to add terminal constant regions ● TIMING ~1 d

1| Run pilot experiments for the PCR amplification of the whole DNA library first at small scale (100 μl) by combining the following components:

Component	Amount	Final concentration
ThermoPol (10×) buffer, including MgCl$_2$	10 μl	1×
Water	to 100 μl	
dNTP mix, 5 mM each	4 μl	0.2 mM
Forward primer (50 μM)	2 μl	1 μM
Reverse primer (50 μM)	2 μl	1 μM
DNA library	e.g., 0.5 pmol	e.g., 5 nM
DeepVent Polymerase (2,000 U ml⁻¹)	1.25 μl	25 U ml⁻¹

2| Amplify the library with the following conditions:

Cycle	Denaturation	Annealing	Extension
1	94 °C for 3 min		
2–10	94 °C for 30 s	55 °C for 45 s	72 °C for 1 min

3| Draw aliquots of the reaction at the end of each PCR cycle and analyze the aliquots by agarose gel electrophoresis[38]. Identify the required number of cycles to achieve about tenfold amplification.

▲ **CRITICAL STEP** Do not amplify less than tenfold. If the DNA is amplified tenfold, 90% of the total DNA is modified with the new ends. The DNA library should run as a single band in the gel. Ensure that the DNA library used for this PCR does not contain primers from previous PCR reactions. Otherwise, purify DNA library before PCR by agarose gel electrophoresis[38]. If necessary, optimize PCR conditions for the specific primer pair[38].

4| Repeat the PCR reaction (Steps 1–2) on a preparative scale by combining components at the same ratio for a 30 ml reaction. Aliquot the reaction into three 96-well PCR plates and amplify for the determined number of cycles.

5| Transfer all 30 ml of the PCR reaction into one 50 ml tube.

6| Add half the volume of phenol, pH 8.0.
! CAUTION Phenol and chloroform are toxic; always wear goggles and gloves, handle carefully and work in a fume hood.

7| Shake vigorously and centrifuge at ~2,000g for 30 s to separate two phases.

8| Pipette the upper phase (aqueous) that contains the DNA into a fresh tube.

9| Extract the phenol phase remaining in tube from Step 7 by adding half the volume of water and repeat Step 7.

10| Repeat Step 8 and combine the two aqueous upper phases.

11| Extract the combined aqueous phase three times with twice the volume of chloroform following the same procedure as described in Steps 7–8.
! CAUTION Phenol and chloroform are toxic; always wear goggles and gloves, handle carefully and work in a fume hood.

12| Concentrate the aqueous phase by adding four times its volume of 1-butanol; mix and centrifuge as described in Step 7 and discard the upper phase that contains 1-butanol.

13| Precipitate the DNA by adding one-tenth of the total volume of KOAc (2.5 M, pH 5.5), mix, add 2.5-fold the volume of ethanol and mix again. Centrifuge for 10 min at 13,000g. Discard the supernatant and air-dry the pellet.

14| Dissolve the pellet in 1 ml of 50 mM NaCl.

15| Quantify the DNA library by agarose gel electrophoresis[38] by comparing with a mass standard. A reasonable yield is >10 μg of DNA per ml of PCR reaction.
▲ **CRITICAL STEP** Quantifying DNA by UV absorption instead is not possible because of the presence of unused primers.
■ **PAUSE POINT** DNA can be stored at −20 °C for several months.

Transcribe DNA library into RNA ● TIMING 1 d
16| Combine the following components and incubate for 3–12 h at 37 °C:

Component	Amount	Final concentration
DNA library (from Step 15)	200 pmol	200 nM
Water	to 1 ml	
T7 buffer (4×)	250 μl	1×
DTT (1 M)	40 μl	40 mM
BSA (1 mg ml^{-1})	100 μl	100 μg ml^{-1}
NTP mix, 25 mM each	200 μl	5 mM each
Inorganic pyrophosphatase (100 U ml^{-1})	10 μl	1 U ml^{-1}
RNAsin (40,000 U ml^{-1})	5 μl	200 U ml^{-1}
T7 RNA Polymerase (50,000 U ml^{-1})	60 μl	3,000 U ml^{-1}

17| Add 80 µl of EDTA (500 mM) and 320 mg of urea (solid) and vortex to dissolve the urea.

18| Heat the solution to 94 °C for 5 min and purify the RNA by 4% denaturing PAGE[38]. Visualize the RNA band by UV shadowing[40], cut out the band and extract the RNA into 0.5× TBE buffer using an electroelution system by following the manufacturer's instructions. Alternatively, the RNA can be eluted by the crush-and-soak method similar to a previously described protocol[41].

19| Precipitate RNA exactly as described in Step 13. Dissolve the pellet in 300 µl of water and measure the absorption at 260 nm to determine the concentration.
■ **PAUSE POINT** RNA can be stored at −20 °C for several months.

Modify the 3′ end of RNA with puromycin ● **TIMING** 2 h
20| This step uses a photo-crosslinking reaction with psoralen and is performed similar to the method described in the study by Kurz *et al.*[36]. Combine the following components:

Component	Amount	Final concentration
RNA library (from Step 19)	4.5 nmol	3 µM
XL buffer (10×)	150 µl	1×
Puromycin-containing oligonucleotide (100 µM)	112.5 µl	7.5 µM
Water	to 1.5 ml	

21| Aliquot the reaction into PCR tubes and heat in a PCR machine to 70 °C for 3 min, then cool to 25 °C over 5 min.
▲ **CRITICAL STEP** This procedure will anneal the puromycin-containing oligonucleotide to the RNA.

22| Transfer 100-µl aliquots into an open Costar 96-well plate that rests on ice. Irradiate with the specified handheld UV lamp at 365 nm for 20 min from a distance of ~1 cm.
! CAUTION Direct viewing of UV rays can damage eyes.

23| Precipitate the RNA as described in Step 13. Wash the pellet with 100 µl of 70% (vol/vol) ethanol and air dry. Dissolve the pellet in 225 µl of water.
▲ **CRITICAL STEP** The procedure typically crosslinks ~50% of the RNA, and the solution therefore contains ~10 µM RNA that is modified with puromycin. Crosslinking efficiency can be determined by denaturing PAGE[38].
■ **PAUSE POINT** The puromycin-modified RNA can be stored at −20 °C for several months.

Generate the mRNA-displayed protein library by *in vitro* translation ● **TIMING** 1.5 h
24| Combine the following components and incubate for 30–60 min at 30 °C:

Component	Amount	Final concentration
Puromycin-modified RNA (10 µM) from Step 23	200 µl	0.2 µM
Water	to 10 ml	
KCl (2.5 M)	400 µl	100 mM
MgOAc (25 mM)	360 µl	0.9 mM
Amino acid mix (minus Met)	800 µl	25 µM of each amino acid
Methionine, L-[35S] (8.6 µM; e.g., 800 µCi)	80 µl	0.07 µM
Rabbit reticulocyte lysate	4 ml	40% (contains ~2 µM Met)

▲ **CRITICAL STEP** Read the manufacturer's instructions for the *in vitro* translation system. Samples containing reticulocyte lysate cannot be centrifuged. Reticulocyte lysate and ^{35}S-methionine should not be thawed and refrozen repeatedly as otherwise the translation yield will decrease substantially. Translation yield is dependent on the concentration of potassium, magnesium and RNA template and should be optimized for each new template library. Because the rabbit reticulocyte lysate is the most expensive component in the whole protocol, perform the translation first at small scale as a pilot experiment (e.g., 50 μl) to optimize conditions for specific library and analyze yields by SDS-PAGE gel (see Step 38).

25| Add the following components, incubate for 5 min at room temperature and save an aliquot of the sample (2%) for quality control (**Box 3**):

Component	Amount	Final concentration (total of Steps 24 and 25)
KCl (2.5 M)	2,360 μl	531 mM
MgCl$_2$ (1 M)	640 μl	50 mM

Purify mRNA-displayed proteins ● TIMING 6 h

26| Transfer the translation reaction from Step 25 into a 50-ml tube that contains oligo(dT)cellulose-binding buffer to yield a tenfold dilution and oligo(dT)cellulose (100 mg oligo(dT)cellulose per ml of translation reaction).

27| Incubate the slurry with rotation at ~8 r.p.m. at 4 °C for 15 min and transfer to a chromatography column.

28| Wash the oligo(dT)cellulose on the column with 3 × 2 ml (i.e., three washes of 2 ml each) of oligo(dT)cellulose-binding buffer, and thereafter with 3 × 2 ml oligo(dT)cellulose wash buffer.

29| Elute the mRNA-displayed proteins with 6 × 1 ml of oligo(dT)cellulose elution buffer. Filter elution fractions through a 0.45-μm centrifugal filter to remove small amounts of oligo(dT)cellulose particles. Save aliquots of flow-through and elution (2%) for quality control (**Box 3**).
? TROUBLESHOOTING

30| Prewash 50 μl of anti-Flag M2-agarose affinity gel (Flag resin) with 2 × 1 ml of Flag clean buffer and 3 × 1 ml of Flag-binding buffer in a 1.5-ml tube in a batch fashion; centrifuge the slurry at 2,000*g* for 30 s and pipette off the supernatant.

31| Mix the oligo(dT) eluate with 10× Flag-binding buffer to yield a concentration of 1× Flag and incubate with prewashed Flag resin for 1–2 h with rotation at ~8 r.p.m. at 4 °C.

32| Transfer the slurry to a chromatography column. Wash the Flag resin with 6 × 1 ml of Flag-binding buffer.

33| Elute the mRNA-displayed proteins with 2 × 100 μl of Flag-binding buffer containing two equivalents of Flag peptide each for 20 min with rotation at ~8 r.p.m. at 4 °C (one equivalent of Flag peptide saturates both antigen sites of the antibody resin; see manufacturer's instructions). Save aliquots of flow-through and elution (2%) for quality control (**Box 3**).

BOX 3 | QUALITY CONTROL BY QUANTIFYING RADIOLABELED ALIQUOTS

Use half of the collected aliquots and store the remaining half at −20 °C for later use in gel electrophoresis (Step 38).
Quantify the efficiency of individual steps of the procedure by measuring the ^{35}S-methionine-labeled samples by scintillation counting of the aliquots. Use Cerenkov counting for all aliquots taken after ^{32}P-labeling of the cDNA by RT. (See also ANTICIPATED RESULTS.)
Calculate the number of mRNA-displayed proteins (n_{Prot}) after oligo(dT) purification (PROCEDURE Step 29) by using the following formula: $n_{Prot} = N_A \times (c_{Met} \times f_{Met\ inc.} \times volume_{TL}) / number_{Met}$ (N_A = Avogadro constant, c_{Met} = total concentration of methionine in translation, $f_{Met\ inc.}$ = fraction of radioactivity (^{35}S-methionine) incorporated into mRNA-displayed proteins, $volume_{TL}$ = volume of translation reaction, $number_{Met}$ = number of methionines per protein).
Use the number of mRNA-displayed proteins after oligo(dT) and the efficiencies of all subsequent steps to calculate the number of molecules that were used for the actual reaction Step 37. As an example, we found that in round 1 of the ligase selection, 4×10^{13} mRNA-displayed proteins were obtained after oligo(dT) purification and 4×10^{12} reverse-transcribed and purified molecules were used for the reaction step with HO-substrate-(PC)-biotin and splint.

Reverse-transcribe RNA of mRNA-displayed proteins into cDNA ● TIMING 1 h

34| Combine the following components and incubate at 42 °C for 30 min:

Component	Amount	Final concentration
mRNA-displayed proteins (from Step 33)	200 µl	e.g., 5 nM
RT primer (modified with substrate A, 10 µM)	2 µl	50 nM
Water	to 400 µl	
Tris, pH 8.3 (1 M)	20 µl	50 mM
MgCl$_2$ (1 M)	1.2 µl	3 mM
2-Mercaptoethanol (1 M)	4 µl	10 mM
dC,G,TTP mix (5 mM each nucleotide)	40 µl	each 0.5 mM
dATP (0.5 mM)	4 µl	5 µM
RNAsin (40,000 U ml^{-1})	1 µl	100 U ml^{-1}
Superscript II (200,000 U ml^{-1})	1 µl	500 U ml^{-1}
α^{32}P-dATP (3 µM; e.g., 50 µCi)	5 µl	0.0375 µM

▲ **CRITICAL STEP** Components need to be combined in the order listed in the table. After the addition of water, mix well by pipetting up and down and continue by adding the next components. After the addition of Superscript II, mix well and immediately transfer a 10-µl aliquot into a second tube (control). Without delay, add the final component (α^{32}P-dATP) to the main RT reaction and mix. Incubate both main and control RT reactions at 42 °C for 30 min. The control reaction will be used as a non-α^{32}P-labeled RT comparison for the SDS-PAGE gel electrophoresis in Step 38. Save aliquots of both RT reactions (2%) for quality control (**Box 3**).

Dialyze and purify reverse-transcribed mRNA-displayed proteins ● TIMING 12 h

35| Dialyze the RT reaction sample in a Slide-A-Lyzer dialysis cassette according to the manufacturer's instructions two times (>2 h and overnight) against Flag-binding buffer at a ratio of 1/1,000. Save an aliquot (2%) for quality control (**Box 3**).
▲ **CRITICAL STEP** This dialysis step removes the Flag peptide from the first Flag affinity purification.

36| Incubate the dialyzed sample with 50 µl beads of anti-Flag M2-agarose affinity gel. Proceed as described for the first Flag affinity purification (Steps 30–33). Save aliquots of flow-through and elution (2%) for quality control (**Box 3**).
▲ **CRITICAL STEP** This second Flag affinity purification separates the reverse-transcribed mRNA-displayed proteins from excess RT primer and reverse-transcriptase enzyme.

Isolate cDNAs encoding enzymatically active proteins (selection) ● TIMING 2–10 h

37| Add 5× selection buffer to the Flag elution from Step 36 to yield a final concentration of 1×. To start the reaction, incubate this solution with 2 µM HO-substrate-(PC)-biotin and 3 µM splint at room temperature for the desired time. Stop the reaction by adding EDTA to a final concentration of 10 mM. Save an aliquot (2%) for quality control (**Box 3**).
▲ **CRITICAL STEP** The choice of reaction conditions depends on the particular reaction of interest. In general, for the early rounds of selection, longer incubation times could be chosen to ensure that even enzymes with low activity will survive the selection. For later selection rounds, the time can be shortened to increase the selection pressure and favor faster enzymes. As an example, to select for RNA ligases, the incubation times were: overnight for the first 9 rounds, 1 h for rounds 10–12 and 5 min for the following rounds.

38| For quality control, analyze all collected radioactive aliquots by gel electrophoresis on a denaturing 7.5% Tris-HCl SDS-PAGE gel in Tris/Glycine/SDS-running buffer according to the manufacturer's recommendation. For example, load the following amounts of mRNA-displayed protein samples after the respective steps: 1 µl translation reaction (from Step 25), 10 µl oligo(dT) elution (from Step 29), 0.5% Flag elution (from Step 33), 0.5% RT control (from Step 34), 0.1% RT reaction (from Step 34), 0.1% dialysis (from Step 35), 0.2% Flag 2 elution (from Step 36) and 0.2% reaction (from Step 37). Visualize the

Figure 4 | Autoradiogram of an electrophoresis gel of radiolabeled mRNA-displayed proteins. This gel was prepared with samples from the selection for RNA ligase enzymes[24]. mRNA-displayed proteins are labeled 'mRNA-protein'; mRNA-displayed proteins after reverse transcription (RT) are marked as 'cDNA-mRNA-protein'; and 'free protein' refers to translated proteins that were not covalently linked to their mRNA and therefore removed by oligo(dT) cellulose column purification. The numbers shown in parentheses represent the respective PROCEDURE step after which each sample was taken. Samples in the first four lanes on the left are labeled by [^{35}S] methionine in the protein. Samples in the last four lanes on the right are labeled by both [^{35}S] in the protein and [α^{32}P] dATP in the cDNA. A prestained protein weight marker was used and its image was superimposed on the autoradiogram, as the weight marker itself was not radiolabeled.
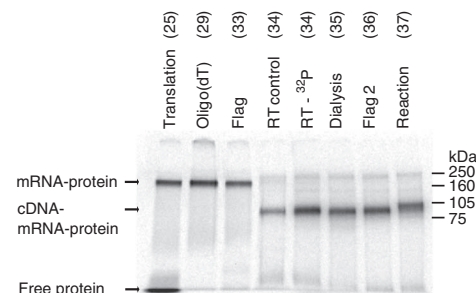
bands by autoradiography. Quantify the radioactive bands to determine the efficiency of each purification step. A representative gel is shown in **Figure 4**.

▲ **CRITICAL STEP** Note that because of the unique nature of the mRNA-displayed proteins, standard predictions of the electrophoretic mobility of either nucleic acids or proteins do not apply. In our experience and for reasons not fully understood, the mobility of mRNA-displayed proteins generally increases after RT. As an example, the electrophoretic mobility of mRNA-displayed proteins encoding 93 amino acids is equivalent to a ~200-kDa protein. After RT, the mobility of the mRNA-protein-cDNA complex increases to ~80 kDa.

■ **PAUSE POINT** Gel electrophoresis can be carried out immediately after Step 37 or any time later as all aliquot samples can be stored at −20 °C.

▲ **CRITICAL STEP** Do not heat-denature samples before loading them onto gel.

39| Wash 350 µl of immobilized streptavidin-agarose beads with 1 ml of PBS buffer and incubate beads with 1 ml of PBS buffer including 2 mg ml^{-1} tRNA, with rotation at ~8 r.p.m. for 10 min.

40| Centrifuge the slurry at 2,000*g* for 30 s and pipette off the solution. Wash beads with 1 ml of PBS in the same manner.

41| Use the scintillation counter according to the manufacturer's instructions to Cerenkov-count the radioactivity of the whole reaction solution from Step 37 (cDNA is ^{32}P-labeled) and incubate the solution with the pretreated streptavidin agarose from Step 40 for 20 min with rotation at ~8 r.p.m.

42| Transfer slurry to a chromatography column and wash the streptavidin agarose with 5 ml SA-binding buffer, 8 ml of SA urea wash buffer, 8 ml of SA basic wash solution and 3 ml of water, added in 1-ml portions. Cerenkov-count all fractions to determine the efficiency of the wash process.

▲ **CRITICAL STEP** It is important to minimize the fraction of cDNA that binds nonspecifically (not by means of biotin) to the streptavidin agarose beads. To achieve this goal, use extensive washes as the biotin-streptavidin binding is very stable and can withstand a variety of stringent buffers. The nonspecific binding should be determined in a pilot experiment and should ideally be less than 0.1%.

43| For the first round of selection, follow option A of this step to avoid any loss of cDNA. For round 2 and thereafter follow option B, which not only results in a more specific release of the immobilized cDNA but also causes loss of material as the efficiency of the photocleavage is only ~30%.

(A) First-round selection
  (i) Transfer the streptavidin agarose from Step 42 directly into the PCR reaction (Step 44) at a ratio of 50 µl streptavidin agarose beads per 1 ml PCR.

(B) Second and further rounds of selection
  (i) Transfer the streptavidin agarose beads from Step 42 in 100-µl aliquots of a 50% PBS slurry to a Costar 96-well plate.
 (ii) Seal wells with microtiter sealing tape that is nonabsorbent at 365 nm.
(iii) Irradiate the slurry with a UV lamp at 365 nm from a 1 cm distance for 15 min while gently shaking the slurry to prevent settling of the beads.
     ▲ **CRITICAL STEP** This irradiation selectively breaks the PC linker between the biotin and the cDNA and releases the cDNA from the beads.
     ! **CAUTION** Direct viewing of UV rays can damage eyes.
 (iv) Separate the beads by centrifugal filtration and measure the radioactivity of the cDNA solution by Cerenkov counting. The fraction of cDNA retrieved with respect to the total cDNA before immobilization (Step 41) is used to monitor the progress of the selection (**Fig. 5**).

PROTOCOL

Amplify selected cDNAs by PCR ● TIMING 4 h

44| Prepare a pilot PCR reaction at a 50 µl scale using the ratios of components shown in Step 47 and run the PCR according to Step 48 for 30 cycles while taking aliquots after 16 cycles and every other cycle thereafter.

45| Analyze aliquots by agarose gel electrophoresis[38].

46| Choose the number of cycles that yield the most intense single product band in the gel without any other bands and carry out the 1 ml scale PCR reaction for the chosen number of cycles. Include a PCR control reaction without cDNA template, which should not yield any PCR product.
? TROUBLESHOOTING

Figure 5 | Selection progress. The graph shows the fraction of cDNA after each round of selection that is immobilized and subsequently photocleaved and eluted (see Step 43). Data used in this graph were taken from the selection for RNA ligase enzymes[24].

47| To amplify selected cDNAs on a preparative scale, combine the following components:

Component	Volume	Final concentration
PCR buffer (10×)	100 µl	1×
MgCl$_2$ (50 mM)	30 µl	1.5 mM
dNTP mix (5 mM each)	40 µl	0.2 mM
Forward primer (50 µM)	20 µl	1 µM
Reverse primer (50 µM)	20 µl	1 µM
Selected cDNA (on beads or photocleaved, from Step 43)		
Taq DNA polymerase (5,000 U ml^{-1})	5 µl	25 U ml^{-1}
Water	to 1 ml	

48| Amplify the cDNA using the following conditions:

Cycle	Denaturation	Annealing	Extension
1	94 °C for 3 min		
2 (up to 30 as determined in Step 46)	94 °C for 30 s	55 °C for 30 s	72 °C for 1 min

49| Perform a phenol/chloroform extraction of the PCR product and precipitate the DNA with ethanol as described in Steps 6–11 and 13, respectively.

50| Dissolve pellet in 100 µl of 50 mM NaCl and quantify DNA by agarose gel electrophoresis[38].
■ PAUSE POINT DNA can be stored at −20 °C for several months.

Repeat cycles of selection and amplification ● TIMING 3–60 d

51| Use the DNA from Step 50 as input for the next round of selection starting again at Step 16. Perform round 2 and subsequent rounds by following Steps 16–50 as described, except for the following changes: at Step 16, use a final DNA template concentration of 20 nM and downscale the transcription reaction to a total of 500 µl; at Step 20, downscale the photo-crosslinking reaction to 300 µl; at Steps 24–25, downscale the translation reaction to 2 ml using 20 µl of ^{35}S-methionine; finally, at Step 39, use only 200 µl of streptavidin-agarose beads.

Repeat the cycle of selection and amplification (Steps 16–50) until the library is dominated by enzymatically active proteins as indicated by a substantial increase in the amount of cDNA immobilized (and photocleaved) after a round of
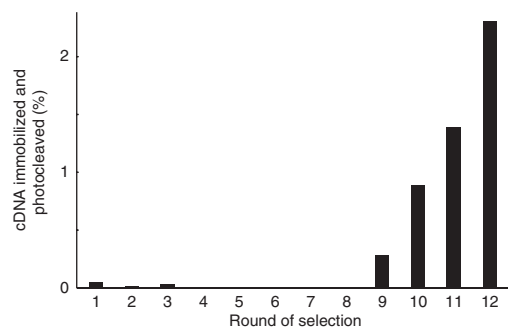
selection (Step 43; **Fig. 5**). Alternatively, the DNA from Step 50 can be subjected to mutagenesis before each round to enable *in vitro* evolution (**Box 2**).

▲ **CRITICAL STEP** The number of selection rounds necessary to reach the substantial increase in immobilized cDNA varies and depends on the enrichment achieved in each round, as well as on the number of active enzymes in the starting library. During the selection of an RNA ligase from a zinc-finger library, this marked increase of cDNA occurred after round 9 and was about 20-fold above background. The selection can be continued until this amount does not increase any further, indicating that no additional enrichment is achieved.

? TROUBLESHOOTING

52| Identify the selected enzymes by cloning and sequencing the DNA after the final round of selection using the TOPO TA Cloning Kit according to the manufacturer's recommendations and any DNA sequencing service.

53| Assay individual enzymes for the desired enzymatic activity. The nature of the specific assay depends on your chosen reaction. In the case of the RNA ligation that is described here as an example, the assay was performed as reported previously[24].

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
29	Elution yield lower than expected	Low translation yield	Use fresh reticulocyte lysate and methionine
		RNA degraded	Analyze aliquots of sample by gel electrophoresis as in Step 38; if degradation is confirmed, adhere more strictly to critical suggestions in REAGENT SETUP
46	No template control PCR yields product band	PCR components contaminated with DNA	Repeat with new PCR components
51	Amount of immobilized cDNA not increasing	Library not sufficiently enriched yet	Continue selection for additional rounds
		No activity in starting library	Start from different library

● **TIMING**
Steps 1–15, Modify DNA library by PCR to add terminal constant regions needed for mRNA display: ~1 d
Steps 16–19, Transcribe DNA library into RNA: 1 d
Steps 20–23, Modify 3′ end of RNA with puromycin-containing oligonucleotide: 2 h
Steps 24 and 25, *In vitro*-translate puromycin-modified RNA to generate mRNA-displayed protein library: 1.5 h
Steps 26–33, Purify mRNA-displayed proteins using oligo(dT)cellulose and anti-Flag affinity columns: 6 h
Step 34, Reverse-transcribe mRNA-displayed proteins into cDNA: 1 h
Steps 35 and 36, Dialyze and purify by second anti-Flag affinity column: 12 h
Steps 37–43, Isolate cDNAs encoding enzymatically active proteins (selection): 2–10 h
Steps 44–50, Amplify selected cDNAs by PCR: 4 h
Step 51, Repeat cycles of selection and amplification: 3–60 d
Step 52, Clone and sequence selected enzymes: 2 d
Step 53, Assay activity of individual proteins: 1–2 d

ANTICIPATED RESULTS
This procedure can generate enzymes *de novo* from a library based on a non-catalytic scaffold. As discussed above (Step 51), it is difficult to predict the number of rounds of selection and amplification that are necessary to enrich the new enzymes from the vast excess of inactive proteins to a level at which the enzymatic activity can be first detected. In the case of the RNA ligase example, nine rounds were needed (**Fig. 5**). After subsequent *in vitro* evolution, the novel enzymes accelerated the ligation reaction at least 2 million-fold.

In a typical oligo(dT) purification, the fraction of [^{35}S]-methionine that is incorporated into the mRNA-displayed protein ranges between 0.005 and 0.04. This corresponds to a yield of >10^{12} mRNA-displayed proteins per ml of *in vitro* translation reaction. This yield depends on the particular protein library and generally decreases with increasing protein length. During an anti-Flag affinity purification, usually between one-quarter and three-quarters of the mRNA-displayed proteins can be eluted from the column.

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