EURx Molecular Biology Products

XELEX RNA Core Kit

eXtended systematic Evolution of Ligands by EXponential enrichment

Modular SELEX Kit –
For RNA aptamer selection, for binder enrichment and for *in vitro* evolution.

This kit requires separate purchase of the Apt-Get T7 RNA Transcription Kit.

For experienced research staff only.
Requires advanced skills.

Cat. no. E3660

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For laboratory use only.
Not for drug, household or other uses.

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Kit – Package Contents

List of Kit packages and order numbers:

Cat. No.	Kit	Package Content
E3660-01	XELEX RNA Core Kit, 50 RNA selections (this kit)	50 RNA selection preparations
E3652-01	XELEX DNA Core Kit – Analysis Unit (for analysis and evaluation of aptamer enrichment)	25 RNA analysis preparations
E0905-02	Apt-Get T7 RNA Transcription Kit (2'-fluoro-modified pyrimidines) 50 reactions (50 µl each)	for 50 RNA selections
E0901-03	T7 RNA Transcription Kit (non-modified NTPs) for XELEX RNA Core Kit 50 reactions (50 µl each)	for 50 RNA selections

The XELEX RNA Core Kit (E3660-01) requires separate purchase of the → XELEX DNA Core Kit – Analysis Unit (Cat. no. E3652-01) and one of the listed T7 RNA transcription kits: Apt-Get T7 RNA Transcription Kit, Cat. No. E0905-02 (with 2'-fluoro modified pyrimidines; recommended) or 'plain' T7 RNA Transcription Kit, Cat. No. E0901-02 (with non-modified NTPs), respectively).

Package Contents XELEX RNA Core Kit (this kit):

- RNA spin columns (optimized towards recovery of small RNA molecules <100 nt in size),
- RNA purification buffer set, specifically optimized towards RNA aptamer purification,
- SELEX - binding and washing buffer (concentrated),
- 1-step RT-PCR enzyme set, compatible with emulsion RT-PCR and modified NTP aptamers,
- emulsion components,
- DNA purification spin columns for emulsion RT-PCR purification,
- DNA purification buffer set for emulsion RT-PCR purification,
- random library T7-Lib-Bank 40 (with T7 promoter region),
- 5' and 3' amplification primers (for library amplification), including a T7 RNA polymerase compatible T7-Lib-Bank40 library primer (T7-Bank40-FPri),
- *Taq* DNA polymerase and 10x buffers for initial conversion of ssDNA library to dsDNA and for scale-up emulsion PCR, if necessary.

Package Contents Add-On: T7 RNA Transcription Kits for XELEX RNA Aptamer Selection:

- T7 RNA transcription components
    - SELEX-optimized T7 RNA polymerase mutant (for modified or non modified NTP sets), complete enzyme preparations:
    - complete, optimized buffer set,
    - NTP sets:
        - non-modified (with cat no. E0901-02), or
        - 2'-fluoro modified pyrimidines, non-modified purines (with cat no. E0905-02),.
    - and accessories set.

Package contents XELEX DNA Core Kit - Analysis Unit (Cat. No. E3552):
Required components for protocols listed in section III of this manual.

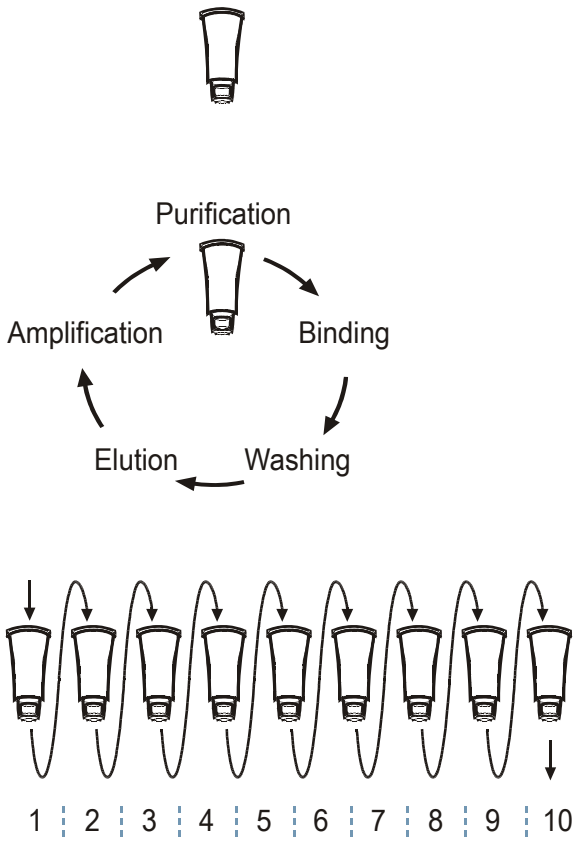
- Diversity Standard Set Bank 40: Standards 0N, 4N, 8N and 12N
- DNA Purification Spin Columns for Emulsion PCR Purification
- DNA Purification Buffers for Emulsion PCR Purification
- 5' and 3' Amplification Primers (for library and diversity standard amplification)
- 10x DA buffer (DNA- and nuclease-free) [for DiStRO assay only]
- Good Streptavidin Binder (positive control aptamer)
- Weak Streptavidin Binder (negative control aptamer)

Required components that are not supplied as part of the core kit, but are available as separate add-on package for maintaining maximum flexibility due to specific experimental requirements:

Amplification add-on (choose an appropriate alternative):

- *Taq* DNA polymerase (Cat. No. E2500) or
- Mutagenesis / Error Prone DNA Amplification (future release add-on) or
- NASBA + RNA Purification (future release add-on).

Definitions



1 selection preparation = 1 selection prep =
1 DNA purification step per single selection target

1 selection round =
1 complete reaction cycle per single target

1 DNA spin column (= 1 selection prep) required
per 1 selection round and per 1 selection target

1 selection process =
10 adjacent selection rounds per single target

10 DNA spin columns (= 10 selection preps) required
per 10 selection rounds and per 1 selection target

Figure 1: Definition of frequently used terms throughout this manual and introduction to experimental design for the selection unit of this kit.

Kit Description

The XELEX RNA Core kit is the central part of a modular SELEX kit system. It contains all required reagents for conducting RNA based SELEX reactions. Specific add-on packages provide further functionality to fine-tune selection protocols towards specific experimental requirements. The XELEX RNA Core Kit has been specifically designed towards selection of stabilized RNA aptamers. Introducing an optimized protocol and a streamlined workflow, the kit allows for selection of highly target specific RNA aptamers at a strongly reduced workload. The accompanying protocol speeds up the entire selection process considerably and makes the kit a premier choice for setting up semi-automated high-throughput selection processes.

As compared to their DNA counterparts, RNA aptamers exhibit improved secondary structure binding capabilities. RNA aptamers are structurally different from their DNA counterparts. Thus, DNA and RNA aptamers sharing the same sequence remain structurally quite different and may exhibit completely different binding properties. Furthermore, secondary structures formed by RNA aptamers may interact more tightly with target structures as compared to DNA aptamers.

In direct comparison to the protocol provided with the XELEX DNA Core Kit, the protocol for the XELEX RNA Core Kit adds further complexity to the SELEX protocol. However, since RNA aptamers are often reported as being more efficiently and rapidly selectable, experimenters often experience RNA selections as more straightforward compared to DNA aptamer selections. Usage of modified NTPs, such as 2'-fluoro or 2'-O-methyl modified NTPs, protect RNA aptamers against nuclease attack and warrant sufficient stability for aptamer applications within biological fluids such as human or animal sera.

The provided protocol is divided in two parts, an RNA-based part for the actual selection process, and a DNA-based part for monitoring library diversity and for quality control (for an overview, see figure 3, page 14). Within each selection round, the RNA-SELEX procedure switches between both parts, between (1) the RNA level ("*Realm of RNA*") which covers the actual selection process, and (2) the DNA-level ("*Realm of DNA*"), which contains the required steps (a) for library re-amplification / regeneration, (b) for monitoring library integrity and quality as well as (c) for measuring certain diversity parameters.

A brief protocol overview: RNA aptamer library generation initiates from the supplied, high quality ssDNA library comprising a well balanced random sequence stretch. Conversion into dsDNA is achieved either by 2nd strand synthesis / primer extension (1st SELEX round) or by emulsion RT-PCR (eRT-PCR; 2nd and all follow-up rounds). During eRT-PCR, a T7 RNA polymerase promoter is incorporated into each PCR amplicon. Following DNA purification, the dsDNA library is converted to an RNA library via T7 *in vitro* transcription, thus entering the procedures' "realm of RNA". Any remaining DNA is removed via purification by a specifically adapted RNA spin column, additionally optimized towards purification of ~80mers with high efficiency and recovery. The actual SELEX procedure, namely all binding, washing and elution steps, respectively, are conducted within the RNA realm. Following elution, the enriched RNA library is reverse transcribed to DNA and subsequently amplified by PCR using a proprietary, emulsion-compatible 1-step RT-PCR reaction step, switching the SELEX procedure from RNA- back to DNA-level, and thus entering the procedure's "realm of DNA" again. 1-step eRT-PCR regenerates dsDNA and re-incorporates T7 promoter sites for subsequent T7 *in vitro* transcription. Following subsequent DNA purification, determination of DNA concentration and storage of a backup, a new selection round starts by T7 transcription of dsDNA to RNA.

Progress of the selection is mostly measured within the realm of DNA. When completing each selection round, a number of routine measurements for measuring the regenerated dsDNA library's specificity and integrity is conducted (namely agarose gel check, quantitation by spectrophotometric measurement, as well as determination of selected parameters such as post-PCR DNA output yield and optional O/I ratio analysis). Measurements on DNA-level also include most analyses only to be conducted upon completion of all selection rounds (namely DiVE or DiStRO assays for measuring reduction of library diversity with progressing enrichment of good binders, as well as Next Generation Sequencing or molecular cloning approaches for identification of good binders).

In contrast, all binding analyses are performed on RNA level using the polyclonal and clonal FLAA assays. FLAA assays must not be performed on DNA level, since, as previously mentioned, DNA aptamers and RNA aptamers with identical sequences generally result in different structural and target - binding properties. For further information on the above mentioned assays as well as for detailed protocols, consult the analysis unit part of the → XELEX DNA Core Kit manual (E3652).

General Protocol

Preparation

1. **Immobilization of Selection Target to Magnetic Beads**
   Immobilize selection target to magnetic beads. Commonly by biotinylation and on streptavidin-coated beads. Alternatively, immobilization of proteins to magnetic beads via Sortase. (Cat No. E4400-01). For alternate immobilization methods see manual → XELEX DNA Core Kit.

2. **Random Oligonucleotide Library**
   Generate a randomized oligonucleotide library (when not using the provided T7-Lib-Bank40 library).

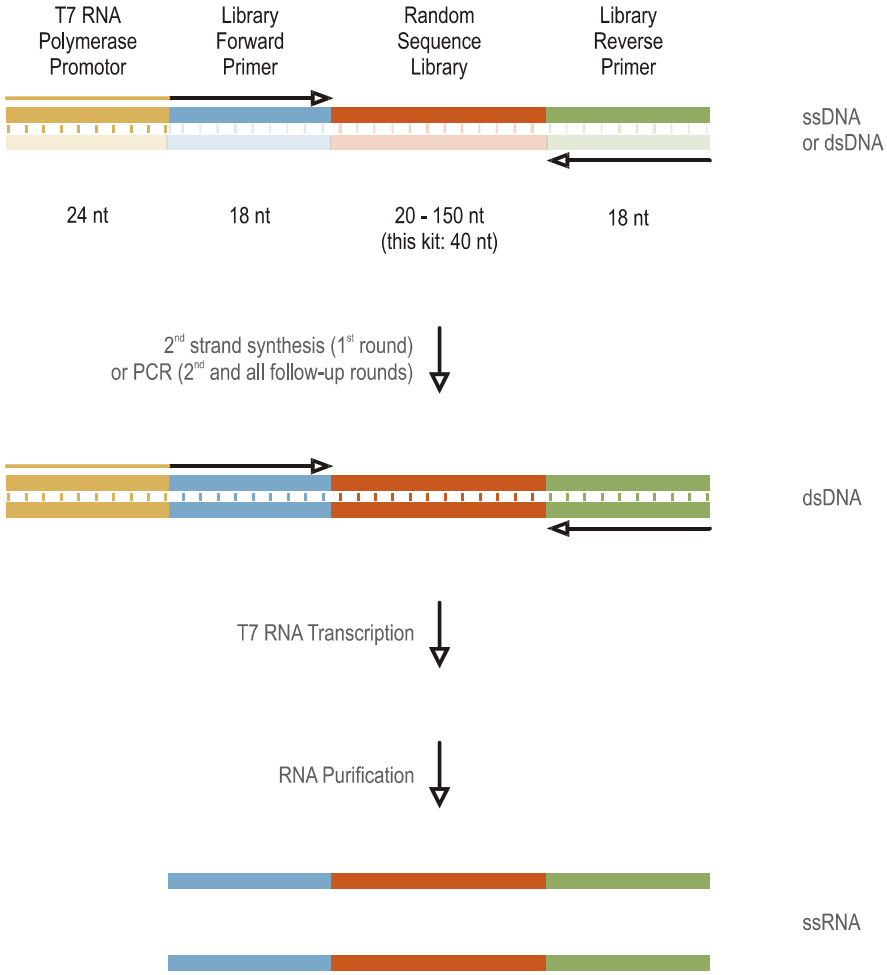


Figure 2: Conversion of oligonucleotide libraries from ssDNA to ssRNA by 2nd strand synthesis and by T7 *in vitro* RNA transcription. A complete T7 promoter sequence is generated either by second strand synthesis (1st SELEX round) or by PCR using an extended primer (2nd and all follow-up SELEX rounds). Library structure: A random sequence stretch of 20-150 nt length (most commonly 40 nt) is flanked by two constant primer binding sites required for PCR amplification. One of both library amplification primers is 5'-extended by a T7 RNA polymerase promoter sequence, which serves as recognition site for transcription initiation by T7 RNA polymerase upon amplification. Expected length of PCR amplicon (dsDNA): 106 bp. Expected length of RNA transcript (ssRNA): 84 nt (since T7 RNA polymerase promoter sites are not transcribed to RNA).

3. 2nd Strand Synthesis by Taq DNA Polymerase-Mediated Primer Extension

- Start with 150 nmol (~5 µg, ~10^{14} ssDNA template copies) ssDNA oligonucleotide library.
- Assemble a library / primer premix as follows, but do not yet include any dNTPs or *Taq* DNA polymerase:

10 x PCR Buffer B (1.5 mM $MgCl_2$ final)	1 x	10 µl
RT-Bank40-RPri	1 µg (~150 pmol)	1,5 µl
Library T7-Lib-Bank40	30 pmol, 5 µg, up to 10^{14} copies	10 µl
Sterile, DNA-free H_2O	76 µl	@97 µl

Incubate the library / primer premix in a thermal cycler block:

Denaturation	95°C	180 sec
Touchdown	95°C → 55°C	dT 1°C/ 90 sec
Hold	4°C	

Note 1: Snap Cool. Best results are obtained, when, at the end of the touchdown step, reaction vessels are directly and immediately transferred from the 55°C heated thermal block onto wet ice. The PCR cycler's ramp from 55°C to 4°C cools at a much slower rate as compared to snap cooling on wet ice. Slow cooling can increase the likelihood of partial intermolecular refolding by secondary-structure rich aptamers.

Note 2: Reaction Kinetics, Allow Enough Time for Re-annealing. See page 9, note 6 for further information.

- Put reaction on wet ice.
- Complete the primer extension reaction assembly by adding the following components to the library / primer premix and by gently mixing (pipetting):

dNTP mix [5 mM]	400 µM	2 µl
Thermostable DNA polymerase [5 U/µl]	1.25 U	0.25 µl
Total reaction volume		***100 µl***

Note 1: Gently mix by pipetting prior to thermal incubation. Avoid collecting *Taq* DNA Polymerase at the bottom of the reaction tube.

Preheat thermal cycler to 72°C and transfer reaction mixture quickly from ice to the preheated thermal cycler block (“Hot Start”).
Continue with the actual primer extension reaction as follows:

Primer extension	72°C	20 min
Hold	4°C	∞

Note 1: From ssDNA to dsDNA. ssDNA library is converted into a dsDNA library, which in turn serves as an efficient template for T7 RNA polymerase mediated *in vitro* transcription.

Note 2: Non-Emulsified PCR. Primer extension is conducted as non-emulsified, "open" reaction. The assay converts ssDNA to dsDNA without PCR amplification which could lead to amplification bias.

Note 3: Only one single primer is required. Contrary to PCR, primer extension requires presence of only one single primer.

Note 4: Secondary structures. Usage of *Taq* DNA polymerase for primer extension under thermostable conditions minimizes problems with potential secondary structures (as compared to Klenow-Fragment / T4 DNA polymerase mediated second strand synthesis).

Note 5: Ensuring Quantitative Synthesis by Spectrophotometric Measurement (A260). Upon completion of the reaction, ensure that the reaction has proceeded quantitatively. In a complete quantitative reaction, each single ssDNA molecule is converted to a dsDNA molecule. Quantitative verification is possible either by spectrophotometric measurement or by capillary electrophoresis (as provided through commercial systems, such as the Agilent Bioanalyzer). Following primer removal via initial DNA purification (page 10), conduct spectrophotometric measurement (260 nm, A_{260}) to determine concentration of dsDNA. Calculate and compare DNA concentration by converting from A_{260} values.

dsDNA: $A_{260} = 1 \approx 50$ µg/ml (Concentration of dsDNA library *after* primer removal)
ssDNA: $A_{260} = 1 \approx 37$ µg/ml (Concentration of initial ssDNA library without primers)
30 pmol ssDNA library T7-Lib-Bank40 (page 20) = ~ 1 µg ssDNA

Note 6: Optimizing Guidelines. If quantitative second strand synthesis was not achieved, the most promising parameter for optimization is to extend the touchdown time for re-annealing within the library primer premix. In case the touchdown time has been chosen too short, e.g. 30 sec per 1°C temperature difference, the formation of non-completely hybridized template DNA strands is occasionally observed. The safe bet is to always allow sufficient time for ensuring proper and complete re-annealing.

Note 7: It is not recommended to replace initial 2nd strand synthesis by PCR amplification. The initial library is too diverse to allow for artifact-free PCR amplification, even when performing PCR within emulsions (see page 40 for a visual demonstration of typical PCR artifacts). During the first selection round, overall library diversity is reduced by many orders of magnitude. Only upon reduction of diversity, artifact-free PCR can be achieved. Thus, artifact-free PCR, ePCR or eRT-PCR of a random DNA library cannot be performed before conducting a first selection step. Consequently, a 2nd strand synthesis step at elevated temperatures (for reduction of secondary structure based bias) is conducted to generate dsDNA without performing an amplification reaction.

4. DNA Purification of Initial dsDNA Library

- Apply 30 µl of activation **Buffer** DX onto the spin-column (do not spin) and keep it at room temperature until transfer of mixture to spin-column.

Note 1: Addition of Buffer DX onto the center of the resin enables complete wetting of membranes and maximal binding of DNA.

Note 2: The membrane activation should be performed before starting the isolation procedure.

- Add 400 µl of orange-colored buffer **Orange** DX to 1 volume of DNA sample (100 µl of primer extension reaction, page 8) and mix.

Note 1: The maximum volume of DNA sample is 250 µl.

- Transfer the mixture to a spin-column/receiver tube assembly.
- Spin down in a microcentrifuge at 11,000 x g (12,000 rpm) for 1 minute.
- Remove spin column, pour supernatant off and put spin-column back on top of the receiver tube.
- Add 500 µl of buffer **Wash** DX2 and spin down at 11,000 x g (12,000 rpm) for 1 minute.
- Remove spin column, pour supernatant off and put spin-column back on top of the receiver tube.
- Add 500 µl of **Wash** DX2 buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
- Remove spin column, pour supernatant off and put spin-column back on top of the receiver tube.
- Spin down at 11,000 x g (12,000 rpm) for 1 minute to remove any residual traces of Wash-DX buffer.
- Transfer spin-column to a new receiver tube (1.5-2 ml) and add 40-100 µl of RNase **free water** directly onto the membrane to elute bound DNA.
- Incubate spin-column/receiver tube assembly for 2 minutes at room temperature.
- Spin down at 11000 x g for 2 minutes.
- Remove spin column, cap the receiver tube. Isolated DNA is ready for initial T7 *in vitro* transcription (page 21). When not continuing immediately, it is safe to store DNA at 2-8°C (short term) or (for long term storage preferred) at -20°C.

Note 1: Use RNAse-free **reaction** vessels for collecting the sample. It is recommended not to autoclave reaction vessels, since autoclaving might result in RNase contamination. Modern high quality plastic reaction vessels are manufactured under RNase-free conditions. If stored properly (e.g. under dust free conditions, in clean glass trays) and handled carefully (always use a clean forceps to take reaction vessels from the glass tray), tubes do not require additional treatment.

Note 2: **RNase Free Water Blank**. Remember to use RNase free water, not pure water, as blank when determining DNA concentration by spectrophotometric measurement.

First RNA-SELEX Round

Binding conditions for the first round are chosen comparably permissive and relaxed, allowing to retain most binders that compete with the vast majority of unspecific binders.

5. Initial T7 Transcription
   - Transcribe dsDNA library to RNA according to the T7 RNA transcription protocol on page 21. Reaction volume for the first SELEX round: 100 µl.

6. Initial Purification of Transcribed RNA
   - Purify transcribed RNA using the RNA spin column and buffer set provided with this kit according to the instructions given on page 23.

7. Binding
   - Start with 400 pmol - 2 nmol (10 – 50 µg) transcribed oligonucleotide library. Add 50 µl [10x] SELEX buffer (=1/10 final volume) and fill up with nuclease-free water to 500 µl final volume.
   - Add oligonucleotide library to magnetic beads *with* immobilized target structure (10x molar amount corresponding to a tenfold binding capacity of beads; max. 200 µl).
   - Incubate for 30 min to 1 h. Mix beads gently to prevent sedimentation (e.g. by gentle shaking or by gently moving the suspension through a pipette in 5 min intervals).
   - Incubation temperature: Room temperature for analyses, 37°C for *in-vivo* targets.

8. Washing
   - 1 x with 1 ml SELEX Buffer; apply relaxed and permissive conditions (see page 19).

9. Elution
   - One of elution strategies 1 – 4 (see protocol on page 26).

10. Amplification of Binders
    - One-step emulsion RT-PCR (see protocol on page 31).

11. Measurement of Efficiency
    - Determine reaction yield [µg DNA of obtained PCR product].
    - Perform an agarose gel check for verifying presence of correctly sized PCR products (to save some time during semi-automated SELEX procedures, run this check in parallel while already starting with T7 RNA transcription of the follow-up selection round).
    - Optional: Calculate Output-/Input (O/I-) Ratio (DNA amplicon, as described in the → XELEX DNA Core Kit).

Second and All Follow-Up RNA-SELEX Rounds

Adjust binding conditions of follow-up rounds stepwise with increasing stringency (see page 19).

12. T7 RNA Transcription of dsDNA Library

- Transcribe dsDNA library to RNA using the T7 RNA transcription protocol on page 21. Reaction volume for the second and all follow-up SELEX rounds: 25 µl.

13. Purification of Transcribed RNA

- Purify transcribed RNA using the RNA spin column and buffer set provided with this kit according to the instructions given on page 23.

14. (Optional step: Counterselection)

This optional, but often required step selects against non-specific binders. Counterselection is highly recommended for selection towards biotin-immobilized targets on streptavidin coated beads.

- Mix RNA (dissolved in water or elution buffer) with 50 - 100 µl [10x] SELEX buffer (=1/10 final volume) and fill up with nuclease-free water to 500 - 1000 µl final volume.
- Denature transcribed RNA at 70°C for 3 min, then put immediately on ice.
- Add to magnetic beads without any immobilized target structure (1/10x volume, as compared to step 1, no less than 20 µl; the absolute minimum for useful work is 10 µl). Binders with high affinity to the coated surface of magnetic beads or to conjugates (instead of high affinity to the actual selection target) are captured during this step and removed from the further selection process.
- Incubate 30 min to 1 h. Incubation temperature: Target environment temperature for aptamer usage, e.g: room temperature for analyses, 37°C for *in-vivo* targets.
- Capture magnetic beads with a magnet. Remove supernatant for use in the further selection process. Discard magnetic beads containing non-specific binders.

15. Binding

- Start with 1 - 3 µg purified RNA from step 7 or, following an optional counterselection step, start with the supernatant from step 8 (in the latter case, RNA is already dissolved in [1x] SELEX buffer). Mix RNA (in water or elution buffer) with 50 - 100 µl [10x] SELEX buffer (=1/10 final volume) and fill up with nuclease-free water to 500 - 1000 µl final volume. It is important to dissolve RNA in the presence of proper ionic concentration prior to denaturation for obtaining correct (re-)folding behavior.
- Denature RNA at 70°C for 3 min, then put immediately on ice.
- Add to magnetic beads *with* immobilized target structure (1/10x volume, as compared to step 1; approximately corresponding to the binding capacity of beads; no less than 20 µl; the absolute minimum for useful work is 10 µl).
- Add 500 µl 1x SELEX Buffer.
- Incubate approx. 30 min (shorten incubation time as compared to step 1). Mix beads gently to prevent sedimentation. Incubation temperature: Target aptamer environment temperature, ,e.g. room temperature for analyses, 37°C *for in-vivo* targets.

16. Washing
    - *Selection round 2:* 2 x with 500 µl – 1 ml SELEX Buffer
    - *Selection round 3:* 3 x with 500 µl – 1 ml SELEX Buffer
    - *Additional selection rounds*: Variable, roughly one additional washing step per additional selection round, dependent on selection progress.

17. Elution
    - One of elution strategies 1 – 4 (see page 26).

18. Amplification of Binders
    - Emulsion RT-PCR (see page 28 ff.)

19. Measurement of Efficiency
- Determine reaction yield [µg DNA of obtained PCR product].
- Perform an agarose gel check for verifying presence of correctly sized PCR products (to save some time during semi-automated SELEX procedures, run this check in parallel while already starting with T7 RNA transcription of the follow-up selection round).
- Optional: Calculate Output-/Input (O/I-) Ratio (DNA amplicon, as described in the manual of the → XELEX DNA Core Kit, Analysis Unit).

20. Cycling / Performing Additional Selection Rounds
- Store 50 - 90 % of amplified nucleic acid as backup and for quality control purposes. Use 10 – 50 % of amplified nucleic acid as starting material for the follow-up selection round. Start the next selection round by continuing with step 12. Upon completion of ten selection rounds, perform library quality control, followed by identification and characterization of aptamers, as described in section III (manual → XELEX DNA Core Kit, Analysis Unit). If analyses still point to high library diversity upon completion of selection round 10, it may be necessary to conduct further selection rounds for enrichment of good binders. When analyzing libraries by Next Generation Sequencing approaches, as few as three to five selection rounds in total may suffice for identification of good binders.

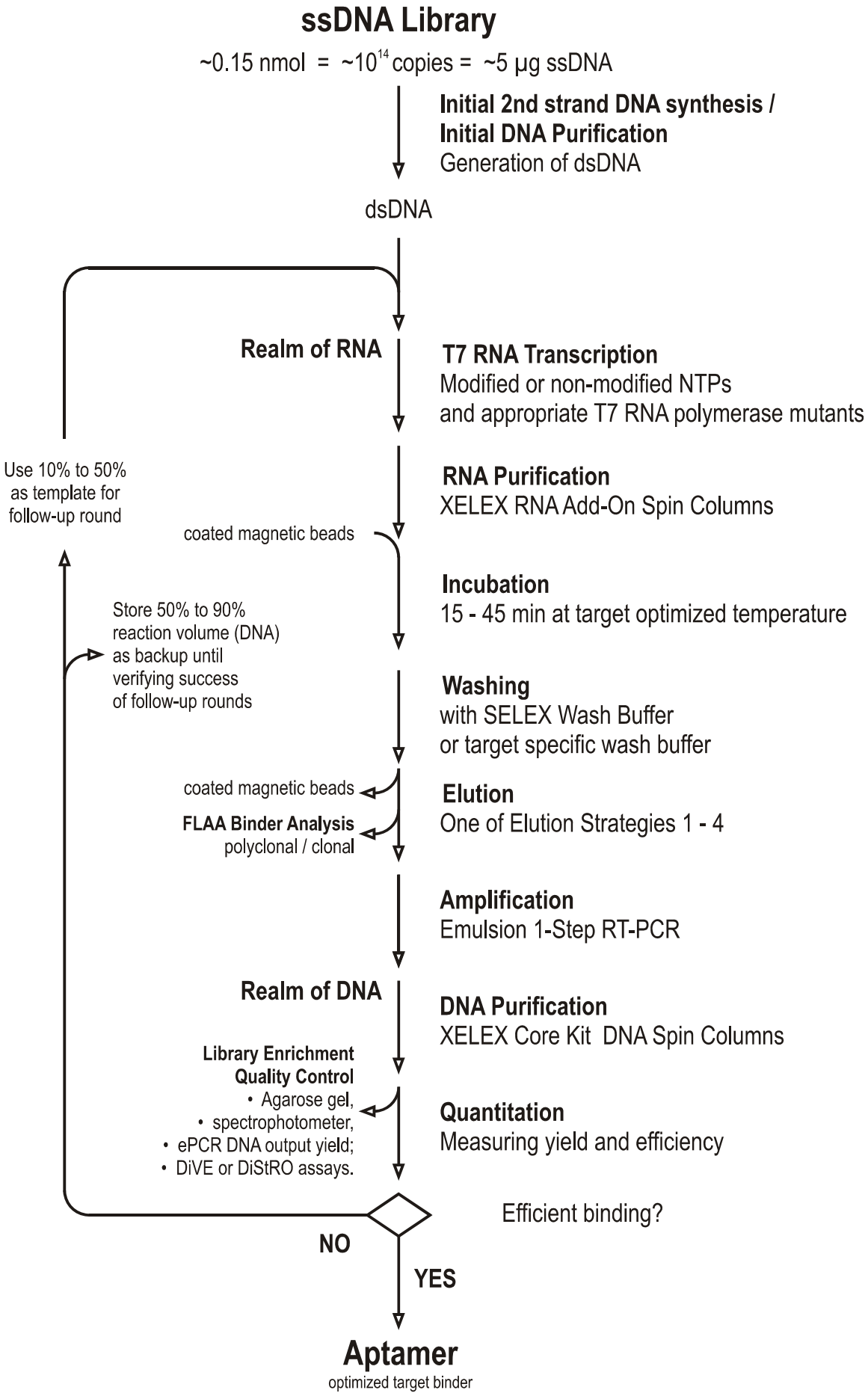


Figure 3: From library to RNA aptamer: Schematic overview of the RNA-SELEX work flow. SELEX is a cyclic process. A “selection round” refers to one completed cyclic sequence of subsequent reaction steps. Each RNA selection round is subdivided into two 'realms', (1) a RNA realm (for selection and enrichment of good binders) and (2) a DNA realm (for library re-amplification, for performing most library-related quality controls, and, finally, for aptamer sequence determination via Next Generation Sequencing or molecular cloning).

Measurement of selection Efficiency

- Determine reaction yield [µg nucleic acid] from the amplification step (DNA or RNA output yield). Keep record of the required number of cycles for amplification of 1 – 5 µg nucleic acid from bead eluate. Both values serve as indirect measurement of the O/I ratio.
- Optional: Determine output-/input (O/I-) ratio and compare to the amplification yield and number of required amplification cycles from the previous cycle. Adjust conditions for follow-up selection round accordingly .
  - *DNA / RNA output yield or O/I ratio increase:* Increase reaction stringency parameters
  - *DNA / RNA output yield or O/I ratio remain constant:* Maintain reaction stringency parameters
  - *DNA / RNA output yield or O/I ratio decrease:* Relax reaction stringency parameters, apply less stringent reaction conditions

For additional information on efficiency measurement, consult the manual for the → XELEX DNA Core Kit, Analysis Unit (section III).

	Input	Reaction Parameters					Output
Round	Input RNA [µg] (T7 *in vitro* transcript, 84 nt)	RNA [pmol] * (T7 *in vitro* transcript, 84 nt)	Beads + Target Structure ** [µg] | [pmol]	Binding Incubation Time [min]	Washing Steps	Number of PCR Cycles	Output - Yield [µg] ***
1	5.0	186	1000 | 70	90	1x 500 µl	15	7.2
2	3.4	126	500 | 35	60	1x 500 µl	15	2.8
3	1.8	67	500 | 35	45	2x 500 µl	15	1.9
4	0.9	33	500 | 35	45	2x 500 µl	15	0.9
5	0.4	15	500 | 35	45	2x 1000 µl	15	2.3
6	1.1	41	500 | 35	45	2x 1000 µl	14	3.5
7	2.6	97	500 | 35	30	2x 1000 µl	12	4.5
8	3.0	112	500 | 35	30	3x 1000 µl	10	3.6
9	2.2	82	500 | 35	20	3x 1000 µl	9	2.9
10	2.0	74	200 | 14	15	3x 1000 µl	8	3.3

Table 1: SELEX example protocol. Values serve as orientation for own experiments and require adjustment to conditions for the planned selection. The protocol illustrates the strategy of increasing stringency by adjusting binding and washing conditions step-by-step with each additional selection cycle. In this example, all stringency parameters listed below (see page 19) are varied, such that overall stringency of binding conditions increases with each selection round. For the purpose of illustration, in this example binding conditions for each selection round varies slightly from the conditions for each previous round. The number of PCR cycles are reduced in rounds 6 to 10, simply to illustrate the fact, that upon proceeding enrichment of binders during late selection rounds, and hence upon increasing RNA concentrations in bead eluate, fewer amplification cycles are required for library regeneration. It is safe, however, to apply the maximum number of 15 PCR cycles for emulsion RT-PCR during late selection rounds as well, since emulsion reactions efficiently prevent amplification bias and undesired formation of artifacts. In practice, the decision for applying increased stringency conditions during follow-up selection round depends on comparing the amount of DNA output between current and previous selection rounds.

* Calculated for the T7 RNA transcript of oligonucleotide T7-Lib-Bank40 (no T7 promoter sequence present), with an average molar mass (MM) of 26880 g mol^{-1}.

** In this demonstration example, 200 µg magnetic beads correspond to 20 µl bead suspension and 14 pmol bound target structure. All values must be adjusted to actual experimental requirements.

*** DNA output yield: The DNA output yield is a *relative* indicator for the success of the selection process. It is measured by spectrophotometric measurement of DNA yield following DNA amplification and subsequent purification. Compared are (a) the number of PCR cycles required for amplification of 1 – 3 µg from bead eluate and (b) the amount of DNA obtained after amplification to the corresponding values from previous selection rounds. Both values are approximately proportional to the output / input ratio mentioned earlier. Good selection progress: DNA output yield remains constant or increases. Bad progress: DNA output yield decreases. If, in spite of increasing reaction stringency, the DNA output yield remains constant or increases (i.e. if in each round as much as or more output nucleic acid per µg input nucleic acid is obtained, as compared to the previous round), maintain or even increase specificity by adjusting one or more of the stringency factors of the reaction (see page 19, stringency factors). In case DNA output yield decreases, relax one or more of the stringency factors for the follow-up round. Note: The DNA output yield is known to be variable and is highly experiment as well as selection target dependent. Unfortunately it is not possible to assign any absolute value as quality indicator, e.g. such as a minimum threshold value (in the sense of “below this threshold value the selection does not work”) to this ratio.

Section II: Selection Kit Unit, RNA SELEX Protocol - Reaction Steps in Detail

General Considerations

Aptamer binding to target molecules occurs exclusively by non-covalent interactions and thus is in principle fully reversible. Four factors contribute to aptamer - target molecule binding: (1) hydrogen bonding, (2) electrostatic interactions, (3) hydrophilic / hydrophobic interactions and (4) Van-der-Waals forces. The overall quality of aptamer binding to their respective target molecules (ligands) is described by their respective dissociation constant (K_d). Good binding aptamers and their target molecules are characterized by low K_d values, indicating that only low target molecule concentrations are required for occupying half of the aptamer binding sites. The lower the dissociation constant, the tighter the binding of aptamer to target and thus the higher the affinity of an aptamer for its target molecule.

Choosing the Appropriate Bead Size

Beads for immobilization of target structures are available in various sizes. Most commonly, beads measure between 0.1 and 10 µm in diameter. The recommended bead size for this kit is 1 µm.

The bead volume increases by the power of three with increasing bead diameter, whereas the bead surface increases only by the power of two. For an identical amount of beads with respect to bead weight, beads with small diameters possess a much larger surface area and thus a higher binding capacity.

Furthermore, following the binding and washing steps outlined below, beads with diameters of 1 µm and less can be used directly for PCR. Dependent on the selection target and overall selection progress, it is often not necessary to perform a separate elution step. If you are not sure whether beads interfere with the PCR reaction, test with a control template before conducting the SELEX experiment.

SELEX Buffer Composition

1x SELEX Buffer Composition (buffer for all binding, washing and elution steps) is a physiological buffer and contains

NaCl	140 mM
KCl	2 mM
$MgCl_2$	5 mM
$CaCl_2$	2 mM
Tris pH 7.4	20 mM
Tween 20	0.05 % [v/v]

optional 10 µg tRNA (Blocking) or Salmon Sperm DNA

SELEX buffer formulation might require further adjustment or replacement with other buffer systems in case aptamers are designed for function within non-physiological environments.

Immobilization of the Target

Choosing an efficient immobilization strategy depends on considering all relevant properties of the target structure to be immobilized. Most commonly, one of the following strategies is appropriate:

- *Streptavidin-coated microbeads*: The target structure must either contain a covalently bound biotin or requires coupling via a biotinylated conjugate. Not compatible with alkaline pH conditions. Caution: Care must be taken to distinguish between aptamers binding to the target structure of interest and aptamers binding to the streptavidin coating of microbeads. When choosing this immobilization strategy, it is recommended to perform a counter-selection step as outlined below (page 25). Several methods exist for adding biotin to a selection target, for example via *in-vivo*-biotinylation, via chemical coupling to lysine or amino groups, as well as via biotinylated conjugates such as sugars, proteins, substrate analogues and more. Many biotin-coupled conjugates are commercially available from third parties, allowing to attach a wide variety of chemical substances to biotin-coupled conjugates for immobilization on streptavidin-coated surfaces. For coupling the selection target to one of these conjugates, follow the accompanying manual that shipped with the respective product. Following immobilization, *briefly* add 1 µM biotin to beads plus immobilized targets for saturating any unbound streptavidin. This prevents potential non-specific binding of oligonucleotides to streptavidin. Do not expose streptavidin-biotin complexed targets with free biotin for extended periods, since free biotin exhibits higher affinity towards streptavidin as compared to conjugate-bound biotin. Thus, biotin labeled targets might get displaced by free biotin, when exposed for extended time periods.
- *Carboxy-coated microbeads*: Suitable for many proteins and for certain non-protein targets. Immobilization proceeds via carbodiimide activation of immobilized carboxyl-groups. The target structure must carry a free amino group. The reaction is conducted in 2-(N-morpholino)ethanesulfonic acid (MES) buffer [0.1 M] under relative mild conditions at pH values between 4.0 – 6.5.
- *Amine-coated microbeads*: Compounds with aromatic rings are immobilized to amine-coated beads via formamide condensation (Mannich reaction) at pH 4.5 - 5. The target molecule is bound to immobilized amine groups via formation of very stable covalent bonds. Since the reaction proceeds in pure water as well as in ethanol-water solutions [50 % v/v], coupling of certain substances, which may not be soluble in pure water, is feasible. It is advisable to passivate remaining amine groups, because nucleic acids might unspecifically attach to positive charged groups.
- *Oligoglycine-coated microbeads*: Protein targets only. Target proteins are C-terminally coupled by sortase (Cat No. E4400). Proteins are immobilized under mild, physiological conditions, thus mostly retaining native protein conformation and functionality. Immobilization requires the presence of a sterically accessible LPXTG recognition motif at the coupling site and the absence of an accessible (exposed) motif within the target protein. Caution: Efficiencies of sortase mediated protein ligation reactions are extremely variable and are highly dependent on substrate protein structure as well as reactant concentrations.
- *His(6)Tag* for Ni-NTA binding. Protein targets only. As with Sortase-mediated immobilization, proteins are immobilized under mild, physiological conditions, thus mostly

retaining native protein conformation and functionality. Non-laborious method, but requires presence of a polyhistidin tag at the protein of interest. Contrary to sortase-mediated immobilization, covalent bonds are not formed. Binding is mediated solely by “weak”, non-covalent interaction.

For very small target molecules / proteins, coupling to the solid surface via a spacer peptide stretch may be necessary to improve accessibility of the target to binding nucleic acids.

Stringency Factors in RNA-SELEX

Regulate the reaction stringency by adjusting the following parameters to the reaction yield:

- Amount of RNA introduced to the reaction step.
  - *Low stringency:* Large RNA amounts
  - *High stringency:* Low RNA amounts.
  - *Defaults:* 10 µg RNA for the first round,
    1- 3 µg or 10 %-50 % volume of previous amplification for follow-up rounds.

- Amount of magnetic beads introduced to the reaction.
  - *Low stringency:* Large magnetic bead volumes.
  - *High stringency:* Low magnetic bead volumes.
  - *Defaults:* 100 µl (or 1000 µg) magnetic beads for the first round,
    decrease stepwise to 20 µl (or 200 µg) for follow-up rounds.
    The absolute minimum volume for useful work is 10 µl.

- Duration of RNA binding to magnetic beads.
  - *Low stringency:* Long, extended binding time.
  - *High stringency:* Short binding time.
  - *Defaults:* 30 min to 90 min for the first round.
    15-30 min for follow-up rounds.

- Number of washing steps.
  - *Low stringency:* One single washing step.
  - *High stringency:* Two or more washing steps.
  - *Defaults:* One washing step in the first round.
    One additional washing step for each additional follow-up round, respectively.

- Temperature and salt content
  - *Low stringency:* room temperature and physiological buffer.
  - *High stringency:* 37°C and high salt (depending on specific application requirements: 0.5 M NaCl or other salts, such as $MgCl_2$ for Watson-Crick base pairing; K^+ (important, since G-quadruplex structures exhibit more pronounced stability with K^+ as compared to Na^+; Ca^{2+}, dependent on target specific requirements).
  - *Defaults:* Conditions of low stringency. Adjusting stringency conditions as close as possible to the conditions in the final application environment is important for the selection of functional and efficient aptamers.

For the first selection round, choose stringency of reaction conditions much lower as compared to follow-up selection rounds. During each follow-up round, increase stringency gradually with increasing reaction yield and hence with increasing DNA output yield (selection progress is measured on DNA level, see table 1, page 16). The higher the increase in DNA output yield, the higher the increase in stringency that can be applied to reaction conditions. In case of a decreasing DNA output yield, as compared to the previous selection round, apply adequately relaxed stringency conditions to allow a larger population of binders being retained.

Oligonucleotide Library and Primer Design

For practical purposes, oligonucleotide libraries with 40 random bases (universal library size: 4^{40} ~ 1.2 x 10^{24} different molecules; see appendix of manual → XELEX DNA Core Kit) are most efficient for generation of random nucleotide libraries.

The oligonucleotide T7-Lib-Bank40 is a well-tested random 40mer library. Both, 5'- and 3'-ends of each random 40mer are flanked by two defined priming sites for reverse transcription and for subsequent PCR amplification.

T7 RNA polymerase does not transcribe the promoter, hence the promoter sequence is temporary lost during the transcription step. The T7 promoter sequence is regenerated later in the PCR amplification step through integration of the promoter sequence within the PCR primerT7-Bank40-FPri.

T7-Lib-Bank40

```
5'- AATGCTAATACGACTCACTATAGGAAGAAAGAGGTCTGAGACATT – N40 –
    CTTCTGGAGTTGACGTTGCTT-3'
```

106 nt (45 nt + 40 nt + 21 nt); average molar mass = 32761 g mol^{-1}, 1 µg oligonucleotide = 30.5 pmol

T7-Bank40-FPri (5'- primer, for PCR amplification of T7-Lib-Bank40)

```
5'- AATGC TAATACGACTCACTATAGG AAGAAAGAGGTCTGAGACATT-3'
```

45 nt; molar mass = 13969 g mol^{-1}, 1 µg oligonucleotide = 71,6 pmol; T7 promoter region marked in blue color

RT-Bank40-RPri (3'- primer, for RT and for PCR amplification of T7-Lib-Bank40)

```
5'-AAGCAACGTCAACTCCAGAAG -3'
```

21 nt; molar mass = 6476 g mol^{-1}, 1 µg oligonucleotide = 154,4 pmol

T7 transcription start:

```
5'- AATGCTAATACGACTCACTATAGGAAGAAAGAGGTCTGAGACATT – …
                          ↑ = +1; run-off-transcript: 23 nt + 40 nt + 21 nt
```

T7 library transcript sequence:

```
5'- GGAAGAAAGAGGTCTGAGACATT – N40 – CTTCTGGAGTTGACGTTGCTT -3'
(84 nt total length = 23 nt + 40 nt + 21 nt)
```

T7 RNA Transcription

dsDNA library is transcribed to RNA by use of T7 RNA polymerase. Depending on the target application of the aptamer, use one of the following T7 RNA transcription kits (available separately, cat nos. E0905-02 (with 2'fluoro modified pyrimidines; recommended) or E0901-02 (non-modified NTPs; only applicable if resistance against RNase degradation is not required for the aptamer target environment).

- *Non-modified NTPs:* Generation of non-modified RNAs. Not applicable for usage within animal sera or biological liquids (lack of stability due to RNase susceptibility). Applicable for non-biological fluids or for "intramers".
- *NTPs with 2'-fluoro modified pyrimidines:* Increased resistance against nucleolytic digestion (RNase A recognizes and cuts behind pyrimidine sites). Exhibit pronounced stabilizing effects towards RNA secondary structure, due to increased impact of stacking forces. Improved secondary structure stability, e.g. as compared to 2'-amino modified NTPs. Safety issues might apply for *in-vivo* usage, since it cannot be excluded that fluoro-modified nucleotides get recycled into host DNA (Burmeister et al. 2005).

Below, an example protocol for the Apt-Get T7 RNA Transcription (Cat. no. E0905) kit is given. When using T7 RNA polymerases or kits from third parties, consult the respective product manual.

Reaction assembly should be performed at room temperature (not on ice). This prevents any precipitation of template DNA due to spermidine contained in the 5x reaction buffer.

T7 RNA polymerase 5x reaction buffer	5 µl	20 µl
NTPs mix (2'F-Py), 25 mM each	1.5 µl	6.0 µl
Amplified library DNA template	1.0-2 µg	40 - 50 µl
Apt-Get T7 RNA polymerase	0.5 µl	2 µl
RNase-free water	@25 µl	@100 µl
Total Reaction Volume	25 µl	100 µl

Note 1: Reaction Volume. For the initial, first SELEX round, assemble a total volume of 100 µl T7 *in-vitro* transcription reaction. For the second round and for all subsequent rounds, a total reaction volume of 25 µl is sufficient.

Incubate up to 2 hours at 37°C, then check transcription on an appropriate denaturing polyacrylamide gel.

Load 5 µl of reaction mixed with 3 µl of RNA loading buffer.

Note 1: High purity of template DNA is very important for obtaining high reaction yields. If run off transcription is applied, be sure there remains no RNase A contamination that could be due to insufficient DNA purification or to RNase contaminated buffers / reaction vessels / plastic ware etc. In case, T7 template DNA is a PCR fragment, remove primers (Recommended procedure: Purification from agarose gels using e.g. EURx GeneMatrix AgaroseOUT DNA Kit, Cat. No. E3540) and confirm DNA homogeneity on an agarose gel.

Note 2: Template Amount. Use 50 % of ePCR-amplified DNA library (25 µl) as template. Keep the remaining 50 % (25 µl) of DNA-library as backup (-20°C).

Note 3: No Transcription of T7 Promoter Sequence. The T7 RNA polymerase promoter sequence stretch is recognized by T7 RNA polymerase, but is not transcribed to RNA. Transcription initiates at the 3'-terminal GG nucleotides of the 5'- …TA ATA CGA CTC ACT ATA GG -3' T7 RNA promoter sequence. Therefore, the 106 bp dsDNA PCR amplicon is transcribed into 84 nt ssRNA RNA transcripts ("forward" strand only). The 5'-terminal nucleotide sequence of the final transcript is 5'- GG AAG AAA GAG GTC TGA GAC ATT -3'. T7 promoter sequences are re-introduced during each RT-PCR library reamplification step via primer T7-Bank40-FPri.

Note 4: For Selection of Nuclease-Resistant Aptamers, Use Modified NTPs Solely. When aiming at selection of nuclease-resistant RNA aptamers with modified nucleotides, perform selections by using modified nucleotides right away. Aptamers composed of modified NTPs exhibit different binding and secondary structure behaviour as compared to aptamers built from non-modified NTPs. Post selection switching from non-modified to modified NTPs requires laborious combinatorial work for determination of nucleotide positions that might tolerate introduction of modifications while not significantly affecting aptamer binding behavior, thus leading to non-optimal results.

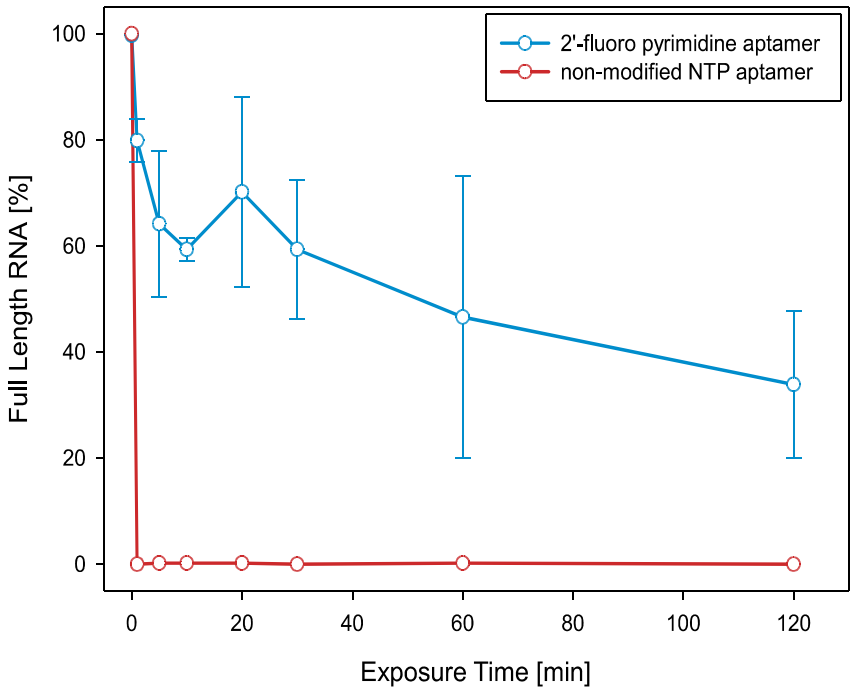


Figure 4: Comparing stability and RNase resistance of 2'-fluoro-pyrimidine modified aptamers vs. aptamers derived from non-modified NTPs. Half-lifes of a 2'F-pyrimidine modified (2'F-Py) RNA aptamer and a RNA aptamer with identical sequence from non-modified NTPs were determined and compared within a nuclease-rich environment of cell culture medium containing animal sera. 2'F-Py RNA aptamers (blue) and non-modified RNA aptamers (red) were incubated in cell culture medium containing fetal bovine serum. Samples were drawn at the indicated time points (X-axis). Nucleic acids were recovered. Radiolabelled full length aptamers were separated by non-denaturing PAGE. Band intensities of full length aptamers were visualized and quantified by autoradiography. The half life of non-modified RNA aptamers within nuclease rich environments such as animal sera is too short to measure – RNA is degraded immediately upon addition to the cell culture medium. Half-life of the 2'-F Py modified aptamer is in the range of 60 min for this particular sequence. Generally, half-life of 2'-F Py aptamers is strongly depended on individual sequence and secondary structure features, and thus may vary in a broad range. For certain 2'F-Py aptamers, half lifes were reported to exceed 24 hour time-frames. (Data from Meyer C., Berg K. et al. (2014) RNA Biology 11 (1), 1–9).

RNA Library Purification

Note 1: During Selection Round 2 to Round Last-But-One, RNA Purification may be omitted. See page 25, note 3 ("During selection rounds 2 to last-but-one, RNA purification may not be required") for further details.
Note 2: Column Binding Capacity. The total RNA binding capacity is 100 µg per spin-column.
Note 3: Avoid overloading the mini columns. Overloading will significantly reduce yield and purity and may block the mini columns.
Note 4: Store Bottles Tightly Closed. All solutions should be kept tightly closed to avoid evaporation and resulting components concentration changes. Store the kit components at 2-8°C.

1. Add 3 volumes (150 µl) buffer RL to 1 volume of T7 RNA transcription assay (50 µl). Mix thoroughly.

2. Carefully transfer the supernatant to the homogenization spin-column placed in a 2 ml receiver tube. Centrifuge at maximum speed for 2 minutes.

Note 1: Centrifugation through the homogenization spin-column filtrates and homogenizes the lysate and removes DNA.
Note 2: DNA (library) remains bound to the matrix of the homogenization spin column. Recovering DNA library, if once required, is possible by elution either with pure water or with dilute buffers, such as 1 x TE buffer.

3. Add 1.2 volumes of 96-100 % [v/v] ethanol to the flow-through. Mix thoroughly by pipetting. Do not centrifuge.

Note 1: For example, add 240 µl ethanol to 200 µl of flow through.
Note 2: A precipitate may form after addition of ethanol.

4. Apply the sample, including any precipitate to the RNA binding spin-column. Centrifuge for 1 minute at 11,000 x g (approx. 12,000 rpm on standard laboratory centrifuges).

5. Remove the spin-column, pour off supernatant and put spin-column back on top of the receiver tube.

6. Add 600 µl of Wash miRNA buffer to the RNA binding spin-column and centrifuge at 11,000 x g (approx. 12,000 rpm) for 1 minute.

7. Remove the spin-column, pour off supernatant and put spin-column back on top of the tube.

Note 1: DNase I on-column digestion is neither required nor possible. Since RNA molecules are short, any attempt to wash columns for DNase I removal would co-elute short RNAs as well. Furthermore, DNase digestion is not required: First, the RNA spin columns differentiate very precisely between DNA and RNA. Second, even if there should remain any traces of DNA after spin column purification, these DNA traces would be most likely part of DNA-RNA hybrid molecules. As part of double-stranded hybrid molecules, any contaminating DNA would not show pronounced tendencies for secondary structure formation and thus would most likely not interfere with target binding. Most likely, any traces of contaminating DNA, if present at all, would be lost in subsequent binding and washing steps during the follow-up SELEX round.

8. Centrifuge at 11,000 x g for additional 1 minute to remove any residual wash buffer.

9. Place spin-column into new receiver tube (1.5-2 ml) and add 40-80 µl RNase-free water directly onto the membrane.

   Note 1: It is not necessary to close the tube at this step.

10. Centrifuge for 2 min at 11,000 x g.

11. Remove spin-column, cap the receiver tube. The RNA library is ready for the selection steps. Quality controls: Spectrophotometric measurement (A260 / A280), denaturing PAGE gel or either a 'plain', standard agarose gel check; to save time, run gel check in parallel while performing the actual SELEX procedure, i.e. during binding, washing and elution of RNA to beads)
    For long term storage, store samples at -20˚C or below until further usage.

    Note 1: Expected length of the purified RNA library is 84 nt, since T7 promoter sequences of PCR amplicons are recognized by T7 RNA polymerase, but are not transcribed. Due to secondary structure formation of ssRNA, correct size estimation would require laborious PAGE electrophoresis under fully denaturing conditions. An agarose gel electrophoresis check provides a quick alternative to denaturing PAGE gels. Correct size estimation is performed later, when switching back to dsDNA level, following the 1-step emulsion RT-PCR step. Since ssRNA will form secondary structures under non-denaturing conditions, agarose gel electrophoresis will not show a clear sharp RNA band, but a slight, blurred smear.

    Note 2: Single-stranded RNA is sensitive to repeated freeze-thaw cycles. To avoid any loss of single-stranded RNA through freeze-thaw cycles, it is recommended to either proceed with the selection steps immediately or to store RNA over night at 4°C in RNase free vessels. High quality plastic reaction tubes are usually RNase-free, due to automated manufacturing processes. When stored properly and handled carefully, tubes do not require any additional, special treatment Safe handling includes e.g. to store tubes in an RNase free glass beaker with lid, and to use RNase-treated, clean forceps to draw individual reaction tubes from the beaker. Contrary, autoclaving might introduce RNases due to aerosol formation. In addition, certain RNases are extremely heat tolerant, and RNases are not completely inactivated by autoclaving. Long term storage of purified RNA library is not recommended. For long term storage of libraries (e.g. backups), we recommend to keep backup aliquots from eRT-PCR amplicons and to store libraries in dsDNA format.

Note 3: During Selection Round 2 to Round Last-But-One, RNA Purification May Not Always Be Required. The RNA spin column purification step described above may not be strictly required, at least for some targets and during late SELEX rounds. At this point of the protocol, a large fraction of DNA is present as double stranded DNA and ss-RNA mixture. With respect to target binding capabilities, double-stranded DNA is known to be a poor competitor for single stranded RNA. Any remaining DNA (e.g. the amount of DNA strand not transcribed to RNA), as well as short ssDNA primers, are poor binding competitors of RNA aptamers. Thus, any remaining DNA would not participate significantly in competitive target substrate binding. Therefore, the described RNA library purification may not be a strictly required step between selection round 2 and the last-but-one selection round (e.g. round 9, if 10 rounds in total are conducted). During those "intermediate" selection rounds, it may be sufficient to either conduct the RNA purification step only occasionally (preferably during the earlier cycles, while omitting this step during late cycles), or even to omit the step completely. Keep in mind, that the RNA purification step is a mandatory, strictly required step (1) following the first selection round (for complete removal of remaining ssDNA primers from 2nd strand synthesis) and, of course, (2) following the very last selection round, immediately preceding RNA binder characterization and identification. Benefits of omitting the RNA purification step: Some streamlining of the process between round 2 and the last but one round. A certain degree of time and labour saving. Potential drawbacks: Probably not recommendable for all targets. It is not entirely clear which targets may tolerate skipping the RNA purification step and for which targets a non-neglectable impact on the course of the SELEX enrichment process has to be taken into account. Increased risk of artifact formation (see page 40). An inefficient reverse transcription step may also favor DNA carryover in absence of RNA binding and/or retention. As RNA spin column purification is rapid and straightforward and there is only few savings in time and labour connected with omitting the RNA purification step. Aside from time saving and overall process streamlining, there is little additional benefit in omitting the RNA purification step. In case target binding specificity should decrease, or if artifacts such as improperly sized aptamers are formed during the following eRT-PCR reamplification step, it is highly recommended to always conduct the RNA purification step described above. Consequently, if in doubt, we recommend to always conduct the RNA purification step. When skipping the RNA purification step, inactivate T7 RNA polymerase now by heating for 5 min to 60°C (optional addition of EDTA removes free Mg^{2+} ions and thus aids in preventing RNA degradation).

Optional Selection Step: Counter-Selection

This step selects against non-specific binders. During this optional step, non-specific binders to coatings of magnetic beads, such as streptavidin, are captured and removed from the further selection process, thus increasing the ratio of good binders to total binders in the DNA pool. In many application scenarios, for example during selections on streptavidin-coated beads with biotinylated probes, a counterselection step is strictly required to prevent selection of streptavidin-specific binders. Other coated surfaces may as well lead to unwanted enrichment of aptamers with high affinity to microbeads coating instead of target structure. If unsure, include a counterselection step starting from the second selection round. See also page 12.

Binding

In a typical binding step, 1 - 3 µg of purified RNA are bound to magnetic beads in the presence of 500 – 1000 µl 1x SELEX buffer. With increasing number of selection rounds, the binding time is typically decreased step by step from 90 min during early selection rounds to 15 min during final selection rounds. The molar relation of RNA to maximum bead binding capacity ranges typically between 10 fold (early selection rounds) to 1 fold (late selection rounds). Incubation temperature is selected according to the properties of the target application: For *in vitro* use of aptamers, choose e.g. 25°C, for *in vivo* use select e.g. 37°C. See also page 12.
During the early selection rounds, RNA is supplied in large excess and thus high competition of RNA molecules for target binding sites is maintained. Use an extended binding time of 30 to 90 min for the first selection round to retain a large variability of binders. During follow-up rounds, decrease binding time to 15 min for favoring conditions of increasing specificity towards good binders. Varying the time frame for RNA binding (but not the binding temperature) is one means to regulate stringency of the SELEX process (see page 19).
Choose an appropriate binding temperature according to the requirements of the targeted reaction environment for the aptamer. Examples:

- For typical *in-vitro* use, bind at room temperature (25°C).
- For *in-vivo* use, apply body temperature of target organism or environment (e.g. 37°C).

Washing

Wash beads with 1 ml 1x SELEX buffer per washing step.
Temperature: Choose the same temperature for binding and washing steps.

- For *in-vitro* use, wash at room temperature (25°C).
- For *in-vivo* use, apply body temperature of target organism or environment (e.g. 37°C).

To increase stringency and specificity, add one additional washing step per selection round (max. four washing steps). To relax stringency, reduce the number of washing steps. Varying the number of washing steps (but not the washing temperature) is one means for regulating stringency (see page 19).

Elution

Four general strategies are available for elution of good RNA binders from their target structures. Efficiency of each step may vary with respect to the nature of the target structure. Note, that in strategies 2 and 3, respectively, RNA removal must proceed quickly to prevent re-attachment of good binders to the bead immobilized target structure, resulting in *de facto* loss and counterselection against good binding molecules. Note also, that certain SELEX techniques such as Cell-SELEX, are not compatible with bead technology and may require application of advanced elution methodology. Apply one of the following elution strategies:

1) Heat

- Fix beads to one side of tube using a magnetic device and remove any remaining SELEX buffer.
- Heat beads in 50 - 100 µl dest. H_2O or 1x TE to 70°C – 80°C for complete denaturation (add 2 mM EDTA, only if RNA is to be purified prior to subsequent PCR amplification).
- Fix beads to one side of tube using a magnetic device, quickly (!) pipet the supernatant without any beads to a new reaction tube.
- Use 25 µl or up to 40 µl of eluate (half of total eluate volume, corresponding to 1 – 3 µg RNA) for subsequent direct eRT-PCR amplification from eluate. Alternately, precipitate RNA, or add one volume of nuclease free water (e.g. 50 µl) and three volumes (e.g. 150 µl) of buffer RL and purify on RNA spin columns (see page 23, or use the RNA/miRNA Purification Kit, Cat. No. E3599).

Note 1: RNA Purity Considerations. Spin column purification of RNA gives optimal results but is usually not required. Direct eRT-PCR amplification from eluate is straightforward, fast and gives satisfactory results.

2) SDS (2 % [w/v])

- Incubate beads in 2 % [w/v] SDS and heat to 70°C – 80°C.
- Fix beads to one side of tube using a magnetic device, quickly (!) transfer the supernatant without any beads to a new reaction tube.
- Precipitate RNA. Alternately, add one volume of nuclease free water (e.g. 50 µl) and three volumes (e.g. 150 µl) of buffer RL and purify on RNA spin columns (see page 23).

3) Competitive elution with either natural ligand of immobilized target for specific displacement or excess amount of free target (e.g. free biotin as competitor to biotin-coupled selection targets). Both approaches elute the immobilization target along with bound aptamers. Since the eluate serves as template for the subsequent amplification step, the elution target must not exhibit any inhibitory effects on the amplification step. The latter approach may fail to enrich high affinity binders.

Note 1: Keep a Backup. Use 50 % of the elution volume as template for amplification. Keep the remaining 50 % as backup until successful completion of the SELEX process.

Note 2: To prevent autohydrolysis of DNA during storage of backups, store backups in dilute buffers (1x TE or, for assays sensitive to presence of primary amines, with phosphate buffers).

Generally, due to small amounts of eluted nucleic acids, quantification at this step is only possible by 1-step real-time RT-qPCR or via a radioactivity assay of a previously labeled library. Furthermore, the eluate containing enriched binders is too precious to sacrifice for nucleic acids concentration measurement. Thus, we recommend not to determine the concentration of retained RNA at this point. A more straightforward approach is to determine the concentration of amplified and purified PCR product only upon completion of the following eRT-PCR amplification and subsequent DNA purification steps.

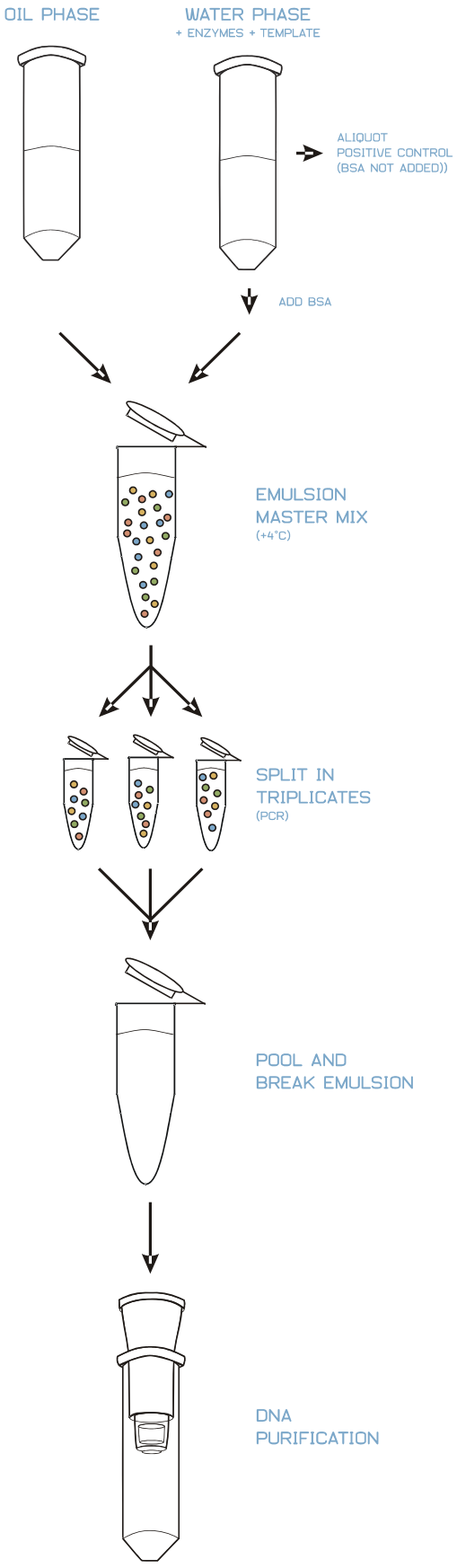


Figure 5: General work flow for emulsion RT-PCR (eRT-PCR). Sample volume of the water phase is 50 µl. Total reaction volume increases due to addition of the oil phase. Thus, for thermocontrolled reaction conditions (e.g. ePCR), the total reaction volume is split into triplicates.

Emulsion RT-PCR Amplification - General Considerations

An important precaution for maintaining high diversity of the oligonucleotide pool throughout the selection process is to conduct all amplification steps within emulsions. Emulsion RT-PCR (eRT-PCR) ensures that each oligonucleotide can amplify only at the expense of the resources contained within each micelle to which each oligonucleotide is confined. In contrast to single-phase RT-PCR assays, emulsion RT-PCR prevents undesired run-off amplification of molecules with poor binding properties but with high amplification efficiency, avoids diversity shifts and reduces amplification bias efficiently.

Additionally, emulsion PCR (ePCR) helps maintaining correct amplicon sizes. Due to unwanted interactions of extended random sequence stretches, amplification artifacts with different lengths may form in non-emulsified PCR reactions. ePCR strongly reduces uncontrollable interactions of random sequence templates by refining only a few template molecules within one micelle. This eliminates the need for post-amplification gel electrophoresis and gel excision of correctly sized amplicons.

Thus, contrary to non-emulsified, “open” PCR assays, e-PCR allows to always perform the maximum numbers of PCR cycles while still maintaining high library diversity and preventing runoff amplification of non-optimal binders. End-point amplification will give maximum PCR yield while still retaining maximum library diversity as well as correctly sized amplicons.

During the eRT-PCR amplification step, the amount of nucleic acids required for the follow-up selection round has to be regenerated. As starting material for T7 RNA transcription, 0.5 – 2 µg of dsDNA is required, plus an identical amount of dsDNA for backup and for quality control purposes. In total, 1 – 4 µg of dsDNA should be regenerated during the eRT-PCR amplification step. In traditional SELEX amplification protocols, a variable number between five and 20 PCR cycles is conducted, depending on the amount of recovered DNA during each selection round, respectively. For ePCR and eRT-PCR, it is not necessary to apply variable numbers of PCR cycles, to keep the total number of

PCR cycles as low as possible and to fine-tune the number of PCR cycles. Simply conduct the maximum number of of 15-20 PCR cycles to gain maximum amplification yield. Undesired PCR errors such as amplification bias, PCR artifacts and runoff-transcription of undesired molecules are confined to the resources enclosed within single micelles solely and cannot propagate across micelle borders. Non-target-specific PCR artifacts are not amplified to abundance and are likely lost during the following selection round. Keep in mind, that the amount of chemicals (e.g. dNTP) per micelle is limited and usually exhausted after 15 cycles. If only few product is generated following 15 - 20 PCR cycles, conduct a scale up PCR (see ePCR protocol in the → XELEX DNA Core Kit; refer also to page 30, step 3, note 2).

If nonetheless further adjustment of PCR cycles is desired, there is a rule of thumb: During the late stages of the selection process, good binders are enriched in increasing numbers and thus the number of required PCR cycles declines with increasing numbers of bound molecules which in turn serve as PCR templates. Thus, the lower the number of required PCR cycles, the more the selection process has come to an end. If a normal, non-emulsified control (“open”) PCR is performed in parallel, check an aliquot of an intermediate amplification step by 'standard' (non-denaturing) agarose gel electrophoresis or by non-denaturing PAGE. If no or little product is visible, conduct additional PCR steps (for details, see page 34, note 4).

Note 1: Micelles and Reverse Micelles. By standard convention, water-in-oil inclusions are referred to as "reverse micelles", whereas the term "micelle" in a strict sense corresponds solely to oil-in-water emulsions, such as milk. For the sake of readability, the term "micelle" will be used as an abbreviation for "reverse micelles" and thus for water-in-oil inclusions throughout this manual.

General Outline of the Emulsion RT-PCR Protocol

1. Assemble RT-PCR reactions in a total volume of 50 µl as outlined below (page 31).

2. From 50 µl elution volume, use
   - 45 µl bead eluate as template for emulsion PCR
   - 5 µl bead eluate as template for open control PCR

   Note 1: Keep a Backup. Always keep 50 % of any purified PCR reaction as backup and for quality control purposes until having finished the complete SELEX process. In case of errors or continuous decrease of PCR yield or complete loss of DNA, it might prove handy to have a backup from previous cycles to continue from, rather than having to start a new SELEX process from the very beginning.

3. Conduct 15 – 20 cycles PCR as described below (see page 33). Adjust cycle count adequately to amplify 0.5 to 3 µg of dsDNA as starting material for the next selection round.

   Note 1: Resource Depletion in Micelles. The resources confined within single micelles deplete within 15 - 20 cycles. Performing more than 15 - 20 cycles will not lead to a further increase in reaction yield. Upon depletion of chemicals, DNA will denature and reanneal, but will cease to amplify any further. In case higher DNA yield is desired, conduct a scale-up PCR: (1) Break the emulsion as outlined below (page 34), (2) purify DNA by spin column purification and (3) use as much of the purified DNA as possible for setting up a second emulsion PCR.

   Note 2: Maximum 2x 20 PCR Cycles. Do not conduct more than a total of 30-40 PCR cycles (= 2 x 15-20 emulsion PCR cycles) for reamplification of 1-3 µg library DNA from bead eluate. The more PCR cycles are required for amplification, the less DNA molecules were left over from the bead elution step, the lower the diversity of molecules to select from during follow-up rounds. Example calculation: 20 PCR cycles amplify template DNA molecules by a factor of 2^{20} = 1048576 ≈ 10^6 = 1 million. 30 PCR cycles amplify roughly by 10^9, 40 by 10^{12} etc… Starting with 10 µg of DNA library would select from approximately 10^{14} different molecules. In case 40 PCR cycles would be required to detect any band during an agarose gel check, selection would have started from a pool of as few as 100 DNA molecules left over from the bead elution step. This extremely low diversity would lead most likely to poor and non-specific enrichment. If more than 30-40 cycles are required for reamplification of 1-2 µg library DNA, discard the assay, check all selection and DNA purification steps and restart from a backup bead eluate of the last known-to-work selection step.

4. Purify PCR products on spin columns (see page 35).

5. Quality control: Verify the proper size of the PCR product by 'standard' (non-denaturing) agarose gel electrophoresis or by non-denaturing PAGE gel electrophoresis.
   Quantify PCR yield by spectrophotometric measurement (see page 36).
   - High efficiency: > Continue with the next selection round.
   - Low efficiency: > Troubleshooting

6. Keep note of the number of PCR cycles required for PCR amplification as well as the amount of DNA obtained after reamplification. Compare these numbers between selection rounds to verify proper progress of the selection process. For an example see table 1, page 16.

Emulsion RT-PCR – Detailed Protocol

For all RNA aptamer selections, this protocol replaces the emulsion PCR protocol steps in the → XELEX DNA Core Kit.

Setting up the Emulsion

1. Create *Oil Surfactant Mixture* (200 µl per reaction):

~73 %	Emulsion Component 1	220 µl
~7 %	Emulsion Component 2	20 µl
~20 %	Emulsion Component 3	60 µl

   - Mix thoroughly by vortexing.
   - Keep on crushed ice until further usage.

Note 1: Viscosity. Emulsion Component 2 is very viscous. Mixing of all components is greatly facilitated, when adding Emulsion Component 2 not as first component to the empty reaction tube.

Note 2: Precipitates. Do not use any precipitate that may occasionally form in Emulsion Component 1.

Note 3: Best Use Before Expiry Date. Do not use surfactant solutions beyond shelf life.

Note 4: Oil Surfactant Stability Issues. Do not store assembled Oil Surfactant Mix for more than two days.

Note 5: Oil Mix. Prepare an oil surfactant master mix for minimizing pipetting errors due to small volumes.

Note 6: Use precut pipette tips for a proper transfer of highly viscous components (such as component 2). Use a clean and sterile scissor for cutting off the ends of pipette tips, resulting in a larger pipette tip diameter and an easier transfer.

Note 7: Assemble at RT. Due to the high viscosity of components, we recommend assembling the Oil Surfactant Mixture at room temperature, followed by cooling of the readily assembled mixture to +4°C.

Note 8: Volume Scaling. In a standard reaction, use 300 µl *Oil Surfactant Mixture* per 50 µl enzymatic water phase. In case water phase volume does not equal 50 µl, adjust *Oil Surfactant Mixture* volume proportionally.

Note 9: Variability in Oil Phase Composition. To a certain extent, the proportions of all three components may vary slightly for obtaining emulsions with different properties. Large deviations from the above given compound composition may result in unstable emulsions.

Note 10: Thermostability. All emulsion components are thermostable (well above 100°C). However, depending on buffer conditions of the water phase, we recommend not to heat above 95°C to prevent emulsion instability.

2. Create *PCR Water Phase*:
   Mix PCR sample on ice. A single typical emulsion PCR reaction contains a water phase of 50 µl. An "open", non-emulsified control reaction requires 12.5 µl of volume. For reaction assembly, prepare a 62.5 µl master reaction volume and transfer 12.5 µl of master reaction volume to a separate tube. The remaining 50 µl reaction volume serve as RT-PCR water phase. Mix per 50 µl and per 62.5 µl water phase volume, respectively:

2x Master Buffer Mix	25 µl	31.25 µl
5'-Primer (T7-Bank40-FPri) [100 µM]	1 µl	1.25 µl
3'- Primer (RT-Bank40-RPri) [100 µM]	1 µl	1.25 µl
RNA [10 ng-2 µg]	x µl	x µl
Master Enzyme Mix	2 µl	2.5 µl
RNase free water	to 50 µl	to 62.5 µl
Total Reaction Volume:	50 µl	62.5 µl

Gently mix the components by pipetting. Spin down briefly to collect the liquid at the tube bottom.

Note 1: Non-Emulsified Control PCR. For control purposes, it is recommended to set up an non-emulsified ("open") PCR control reaction (volume: 25 µl) in parallel to the emulsified PCR assay .

Note 2: Precautions for Non-Emulsified Control PCR. When running an "open" (non-emulsified) control reaction (recommended), proceed as follows:

(1) Prepare a RT-PCR master mix.

(2) Retain an aliquot for setting up the non-emulsified reaction control.

(3) Proceed setting up the emulsion reaction as described below.

Note 3: ePCR Template DNA Amount. For RNA transcribed from oligonucleotide T7-Lib-Bank40 (106 nt, RNA, MW ~324 g nt^{-1} mol^{-1}), ~0.58 µg RNA correspond to approx. 10^{13} template DNA molecules. At a total of 10^8 to 10^9 micelles per emulsion reaction, this corresponds to 10^5 to 10^6 DNA molecules per micelle. The high amount of template RNA helps to ensure that an as large as possible diversity of molecules is introduced as basis for selection reactions. For comparison: ~58 pg oligonucleotide T7-Lib-Bank40 would correspond to 10^9 copies.

Note 4: 1-Step RT-PCR and Compatibility with Emulsions: Many OneStep RT-PCR kits from third parties are known to be incompatible for RT-PCR within emulsions and are known not to work in this assay.

3. Create *Emulsion Reactions*.

- Mix 300 µl prechilled (wet ice) Oil Surfactant Mixture
- Add 50 µl prechilled (wet ice) PCR Water Phase.
- Mix PCR assay in a cold room using vortexer for 5 min at max speed or, alternatively, use a bead beater (Caution: Just mix the liquid sample thoroughly, do not use glass beads or similar).
- Dispense each aliquot in equal amounts to three empty, thin-walled PCR tubes ("triplicates").
- Perform regular RT-PCR program in a standard PCR cycler.

Note 1: Temperature Limits. Do not exceed denaturing temperatures of 95°C, to avoid destabilization of the emulsion, resulting in possible phase instability and separation.

Note 2: Variability in Oil Phase Composition. Within a certain range the relative amount of Oil Surfactant Mixture to PCR Water Phase may vary. This can affect micelle size and, as a consequence, have an impact on both DNA yield (positively correlated to micelle size) and reaction specificity (negatively correlated to micelle size). Exceeding the permissible range results in unstable emulsions, prone to spontaneous phase separation.

Note 3: Reaction Volumes. Each of the triplicate PCR tubes that were derived from one PCR assay should now contain approx. 113 µl of PCR emulsion reaction, corresponding to approx. 17 µl pf PCR Water Phase.

Note 4: Limited Resources. The resources of micelles are limited. For example, typical ePCR reactions deplete available resources per micelle within max. 15 cycles, dependent on composition of the emulsion, i.e. on micelle size. For a further increase of eRT-PCR yield (e.g. when starting from small template RNA amounts), conduct a scale-up ePCR: (1) Break the emulsion as outlined below (page 34), (2) purify DNA by spin column purification and (3) use as much of the purified DNA as possible for setting up a second emulsion PCR (not an eRT-PCR!). Do not conduct more than a total of 30 (=2 x 15) PCR cycles.

PCR Cycling Parameters

Always conduct the maximum number of 15 – 20 amplification cycles per selection round,. Reaction yield must equal or exceed 50 - 130 pmol (= 1 - 3 µg) of purified PCR product as starting material for introduction in the following selection round. In case (much) less than 50 – 130 pmol DNA is obtained, purify PCR product as described below (page 35) and use purified DNA as template for setting up a second, "scale-up" emulsion PCR reaction (again, conduct the full amount of 20 cycles).

	Reverse Transcription	54°C	30 min
	Initial Denaturation	94°C	5 min
20 cycles			
	Denaturation	94°C	60 sec
	Annealing	55°C	60 sec
	Extension	72°C	180 sec
	Final Extension	72°C	300 sec
	Hold	+4°C – +8°C	∞

Introduce approximately 50 - 130 pmol (= 1 – 3 µg) of purified dsDNA as input for the second and all follow-up selection rounds (remember to store an aliquot of input DNA as backup). Depending on overall reaction progress during follow-up rounds and upon progressive enrichment of good binders, dsDNA input is pronouncedly reduced in follow-up selection rounds.

Note 1: Good reaction progress is indicated by efficient PCR and high yield. Template DNA is enriched by a factor of 10^9 by 20 PCR cycles. As a rule of thumb: Amplification of sufficient starting material for the follow-up round starting from bead eluate within 20 PCR cycles indicate good reaction progress. The selection progress should be viewed with increasing skepticism with numbers of required PCR cycles approaching 30-40. If more than 30-40 PCR cycles (= 2 x 15-20 emulsion PCR cycles) are required for library re-amplification, it is recommended to discard the amplificate and to re-start the selection from a backup aliquot of a previous selection round.

Note 2: Low Yield Troubleshooting. In case the library is not re-amplificable from bead eluate within 20, max. 30-40 PCR cycles check the following error sources: (1) Template DNA purification is inefficient or DNA is lost completely; (2) PCR fails to amplify DNA; (3) selection target is not immobilized properly to microbeads; (4) selection does not proceed specifically against the target but proceeds non-specific or "false" specific against coated microbead components such as streptavidin; (5) binding specificity, as selected for during previous selection rounds might accidentally have been lost due to misconducted binding, washing or elution steps or application of inappropriate selection conditions.

Note 3: Low Yield and Scale-Up ePCR. The total number of micelles in the assay is 10^8 to 10^9. In case each micelle hosts at least one or more template DNA molecules, all resources contained within the PCR water phase can contribute to maximize the yield of PCR amplification. Under these conditions, maximum PCR yield is achieved. Due to inherent properties by Poisson distribution of template DNA molecules to micelles, this requires a minimum of approx. 10^9 to 10^{10} template DNA molecules. In case a fraction of micelles does not host any DNA template molecule, the PCR reagents in the enclosed volume cannot contribute to overall PCR yield. Thus, beyond a certain threshold value of approx. 10^9 to 10^{10} template DNA molecules per total reaction, PCR

yield will drop sharply with decreasing template DNA molecule counts (see appendix, page 49). In case the initial number of template DNA molecules drop beyond the detection limit of emulsion PCR (approx. 10^8 molecules), the first emulsion PCR may end with non-detectable amounts of PCR product, indicating that the number of DNA molecules introduced to emulsion PCR was less than 10^8. In this case, break the emulsion as outlined below, purify DNA and restart a second emulsion PCR. If the second emulsion PCR fails to generate detectable amounts of PCR product as well, there is no use in further trying. Discard the amplificate and restart emulsion PCR from the last known-to-work backup of a previous selection round. Try to apply somewhat relaxed stringency conditions.

Note 4: An unconventional way for ensuring that the number of PCR cycles suffices to maintain library re-amplification: Stop the PCR after completion the maximum number of 15 - 20 cycles, place both emulsified and open PCR assays on wet ice or in the fridge, and check an aliquot of the open control PCR by agarose gel electrophoresis. Optionally, purify an aliquot of the PCR assay, and determine by spectrophotometric measurement, whether DNA concentration is sufficient. If not, conduct a scale-up PCR with additional PCR cycles (max. 30).

Breaking the Emulsion

4. Pool the corresponding triplicates of each ePCR assay into a single 2 ml reaction tube. Break emulsion by adding 1.0 ml 2-butanol (or butanol). Mix by vortexing

Note 1: Addition of 2-butanol (or butanol) results in a merger of the water and the oil phase either in two distinct phases or in one single phase. During this step the visual appearance of the emulsion changes from milky, white to clear, transparent.

Note 2: Troubleshoting - Lack of Phase Separation. In some cases no phase separation is obtained. If only one single, but clear and transparent phase is obtained, continue with purification as described below. If the solution remains milky, the emulsion has not opened completely. In this case DNA cannot bind properly to the column matrix and all attempts to purify DNA will lead to poor results. Try to improve breaking the emulsion by vortexing thoroughly and / or by stepwise addition of 2-butanol.

Note 3: Reaction Volumes. At this point, each 2 ml plastic tube should contain 50 µl PCR Water Phase, 200 µl Oil Surfactant Mixture and 1000 µl 2-butanol (or butanol).

5. Add 400 µl of orange-colored Orange-DX buffer to the solution.
   Mix opened emulsion solution by gentle agitation (e.g. on a rotator for 2 min).
   Centrifuge for 2 min at 16 000 x g (approx. 14 000 rpm) for phase separation.

6. Remove most of the the yellow colored organic phase. Since both, water phase and interphase will be used for spin column purification, it is recommended to leave a small rest volume of the organic phase on top of the interphase. All organic phase remains will be completely removed during the follow-up spin column purification.

Note 1: Colored Organic Phase. Phases are separated into a water phase containing DNA and a organic phase. The yellow color supplied with buffer Orange-DX moves to the organic phase. Thus, for DNA purification, the colorless water phase will be used, whereas most of the yellow colored organic phase is discarded.

Note 2: Low DNA Yield Measurements. In case of low DNA yield, pass the entire liquid through the DNA binding column (i.e. water phase, interphase and organic phase). Spin columns are tolerant for contact with the organic phase. To prevent spills, transfer no more than 600µl of liquid, centrifuge and discard the flow-through before applying the remaining liquid to the spin column.

Note 3: Optional Quick Gel Check. An aliquot of the water phase may be used for a quick reaction control on an agarose gel. Please keep in mind, that for now migration inconsistencies such as gel retardation may occur, because DNA-binding proteins are not yet removed. This step is a purely optional step for the impatient. It is more important to thoroughly check integrity and specificity of RT-PCR products following the DNA purification step described below. See page 39.

7. Apply 30 µl of activation Buffer DX onto the spin-column (do not spin) and keep at room temperature until transferring the mixture to the spin-column.

Note 1: Buffer to Membrane Center. Addition of Buffer SF onto the center of the resin enables complete wetting of membranes and maximal binding of DNA.

Note 2: Activate Before Start. The membrane activation should be done before starting isolation procedure.

8. Pour mixture (aqueous phase + interphase; max. 600 µl) into a spin-column/receiver tube assembly.

Note 1: Column passage of organic and water phase. It is possible to add the both liquid phases to the spin column. Spin columns tolerate the passage of the organic phase. Subsequently add aliquots of 600 µl maximum volume to the spin column and remove the flow-through following each centrifugation step.

9. Spin down in a micro-centrifuge at 11,000 x g (12,000 rpm) for 1 minute.
10. Remove spin column, discard flow-through, put spin-column back on top of the tube.
11. In case total volume of aqueous phase + interphase exceeds 600 µl: Repeat steps 8 – 10 using the same spin column.
12. Add 500 µl of Wash-DX2 buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
13. Remove spin column, discard flow-through, put spin-column back on top of the tube.
14. Add 500 µl of Wash-DX2 buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
15. Remove spin column, discard flow-through, put spin-column back on top of the tube.
16. Spin down at 12,000 rpm for 2 minutes to remove remaining traces of Wash-DX2 buffer.
17. Place spin-column into new receiver tube (1.5-2 ml). Add 70-120 µl of Elution-DX buffer to elute bound DNA.

Note 1: Add Buffer to Membrane Center. Addition of eluting buffer directly onto the center of the membrane improves DNA yield.

Note 2: Hot Elution. Elution with buffer heated to 80˚C helps to increase efficiency of DNA recovery.

Note 3: Use Elution-DX Buffer. For elution of DNA the Elution-DX buffer is highly recommended. The buffer is prepared using ultra-pure water with trace addition of buffering compounds. Elution-DX buffer will not interfere with subsequent DNA reaction steps.

Note 4: Reduced Elution Volume. It is possible to reduce the volume of eluting buffer below 50 µl (no less than 20 µl). However, recovery of DNA will gradually decrease.

Note 5: Elution Buffer Volumes. For all but the last selection rounds, elute with 60 µl elution buffer to obtain a total volume of >50 µl eluate (3-5 µl for spectrophotometric measurement, 3-5 µl for agarose gel check, 15-25 µl for backup purposes and 15 µl as input DNA to the follow-up selection round). For the last selection round, elute with 110 µl to a total volume of >100 µl eluate (2 x 200-300 ng of dsDNA required for either DiVE or DiStRO assay analysis in duplicates, respectively; 2 x 300-500 ng of dsDNA required for duplicate FLAA analysis plus a sufficient amount of DNA for either performing Next Generation Sequencing analysis or for molecular cloning steps, respectively).

18. Incubate spin-column/receiver tube assembly for 2 minutes at room temperature.
19. Spin down at 11,000 x g (12,000 rpm) for 1 minute.

20. Remove spin column, cap the receiver tube. Isolated DNA is ready for analysis / manipulations and for use as input DNA for follow-up selection rounds. It can be stored at 4°C (short-term) or at -20°C (long-term, preferred).

Note 1: **Store a Backup Now.** Remember always to keep 50 % of any purified PCR reaction as backup until having finished the complete SELEX process. In case of errors or continuous decrease of PCR yield or complete loss of DNA, it might prove handy to have a backup from previous cycles to continue from, rather than having to start a new SELEX process from the very beginning.

Note 2: **Store a DNA Library Backup, Not a RNA Library Backup.** Backups of libraries are rather stored in dsDNA format than in ssRNA format (following T7 *in vitro* transcription), since ssRNA is much more susceptible to freeze-thaw-cycles as compared to dsDNA.

Note 3: **Quality Check.** Perform a quality control by agarose gel electrophoresis or by PAGE gel electrophoresis. Verify that RT-PCR amplicons form a sharp distinct band of the expected size. In case of artifact formation, discard the RT-PCR product and restart from the last-known-good RT-PCR amplicon form previous selection rounds. See page 39.

Following elution, check concentration of amplified DNA. Minimum PCR yield as starting material for follow-up SELEX rounds is 1 µg PCR-DNA per 50 µl volume, corresopnding to A_{260} = 0.4 (see page 33). The higher the DNA concentration, the higher the number of good binders and hence the closer the end of the selection process.

Monitoring the Selection Progress

SELEX is a complex method requiring the experimenters to develop their own experience and intuition for correctly adjusting reaction parameters throughout the selection process. The progress of the selection process requires monitoring of the reaction yield after each selection round and, deducing from these values, adjusting stringency parameters for the follow-up selection round. Additionally, upon completion of all selection rounds, a comparative analysis of amplified nucleic acid aliquots obtained during each selection round is performed to monitor and verify the continuous decrease in library diversity and the corresponding increase in good binder enrichment.

Since there is a large variation with respect to the requirements of different selection targets, there are neither general guidelines nor distinct values that can serve as reliable guide parameters. Rather, the researcher has to follow general tendencies by comparing output per selection round relative to input. Depending on nature and requirements of the target structure, experimental design needs careful adjustment to the progress of the selection. The success of each selection round has to be monitored, protocoled and carefully compared in the light of the results from previous rounds. For an example see table 1, page 16.

Several variables differ between SELEX experiments and selection targets. Parameters influencing the SELEX process include general properties of the selection target, such as charge, structure and stability, as well as reaction-specific parameters such as the strategy chosen for target immobilization, length of the oligonucleotide library and the diameter and coating of magnetic particles.

Thus, the efficiency of each SELEX process is highly target-dependent. Therefore, it is not possible to draw valid conclusions by comparing parameters from different SELEX experiments. In practice, three parameters are of special interest for measuring overall reaction progress between selection rounds:

1) Output yield of the PCR amplification (measured after purification),
2) Diversity of the oligonucleotide library after each selection round,
3) Output / input Ratio (O/I Ratio).

Measurement of (1) is by spectrophotometric concentration determination (A260 absorption), suitable assays for (2) are described in section III contained within the → XELEX DNA Core Kit manual (Analysis Unit). The O/I ratio is probably the most difficult to measure parameter for determining reaction conditions of follow-up selection rounds. Fortunately, measurement of the O/I ratio is most often not required. In principle, the O/I ratio is determined by dividing the amount of DNA after completion of a selection round to the amount of starting material introduced in the respective selection round. In practice, the O/I ratio is often not monitored at all or at best monitored indirectly (see below for further details).

Both, the O/I ratio and the DNA output yield are equivalent useful parameters for monitoring selection progress and for setting up appropriate follow-up round stringency parameters (compare table 1, page 16 and page 36). In emulsion PCR, amplification proceeds until reaction components confined within micelles are exhausted, regardless of the number of template molecules initially present. Since emulsion PCR minimizes the impact of PCR-bias based diversity shifts, there is no need to vary the number of PCR cycles between selection rounds. Simply conduct ePCR with maximum cycle numbers. Under these conditions, amplification in template-containing micelles will always reach the same end point. Any differences in PCR yield are solely caused by “empty” (non-template DNA containing) micelles. The enclosed volume of “empty” micelles contains a share of PCR water phase that can not generate amplicons and does not contribute to total PCR yield. Primary cause for low PCR yield is the presence of a large number of empty micelles, indicating that the number of template DNA molecules has dropped well below the number of micelles in emulsion PCR. In consequence, the amount of library DNA molecules required for the follow-up reaction step would not regenerate completely during the amplification step, pointing to rather high template losses during the previously conducted selection steps: Either stringency conditions were chosen too high during the previous binding and washing steps (resulting in poor DNA recovery), or there exist potential problems during the elution step.

Analyses to be performed following each selection round

- Carefully protocol selection and amplification parameters as outlined in table 1 (page 16). Keep notes, compare and set these values in relation to the corresponding values obtained during earlier selection rounds.
- Agarose / PAGE gel check: After reamplification, the size of amplification product is verified on a 3 % [w/v] 'standard' (non-denaturing) agarose gel or on a non-denaturing PAGE gel. In case of aberrant band size, discard the amplified PCR product and restart selection from an amplificate backup from the last known-to-work selection round. Due to secondary structure formation, single stranded DNA and RNA form non-distinct bands during non-denatured agarose gel electrophoresis and show different migration behavior as compared to double stranded DNA. Tip: Running routine agarose / PAGE gel checks in parallel, while already conducting the follow-up selection round may often helps to save valuable time.
- Post-PCR DNA output yield: Following each selection round, determine the amount of DNA following PCR amplification and purification by spectrophotometric measurement. Between all cycles of a given selection reaction for one specific target (but not between selection reactions for different targets), DNA amplification output yields obtained after different selection rounds are well comparable. Given the number of PCR cycles remains constant: If the DNA output yield increases or remains constant at least, increase stringency of the reaction. In case the DNA output yield decreases, relax the stringency conditions during follow up rounds. If the DNA output yield continues to decrease and nucleic acids of aberrant size appear during gel electrophoresis, discard the current assay and return to the last selection round that gave positive results.
- Output / input (O/I) ratio (optional, only required for troubleshooting purposes): The bead eluate contains only minute amounts of bound nucleic acids. Thus, if not aiming at direct radioactive measurement, it is mandatory to conduct an amplification step for obtaining analyzable amounts of library DNA. In practice, there exist two approaches for indirect O/I ratio measurement: (1) A rough but quick estimate, accurate enough for most applications: Following elution, determine the number of PCR cycles required to reamplify sufficient amounts of DNA for the follow-up round (up to approx. 2-3 µg DNA are required per round). Protocol and compare the number of PCR cycles necessary for reamplification as well as the amount of DNA obtained after reamplification. (2) The amount of template DNA in bead eluate is quantified more accurately (albeit more time-consuming) by Real Time PCR analyses of defined bead eluate aliquots. To save valuable time, keep an aliquot of bead eluate for Real Time PCR analyses and conduct Real Time PCR analyses upon completion of all 3 – 10 or 15 selection rounds in parallel. Most often, laborious (O/I) ratio determination is not required: The DNA output yield mentioned above provides enough information to decide, whether binding and washing stringency conditions for the follow-up selection round need to increase, remain constant or have to be relaxed, respectively.

Analyses to be performed upon completion of all selection rounds

(typically after 3 to 10 or up to 15 selection rounds):

- Optional output / input ratio determination by RealTime PCR analyses (enzymatic; target: non-amplified bead eluate aliquots)
- S1 nuclease (DiVE) assay (enzymatic; target: reamplified library aliquots)
- RealTime diversity assay (DiStRO) (non-enzymatic; target: reamplified library aliquots)
- Binder assay studies (FLAA) (target: reamplified library aliquots, aptamer clones)

Note 1: **Save Time and Effort.** The described assays are highly useful for monitoring reaction progress and for library quality control, but are time consuming. Therefor it is recommended to complete all 3 to 10 selection rounds without interruption for performing the described measurements. Only upon completion of the entire selection process, aliquots of the amplified bead eluate backups obtained during each selection round are analyzed in parallel, as described in the → XELEX DNA Core Kit manual, section III (Analysis Unit). Analysis results allow determination of interesting selection rounds to be analyzed in close detail (usually this will be the last or the last known-to-work selection round).

Protocols for the DiVE, DiStRO and FLAA assays, respectively, are given in the → XELEX DNA Core Kit manual, section III (Analysis Unit).

It is mandatory to monitor the RT-PCR amplicons for the formation of artifacts, appearing as additional bands or non-specific smear. This may be achieved either by non-denaturing PAGE gels, or by non-denaturing, 'standard' agarose gel electrophoresis. If there are any doubts on the specificity of synthesized RT-PCR amplicons, it is recommended to discard the amplicon in question and restart from a backup of the last-known-good amplification product. Any small errors propagate quickly and tend to accumulate during later selection rounds. Thus, it is better to decide on time to restart from a clean amplification product in order to save a lot of futile and extra work.

High quality RNA is indicated by sharp, precisely defined bands. Low quality RNA is identified by smeared bands.

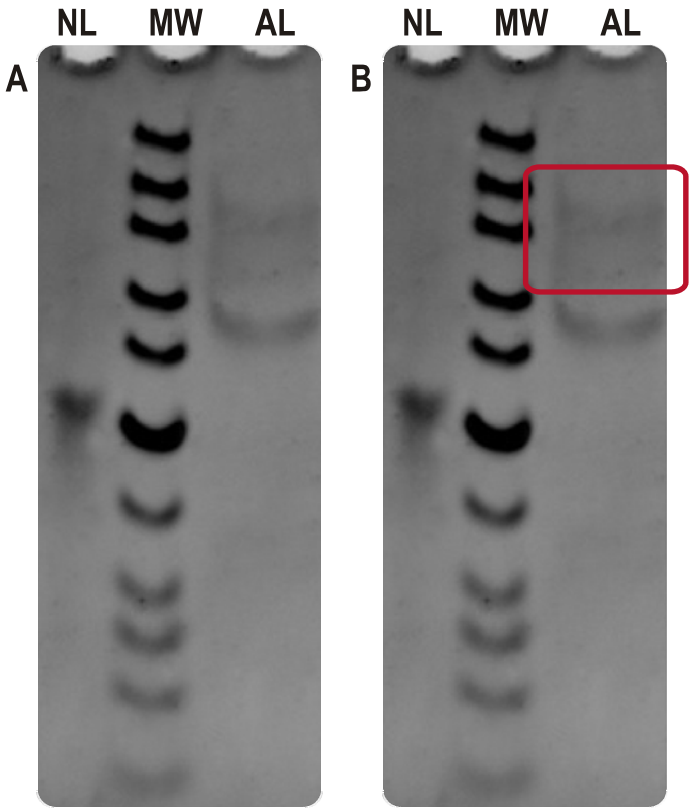


Figure 6: Demonstrating the looks of typical PCR / ePCR artifacts. This gel picture demonstrates the optical appearance of typical SELEX PCR artifacts following gel electrophoresis on a non-denaturing PAGE gel. PCR artifacts appear as “ghost bands”. ePCR is supposed to result in amplification of one single, clean band. PCR artifacts may occasionally form during non-balanced PCR amplification of highly diverse libraries.
Generally, the higher the library diversity and the denser the concentration of template DNA, the more likely is the formation of PCR artifacts. Initial library diversity, preceding the first selection and elution step, is extremely high. Immediately following the very first selection and elution step, library diversities drop sharply by several orders of magnitude, and decreases further over time with proceeding selection and binder enrichment during later selection rounds. Consequently, any PCR library amplification preceding the first selection round, when library diversity is at its very highest, is most prone to PCR artifact formation. Artifacts may form during later selection rounds as well, mostly when performing non-emulsified ePCR reactions. The provided ePCR amplification kit is much less prone to PCR artifact formation.
Adequate measurements: In case any appearance of similar PCR artifacts is observed, the artifact-prone assay should be discarded and a new amplification should be performed, starting from a backup of the last known good library enrichment from previous selection rounds. Once formed, PCR artifacts will inevitably accumulate in further selection rounds. Thus, it is not advised to make any further usage of any artifact-laden library amplificates. Lanes NL: Non-amplified, single stranded DNA library. MW: Molecular weight marker. AL: PCR-amplified, double stranded library with typical PCR artifacts (“ghost bands”, highlighted in the red rectangle on the copy of the gel picture at the right hand side). A: Gel photo. B: Copy of gel photo with PCR artifacts highlighted by a red rectangle. Photo reproduced with kind permission of Florian Mittelberger, University of Hamburg.

Optional Step: Scale-up Emulsion PCR

In case less than 0.1-0.5 µg of dsDNA amplicons are obtained during the initial eRT-PCR step, an additional scale-up ePCR step may be required to regenerate the required amount of enriched library DNA for the follow-up selection round. For increasing reaction yield, set up an emulsion PCR (without preceding RT step) on top of the emulsion eRT-PCR described on page 31. Never perform more than one scale-up ePCR amplification per selection round (see page 30, step 3, note 2, as well as PCR Cycling Parameters, page 33, note 3).

Following the initial eRT-PCR step, the emulsion is broken by addition of 1.0 ml 2-butanol and DNA is purified with spin columns (XELEX Core Kit - Emulsion & DNA Spin Column Set, Cat. No. E3653) as described in the → XELEX DNA Core Kit manual. The pre-amplified template DNA, ideally close to 10^9 copies per reaction or 10-50 % of total micelle count, is then used for a second scale-up PCR. This follow-up PCR is performed with identical primers and with the desired number of amplification cycles (typically 15 – 20 cycles). As opposed to conventional PCR, the creation of PCR artifacts during scale-up PCR is effectively inhibited by the compartmentalization of individual template DNA molecules to separate reaction compartments.

Additionally required components for scale-up emulsion PCR:

- Micellula DNA Emulsion & Purification Kit (Cat. No. E3600)
- *Taq* DNA Polymerase (Cat. No. E2500) plus dNTP set (Cat.no. E2800);
  or alternatively *Taq* PCR Master Mix (Cat. No. E2520)

Backups

For each selection round, keep backups of

- 50 % of bead eluate (from the elution step, non-amplified),
- 50 to 90 % of amplified libraries (from the amplification step).

The material is required for library analysis, quality control and as a backup, if any follow up selection round would not work as expected and a fallback to the last known-to-work selection round becomes necessary. Store backups at -20°C until finishing the entire selection process, or better until identification and characterization of good binding aptamers. For long term storage, use dilute buffers such as 1 x TE or phosphate buffers (for amine sensitive assays) rather than pure water to prevent autohydrolysis of DNA.

Having a Break / Scheduled Interruptions

A skilled, experienced experimenter accustomed to the protocol may manage to accomplish one selection round per working day, although these particular working days could become long and the working schedule tight. Since the SELEX process consists of many individual SELEX rounds, each equivalent to one working day, an interruption of the SELEX process is inevitable. Therefor it is important to provide specific points, where the SELEX process can be interrupted.

The recommended point of interruption is upon purification of re-amplified dsDNA library (after having finished with the DNA purification protocol described on page 35). This is the only point, where long-term storage of an intermediate product is recommended (e.g. library backups of intermediate SELEX rounds). Purified dsDNA is stable for long term storage at -20°C.

Short-term interruptions are as well possible after the following steps

- Following the RNA library purification step described on page 23. Store RNA in an RNase free vessel at 4°C. Do not freeze, since RNA is sensitive to freeze-thaw cycles. Short-term storage only. See also page 24, note 2.
- After setting up and running eRT-PCR (described on page 31). It may prove convenient to set up and run eRT-PCR over night and continue with DNA purification on the next day. Upon completion of PCR, set the cycler on hold to cool the assay either to +4°C or to +8°C. Cooling to +4°C put a lot of stress on the Peltier elements of the PCR cycler and, when applied frequently, might result in an early wear-out of those expensive elements. Thus, a temperature hold at +8°C to +10°C might prove advantageous. If possible, it is recommended not to keep the eRT-PCR assay on the cycler over night. It is even better to wait until PCR is finished, to proceed with the subsequent DNA purification step (page 23) and to store the purified dsDNA, which is devoid of any enzyme.

Controls

One important issue regarding controls: As SELEX is a complex procedure, selection success is not guaranteed, even if aptamers towards chemically similar targets or to molecules with related structure have been selected before: For some target molecules it is quite simple to find good binding aptamers with low dissociation constants (K_d). For some target molecules selection proves increasingly difficult while for some other target molecules it appears to be impossible to select for any good binding aptamer. Thus, inclusion of both *well designed* positive and negative controls are mandatory. The manual includes hints, as to which controls should be included to verify proper selection progress. Due to the different nature of selection targets and possible protocol modifications, these suggestions should not be considered as a complete list. This manual cannot relieve the researcher from giving some thoughts on appropriate positive and negative controls. Often, absent or poorly designed controls may create the false impression of good selection progress, whereas in fact only accumulation of poor or non-specific binders is measured. (For example, selection for specific aptamers to (improperly purified) DNA- or RNA- binding proteins might also enrich complementary nucleic acid sequence stretches against DNA or RNA fragments).

Suitable controls ensure monitoring whether selected aptamers do actually bind solely to chosen the selection target and do not

- bind to coated surfaces (e.g. streptavidin-coated beads),
- bind to the conjugates used for coupling target structures to coated surfaces,
- bind to potential co-purifying contaminants of non-completely purified selection target molecules (e.g. nucleic acids fragments that were possibly retained during isolation of target molecules). It may require a good amount of careful thought to imagine potential error sources and design appropriate controls for the selection target of interest.

Caution: Always be critical with data interpretation. False conclusions may be drawn from experimental data and non-target-specific enrichment may erroneously be taken for good selection progress.

Another important issue is to consider other target specific controls, which may be included. Since there are many potential targets for aptamer selection, each with their own peculiarities, it is not possible to explicitly mention all required, target-specific controls for each individual selection reaction setup. Many potential sources of errors may hide in the process, each of which might require application of suitable controls.

Section III: Identification and Characterization of Aptamers

Library quality control and characterization is performed at DNA level, using amplified PCR products (dsDNA) as starting material. Therefore, the protocols described within section III (Analysis Unit) of the → XELEX DNA Core Kit manual apply to RNA aptamer selections accordingly

Aptamer binding analysis, such as the FLAA assays described in the → XELEX DNA Core Kit – Analysis Unit (section III), must be performed on RNA level, not DNA level, for obvious reasons. Use RNA-specific dyes for FLAA binding measurement and quantification, such as RiboGreen.

Note, that the Streptavidin positive and negative controls included with the → XELEX DNA Core Kit – Analysis Unit are of limited use, when working with RNA aptamers.

Appendix

Formulas

Calculating the molar mass (MM) of single-stranded DNA (ssDNA)

Method 1: For single stranded DNA molecules with roughly equal distribution of all nucleotides, molar mass is easily estimated with this formula:

$$MM\ [g\ mol^{-1}] = ssDNA\ length\ [nt] \cdot 308\ [g\ mol^{-1}\ nt^{-1}]$$

The mM of oligonucleotide T7-Lib-Bank40 (106 nt, ssDNA library + T7 promoter) calculates to 32648 g mol^{-1}.

For single stranded RNA molecules with roughly equal distribution of all nucleotides, molar mass is determined accordingly:

$$MM\ [g\ mol^{-1}] = RNA\ length\ [nt] \cdot 320\ [g\ mol^{-1}\ nt^{-1}]$$

The mM of oligonucleotide T7-Lib-Bank40 (84 nt, T7 RNA transcript, no T7 promoter) calculates to 26880 g mol^{-1}.

Method 2: For ssDNA with pronounced non-equal-distributed nucleotide composition (e.g. large excess of purins or pyrimidines), an elaborate calculation may give slightly more accurate results (all units in [g mol^{-1}]):

I. Count the occurrence of each base in the ssDNA molecule. Example: oligonucleotide T7-Lib-Bank40 is composed of 20 A's, 11 C's, 16 G's, 19 T's and 40 N's (N's are estimated to consist of equimolar amounts of A. C, G, and T, respectively).
II. According to Doležel *et al*. Cytometry, 2003, Vol. 51A, 2, 127-8. the mM for single deoxynucleotide monophosphates are (A: 331.2213); (C: 307.1966); (G: 347.2207); (T: 322.2079); (N: 326.9616; on average), respectively. Thus the mM of the sum of all nucleotide monophosphates in oligonucleotide Bank-40 calculates to:

    20 x 331.2213 (A) + 11 x 307.1966 (C) + 16 x 347.2207 (G) + 19 x 322.2079 (T) + 40 x 326.9616 (N) = 34759.5339 [g mol^{-1}]

III. For all nucleotides but the 5'-nucleotide, the loss of one H_2O upon phosphodiester bond formation must be taken into account (-18.0125 each bond). In a ssDNA molecule with N nt's (e.g. 106 nt's), there are N-1 phosphodiester bonds (e.g. 105). Thus, for oligonucleotide T7-Lib-Bank40, 105 x 18.0125 have to be subtracted from the MM-value calculated in step 2.
IV. Under physiological conditions, phosphates are acidic and H^+ will dissociate from all nucleotide phosphates. The mM of an oligonucleotide consisting of N nt (e.g. 106 nt) is lowered by N x (e.g. 106 x) 1.0079.
V. Taking together steps 2, 3 and 4, the mM of oligonucleotide Bank-40 calculates to:
    34759.5339 - 105 x 18.0125 - 106 x 1.0079 = 32761.384 [g mol^{-1}]

For the RNA transcript of T7-Lib-Bank40 (84 nt, RNA,11 A's, 6 C's, 14 G's, 13 U/T's, and 40 N's, respectively), method 2 calculates a MW of 26034.9452 g mol^{-1} (as compared to 26880 g mol^{-1} using method 1).

For oligonucleotide T7-Lib-Bank40 (106 nt, DNA) and for it's T7 *in vitro* RNA transcript (84 nt, RNA), the values calculated by both methods differ by approx. 0.3 %, which is neglectable for the practical purposes outlined here. In most cases, the simple formula in method 1 gives sufficient precision.

The molecular weight of ribonucleotides differs from the MW of deoxyribonucleotides. MWs for NMPs are AMP 347.2207, CMP 323.196, GMP 363.2201, and UMP 324.1808 g mol^{-1}, respectively. Taking into account that the average weight of a NTP in a random RNA sequence stretch is 12.4928 g nt^{-1} mol^{-1} higher as compared to random ssDNA molecules, the average molecular weight per NTP is 320.43135 g mol^{-1}. This value can be converted to an absolute value by multiplication with the mass of an atomic unit, which equals one-twelfth of the weight of an ^{12}C atom, or 1.660539×10^{-24} g (Doležel *et al*. Cytometry, 2003). Thus, the molecular weight for an average, random nucleotide is 0.532 x 10^{-9} pg for (ss)-RNA and 0.511 x 10^{-9} pg for ssDNA. The conversion factor for converting pg nucleic acid to number of bases is for ssRNA 1.879 x 10^9 bases per pg RNA and for single-stranded DNA 1.956 x 10^9 bases per pg ssDNA.

Calculating DNA Copy Numbers for single stranded DNA:

$$Copy\ Number\ [molecules] = \frac{DNA\ amount\ [\mu g] \cdot 6.022 \cdot 10^{23}\ [molecules\ mol^{-1}]}{ssDNA\ length\ [nt] \cdot 308\ [g\ mol^{-1}\ nt^{-1}] \cdot 10^{6}\ [\mu g\ g^{-1}]}$$

$$= \frac{DNA\ amount\ [\mu g] \cdot 1.96 \cdot 10^{15}\ [molecules\ nt\ \mu g^{-1}]}{ssDNA\ length\ [nt]}$$

Note 1: The value for the expression (ssDNA length [nt] x 308 [g mol^{-1} nt^{-1}]) can be replaced with a calculated molecular mass value of higher accuracy, as outlined above (see Calculating the molar mass of single-stranded DNA).

Note 2: For calculating *DNA copy numbers from double stranded DNA (dsDNA)*, replace the value of 308 [g mol^{-1} nt^{-1}] for ssDNA with 616 [g mol^{-1} bp^{-1}] for dsDNA. and ssDNA length [nt] with dsDNA length [bp].

Calculating RNA Copy Numbers for single stranded RNA:

$$Copy\ Number\ [molecules] = \frac{RNA\ amount\ [\mu g] \cdot 6.022 \cdot 10^{23}\ [molecules\ mol^{-1}]}{RNA\ length\ [nt] \cdot 320\ [g\ mol^{-1}\ nt^{-1}] \cdot 10^{6}\ [\mu g\ g^{-1}]}$$

$$= \frac{RNA\ amount\ [\mu g] \cdot 1.88 \cdot 10^{15}\ [molecules\ nt\ \mu g^{-1}]}{RNA\ length\ [nt]}$$

Calculating pmol ssDNA from µg

$$ssDNA\ [pmol] = \frac{DNA\ amount\ [\mu g]}{DNA\ length\ [nt] \cdot 308\ [g\ mol^{-1}\ nt^{-1}]} \cdot \frac{10^{12}\ [pmol\ mol^{-1}]}{10^{6}\ [\mu g\ g^{-1}]}$$

Example: DNA amount = 1.0 µg, ssDNA length = 106 bp

$$ssDNA\ [pmol] = \frac{1.0\ [\mu g] \cdot 10^{12}\ [pmol\ mol^{-1}]}{106\ [nt] \cdot 308\ [g\ mol^{-1}\ nt^{-1}] \cdot 10^{6}\ [\mu g\ g^{-1}]} = \frac{1.0}{(106 \cdot 308)} \cdot 10^{-6}\ mol = 30{,}6\ pmol$$

Calculating pmol RNA from µg

$$RNA\ [pmol] = \frac{RNA\ amount\ [\mu g]}{RNA\ length\ [nt] \cdot 320\ [g\ mol^{-1}\ nt^{-1}]} \cdot \frac{10^{12}\ [pmol\ mol^{-1}]}{10^{6}\ [\mu g\ g^{-1}]}$$

Example: RNA amount = 1.0 µg, RNA transcript library length = 84 bp

$$RNA\ [pmol] = \frac{1.0\ [\mu g] \cdot 10^{12}\ [pmol\ mol^{-1}]}{84\ [nt] \cdot 320\ [g\ mol^{-1}\ nt^{-1}] \cdot 10^{6}\ [\mu g\ g^{-1}]} = \frac{1.0}{(84 \cdot 320)} \cdot 10^{-6}\ mol = 37{,}2\ pmol$$

Calculating µg ssDNA from pmol

Calculate the molar mass of single-stranded DNA by one of both formulas described in paragraph “Calculating the molar mass of single-stranded DNA” (page 45) and multiply with appropriate factors for conversion of mol to pmol and from g to µg:

$$ssDNA\ amount\ [\mu g] = ssDNA\ [pmol] \cdot ssDNA\ length\ [nt] \cdot 308\ [g\ mol^{-1}\ nt^{-1}] \cdot \frac{10^{6}\ [\mu g\ g^{-1}]}{10^{12}\ [pmol\ mol^{-1}]}$$

Calculating µg RNA from pmol

Calculate the molar mass of single-stranded DNA by one of both formulas described in paragraph “Calculating the molar mass of single-stranded DNA” (page 45) and multiply with appropriate factors for conversion of mol to pmol and from g to µg:

$$RNA\ amount\ [\mu g] = RNA\ [pmol] \cdot RNA\ length\ [nt] \cdot 320\ [g\ mol^{-1}\ nt^{-1}] \cdot \frac{10^{6}\ [\mu g\ g^{-1}]}{10^{12}\ [pmol\ mol^{-1}]}$$

Calculating Universal Library Size and Weight

Kurd Lasswitz, a german scientist and writer, imagined in his 1901 short story “Die Universalbibliothek” (The Universal Library) the thought of an imaginary universal library. The imaginary library would contain all books that can be printed by systematically recombining a set of 100 printing characters in all feasible sequences, thus generating a truly universal library of all possible literature. Estimating the size of such a universal library ended by juggling with unbelievably large numbers.

In contrast, what is the size and the weight of a truly universal random oligonucleotide library with all possible combinatorial permutations of 20, 40 or 80 random nucleotide positions? In other words, how much ssDNA is required at minimum, if each possible sequence permutation of a random oligonucleotide library is introduced in the selection enrichment? A random nucleotide library consists of four different characters, A, C, G and T, respectively. Thus, in a population of random oligonucleotides with a length of N nucleotides, there are molecules with a maximum of 4^N different sequences as basis for selection of the best binding molecule. As outlined above, the number of oligonucleotides to be introduced in 50 µl SELEX assays is limited to a maximum of approximately 10^{15} nucleotides. Below is a simple formula for the calculation of the minimal weight of a universal library, under the idealized assumption, that the library would contain just one single molecule of each possible permutation (i.e. without any duplicate). The minimal weight of an ideal universal library for a random oligonucleotide with a length of N nucleotides is calculated as follows:

DNA:

$$Ideal\ Universal\ Library\ Weight\ [g] = \frac{308\ [g\ mol^{-1}\ nt^{-1}] \cdot N\ [nt] \cdot 4^{N}\ [molecules]}{6.022 \cdot 10^{23}\ [molecules\ mol^{-1}]}$$

RNA:

$$Ideal\ Universal\ Library\ Weight\ [g] = \frac{320\ [g\ mol^{-1}\ nt^{-1}] \cdot N\ [nt] \cdot 4^{N}\ [molecules]}{6.022 \cdot 10^{23}\ [molecules\ mol^{-1}]}$$

The weight of an ideal universal random library is solely dependent on the value of the number of random positions, N, and, due to the term 4^N, increases sharply with increasing values for N. This holds true for an idealized library owning exactly only one copy of each sequence. Since it is highly unlikely that any randomly picked library would contain just one single molecule of each distinct oligonucleotide, what would be a more realistic estimate for the expected size of a randomly generated library containing at least one molecule of each oligonucleotide? How many oligonucleotides would have to be drawn from an infinitely sized stock to obtain a set of oligonucleotides, which would expectedly contain each possible sequence? Analogous to the well-known "trading cards" problem, is possible to iteratively calculate all numbers of required draws for obtaining each next non repeating oligonucleotide from a hypothetical infinitely sized stock. The expected value for the universal library size containing all possible sequences is a sum of the reciprocals of probabilities for sampling all unique oligonucleotides respectively:

$$\frac{N}{N} + \frac{N}{N-1} + \frac{N}{N-2} + \ldots + \frac{N}{2} + \frac{N}{1} = \sum_{k=1}^{N} \frac{1}{k}$$

with N being the total number of different oligonucleotides. The total sum is well approximated by the term

$$N \log N$$

Multiplying the number of different oligonucleotides by N log N, calculating the sum of all bases and converting the molar mass of the total number of all bases from moles to weight gives the expected value for library size containing all possible sequences.

Example calculation for estimating the expected library size of oligonucleotide T7-Lib-Bank40 (106 nt, ssDNA, 40 randomized sequence positions, 66 invariable, constant sequence positions; for sequence see page 20. The calculation for the 84 nt sized RNA transcript proceeds analogous when using an MW of 320 g nt^{-1} mol^{-1}, and a library length of 84 nt, where 40 nt are randomized positions and the remaining 44 positions remain constant, determined).

Total number of bases per oligonucleotide [nt]	106
Total number of random positions per oligonucleotide [nt]	40
Expectation value for the number of oligonucleotides to be drawn from an infinitely large library for obtaining a complete collection of an oligonucleotide set with all possible N different oligonucleotides	N log N
N: Number of different oligonucleotides with 40 random positions (= size of an ideal, non-redundant universal library; each sequence is contained once and only once)	$N = 4^{40} =$ 1208925819614629174706176 = ~ 1.2×10^{24}
log N: (where log is *logarithmus naturalis*)	log N ~ 55.45
N log N: Expectation value for the required number of oligonucleotides to obtain a complete set of random 40-mers (= expected size of a redundant universal library, expected to contain each possible sequence at least once)	N log N = 6.7×10^{25}
Total number of bases in oligonucleotide: 106 nt = 40 random + 66 fixed	106
Total number of bases in library of expected size [nt] (= total library size [nt]):	106 x 6.7×10^{25} = 7.11×10^{27}
Conversion factor of base pairs (bp; dsDNA) to pg DNA (Doležel *et al*., Cytometry, 2003, Vol. 51A, 2, 127-8)	DNA content [pg] = tot. library size [bp] / (0.978×10^9) [bp pg^{-1}])
Conversion factor of bases (nt; ssDNA) to pg DNA same as above, but coefficient is multiplied by a factor of 2	DNA content [pg] = total library size [nt] / (1.956×10^9) [nt pg^{-1}])
DNA amount [pg] = 7.11×10^{27} [nt] / 1.956×10^9 [nt pg^{-1}] = 3.63×10^{18} [pg] DNA amount [kg] = 3633	

The conversion factor for single stranded RNA is 1.956×10^9 [nt pg^{-1}]). Thus, the expectation value for a universal library size equals for the 106 nt sized RNA transcript 7.11×10^{27} [nt] / 1.956×10^9 [nt pg^{-1}] = ~3.63×10^{18} pg = 3630 kg.

Universal DNA-libraries weight in the ng-scale for random 20mers, in µg-scale for random 25mers, in mg-scale for random 30mers, in (metric) ton-scale for random 40mers, whereas the vast size of a minimal universal random 80mer library (with an estimated mass comparable to the mass of the sun) demonstrates that the diversity of large random libraries (e.g. random 80mers) is completely unaddressable. However, some applications, such as the selection of oligonucleotides with catalytic activity, require screening of libraries with a length of 150 bases and longer (Pollard 2000).

Oligo	Length of random sequence stretch [nt]	Total number of different molecules	Minimal weight of universal library [g]**	Expected weight of universal library [g]***
Random 20mer	20	$4^{20} = 1.1 \times 10^{12}$	1.1×10^{-8}	3.12×10^{-7}
Random 25mer	25	$4^{25} = 1.3 \times 10^{15}$	1.4×10^{-5}	4.99×10^{-4}
Random 30mer	30	$4^{30} = 1.2 \times 10^{18}$	5.9×10^{-2}	7.36×10^{-1}
Random 40mer	40	$4^{40} = 1.2 \times 10^{24}$	2.5×10^{4}	1.37×10^{6}
Random 80mer	80	$4^{80} = 1.5 \times 10^{48}$	6.0×10^{28}	6.63×10^{30}
T7-Lib-Bank40*	40	$4^{40} = 1.2 \times 10^{24}$	4.7×10^{4}	3.63×10^{6}

*Oligonucleotide Bank40 contains an additional defined sequence stretches without permutation.
** Minimal weight of a hypothetical universal library with exactly one single copy per possible sequence.
*** Expected weight value for a library containing each possible sequence at least once.

Adjusting DNA Amount to Total ePCR Micelle Count

This procedure is listed for calibration or troubleshooting purposes only. In SELEX, the determination of total micelle count is entirely optional, since template DNA is always applied in vast excess to total micelle count. The number of individual reaction compartments (micelles) per reaction are roughly estimated by performing dilution series with a known number of template DNA copies. Here is a brief outline of the procedure (which has not to be performed on a regular basis, probably only once, if at all, when establishing proper reaction conditions).

- Prepare an emulsion PCR series as outlined above. Prepare a six-step-dilution series (in duplicate or triplicate), where the ePCR water phase contains the following copy number of template molecules: 1×10^{12}, 1×10^{11}, 1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7} and 1×10^{6} copies, respectively.
- After ePCR, followed by DNA purification, apply equal volumes of PCR products to a 'standard" agarose or to a non-denaturing PAGE gel.
- Determine the dilution step, where the yield decreases sharply. In case template copy numbers largely exceeds micelle count, nearly all micelles host a template molecule and the substrate enclosed by each micelle can contribute to total ePCR yield. As a consequence, the assays with highest template DNA concentration will show a near identical yield. At a certain dilution step, yield decreases sharply. There remain not enough template DNA copies to fill all micelles. Empty micelles, devoid of any template DNA, show no amplification and the volume enclosed by the empty micelle can not contribute to ePCR yield. The fewer template DNA molecules, the less micelles will contain template DNA and the less overall ePCR yield is obtained. This behavior can be used to estimate the total count of micelles in an ePCR assay: Due to the random (Poisson-like) distribution of template DNA molecules to micelles, the number of micelles roughly equals the number of template DNA copies at the point, where emulsion PCR yield shows an approx. yield reduction of 37 % (≈ 1/e).

In conventional (non-SELEX) ePCR approaches, the recommended template DNA copy number corresponds to the lowest concentrated ePCR assay, which still gives maximum yield, i.e. the concentration step immediately preceding the sharp decrease in PCR yield. Note that for SELEX reaction this calibration is most often not required, since the goal is to introduce as much template as possible into selection reactions. Under these conditions, it is ensured that each micelle will host many template DNA molecules to start PCR from.

Calculation of Binding Capacity for Coated Magnetic Beads or Microwell Plates

Example for the calculation of binding capacity on streptavidin-coated bead surfaces (applies analogous to alternate surface chemistry and to surfaces of coated microwell plates):

The nominal binding capacity of biotin molecules per bead, according to the manufacturer supplied information, is given as 1 x 10^{18} moles free biotin per single bead. The binding capacity of free biotin molecules per single bead is calculated as follows:

$$6.23 \cdot 10^{23}\ molecules\ mol^{-1}\ \cdot\ 1 \cdot 10^{-18}\ mol\ biotin\ bead^{-1} = \sim 6 \cdot 10^{6}\ molecules\ biotin\ bead^{-1}$$

If 1 mg of magnetic beads equals ~ 7 x 10^{8} beads, then the binding capacity per mg of beads is:

$$7 \cdot 10^{8}\ beads\ \cdot\ 6 \cdot 10^{6}\ molecules\ biotin\ beads^{-1} = \sim 4.2 \cdot 10^{14}\ molecules\ biotin$$

$$\frac{4.2 \cdot 10^{14}\ molecules\ biotin}{6.23 \cdot 10^{23}\ molecules\ mol^{-1}} = \sim 700\ pmol\ biotin$$

The maximum binding capacity for – potentially large - biotin-to-surface-immobilized target molecules or biotinylated linkers is much lower as compared to the binding capacity for comparatively small free biotin molecules. Depending on the size of the linker or target molecule, respectively, only between 1/3 (2000 pmol) and 1/100 (7 pmol) or less of the nominal binding capacity may be available. Due to large variability in binding capacities, titration of the biotinylated linker and / or the target molecule is required for most applications to optimize the amount of surface-bound targets. Refer to the manual of the streptavidin-coated beads resp. microwell plates to obtain detailed information on binding capacity for the brand of beads resp. plates in use.

References

General

This kit refers to methodologies described in

- Schütze T. et al. (2011) PLoS ONE 6 (12) e29604

General introduction to SELEX

- Pollard J. *et al.* Current Protocols in Molecular Biology (2000) 24.2.1-24.2.24

DiVE – S1 nuclease assay

For monitoring diversity using standard laboratory equipment.

- Lim T. S. et al. (2011) Anal. Biochem. 411: 16–21

DiStRO assay

For monitoring diversity using Real Time PCR equipment.

- Schütze T. et al. (2010) Nucleic Acids Research, 38: 4 e23

FLAA assay

For analysis of binders.

- Wochner A., Glökler J. (2007) BioTechniques 42: 578-582

Modified NTPs

For analysis of binders.

- Burmeister P. E. *et al*. (2005) Chemistry & Biology, 12: 25–33.

Legal Disclaimer:

The XELEX RNA Core Kit is a dedicated kit for simple and reproducible assembly of particle-/ bead-free RT-PCR (eRT-PCR) as well as emulsion PCR (ePCR) assays in emulsion and subsequent DNA purification thereof. In addition to particle-/ bead-free emulsion RT-PCR and PCR, the flexible design of the kit may allow to perform a broad variety of DNA-targeted and other enzymatically catalyzed biochemical reactions within water-in-oil emulsions.

Emulsion-based enzymatic reactions are a broad field of ongoing research, development and innovation. New technologies are developed constantly and some of these are covered by patents. Consequently, some emulsion-based applications, such as bead-based ePCR or eRT-PCR methods or certain *in vitro* expression technologies, represent intellectual property (IP) owned by third parties. Application of such methodologies may require purchase of a separate license from the holder of rights.

Due to the broad field of additional, potential applications, that are not part of this products' primary focus of application, the Micellula DNA Emulsion & Purification Kit does not include any licenses for technologies covered by third parties IP.

XELEX – SELEX RNA Core Kit The XELEX Kit series is a modular kit system covering the entire SELEX work-flow from random DNA library to RNA aptamer: This kit presents an optimized, straightforward standard operating procedure for the whole SELEX process. It consists of a core kit and several add-on packages which add functionality for individual adjustment of the selection process to experimental requirements.

The kit is subdivided into two units, a selection unit and an analysis unit. Whereas the selection unit covers the entire range of the actual SELEX enrichment and aptamer selection procedure from random DNA libraries, the analysis unit deals with all issues related to quality control and initial characterization of both, the enriched libraries as well as of those aptamers identified as good binders.

Many years of experience with SELEX went into development of the streamlined work-flow presented in this manual. Novel approaches help to significantly cut down hands-on time. SELEX is no longer a matter of several months, now it is a matter of weeks. The overall kit design is optimized towards maximum experimenters flexibility. For critical steps within this procedure, outlines for alternate approaches are given. This ensures compatibility of the kit with high end technologies such as semi-automated selections and Next Generation Sequencing, as well as enabling the researcher to conduct SELEX reactions by using no more than standard laboratory equipment.

Depending on the nature of the selection target, the experimenter is free to choose the most gentle and appropriate strategy from different target immobilization methods. Undecided whether DNA or RNA aptamers suit your needs best? No problem, simply perform protocols for DNA and RNA aptamer selections in parallel. Runoff amplification of non-specific but preferentially amplified molecules is efficiently prevented by proven technology of our Micellula emulsion kits. Our rock-solid GeneMatrix technology warrants efficient purification of DNA and RNA aptamers.

Valuable add-ons extend the functionality of the XELEX kit and add specific modifications for fine adjustment of the selection process to experimental requirements. These add-ons include error-prone random mutagenesis amplifications (for introduction of additional diversity during selection cycles), or for Sortase-mediated protein immobilization under physiological conditions (for sensitive target proteins).

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