EURx® MOLECULAR BIOLOGY PRODUCTS

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XELEX DNA Core Kit

eXtended systematic Evolution of Ligands by EXponential enrichment
Modular SELEX Kit – Core Components For DNA aptamer selection and analysis, for binder enrichment and for in vitro evolution.

Cat. no. E3650 / E3651 / E3652

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Table of Contents

Kit – Package Contents

Kit packages and order numbers:

Cat. No.	Kit	Package Content
E3650-01	XELEX Core Kit (Selection + Analysis units), 50 selections + 25 analyses	50 DNA selection preparations + 25 DNA analysis preparations
E3651-01	XELEX Core Kit (Selection Unit), 50 selections	50 DNA selection preparations
E3652-01	XELEX Core Kit (Analysis Unit), 25 analyses	25 DNA analysis preparations
E3600-01	Micellula DNA Emulsion & Purification Kit 50 preparations	Emulsion reagents and DNA purifiaction buffers with columns for ePCR (emultion PCR) for 50 preparations.
E3600-02	Micellula DNA Emulsion & Purification Kit 150 preparations	Emulsion reagents and DNA purifiaction buffers with columns for ePCR (emultion PCR) for 150 preparations.

XELEX DNA Kit Selection Unit

Component	50 preps	Storage temperature
Library "Bank 40"	500 µl / 0,02 nmol/µl	-20 °C
5'-Bank40-Primer	100 µl / 0,1 nmol/µl	-20 °C
3'-Bank40-Primer	100 µl / 0,1 nmol/µl	-20 °C
10 x SELEX Buffer	24 ml	2-8 °C
Buffer SF	1.8 ml	15-25 °C
Emultion Component 1	13.2 ml	15-25 °C
Emultion Component 2	1.2 ml	15-25 °C
Emultion Component 3	3.6 ml	15-25 °C
Orange SFB	15 ml	15-25 °C
Wash SFX	57 ml	15-25 °C
Elution SFX	9 ml	15-25 °C

XELEX DNA Kit Analysis Unit

Component	25 prep	Storage temperature
DivStd-5'-Amp primer	150 µl / 4.5 pmol/µl	-20 °C
DivStd-3'-Amp primer	150 µl / 4.5 pmol/µl	-20 °C
Diversity Standard Bank 40 (0 N)	15 µl / 2.65 pmol/µl	-20 °C
Diversity Standard Bank 40 (4 N)	15 µl / 2.65 pmol/µl	-20 °C
Diversity Standard Bank 40 (8 N)	15 µl / 2.65 pmol/µl	-20 °C
Diversity Standard Bank 40 (12 N)	15 µl / 2.65 pmol/µl	-20 °C
FLAA Positive control	30 µl / 4 pmol/µl	-20 °C
FLAA Negative control	30 µl / 4 pmol/µl	-20 °C
Buffer DX	1.2 ml	15-25 °C
Orange DX	12 ml	15-25 °C
DA Buffer 10x	0.06 ml	2-8 °C
Wash DX1	15 ml	15-25 °C
Wash DX2	19.5 ml	15-25 °C
Elution DX	3 ml	15-25 °C

Xelex DNA Core Kit, Package content differentiators:

XELEX DNA Core Kit – Package Contents	**DNA Spin Column & Buffer Set**	**DNA Selection, Emulsion & DNA Purification Components** • **DNA spin columns,** • **emulsion components** • **purification buffers**	**Library and Library Amplification Primers** • **Bank40 library,** • **PCR primers for library amplification**	**Analysis Components** • **Diversity standards,** • **Diversity standard amplification primers,** • **non-emulsion DNA spin column and buffer set**
Complete Set	Complete sets for both selection & analysis units	Yes	Yes	Yes
Selection Unit	Selection unit only	Yes	Yes	No
Emulsion & DNA Spin Column Set	For use with selection unit only	Yes	No	No
Analysis Unit	Analysis unit only	No	No	Yes

The kit does not contain any enzymes. EURx *Taq* DNA Polymerase (E2500) is the certified enzyme for all PCR amplifications throughout this manual. The use of *Taq* polymerases supplied by third parties is not recommended.

Note: Stability of emulsions is negatively and unpredictably affected by presence of even minor amounts of detergents (e.g. “PCR-enhancers” such as Triton X-100). Such compounds are present in certain DNA polymerase buffers supplied by third parties. Therefore, for emulsion PCR, we strongly recommend relying solely on high quality EURx DNA polymerases (most notably, EURx *Taq* DNA Polymerase, Cat No. E2500). EURx DNA polymerases are highly optimized enzymes, not requiring buffer supplements such as “PCR enhancers” for achieving “optimal” PCR performance. These enzymes ship with 10x buffers that do not contain any detergents or “enhancers” as buffer additives.

Package contents Selection Unit: Required components for protocols listed in section II of this manual.

- SELEX - Binding and Washing Buffer
- Emulsion Components
- DNA Purification Spin Columns for Emulsion PCR Purification
- DNA Purification Buffers for Emulsion PCR Purification
- Random Library Bank 40
- 5' and 3' Amplification Primers (for library and diversity standard amplification)

Package contents Analysis Unit: 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)

Package contents Core Kit (Selection + Analysis Unit):
Complete Kit. All components included within both selection and analysis units are supplied, respectively.

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:

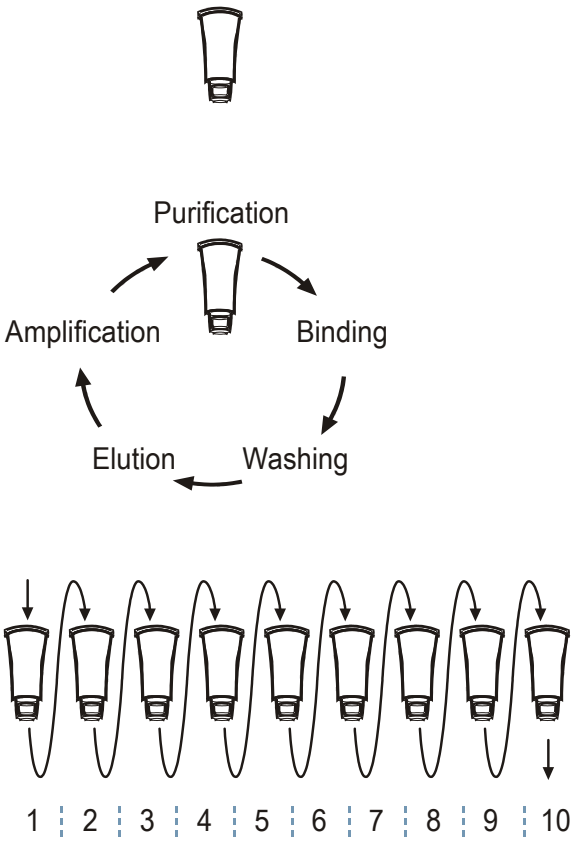
Amplification add-on (choose an appropriate alternative):

- *Taq* DNA polymerase (Cat. No. E2500) or
- Sortase A (Cat. No. E4400)

Not supplied with the kit, provided by user (depending on actual experimental requirements)

- Coated magnetic beads (see page 16 for further information)
- Target-specific, biotinylated conjugate [streptavidin / biotin mediated immobilization only]
- [5 mM] biotin (free, non-coupled) [streptavidin / biotin mediated immobilization only]
- DNA- and nuclease-free H_2O (Cat. No. E0210)
- Depending on target requirements, solutions of [0.5 M] NaCl, KCl, $MgCl_2$, $CaCl_2$ or other salts in DNA- and nuclease-free H_2O for fine adjustment of binding and washing stringency
- 2-butanol (isobutanol) or butanol
- Plastic reaction tubes
- S1 nuclease + 10x buffer (Cat. No. E1335) [DiVE assay only]
- 10x TE and [0.5 M] EDTA (both DNA- and nuclease-free) [DiVE assay only]
- SYBR Green [DiStRO assay only]
- Streptavidin coated microtiter plates [both, polyclonal and clonal FLAA assays]
- DNA binding fluorescent dyes (e.g. Oligreen (ssDNA), Picogreen (dsDNA)) [both FLAA assays]

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. The XELEX DNA core kit assembles a basic set of components, containing all strictly required components for DNA aptamer selections. Thus, overall kit design maintains maximum flexibility and warrants compatibility with all common selection target requirements (e.g. compatibility with different binding-target-to-surface chemistries), as well as with variable experimental design dependent parameters.

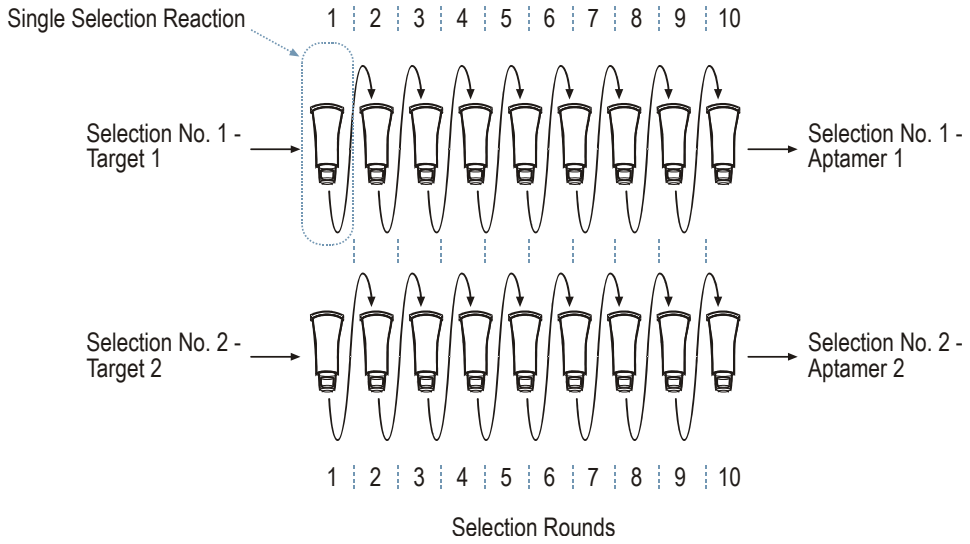


Figure 2: Parallel processing of aptamer selection rounds against different targets. The optional use of semi-automated laboratory equipment enables simultaneous high throughput selections against various targets (see Schütze T. *et al.* (2011) PLoS ONE 6 (12) e29604 for further information).

Kit Description

The XELEX kit is a modular system. It contains a core kit with all required reagents for conducting SELEX reactions. Specific add-on packages provide further functionality to fine-tune selection protocols towards experimental requirements.

The core kit contains oligonucleotides, a combined emulsion and DNA purification system, as well as all required buffers for emulsion creation, selection and DNA purification. Included oligonucleotides are (1) a selection library (40 N), (2) a dedicated diversity standard set, (3) a positive control and (4) amplification primers, respectively. All components are optimized and fine-adjusted for maximum performance and yield. For maximum users flexibility, enzymes are not included, but are available separately as add-on packages. For DNA selections, we recommend either our *Taq* DNA polymerase (Cat. No. E2500).

Additional add-on package is available for sortase-mediated protein immobilization under physiological conditions (for sensitive target proteins) E400-01.

Introduction to SELEX

General Description

SELEX (Systematic Evolution of Ligands by EXponential enrichment) is a selection and *in-vitro* evolution process for aptamer selection and enrichment. Aptamers are single stranded DNA or RNA molecules with sequence-dependent three-dimensional structures. These structures allow distinct molecules to bind to target structures with high specificity and affinity by non-covalent molecular interactions. With respect to certain features aptamers resemble - and, in several applications, are used as a replacement for - antibodies. Additionally, some single stranded nucleic acids with certain sequences exhibit catalytic properties, enabling selections for so-called ribozymes or DNAzymes. Aptamer selection against a broad range of target structures is possible, including proteins, polysaccharides, various large and small organic molecules (drug compounds) and even small metal ions.

Since aptamers are short in length (typically 20 to 60 nt, excluding primer sequences), generation is possible entirely by synthetic procedures, not requiring any cell culture or living animals. The relative small size of the molecules facilitate transfer across cell membranes. Aptamers do not trigger responses of the immune system. Thus, aptamers are extremely well suited as specific binding molecules for pharmaceutical applications, where introduction of antibodies derived from potentially infectious material, such as the blood of laboratory animals, is not desired. Furthermore, large scale synthesis of aptamers for industrial processes is inexpensive and non-laborious. Finally, since SELEX is an *in vitro* process, it is even possible to select for aptamers with binding affinity towards extremely toxic substances.

Reaction Overview

SELEX describes the selective enrichment of ssDNA molecules with high binding affinity to a given target structure from random oligonucleotide libraries. An essential part of the SELEX process is the separation of those oligonucleotides with considerable affinity to the target structure of interest from the vast majority of random oligonucleotides without affinity or with non-specific binding properties. In this process, coined as “partitioning”, the best binding molecules are selected from a total pool of 10^{13} to 10^{15} random oligonucleotides, the maximum number of oligomers to be introduced to the selection process. Since a random library of all possible random 40mers would theoretically contain 4^{40} ($1{,}2 \times 10^{24}$) different sequences, apparently only a small fraction of potential binders is screened per SELEX experiment (see “Calculating Universal Library Size and Weight”, appendix, page 55). To compensate for the necessary initial reduction in diversity, additional variation can be introduced throughout the procedure, e.g. via incorporation of error-prone nucleic acids amplification steps.

Each SELEX process is subdivided into several selection rounds (see pages 6 and 12). The SELEX process is cyclic or iterative in the sense that output of a finished selection round is used as input for the follow-up selection round. Each selection round consists at least of the following reaction steps: Starting from either a random oligonucleotide library (first round) or from re-amplified oligonucleotides derived from a previous selection round (second and all follow-up rounds), ssDNA molecules are bound to the surface-immobilized target of interest. After one to several washing steps for removal of non-specific or weakly binding oligonucleotides from the selection pool, oligonucleotides selected for good binding properties (i.e. binders with a low dissociation constant, K_d) are eluted from the immobilized target. The following amplification step serves three purposes: First, the amount of input nucleic acids required for the next selection round is regenerated. Second, good binders are amplified and enriched in the selection pool. Third, by error-prone amplification, modified amplicons from good binders with potentially improved binding properties are generated and introduced to the selection pool. Amplified oligonucleotides are assayed for diversity and are used as template for the follow-up selection round. Completion of enrichment is detected (1) by decline in overall diversity of the DNA pool and (2) by increase of good binding DNA molecules (“binders”) and hence by increasing elution yield from target-coupled microbeads connected with decline of required PCR amplification cycles. Following completion of all selection rounds, binding properties of enriched oligonucleotide pools are assayed. DNA molecules with good binding properties are identified by DNA sequencing and synthesized oligonucleotides with the determined sequences of good binders are characterized in binding studies (e.g. by surface plasmon resonance). Individual steps for each selection round are explained in more detail in the protocol section below.

SELEX is a complex assembly of methodologies. This kit condenses many man-years of experience with this elaborate technique and helps to circumvent many potential pitfalls when getting started. It can help to reduce but it can not completely abolish all potential error sources. It can do magic, but not miracles. There is no guarantee for successful selection against any particular target structure, even if aptamers to structurally closely related structure molecules are already known. For certain target molecules, good binding aptamers are easily characterized, whereas all attempts to select aptamers for certain other target molecules may fail.

General Protocol

Preparation

1. 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 page 16.
2. Generate a randomized oligonucleotide library (when not using the provided Bank40 library).

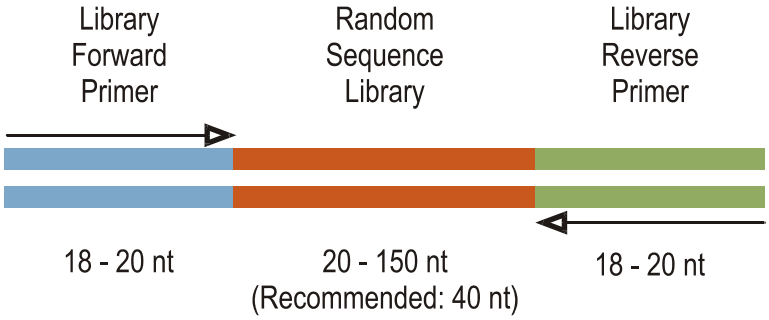


Figure 3: Sketch of a double stranded, random sequence oligonucleotide as starting library for SELEX. A random sequence stretch of 20-150 nt length (most commonly 40 nt) is flanked by two constant primer binding sites required for PCR amplification of target binding DNA between selection rounds. The length of the random sequence stretch depends on the selection purpose. For DNA binding to target structures, 40 bases are recommended. Selection for catalytic molecules such as (deoxy-)ribozymes may require random sequence stretches of 150 bases or longer (Pollard 2000).

First SELEX Round

Binding conditions for the first round are chosen permissive, relaxed, allowing to retain weak binders.

3. **Binding**
   - Start with 400 pmol - 2 nmol (10 – 50 µg) ssDNA 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 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.
4. **Washing**
   - 1 x with 1 ml SELEX Buffer
5. **Elution**
   - One of elution strategies 1 – 4 (see page 20)

6. **Amplification of Binders**
   - PCR with *Taq* DNA polymerase (Cat. No. E2500) (see page 25) or with the Error-prone mutagenesis add-on. NASBA is used for RNA aptamer selections.
7. **Measurement of Efficiency**
   - Determine reaction yield [µg]
   - Optional: Calculate Output-/Input (O/I-) Ratio

Second and All Follow-Up SELEX Rounds

Adjust binding conditions of follow-up rounds stepwise with increasing stringency (see page 17)

8. **(Optional step: Counterselection)**
   This optional, but often required step selects against unspecific binders. Counterselection is highly recommended for selection towards biotin-immobilized targets on streptavidin coated beads.
   - Mix dsDNA (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 amplified dsDNA at 94°C for 3 min, then put immediately on ice for favoring secondary structure formation of ssDNA to dsDNA reannealing.
   - 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: 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 unspecific binders.
9. **Binding**
   - Start with 1 - 3 µg purified dsDNA from step 7 or, following an optional counter-selection step, start with the supernatant from step 8 (in the latter case, dsDNA is already dissolved in [1x] SELEX buffer). Mix dsDNA (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 DNA in the presence of proper ionic concentration prior to denaturation for obtaining correct (re-)folding behavior.
   - Denature dsDNA at 94°C for 3 min, then put immediately on ice for favoring secondary structure formation of ssDNA to dsDNA reannealing.
   - 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: Room temperature for analyses, 37°C *for in-vivo* targets.

10. 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.

11. Elution

- One of elution strategies 1 – 4 (see page 20)

12. Amplification of Binders

- PCR with *Taq* DNA polymerase, error-prone PCR, or NASBA (it is possible to switch between these methods from selection round to selection round)

13. Cycling / Performing Additional Selection Rounds

- Measure efficiency by determining reaction yield and output / input ratio. 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 8 or 9, respectively. Upon completion of ten selection rounds, perform library quality control, followed by identification and characterization of aptamers, as described in section III (page 36). If analyses still point to high library diversity upon completion of selection round 10, it may be necessary to conduct a further five selection rounds for enrichment of good binders. When analyzing libraries by Next Generation Sequencing approaches, as few as three to five selection rounds may suffice for identification of good binders.

Measurement of Efficiency

- (1) Determine reaction yield [µg nucleic acid] from the amplification step (DNA or RNA output yield). (2) Keep record of the required number of PCR 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 (see page 17)
  - *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

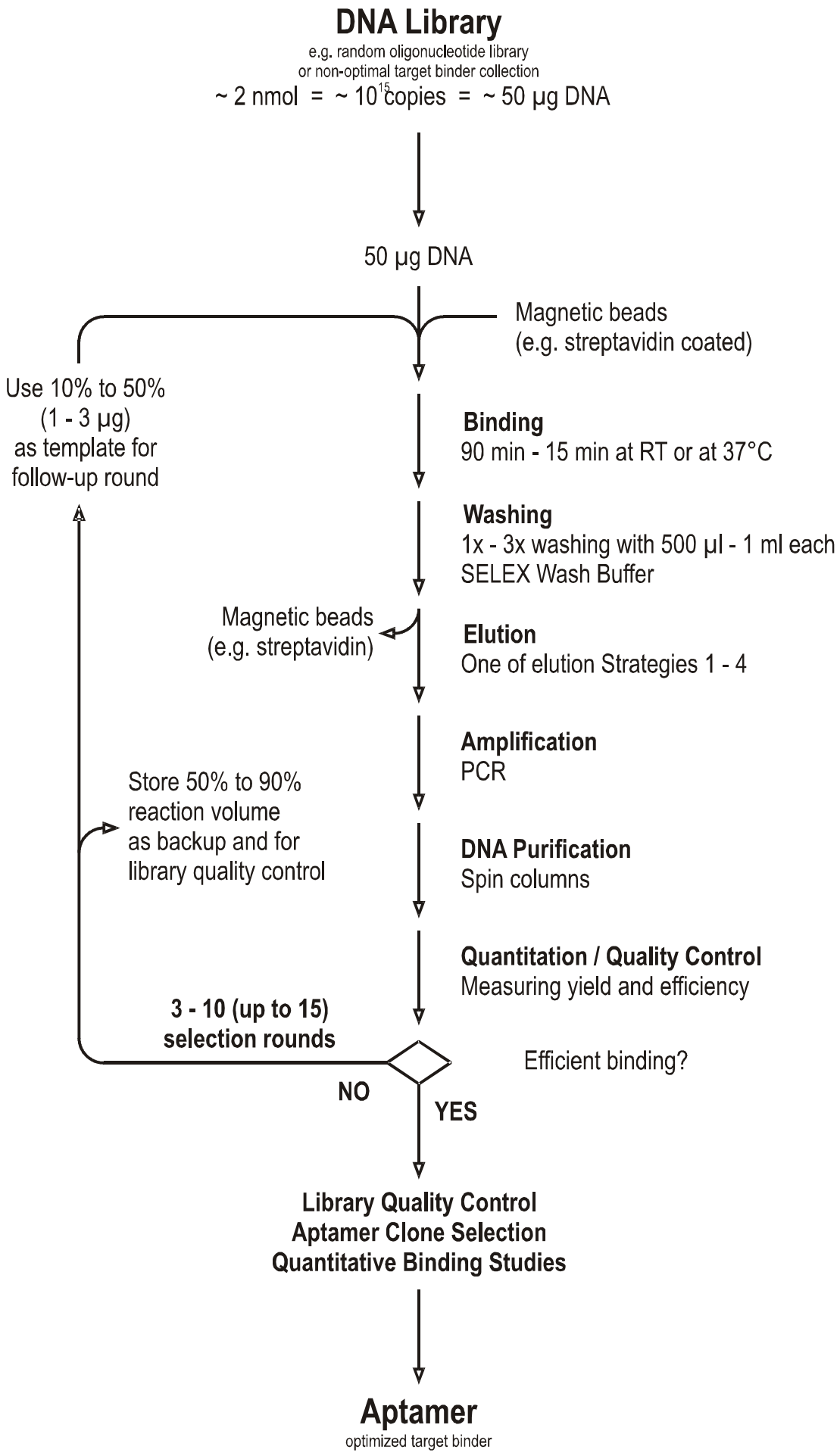


Figure 4: From library to aptamer: Schematic overview of the DNA-SELEX work flow. SELEX is a cyclic process. Each completed cyclic sequence of subsequent reaction steps is referred to as “selection round”.

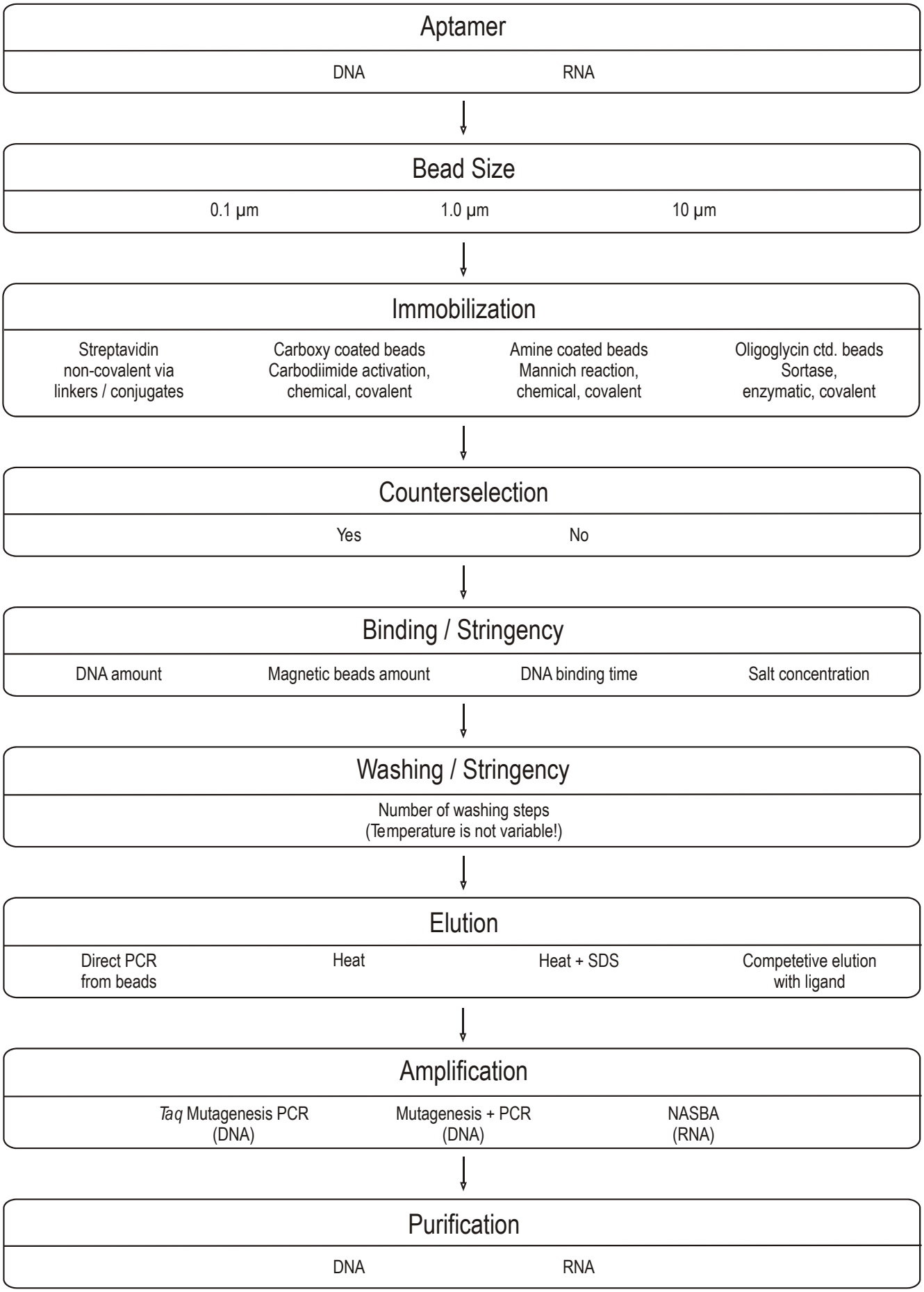


Figure 5: Overview: Alternate possibilities for each reaction step allows fine tuning of the selection process to meet specific experimental requirements. Further details are given in section II (page 15 ff.)

	Input	Reaction Parameters					Output
Round	**Input DNA [µg]**	**ssDNA [pmol] ***	**Beads + Target Structure ** [µg] | [pmol]**	**Binding Incubation Time [min]**	**Washing Steps**	**Number of PCR Cycles**	**Output - Yield [µg] ***
1	50	2140	1000 | 70	90	1x 500 µl	15	7.2
2	3.4	145	500 | 35	60	1x 500 µl	15	2.8
3	1.8	77	500 | 35	45	2x 500 µl	15	1.9
4	0.9	38	500 | 35	45	2x 500 µl	15	0.9
5	0.4	17	500 | 35	45	2x 1000 µl	15	2.3
6	1.1	47	500 | 35	45	2x 1000 µl	14	3.5
7	2.6	111	500 | 35	30	2x 1000 µl	12	4.5
8	3.0	128	500 | 35	30	3x 1000 µl	10	3.6
9	2.2	94	500 | 35	20	3x 1000 µl	9	2.9
10	2.0	85	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 (page 17) 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 DNA 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 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 oligonucleotide Bank40, with an average molar mass (MM) of 23351 g x 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 17). 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 quotient.

Section II: Selection Kit Unit, 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 as low as possible 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 19). 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 unspecific 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 tend to stick to positive charged groups unspecifically.
- *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.

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 SELEX

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

- Amount of ssDNA introduced to the reaction step.
  - *Low stringency:* Large DNA amounts
  - *High stringency:* Low DNA amounts.
  - *Defaults:* 10 µg ssDNA 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 DNA 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 application requirement: 0.5 M NaCl or other salts, such as $MgCl_2$ for Watson-Crick base pairing; K^+ for quadruplex DNA structures; 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 (see table 1, page 14). 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, page 55) are most efficient for generation of a random nucleotide library. It is possible to conduct SELEX using short random oligonucleotides such as 20mers (universal library size: 4^{20} ~ 1.1 x 10^{12} different molecules), but due to steric reasons the variability in binding properties is rather limited as compared to longer random oligonucleotide libraries. Random 80mers (universal library size: 4^{80} ~ 1.1 x 10^{48} different molecules) are rather large oligonucleotides and molecules identified as good binders might, in addition to the targeted affinity, carry additional non-specific or unwanted binding properties. Additionally, a universal library of random 80mers is too large for a complete screening of the entire sequence space (see appendix, page 55). Furthermore, oligonucleotide pools with random sequence stretches exceeding 90 bases in length tend to form self-aggregates, resulting in precipitation upon prolonged incubation (Pollard 2000). However, for certain applications, such as for selection of nucleic acids with catalytic properties, extremely long random oligonucleotides of 150 bases and longer are required (Pollard 2000).

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

Bank-40
5'-TGA CAC CGT ACC TGC TCT – N40 - AAG CAC GCC AGG GAC TAT - 3'
76 nt; average molar mass = 23351 g mol^{-1}, 1 µg oligonucleotide = 42.8 pmol

5' – Bank40-Primer (for PCR amplification of Bank40)
5'-TGA CAC CGT ACC TGC TCT - 3'
18 nt; molar mass = 5473 g mol^{-1}, 1 µg oligonucleotide = 182.7 pmol

3' – Bank40-Primer (for PCR amplification of Bank40)
5'-ATA GTC CCT GGC GTG CTT -3'
18 nt; molar mass = 5544 g mol^{-1}, 1 µg oligonucleotide = 180.4 pmol

Positive Control for Streptavidin Selection (Good Streptavidin Binder, 40-mer; K_d ~ 140 nM)
5'- ATC TCC GAT TGC CCC ACG **ACG C**AG TGG T**CG** G**A**G TTA CTT T -3'
40 nt; molar mass = 11595 g mol^{-1}, 1 µg oligonucleotide = 86.2 pmol

Negative Control for Streptavidin Selection (40-mer; no specific affinity towards streptavidin)
5'- TGA CAC CGT ACC TGC TCT ATG CCT ATC ATG GGC AAC CAC G -3'
40 nt; molar mass = 11533 g mol^{-1}, 1 µg oligonucleotide = 86.7 pmol

The palindromic sequence **ACGC**NNNNNN**CGCA** is a common structure motif for good streptavidin binders. Note that the aptamer does not contain any primer binding sequences.

Optional Selection Step: Counter-Selection

This step selects against unspecific binders. During this optional step, unspecific 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 selecting for 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 10.

Binding

In a typical binding step, 1 - 3 µg of purified ssDNA 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 late selection rounds. The molar relation of ssDNA 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 10.

During the early selection rounds, ssDNA is supplied in large excess and thus high competition of ssDNA 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 DNA binding (but not the binding temperature) is one means for regulating stringency of the SELEX process (see page 17).
Choose an appropriate binding temperature according to the requirements of the targeted reaction environment for the optimal binder.

- 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. 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 17).

Elution

Four general strategies are available for elution of good DNA 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, DNA removal must proceed quickly for preventing 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 – 94°C for complete denaturation (add 2 mM EDTA, only if DNA 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 DNA) for subsequent direct PCR amplification from eluate. Alternately, precipitate DNA or add 400 µl of orange-colored Orange-SFX buffer and purify on DNA spin columns (Short DNA Purification Kit, Cat. No. E3515).

Note 1: DNA Purity Considerations. Spin column purification of DNA gives optimal results but is usually not required. Direct 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 – 94°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 DNA or add 400 µl of orange-colored Orange-SFX buffer and purify on DNA spin columns (Short DNA Purification Kit, Cat. No. E3515 (<100 nt length) or PCR / DNA Clean Up Kit, Cat. No. E3520 (>100 nt length)).

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.

4) Direct PCR from DNA bound to beads (not compatible with emulsion PCR, not recommended!) Introduce beads directly into PCR reactions. Only for 1 µM beads, 10 µM beads are too large.

Dissolve DNA in a final elution volume of 50 - 100 µl in water or in dilute buffers such as 1x TE.

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).

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

Library (Re-)Amplification – Alternate Methods

Throughout the binding, washing and elution steps, the total number of nucleic acid molecules present in the library decreases continuously. Therefore, an amplification step is required for regenerating the necessary amount of nucleic acid molecules for the next selection round. Since poor binders were selectively removed throughout the previous selection steps, re-amplification of the library will regenerate the required number of nucleic acid molecules, albeit in different composition: Good binders are selectively enriched, as compared to the library composition of the previous round.

The simplest method for library reamplification is to perform PCR using *Taq* DNA polymerase as amplifying enzyme. This method is described below. Amplification with *Taq* DNA polymerase followed by selection leads to enrichment of best binders that were initially contained in the starting library aliquot.

It is also possible to perform mutagenesis. Mutagenesis PCR introduces random errors to the amplified library. These amplification errors aid in the *in vitro* evolution process by permanently introducing additional diversity during the selection rounds: A tiny fraction of good binders may experience further improvement in their binding properties upon introduction of beneficial point mutations, thus increasing their competitiveness and gaining further advantage in the enrichment process. In consequence, this may lead to selection of best binders, which were not initially contained in the starting library aliquot.

Alternatively, NASBA is an RNA aptamer selection method. Since the amplification step completes each selection round, it is possible at this step to switch from DNA to RNA selection or *vice versa* between any selection round. 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 of RNA may interact more tightly with target structures as compared to DNA aptamers.

Both latter mentioned methods introduce – by intention - amplification errors more efficiently as compared to *Taq* mediated PCR. Mutagenesis PCR and NASBA continue to evolve enriched binders throughout the selection process and can lead to enrichment and selection of molecules that were not initially present in the library aliquot. In contrast, SELEX with *Taq* DNA polymerase-mediated PCR selects the best binders present in the initial pool.

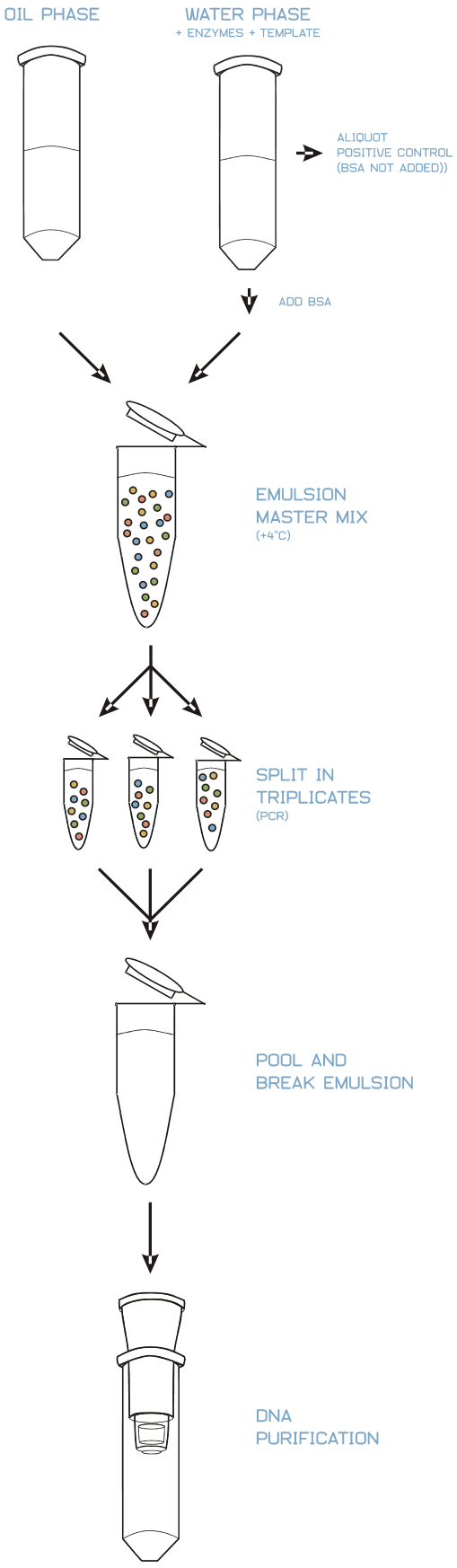


Figure 6: General work flow for emulsion PCR (ePCR). Sample volume of the water phase is 50 µl. Total reaction volume increases due to addition of the oil phase. Thus, for thermocontrolled reaction conditions (e.g. ePCR), the total reaction volume is split into triplicates.

Emulsion 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 PCR (ePCR) 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 PCR assays, ePCR prevents undesired run-off amplification of molecules with poor binding properties but with high amplification efficiency, voids diversity shifts and reduces amplification bias efficiently. Furthermore, ePCR eliminates the need for any size-dependent fractionation of PCR amplicons by gel separation, subsequent gel excision and DNA isolation steps, as required with previous SELEX protocols.

In contrast to other ePCR approaches, template DNA concentration is intentionally chosen rather high in these assays. There are two reasons for confining much more than one template molecule (typically 10^4 to 10^5) within each micelle: First, the focus of ePCR in SELEX processes is not to prevent amplification errors, but to efficiently prevent preferential amplification of a single molecule species at the expense of overall diversity. Second, it is desirable to introduce and maintain an as large as possible diversity throughout the selection process.

Contrary to non-emulsified, “open” PCR assays, ePCR allows to perform the maximum numbers of PCR cycles while still maintaining high library diversity and preventing runoff amplification of non-optimal binders. End-point amplification warrants maximum PCR yield while still retaining maximum library diversity. During the ePCR amplification step the amount of nucleic acids required for the follow-up selection round has to be regenerated. This requires synthesis of at least the required amount of 0.5 µg template DNA as starting material for the next round plus an identical amount for backup and quality control. In total, 1 – 3 µg of DNA are required. Regularly five to 20 PCR cycles are conducted. As opposed to non-emulsified, “open” PCR assays, it is not necessary to keep the total number of PCR cycles as low as possible. Simply conduct the maximum number 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. Consequently, it is not necessary to fine-tune and minimize the number of PCR cycles. Keep in mind, that the amount of chemicals (e.g. dNTPs) per micelle is limited and usually depletes after 15 cycles. If only few product is generated following the maximum number of 15-20 PCR cycles, conduct a scale up ePCR (see page 23, step 3).

If nonetheless further adjustment of PCR cycles is desired, here 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 closer the finalization of the selection process. If a normal, non-emulsified control ("open") PCR is performed in parallel, check an aliquot of an intermediate amplification step by agarose gel electrophoresis. If no or little product is visible, conduct additional PCR steps (for details, see page 27, 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 PCR Protocol:

1. Assemble PCR reactions in a total volume of 50 µl as outlined below (page 25).

2. From 50 µl elution volume (see page 20), 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 26). Adjust cycle count adequately to amplify 0.5 to 3 µg of dsDNA 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 increase 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 27), (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 $2^{20} = 1048576 \approx 10^6 = 1$ 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 unspecific enrichment. If more than 30-40 cycles are required for reamplification of 1-2 µg library DNA, discard the assay, check all previous selection steps as well as all 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 28).

5. Quality control: Verify the proper size of the PCR product by agarose gel electrophoresis. Quantify PCR yield by spectrophotometric measurement (see page 29).
   - 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 14.

Emulsion PCR (*Taq* DNA Polymerase Mediated) – Detailed Protocol

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 out of 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 to assemble the Oil Surfactant Mixture at room temperature, followed by chilling 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, dependent 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. Per 50 µl water phase, mix:

10 x PCR Buffer (with or without $MgCl_2$)	1 x	5 µl
$MgCl_2$ [25 mM], if added separately	1.5 mM (or 1 - 5 mM)	3 µl (or 2-10 µl)
BSA, acetylated [1 mg/ml] (see Note 1)	0.01 mg/ml	0.5 µl
Bank40-5'- Primer [100 µM]	4 µM (or 0.2 - 2 µM)	2 µl
Bank40-3'- Primer [100 µM]	4 µM (or 0.2 - 2 µM)	2 µl
dNTP mix [5 mM]	400 µM	4 µl
Thermostable DNA polymerase [5U/µl]	1.25 U (or 1.25 - 2.5 U)	0.25-0.5 µl
Template DNA (or use DNA bound to magnetic beads as template)	10 pmol (0.5 µg, up to ~10^{13} copies)	
Sterile, DNA-free H_2O		@50 µl

Note 1: BSA Concentration. In most reactions, 0.01 mg/ml EURx BSA (Cat. No. E4020) is the optimum amount for DNA polymerases manufactured by EURx. However, in some reactions, other concentrations of acetylated BSA might be required for a proper coating of the hydrophilic / hydrophobic interface at the micelle border. In case specific experimental requirements request optimization of BSA concentration, test the following BSA concentrations: 0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 mg/ml, respectively. Caution: Excess BSA is an effective inhibitor of PCR reactions. As a consequence, add neither too much nor too less BSA to the reaction.

Note 2: Non-Emulsified Control PCR. It is strongly recommended to set up an non-emulsified ("open") PCR control reaction in parallel to the emulsified PCR assay .

Note 3: Precautions for Control PCR. When running an "open" (non-emulsified) control reaction (recommended), proceed as follows:

(1) Prepare a 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 4: ePCR Template DNA Amount. For oligonucleotide Bank40 (76 nt) 0.5 µg DNA 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 DNA helps to ensure that an as large as possible diversity of molecules is introduced as basis for selection reactions. For comparison: ~0.4 pg oligonucleotide Bank40 would correspond to 10^9 copies.

Note 5: Use Solely EURx DNA Polymerases. PCR buffers from third parties may contain nonionic surfactants such as Triton X-100, which are known to severely affect composition and stability of the emulsion even at low concentrations. We strongly recommend using EURx *Taq* DNA polymerase (E2500), which performs extremely well in this step and is fully buffer-compatible to the described emulsion PCR technique.

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 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 ePCR yield (e.g. when starting from small template DNA amounts), conduct a scale-up ePCR: (1) Break the emulsion as outlined below (page 27), (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. Do not conduct more than a total of 30 (=2 x 15) PCR cycles.

PCR Cycling Parameters

For the first selection round, it is not necessary to amplify the oligonucleotide library prior to selection. Addition of 10 - 50 µg unamplified ssDNA oligonucleotides introduces the maximum possible diversity into the selection process. For all follow up-rounds, conduct the maximum number of 15 – 20 amplification cycles per selection round, depending on reaction yield (and, optionally, on the O/I ratio, see page 31) measured from the previous selection step. 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 28) and use purified DNA as template for setting up a second, “scale-up” emulsion PCR reaction (conduct the full amount of 20 cycles).

	Initial Denaturation	95°C	120 sec
20 cycles			
	Denaturation	95°C	30 sec
	Annealing	55°C	60 sec
	Extension	72°C	180 sec
	Final Extension	72°C	300 sec

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. 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 proceeds not specifically against the target but proceeds unspecific 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, the entire PCR water phase contributes 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 (compare also to page 57). 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 to 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 10 volumes (500 µl) of orange-colored **Orange-SFB** 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 removed during the follow-up spin column purification.

Note 1: Colored Organic Phase. Phases separate into a water phase containing DNA and a organic phase. The yellow color supplied with buffer Orange-SFX 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: Quick Gel Check. An aliquot of the water phase can 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.

DNA Purification

(Alternate protocols replacing this step: NASBA / RNA Purification (future release add-on))

7. Apply 40 µl of activation **Buffer SF** 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.
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, stick 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 600 µl of **Wash-SFX** buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
13. Remove spin column, discard flow-through, stick spin-column back on top of the tube.
14. Add 350 µl of **Wash-SFX** buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
15. Remove spin column, discard flow-through, stick spin-column back on top of the tube.
16. Spin down at 12,000 rpm for 2 minutes to remove traces of **Wash-SFX** buffer.
17. Place spin-column into new receiver tube (1.5-2 ml). Add 50-100 µl of **Elution-SFX** 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-SFX Buffer. For elution of DNA the Elution-SFX buffer is highly recommended. The buffer is prepared using ultra-pure water with trace addition of buffering compounds. Elution-SFX 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.

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.

Following elution, check concentration of amplified DNA. 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 requesting the experimenters to develop experience, feeling 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 conducted 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 exist neither any general guidelines nor any 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. Dependent on nature and requirements of the target structure, experimental design needs careful adjustment to current progress of the reaction. The success of each selection round has to be monitored, protocolled and carefully compared in the light of the results from previous rounds. For an example see table 1, page 14.

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 of this protocol (page 36 ff.). 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 14 and page 29). In emulsion PCR, amplification proceeds until complete depletion of reaction components confined within micelles, regardless of the number of template molecules initially present. Since emulsion PCR minimizes the impact of diversity shifts, there is no need to vary the number of PCR cycles between selection rounds. ePCR is simply conducted with maximum cycle numbers. Under these conditions, amplification in template-containing micelles will always continue until resource depletion. If a micelle hosts at least one template molecule prior to amplification, it will completely fill up with amplicons during ePCR. 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 conducted following each selection round

- **Carefully protocol selection and amplification parameters** as outlined in table 1 (page 14). Keep note, compare and set these values in relation to the corresponding values obtained during earlier selection rounds.
- **Agarose gel check:** After reamplification, the size of amplification product is verified on a 3 % [w/v] agarose gel. In case of aberrant band size, discard the amplified PCR product and restart selection from a 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 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 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) The rough but fast 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 conducted 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 section III, page 36 ff. 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 below (section III, page 38 ff.).

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, in case any follow up selection round does 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 1x TE or phosphate buffers (for amine sensitive assays) rather than pure water to prevent autohydrolysis of DNA.

Controls

One important point regarding controls: Keep in mind, that SELEX is a complex protocol. Furthermore, selection success is not guaranteed, even if aptamers to chemically similar targets or to molecules with related structure were already characterized: For some target molecules it is quite easy to find good binding aptamers with low dissociation constants (K_d). For some target molecules selection proves increasingly difficult and for some 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. Where possible, the manual includes hints, 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 selecion 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.

Thus a warning: Always be critical with data interpretation. It happens quite sometimes that false conclusions are drawn from experimental data and non-target-specific enrichment is erroneously taken for good selection progress.

An important point is to consider other target specific controls, which need to 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. There hide many potential sources of errors, which may require application of suitable controls. And, for your specific selection strategy, there is no one but you who can carefully think of all potential pitfalls and come up with ideas for all suitable control reactions.

Getting Started: Streptavidin Binder as Positive Control

To enable new users getting familiar with the SELEX protocol, we recommend strongly to conduct a test selection with a known-to-work positive control. Performing a test streptavidin selection as a first training helps to get familiar with the technique, to gain first experience in the application and to learn how to interpret the results obtained from measurements following each selection round. A good experiment for getting started with SELEX is to reproduce a classic, known-to-work selection example, the selection for streptavidin binding aptamers. Perform this selection either as stand-alone experiment, or in parallel to other selections. Streptavidin is a good target for verifying, that all experimental procedures are established and work correctly, leading to a successful enrichment of aptamers against the selection target. The main reasons to choose streptavidin binder selection as a positive control are: (1) A common structure motif for good binders, the palindromic sequence ACGCNNNNNNCGCA has been independently determined and confirmed by various research groups. (2) Since streptavidin-coated beads and microwell plates are commercially available, misconducted target immobilization to surfaces is ruled out as error source, thus considerably simplifying the troubleshooting process.

> **Note 1: Always Aim At Good Working Practice.** When working with streptavidin binders, it is mandatory to ensure good, clean working practice. Carry-over contamination of good binders may lead to enrichment of binders in selection processes for other biotin-streptavidin immobilized targets.

The kit includes an optimized aptamer for streptavidin binding as positive control. By measuring the diversity of enrichments with the assays described below, it is possible to determine the relative performance (percentage binding efficiency) of aptamers obtained during the selection process relative to the positive control and thus to check and verify increase of affinity between selected aptamers and selection target throughout the selection process.

Use the following SELEX parameters for a first test streptavidin aptamer selection:

- Target immobilization: None (use streptavidin coated microbeads - this completely eliminates target immobilization as potential source of error);
- Binding and washing parameters: see table 1 (page 14) for orientation (choose values dependent on measured DNA output yield per selection round, respectively);
- Elution method # 1 (perform PCR directly on magnetic beads without any elution step);

- PCR amplification (using *Taq* DNA polymerase) and DNA purification as described in Section II of this manual. Perform ten selection rounds.
- Analyze library and aptamers as outlined in section III (page 36).

Compare the results for your test streptavidin binding assays to assay results obtained with the positive control, the known-to-work streptavidin binder. The control streptavidin aptamer selection is performed either as a separate selection or in parallel to a selection for the target of interest. Progress in binding efficiency of the streptavidin binder is monitored and expressed as relative (percentage) value to the results from binding assays obtained with the good binder. See Section III (Analysis Unit), page 36 ff., for further information. Once you have conducted the first successful binder selection, you have acquired the skills to master further challenges of the selection technique.

The following positive controls are recommended to verify good selection performance:

- Monitor increase of relative target binding efficiency between selection rounds. Use the positive control as reference. Upon finishing the selection, characterize ten enriched binder clones and compare their sequence to the sequence of the positive control and to the common palindromic sequence motif for streptavidin binders, ACGCNNNNNNCGCA.
- Besides measurement of selections, we recommend to spike selections with several dilutions of the good binding aptamer before conducting analyses. Perform a library dilution series (e.g. 1:100, 1:1000 etc …). Per dilution step, prepare the following assays: (A) Library + 1/100 of optimal binder, (B) Only 1/100 optimal binder. Monitor the binding efficiency of diluted libraries. The spiked solutions should reflect the increase resp. decrease of added optimal binder concentration. These controls answer the following questions: Which percentage of optimal binders are detected? Are chemicals (buffers) and selection targets (immobilized streptavidin) working as expected? How specific is the recovery of the library? What percentage of the library is recoverable?

Table 2 (page 35) assembles other suitable positive controls for different target structure classes.

Target Structure Class	Target Molecule	Aptamer: Nucleic Acid Type	Dissociation Constant (K_d)	Reference
Alkaloids	Cocaine	DNA	0,4 – 10 µM; 20 µM	Stojanovic M.N. *et al.* (2001) J Am Chem Soc. 123, 4928-4931
	Theophyllin	RNA	0,3 µM	Jenison R.D. *et al.* (1994) Science 263: 1425-1429
Nucleotides	ATP	DNA	12 µM	Huizenga D.E. and Szostak J.W. (1995) Biochemistry 34: 656-665
	Flavin mono-nucleotide (FMN)	RNA	500 nM	Fan P. *et al.* (1996) J mol Biol. 258 (3) 480-500
Peptides	Rev-Peptid	RNA	20 nM	Xu W. and Ellington A.D. (1996) Proc. Natl. Acad. Sci. USA 93: 7475-7480
Proteins	L-Selectin	DNA	66 ± 34 pM (37°C)	Watson S.R. *et al.* (2000) Antisense Nucleic Acid Drug Dev. 10: 63-75 (2000)
	Thrombin	DNA	25 nM	Bock, L.C. *et. al.* (1992) Nature 355: 564-566
	Immunglobulin E (IgE)	DNA	10 – 35 nM	Wiegand T.W. (1996) J Immunol 157: 221-230.
	Streptavidin	DNA	50 – 105 nM	Wang C. et al. (2009) Acta Biochim Biophys Sin 335–340
Antibiotics	Tobramycin	RNA	3 µm	Wang Y., Rando R.R. (1995) Chem Biol, 2: 281-290
	Tetracyclin	RNA	1 µM	Berens C. (2001) Bioorg Med Chem 9: 2549–2556
	Daunomycin	DNA	20 nM	Wochner A. et al (2008) Anal. Biochem. 373 (1): 34-42
Dyes	Malachite Green	RNA	1 µM	Grate D. and Wilson (1999) Proc Natl Acad Sci U s A. 11: 6131–6136
	Fluorophore HBI (similar GFP)	RNA	464 nM	Paige, JS. *et al.* (2011) Science 333 (6042), 642-646
Virii	Vaccinia-Virus	DNA		Nitsche A. *et al.* (2007) BMC Biotechnol. 7: 48
Cells	*Trypanosoma cruzi*	RNA	40 – 400 nM	Ulrich H. *et al.* (2002) J Biol Chem. 277 (23), 20756–20762

Table 2: Positive controls for selected target molecule classes. Aptamer sequences are not included in this table, since successful experimental application requires further specific information contained in each respectively cited publication. Good binding aptamers are characterized by low dissociation constants, K_d, indicating that only low target molecule concentrations are required for occupying half of the aptamer binding sites.

Section III: Analysis Kit Unit, Identification and Characterization of Aptamers

Once the selection process has completed, nucleotide sequences of good binding aptamers (i.e. binders with a low dissociation constant, K_d) are determined. Here we describe an outline for a recommended experimental work-flow: Following completion of all three to ten selection rounds, aliquots of amplificate backups from each selection round are assayed for decline in diversity (one of the alternate methods described below, DiVE or DiStRO assay) and for the corresponding enrichment of good target binding sequences by measurement of overall library binding efficiency using the FLAA assay (example protocols are provided below). The DiVE, DiStRO and FLAA assays, respectively, serve as quality control for the successful enrichment of binders by measuring diversity reduction of library enrichments. Relative frequencies of the most abundant sequences are in turn determined by random analysis of cloned DNA or by "Next Generation Sequencing". Since the SELEX process selects for the enrichment of molecules with high affinities to the target structure of interest, it is likely to identify good target binding aptamers among the enriched, most abundant DNA sequences (provided a careful evaluation of all included controls has been taken into account). A first selection of 10 to 50 candidate aptamer sequences (“clones”) is screened by FLAA to narrow the range of selection to a few (three to five) promising candidates for further qualitative binder analysis, e.g. by surface plasmon resonance (“Biacore”) characterization. Once the nucleotide sequence of a good binder is determined, the aptamer will be entirely accessible via oligonucleotide synthesis for any future application.

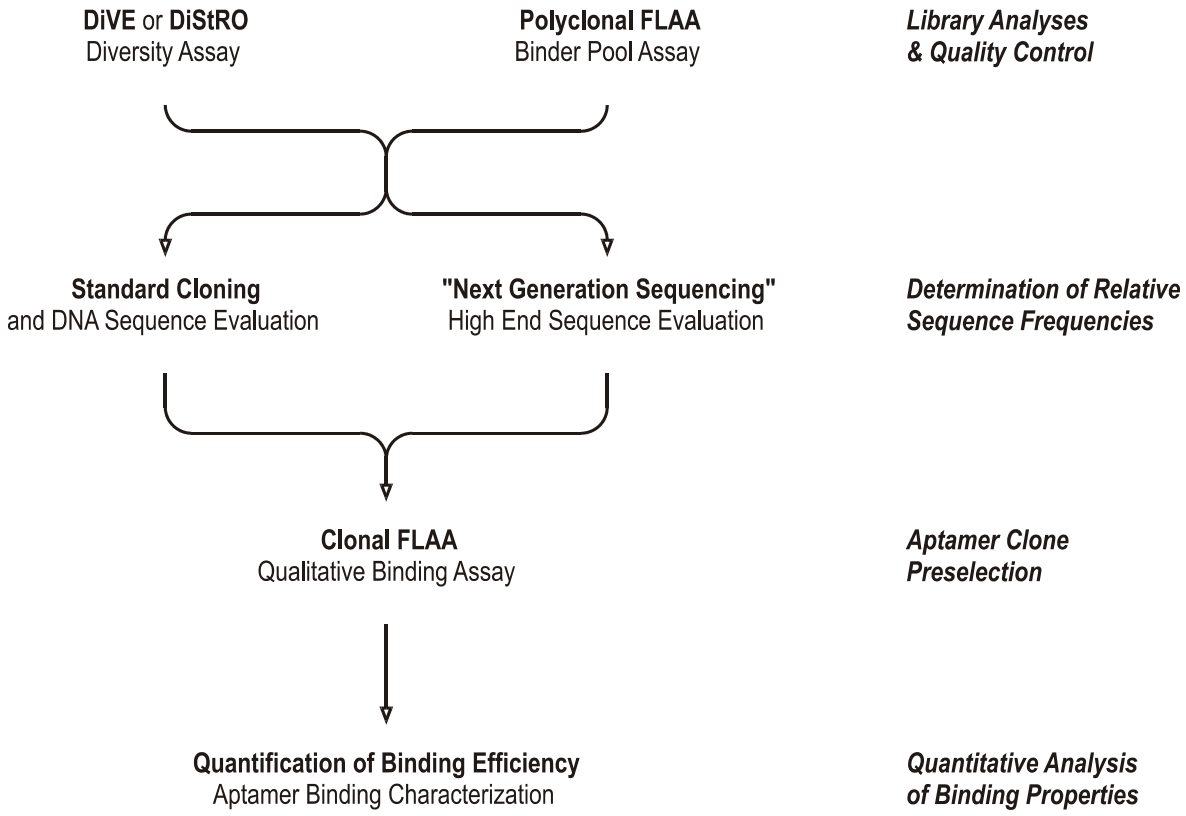


Figure 7: General workflow for monitoring binder enrichment in random libraries, followed by identification and characterization of good binding aptamers. DiVE and DiStRO are alternative assays for estimating library diversity. The FLAA assay gives additional information by monitoring enrichment of binding sequences. Molecular cloning of aptamers and Next Generation Sequencing are alternate methods for determining the relative frequency of enriched aptamer sequences (“clones”) and finally for nucleotide sequences of good binders..

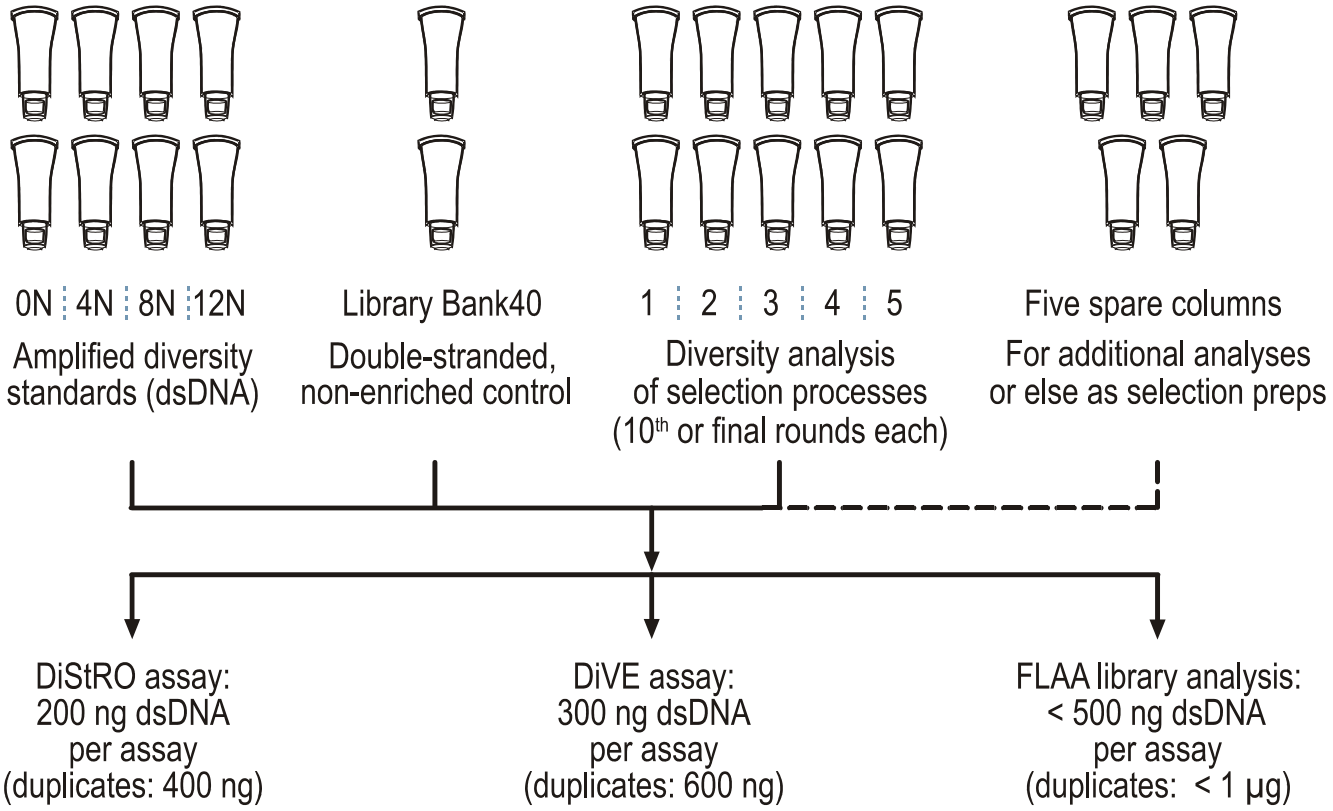


Figure 8: Experimental design of the XELEX analysis kit unit. Among other contents, the analysis unit contains 25 DNA purification preparations. Generally, all assays are conducted at least in duplicate analyses.

Diversity standards:

4 x 2= 8 DNA purification columns are required for initial diversity standard amplification (ssDNA to dsDNA) and subsequent DNA purification. Only 400 – 600 ng of diversity standard are required as a reference per analysis round. dsDNA purified in excess can be stored at -20°C until further usage. The DNA retained from each selection processes final selection round (in many cases, from the 10th selection round) is analyzed by either the DiVE or the DiStRO assay, as well as by the FLAA library analysis (all procedures described below).

Assessment of selection diversity:

For five selections with 10 rounds each, the DNA from the 10th round only is analyzed in duplicate and thus 5 x 2 = 10 DNA spin columns are required for sample measurement.

Non-enriched library Bank40 (PCR amplified for generating double-stranded DNA) serves as non-enriched, high-diversity control for comparison with the diminished diversity of target affinity enriched library samples.

Five DNA spin columns are spare and can be used for analysis of certain intermediate (non-final), interesting selection rounds or else for increasing the number of analyzed replicates.

Three Dimensions of Diversity

Diversity is not a single parameter value. Rather several factors contribute to measured diversity values. Three aspects of diversity are important for library analysis and quality control:

- *Molecular species richness / total number of different molecules:* Assume two libraries, A and B. Library A would consist of 20 molecular species in equimolar amounts, whereas library B would only consist of 11 clones in equimolar distribution of each sequence type. Then library B is considered as less diverse than library A. For most observers, this corresponds to the intuitive understanding of the term diversity. There are two additional important aspects:
- *Relative abundance of clones:* Assume two libraries with the same total number of 11 different molecular species. Library A would contain roughly equal amounts of each species, approximately 9 % per clone. In contrast, library B would consist of as much as 90 % of clone # 1 and only 1 % each of the remaining 10 molecular species, respectively. Then library B would be considered as less diverse than library A.
- *Sequence similarity / differences in sequence distances:* Assume two libraries, A and B, both containing 11 molecular species with different sequences in equimolar amounts. If all 11 sequences in library A would have completely unrelated sequences, not sharing any similarity, whereas library B would consist of 11 closely related and only slightly different sequences, i.e. of variations on a common theme, then library B would appear as less diverse.

It is important to keep in mind, that all three dimensions of diversity contribute to the measurement of library diversity outlined below, albeit to varying extent.

Library Analyses and Quality Control: Measuring Library Diversity

Reaction progress is monitored by measuring the diversity of the oligonucleotide bank. While the selection reaction proceeds, good binders are enriched, and the overall diversity of the oligonucleotide bank library declines accordingly. The decline in diversity and the enrichment of good binders is measured by two alternate approaches:

- *DiVE - S1 nuclease assay (Lim et al. 2011).* This enzymatic assay does not require any sophisticated and expensive equipment and is outlined below. Results require careful evaluation by the experimentator.
- *DiStRO assay (Schütze et al. 2010).* This non-enzymatic assay is fast and straightforward, but requires Real-Time-PCR equipment. Comparing the assay melting curves with melting curves of a set of diversity standards is often considered easier and less interpretation-prone as compared to the DiVE assay. Please note that this assay is not applicable for reactions with only few variable sequence positions (e.g. mutation “hot spots” in conserved sequence stretches).

Both the DiVE and the DiStRO assay make use of the same diversity standard set. Both assays are alternate approaches for measuring diversity. It is entirely sufficient to conduct only one of both assays. If Real Time PCR equipment is available, the DiStRO assay is more convenient and straightforward. Else, the DiVE assay gives similar information by demanding no more than standard laboratory equipment.

Diversity Standard

The diversity standard is a set of double stranded oligonucleotides with varying numbers of random nucleotide positions. Diversity of the oligonucleotide pool correlates exponentially with the number of random sequence positions *(Schütze et al. 2010)*.

- The sample with lowest diversity is a set of two perfectly complementary oligonucleotide strands. For best calibration fidelity, this standard sequence should match the properties of the selection target as close as possible, with respect to length and to conserved sequence stretches such as primer binding sites.
- Introduction of one single randomized position into the standard oligonucleotide sequence leads to formation of four different sequences within the oligonucleotide pool.
- Introduction of further randomized positions increase diversity by a factor 4 per random nucleotide positions.
- Diversity of the oligonucleotide pool thus increases exponentially with each added random position by a factor of $D = 4^N$, where N is the number of randomized sequence positions. One randomized position (1N) results in 4 different sequences, two random positions (2N) in 16, three random positions (3N) in 64, four random positions (4N) in 256 different sequences, etc…
- Diversitiy standards larger than 10N are not required for SELEX. In case, library diversity exceeds 10N, further selection and enrichment is recommended.
- Diversity is measured either by the DiVE assay *(Lim et al. 2011)* or by the DiStRO assay *(Schütze et al. 2010).* Protocols for both assays are provided below.

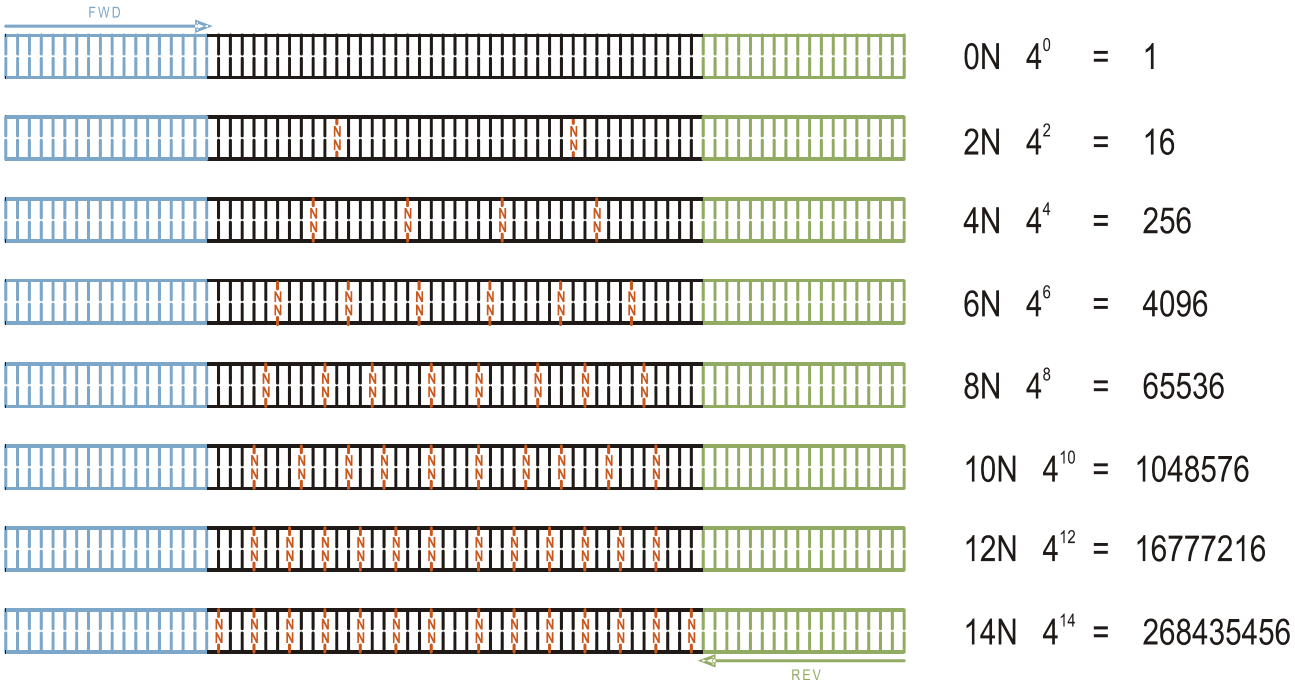


Figure 9: Diversity standard. N denotes the number of randomized sequence positions. Per each additional inserted random sequence position, diversity of the sample increases by a factor of 4. Diversity of the sample pool correlates exponentially with the total number of random sequence positions (Schütze et al. 2010).

Diversity standards are provided as single stranded oligonucleotide DNA. For DiVE (S1-nuclease) and DiStRO (Real Time) assays, respectively, ssDNA requires conversion to dsDNA.

To obtain dsDNA from ss oligo-DNA for each standard, set up separate, non-error prone (!) PCR reactions. In practice, plain *Taq* DNA polymerase (Cat. No. E2500) or proofreading Opti*Taq* DNA polymerase (Cat. No. E2600) amplify with sufficient precision. There is no extra requirement for any dedicated fancy and expensive proofreading PCR enzymes. Amplification of 50 pmol to 2 nmol of each standard in two to six PCR cycles yields ample amounts of DNA for analyses. To avoid introduction of any potential amplification bias to the standard, the amount of template DNA should be chosen as high as possible and the number of PCR cycles should be kept as low as possible. Best results are obtained with two to four PCR cycles. The recommended value is four PCR cycles, the maximum cycle number is five to six. Do not conduct more than six cycles to avoid unpredictable results due to possible amplification bias.

The following non-emulsified, “open” PCR procedure generates ample DNA for analyses:

Material list (components not provided, except where indicated):

- Diversity standard set: 0N, 4N, 8N, 12N (ssDNA, provided with the kit in Bank40 compatible format; change accordingly if libraries with different structural features or lengths are used)
- Diversity standard forward and reverse amplification primers (provided with the kit for amplification of Bank40-compatible diversity standards; change accordingly if libraries in different format are used)
- XELEX DNA spin columns (dsDNA < 100 bp; provided with the kit) or alternately EURx Short DNA Kit (dsDNA < 100 bp; Cat. No. E3515)
- *Taq* DNA polymerase + 10x Buffer B + dNTP mix [5 mM] (Cat. No. EK2500)
- Sterile, DNA-free H_2O, PCR Grade (Cat. No. E0211)

Diversity Standard - PCR Amplification

For each of the supplied diversity standards plus for the DNA library Bank 40, perform the following PCR amplification (in separate tubes, respectively):

10 x PCR Buffer B (with 1.5 mM $MgCl_2$)	1 x	10 µl
DivStd-5'-Amp-Primer [4.5 µM]	1 µg (~45 pmol)	10 µl
DivStd-3'-Amp-Primer [4.5 µM]	1 µg (~45 pmol)	10 µl
dNTP mix [5 mM]	400 µM	8 µl
Thermostable DNA polymerase [5U/µl]	2.5 U	0.5 µl
Template DNA	0.5 µg, up to 10^{13} copies	
Sterile, DNA-free H_2O		@100 µl

	Initial Denaturation	95°C	120 sec
2-6 cycles			
	Denaturation	95°C	30 sec
	Annealing	59°C	30 sec
	Extension	72°C	90 sec
	Final Extension	72°C	300 sec

Note 1: **Non-Emulsified PCR.** PCR is conducted as non-emulsified, open PCR. Due to few PCR cycles, PCR bias is not an issue here.

Note 2: **Optimized PCR Conditions.** The annealing temperature and extension time is adjusted to requirements of OligoBank40-analogous diversity standards, respectively, as supplied with this kit. Usage of oligonucleotide libraries with different primer sequences or different amplicon length may require proper adjustment of PCR conditions.

Note 3: **Required DNA Amount.** The DiVE and DiStRO assays described below require 200 – 300 ng of dsDNA per diversity standard sample and per analysis following each selection round.

Note 4: **Add Library as Control.** Add PCR-amplified library Bank 40 (double-stranded). Amplify single stranded library Bank40 by PCR using the PCR conditions described above, but, instead of using the diversity standard specific primer pair, use both Bank40 - library specific primers (5'-Bank40-Primer and 3'-Bank40 Primer, respectively; see page 18). This assay serves as a control for measuring the original (non-enriched) library diversity and to compare against the reduced diversity of SELEX enrichments. Make sure to use solely Bank40 library specific PCR primers for amplification of oligonucleotide library Bank40. Do not use diversity standard specific primers (DivStd-5'-Amp and DivStd-3'-Amp, respectively). PCR primer sets for amplification of Bank 40 and for all diversity standards were designed to contain subtle differences for avoiding any potential cross-contamination between library and diversity standards, but were fine-adjusted to display corresponding melting / remelting behavior.

Optional step: Remove residual single-stranded DNA and primers by S1 nuclease treatment for 15 min at 55°C in 1x S1-buffer (in most cases, this step gives no further improvement and can be left out).

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

1. Apply 40 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: Add Buffer to Membrane Center. Addition of Buffer DX 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.

2. Add 400 µl of orange-coloured **Orange-DX** buffer to DNA sample and mix.

Note 1: Maximum volume of a DNA sample can not exceed 250 µl.

3. Pour mixture into a spin-column/receiver tube assembly.
4. Spin down in a micro-centrifuge at 11,000 x g (12,000 rpm) for 1 minute.
5. Remove spin-column and discard flow-through.
6. Add 500 µl of **Wash-DX1** buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
7. Remove spin column, pour the supernatant off and stick spin-column back on top of the tube.
8. Add 650 µl of **Wash-DX2** buffer and spin down at 11,000 x g (12,000 rpm) for 1 minute.
9. Remove spin column, pour the supernatant off and stick spin-column back on top of the tube.
10. Spin down at 12,000 rpm for 2 minutes to remove traces of **Wash-DX** buffer.
11. Place spin-column into new receiver tube (1.5-2 ml). Add 50-100 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. The Elution-DX buffer will not interfere with subsequent DNA manipulations, such as DNA sequencing, ligation or restriction digestion, among others.
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.

12. Incubate spin-column/receiver tube assembly for 2 minutes at room temperature.
13. Spin down at 11,000 x g (12,000 rpm) for 1 minute.
14. 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 or (preferred) at -20°C.

DiVE Assay (S1 Nuclease, Agarose Gel Analysis)

The DiVE Assay (Lim et al., 2011) allows monitoring of a DNA molecule pool's diversity by measuring DNA reassociation kinetics via S1 nuclease digestion. A pool of homoduplex dsDNA is denatured and allowed for reannealing under stringent conditions. Depending on sample diversity, annealing of non-homologous DNA leads to formation of varying amounts of heteroduplex DNA containing single stranded loops. Treatment of the reannealed DNA pool with single-strand specific S1 nuclease, followed by agarose gel electrophoresis allows comparison of band intensities from those DNA molecules with the original expected (undigested!) size against an equal amount of reannealed but non-S1-treated control sample (not containing any digested DNA bands). Thus, only the intensity of the largest, undigested DNA band in the S1 assay is compared to its respective undigested control as well as in relation to equal amounts of S1 treated and untreated diversity assay standards (synthetic oligonucleotides of considerable high quality do not generate more than one single band).

For homogenous samples, no or few S1 nuclease digestion is detected. Diverse samples with high proportions of heteroduplex DNA are digested almost to completion. Thus, sequence diversity of the DNA pool is inversely proportional to the difference in band intensity between completely intact, S1 nuclease resistant sample and untreated sample. The smaller the difference in intensity of the non-digested DNA band in the S1 nuclease treated sample vs. the undigested DNA sample, the smaller the diversity of the sample. Quantitation of diversity is achieved by comparison against DNA standards of known diversity (Diversity Standard).

Material list (components not provided, except where indicated):

- Diversity-Standard (dsDNA, PCR amplification of provided ssDNA standard is required; see page 40; provided with the kit as single stranded DNA)
- Amplified and purified DNA pools from each selection-round (dsDNA), minimum 200 ng
- S1-nuclease (Cat. No. E1335)
- S1-buffer 5x (200 mM sodium acetate, 1.5 M NaCl, 10 mM $ZnSO_4$, pH 4.5; provided with S1 nuclease, Cat.No. E1335)
- TE buffer [10x]
- [0.5 M] EDTA

Note 1: Convert ssDNA to dsDNA. Diversity Standard towards OligoBank40 oligonucleotide libraries are provided as ssDNA and have to be amplified under similar conditions as OligoBank40 libraries. When screening oligonucleotide libraries different to OligoBank40, a diversity standard resembling the specific library structure with respect to length, constant sequence stretches such as primer binding sites etc …, must be supplied by the user. None of the other materials required for the DiVE assay are contained in the kit. These components have to be provided by the user .

1. Transfer 200 ng of each diversity standard (dsDNA, PCR amplified) or of the DNA sample, respectively, to separate plastic reaction tubes. For each assay (standard or sample), one tube is treated with S1 nuclease (assay), the other tube remains untreated (control), respectively.

2. Add an appropriate amount of (5x) S1 nuclease buffer to the assay and the control tube. Fill up with nuclease-free water for obtaining a 1x final buffer concentration. Mix thoroughly. The final volumes of assay and control reaction must be identical and the overall volume should be kept as low as possible (approx. final assay volume: ~250 µl).

3. Denature and reanneal both the assay and the control tubes:
   Denaturation: 3 min at 98°C
   Reannealing: 5 min at 65°C

4. Add 1 µl S1 nuclease [1 U/µl] to the assay tube but not to the control tube.

5. Incubate for 30 min at 65°C.

6. Stop S1 nuclease digestion by addition of ~2 µl EDTA [0.5 M, pH 8.0] (final concentration: 2 mM).

7. Analyze an equivalent volume to 20-40 ng of input DNA per slot on an 2 or 3 % agarose gel. Apply assay and control tubes side-by-side.

8. Compare extent of digest to digested diversity standards on an agarose gel for interpolating approximate diversities.

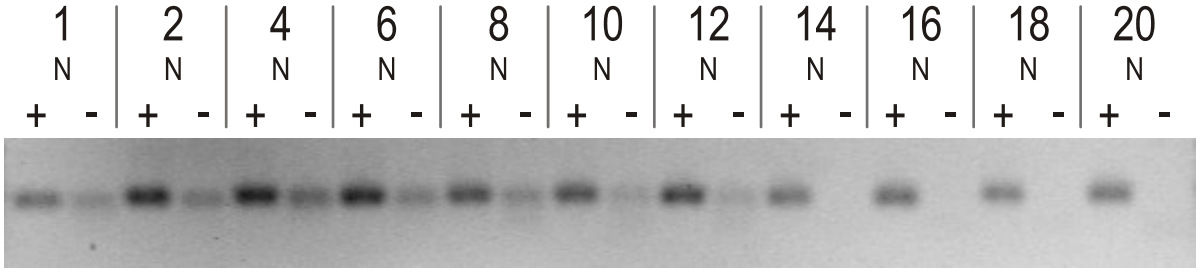


Figure 10: Monitoring the diversity by S1 nuclease digestion of non-denatured (+) or denatured and re-annealed (-) double stranded diversity standard. With increasing diversity, it becomes less likely for DNA molecules to find a perfectly matching complementary molecule for reannealing. Thus the share of non-perfectly matching, reannealed molecules increases, while the share of perfectly matching, reannealed dsDNA molecules decreases. Non-perfectly matching double-stranded DNA is susceptible to S1 nuclease digestion. The degree of S1 nuclease digestibility is a measure for the diversity of the sample, with high N numbers indicating high diversity. Sample diversity is optically compared against diversity of digested standards. N denotes the number of randomized sequence positions. With increasing progress of the SELEX reaction, measured sample diversity decreases, i.e. approaches low N numbers. (Schütze et al. 2010).

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

DiStRO Assay (Real Time Analysis)

Material list (components not provided, except where indicated):

- Diversity standard (dsDNA, 37,5 ng/µl, PCR amplification of provided ssDNA standard required; see page 40; provided with the kit as single stranded DNA)
- Amplified and purified DNA pools from each selection round (dsDNA), minimum 300 ng in max. 8 µl volume
- 10x DA-buffer (200 mM MOPS, 100 mM EDTA, 2,5 M NaCl, 0,3 % Brij-700); provided with the kit
- SYBR-Green

Note 1: Convert ssDNA to dsDNA. Diversity Standard towards OligoBank40 oligonucleotide libraries are provided as ssDNA and have to be amplified under similar conditions as OligoBank40 libraries. When screening oligonucleotide libraries different to OligoBank40, a diversity standard resembling the specific library structure with respect to length, constant sequence stretches such as primer binding sites etc …, must be supplied by the user. None of the other materials required for the DiVE assay are contained in the kit. These components have to be provided by the user.

Method:

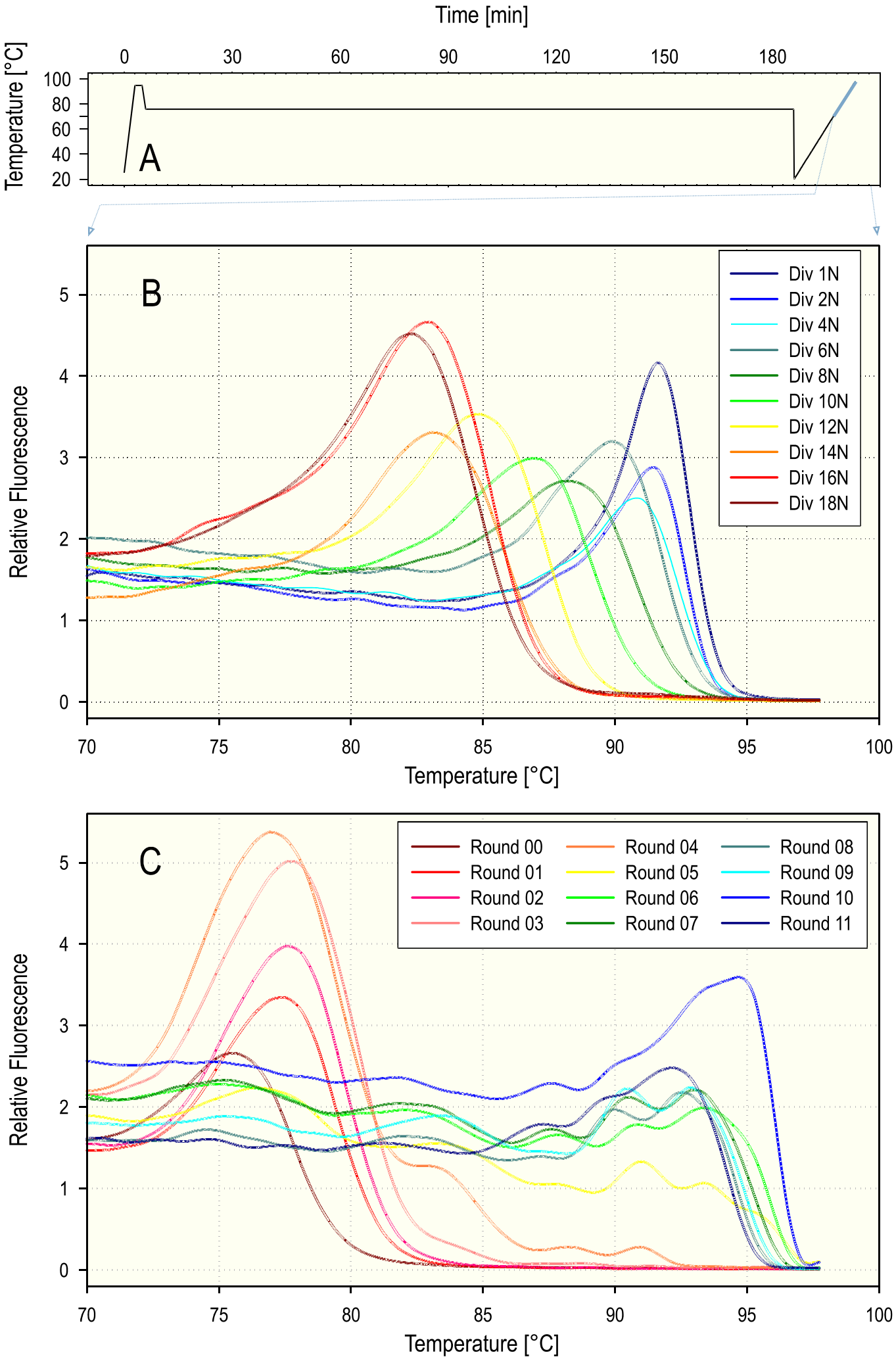
Mix the following components:

300 ng of each diversity-standard or DNA-sample
+ 1 µl DA-buffer (10x)
+ 1 µl SYBR-Green (1:1000 in nuclease free H_2O)
Fill up to 10 µl with nuclease free H_2O

Real-Time-Cycler Program:

Denaturation: 2 min at 95°C
Annealing: 180 min at 76°C (or, for different libraries, 2°C less than calculated Tm)
Take one measurement per minute.
Remelting: Cool to 20°C, then heat by incrementing + 0,5°C per 7 sec
until a temperature of 98°C is reached.
Take one measurement per increment, i.e. per 7 sec.

Figure 11: (page 46) Comparing melting curves of library enrichments to defined diversity standards. (A) Temperature profile of real time assay. The relevant section from remelting curve data during the time interval marked in blue color is displayed enlarged in both graphs below. (B) Example melting curve of diversity standards as measured with the DiStRO assay (Schütze et al. 2010). Single stranded standard oligonucleotides were amplified in four PCR cycles to yield dsDNA. Any remaining ssDNA was digested with S1 nuclease (Cat.No. E1335). 300 ng of dsDNA per standard was heat denatured and allowed to reanneal. The reannealed DNA was reheated from 20°C to 98°C at a rate of + 0.5°C each 7 sec. Highly diverse samples melted at lower temperatures as compared to less diverse samples, because of a higher content of non-perfectly matching heteroduplex DNA. With increasing sample diversity, the probability for reannealing of two perfectly matching oligonucleotides decreases, leading to an increased formation of heteroduplex DNA. The increasing amount of mismatched nucleotides in heteroduplex DNA leads to remelting of dsDNA at lower temperatures, as compared to homoduplex DNA. The higher the amount of homoduplex DNA, the lower the diversity, the higher the observed remelting temperature. Consequently, the remelting temperature decreases with increasing diversity. (B) SELEX experiment. Diversity comparison of reamplified DNA libraries from bead eluate. With increasing progress, good binders are enriched, resulting in a reduction of overall diversity. Diversity is monitored by comparing the peak and the curve progression of sample to diversity standards. In this experiment, the most notable decrease in diversity occurred between rounds 4 and 5. Following 11 selection rounds, diversity is greatly reduced and binders are highly enriched. (Schütze *et al.* 2010, Nucleic Acids Research, 38: 4 e23).


Time [min]
Temperature [°C]
A
B
Relative Fluorescence
Temperature [°C]
Div 1N
Div 2N
Div 4N
Div 6N
Div 8N
Div 10N
Div 12N
Div 14N
Div 16N
Div 18N
C
Round 00
Round 01
Round 02
Round 03
Round 04
Round 05
Round 06
Round 07
Round 08
Round 09
Round 10
Round 11

FLAA Library Analyses and Quality Control: Measuring Enrichment of Binders

The FLAA assay (Wochner & Glökler 2007) is an easy and straightforward method for monitoring enrichment of good binders. This method resembles ELISA assays, except that aptamers take over the role of ELISA's antibodies. A brief outline of the methodology: Depending on specific requirements of the target structure (e.g. protein, glycoprotein, lipid, sugar …) the target is biotinylated by application of target-specific biotin-coupled conjugates. Such conjugates are available from third parties and ship with specific instructions for attaching target to conjugates. The biotinylated target is then immobilized to the surface of coated microwell plates. A negative control plate is prepared, where streptavidin is completely saturated with free biotin. Sample duplicates of target-directed binder enrichments are allowed to bind the immobilized target structure as well as to the negative control. After washing, a single-stranded DNA - specific fluorophore is added to each well. The amount of retained DNA, corresponding to good binders, is then determined by fluorescent measurement and compared to the amount of non-target specifically bound DNA by the negative control. The higher the fraction of good binders per sample, the higher the amount of target bound DNA vs. negative control. High amounts of specifically bound DNA indicate successful enrichment of binders.

This assay is appropriate for (a) monitoring enrichment of good binders in the DNA library throughout the selection process and (b) for comparing binding efficiency of specific DNA aptamers (clones) relative to non-specific clones of similar length. Since fluorescence values are expressed as relative increase in fluorescence against non-specific DNA, negative controls must be included in the binding assays. Appropriate negative controls are: (a) Non-enriched material from the original, non-modified oligonucleotide library for monitoring the enrichment of binders in a DNA library; (b) a non-target binding clone from another selection for testing target affinities of aptamer clones.

Note 1: Qualitative, Not Quantitive. The FLAA assay is not considered as quantitative assay. Rather it is intended solely for monitoring the relative increase in binding efficiency between samples and negative controls following each selection round.

Note 2: No Threshold Values Do Exist. There exists no absolute value that could indicate sufficient binder enrichment. The selection process is finished, when the sequence of good binders has been successfully determined. By next generation sequencing methods, this task is often accomplished after a few selection rounds, often after just three to five selection rounds. Other detection methods, such as screening of plasmid-cloned aptamers, may require further selection and enrichment, typically 10 rounds, sometimes up to 15 rounds. Sufficient binder enrichment is likely indicated, if no additional increase of target-specific vs. non-target specific binders is observed between further selection rounds.

Note 3: Include Appropriate Controls. For correct interpretation of the results, it is mandatory to include appropriate controls to the assay. Appropriate controls would include:

(1) A positive control reaction with a good binding aptamer or a good enrichment of binders (if available).

(2) A negative DNA binder control with a DNA aptamer known to posses no affinity or only weak affinity to the target structure. The aptamer sequence should be of similar length and should possess an arbitrary, but defined sequence. If possible, stretches of fixed sequence, such as primer binding sites for PCR amplification should be present.

(3) A negative beads control with unmodified beads and / or differently coated and passivized beads.

This procedure requires additional components which are not part of this kit and have to be supplied by the user:

- Biotinylated target protein / target structure
- Biotin [5 mM]
- Streptavidin-coated microtiter plates
- DNA binding fluorescent dyes such as Oligreen or Picogreen.

Use duplicates in the assay to avoid errors.

1. *Binder Assay:* Bind the biotinylated target peptide or target structure to the surface of streptavidin-coated microtiterplate wells. Incubate 1 h at room temperature. Add a 10-fold excess of target peptide / structure in relation to the denoted binding capacity.

   Negative control: Add 2 µl biotin [5 mM]. Incubate for 10 min at room temperature.
2. Wash plate twice with 250 µl SELEX buffer [1x].
3. Dilute an appropriate amount of binder DNA in 50 µl SELEX buffer [1x]. Use an amount approx. equal to the denoted binding capacity of the microwell plate. E.g. for streptavidin-coated microplates, 12-20 pmol DNA are a good starting amount; this equals 150-250 ng for a 40-mer or 280-470 ng for a 76-mer, respectively. If unsure, prepare an additional dilution to ensure DNA is not in excess as compared to the number of binding sites (see Appendix, page 54 for calculation of DNA copy numbers from DNA weight [µg]).
4. Heat denature the DNA solution (95°C, 3 min) and immediately cool down on ice to favor ssDNA secondary structure formation over dsDNA reannealing. Transfer the precooled DNA solution to the target coated microtiter plate and incubate over night at 4°C or for one to two hours at room temperature.
5. Discard the DNA supernatant. Wash plate immediately before measurement with 100 µl binding buffer [1x].
6. Add 50 µl Oligreen (or Picogreen) (1:500 in binding buffer) and measure twice after 9 min. (or after 4 min.): Excitation 485 nm, emission 527 nm.

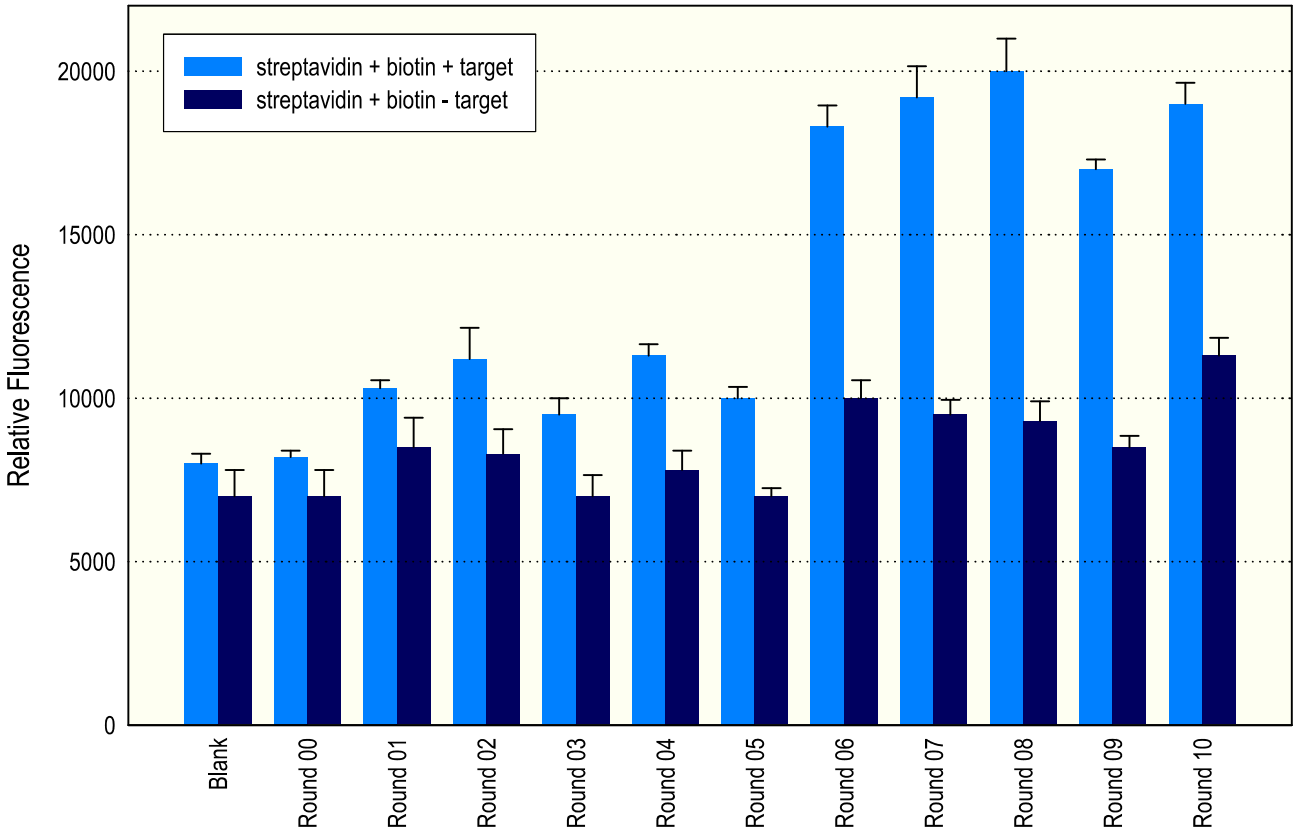


Figure 12: Example evaluation for analysis of binder enrichment using the FLAA assay. For each selection round, relative fluorescences of binder assay (streptavidin + biotinylated target) vs. negative control (streptavidin + biotin) were compared. Slight enrichment of good binders is detected after round 4, good enrichment is detected after round 6.

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

Determination of Sequence Frequencies: Selecting Aptamer Clones

Next Generation Sequencing

The first step of identifying candidate sequences for good binding aptamers is to analyze the relative frequency of enriched aptamer sequences. Using "Next Generation Sequencing" methods, good binder candidates are often detected within the top ten abundant sequences after only three or slightly more selection rounds. Thus, this assay allows detection of enriched good binders at an early stage. Consequently, less selection rounds - and hands-on time - are required. The downside is a requirement for expensive and dedicated equipment as well as trained personnel.

Since Next Generation Sequencing techniques are very high throughput methods, it is possible to analyze library amplificates from many, if not all selection rounds in parallel. For this purpose, supplement library amplificates from each selection round with individual barcodes. Furthermore, bias-free library amplification may be required for generation of sufficient template DNA amounts, e.g. by ePCR using the Micellula Emulsion Kit (Cat. No. E3600). For further information on Next Generation Sequencing sample preparation, consult the manual that shipped with the particular instrument.

Following identification of binder candidate sequences, ssDNA aptamers of abundant binder candidate sequences are synthesized using standard oligonucleotide synthesis technology and are directly applied to the following screening and quantification assays.

Molecular Cloning and Clone Analysis

In case, "Next generation Sequencing" technology is not available, typically 10 selection rounds are required for detection of abundant, enriched binder candidate sequences. Library enrichments from late selection rounds are analyzed as follows: dsDNA amplificate of the last selection round, else from the last known-to-work selection round, is cloned using standard plasmid cloning techniques. 50 - 100 random clones are picked and material from each clone is subjected to quick alkaline lysis (a toothpick tip of material in 10 µl [50 mM] NaOH, 20 % [w/v] SDS; lysis at 95°C for 15 min; fill up to 1 ml with DNA free water; vortex; spin down briefly; use 1 µl as template for PCR amplification). Starting from purified PCR product, single-stranded aptamer DNA is easily generated e.g. (1) by heating and quick cooling on ice, as outlined on page 48, step 4 (recommended); (2) by PCR with a large (approx. 5-10fold) excess of aptamer sense primer to antisense primer; or (3) by the following method:

- PCR amplification using a non-phosphorylated amplification primer for the aptamer-sequence specific strand and a phosphorylated primer for the aptamer-anti-sense strand,
- digestion with Lambda Exonuclease (Cat. No. E1180) for selective digestion of the 5'-phosphorylated aptamer-anti-sense strand, yielding aptamer-sense ssDNA;
- purification of aptamer-ssDNA with DNA spin columns (EURx Short DNA Clean Up Kit for ssDNA <100 bp in length; Cat. No. E3515) following the supplied protocol.

For most applications, method (1) is fast, convenient and entirely sufficient. Additionally, DNA re-association kinetics prove, that melting and reannealing of complementary DNA strands in complex DNA mixtures is a slow enough process to not interfere with aptamer - target structure binding. In complex DNA samples, DNA reassociation rates decrease with increasing DNA library diversity (compare to data of DiVE assay → page 43 ff. and DiStRO assay → page 45 ff., respectively). Thus, apart from heating and snap-cooling, no special precautions for isolation of single strand DNA are necessary. Method (1) has an additional advantage before (2) and (3), since it lacks strand specificity. In case the aptamer “anti-sense” strand (but not the “sense”-strand) would own good binding properties, method (1) would detect this “clone”, whereas methods (2) or (3) could not detect this specific “anti-sense” aptamer.

FLAA Aptamer Clone Preselection

The FLAA binding assay described above may as well serve as a quick and non-expensive prescreening process for good binder candidate aptamers. Instead of reamplified libraries, here individually synthetized oligonucleotides (40mers, ssDNA, without primer binding sites for PCR amplification) are investigated for their binding properties towards the selection target. Following the protocol given on page 47, ssDNA “clones” from aptamers with distinct sequences to be tested serve as starting material (rather than a mixture of DNA aptamers from library enrichments). FLAA

is conducted on coated beads, mostly streptavidin coated beads. The selection target is immobilized via biotin-coupled conjugates to the surface of streptavidin-coated beads. To check for non-target-specific or for streptavidin-specific binding activity, ssDNA of each clone is assayed for its binding activity to a negative control with streptavidin + biotin coated beads, but without selection target.

A good experimental design for clonal FLAA assays includes appropriate controls to ensure, that selected clones do bind specifically to the selection target and do not

- bind to coated surfaces (e.g. of streptavidin-coated beads),
- bind to any conjugate used for coupling target structures to coated surfaces,
- bind to potential co-purifying contaminants of non-completely purified selection target molecules.

The assay is purely qualitative. It is not valid to draw any quantitative conclusion on binding properties from the difference in fluorescence values for binding and control assay. A large difference between fluorescence values for binding vs. control assay does not implicate a high binding activity of the aptamer and vice versa. However, an aptamer is a good candidate for further quantitative analyses such as surface plasmon resonance (e.g. “Biacore”), if fluorescence values for the binding assay are higher as compared to the control assay. Correspondingly, if fluorescence values for the control assay are higher than for the binder assay, further quantitative analyses most often show poor binding properties. Taken together, the FLAA assay should be regarded as purely qualitative assay and provides a simple yes / no answer to the question, whether an aptamer clone should be subjected to further quantitative analysis. FLAA serves as a screening assay to reduce the total number of binder candidates for further (more laborious and expensive) quantitative analyses by a factor of 10.

Good binder candidates are selected by FLAA for compliance with the following criteria:

1. The fluorescence signal strength of the streptavidin-immobilized target is higher than the streptavidin-biotin control.
2. The fluorescence signal strength of binder assay must be higher than the streptavidin blank. Fluorescence signal strength of negative control assay must be higher than the negative control (streptavidin + biotin) blank
3. The difference in binding affinity is higher than the difference measured for the non-specific binder assay compared to its control.

Note 1: Don't Include Primer Sites Within Aptamers. When ordering synthetic oligonucleotides for further analysis by clonal FLAA and quantitative binder studies, remember not to include any constant regions such as PCR primer binding sites. Primer binding sites are not part of the aptamer. Include only the variable part of 40 bases without any constant regions for further binding analysis.

Note 2: Both, Sense and Antisense Strands Matter. Since selection has been performed with dsDNA: Keep in mind, that selected aptamers may exhibit target affinity in “sense” (5'-3') as well as in “antisense” (3'-5') orientation.

Example FLAA protocol (streptavidin selection - positive and negative binder control):

1. *Test Aptamers:* Dissolve 12 - 15 pmol DNA aptamer each in 50 µl 1x SELEX binding buffer (e.g. streptavidin positive and negative binder control, all 40-mers: 12 pmol =~ 150 ng; see appendix for calculation formula).
   Negative control: Dilute 2 µl biotin [5 mM] with 50 µl 1x SELEX binding buffer.
2. Denature all solutions at 95°C for 3 min, then place immediately on ice. Transfer the precooled DNA solution to the target coated microtiter plate (in an amount approx. equal to the denoted binding capacity of the microwell plate, e.g. 12 – 20 pmol DNA; DNA amount must not exceed binding capacity).
3. Incubate 1 h at room temperature.
4. Wash plate three times with 250 µl SELEX buffer [1x].
5. Continue with step 6 of the FLAA library protocol (page 48).

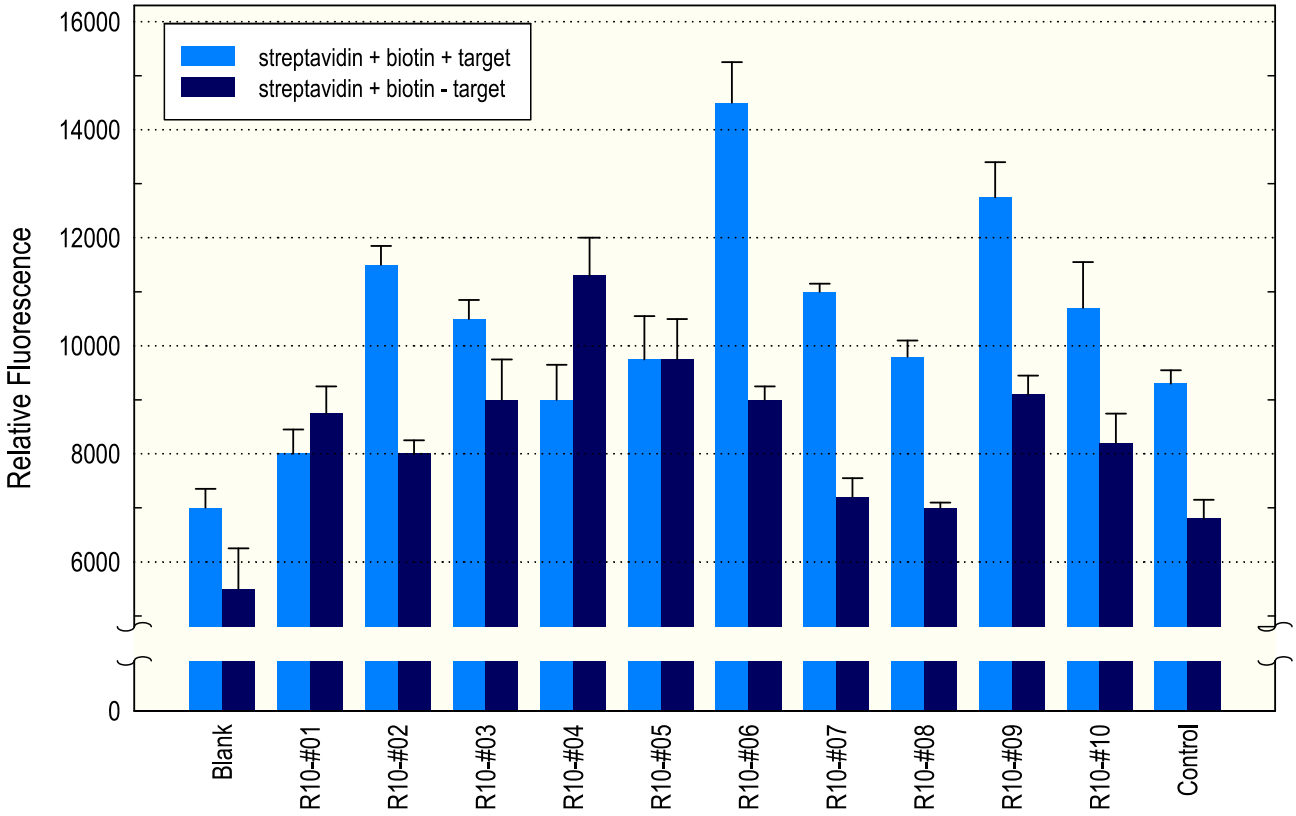


Figure 13: FLAA binder analysis of ten selected aptamer binding clonal sequences ("clones"). Biotinylated binder target was immobilized to streptavidin coated microwell plates. ssDNA aptamers were allowed to bind to immobilized target (streptavidin) or to a negative control (streptavidin + biotin without binder target). Clones from selection Round 10 library were numbered from 01 to 10 (e.g. R10#05 for clone # 5 from round 10). Blank: No DNA. Control: DNA of similar length and structure (e.g. identical primer binding sites), but without any specific affinity to the selection target. Promising selection candidates are clones R10#6 and R10#7 (markedly higher fluorescence values for streptavidin vs. streptavidin + biotin assays). Non-promising candidates are: R10#01, R10#04 (higher fluorescence values for streptavidin + biotin vs. streptavidin; streptavidin fluorescence values are lower than the streptavidin fluorescence values of the non-specific control aptamer) and R10#05 (roughly equal binder vs. control fluorescence values).

Quantitative Analysis of Aptamer Clones

A selection of promising good binder candidates is further analyzed by quantitative binding assays such as surface plasmon resonance (e.g. “Biacore”), following the manuals supplied by the respective manufacturer. Once the sequence of a good target binding aptamer has been unambiguously determined, it will be accessible by oligonucleotide synthesis in virtually any scale, from small to large.

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:

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

Using this formula, the mM of oligonucleotide Bank-40 (76 nt) calculates to 23408 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}]):

1. Count the occurrence of each base in the ssDNA molecule. Example: oligonucleotide Bank-40 is composed of 9 A's, 12 C's, 8 G's, 7 T's and 40 N's (N's are estimated to consist of equimolar amounts of A. C, G, and T, respectively).
2. According to Doležel *et al*. Cytometry, 2003, Vol. 51A, 2, 127-8. the mM for single nucleotide 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:

   9 x 331,2213 (A) + 12 x 307,1966 (C) + 8 x 347,2207 (G) + 7 x 322,2079 (T) + 40 x 326,9616 (N) = 24779,0368

3. 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. 76 nt's), there are N-1 phosphodiester bonds (e.g. 75). Thus, for oligonucleotide Bank-40, 75 x 18.0125 have to be subtracted from the MM-value calculated in step 2.
4. 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. 76 nt) is lowered by N x (e.g. 76 x) 1.0079.
5. Taking together steps 2, 3 and 4, the mM of oligonucleotide Bank-40 calculates to:
   24779,0368 - 75 x 18.0125 - 76 x 1.0079 = 23351,4989

For oligonucleotide Bank-40, the values calculated with both methods differ to less than 0.3 %, which is neglectable for the practical purposes outlined here. In most cases, the simple formula in method 1 gives sufficient precision.

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 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 = 76 bp

$$ssDNA\,[pmol] = \frac{1.0\,[\mu g] \cdot 10^{12}\,[pmol\ mol^{-1}]}{76\,[nt] \cdot 308\,[g\ mol^{-1}\ nt^{-1}] \cdot 10^{6}\,[\mu g\ g^{-1}]} = \frac{1.0}{(76 \cdot 308)} \cdot 10^{-6}\ mol = 42{,}7\ pmol$$

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 54) 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 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.

What, in contrast, 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? Or, 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 exist 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:

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

The weight of an ideal universal random library thus 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? One approach, analogous to the well-known "trading cards" problem, is 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

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 Bank40 (sequence see page 18)

Total number of bases per oligonucleotide [nt]	76
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 = $\sim 1.2 \times 10^{24}$
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)	$N \log N = 6.7 \times 10^{25}$
Total number of bases in oligonucleotide: 76 nt = 40 random + 36 fixed	76
Total number of bases in library of expected size [nt]:	$76 \times 6.7 \times 10^{25} = 5.09 \times 10^{27}$
Conversion factor of base pairs (bp; dsDNA) to pg DNA (Doležel *et al*., Cytometry, 2003, Vol. 51A, 2, 127-8)	DNA content [pg] = 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] = library size [nt] / (1.956×10^9) [nt pg^{-1}])
DNA amount [pg] = 5.09×10^{27} [nt] / 1.956×10^9 [nt pg^{-1}] **= 2.60×10^{18} [pg]** **DNA amount [kg] = 2602**	

Universal libraries weight in the ng-scale for random 20mers, in µg-scale for random 25mers, in mg-scale for random 30mers, in kg-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	$4^{20} = 1.1 \times 10^{12}$	$1{,}1 \times 10^{-8}$	$3{,}12 \times 10^{-7}$
Random 25mer	25	$4^{25} = 1.3 \times 10^{15}$	$1{,}4 \times 10^{-5}$	$4{,}99 \times 10^{-4}$
Random 30mer	30	$4^{30} = 1.2 \times 10^{18}$	$5{,}9 \times 10^{-2}$	$7{,}36 \times 10^{-1}$
Random 40mer	40	$4^{40} = 1.2 \times 10^{24}$	$2{,}5 \times 10^{4}$	$1{,}37 \times 10^{6}$
Random 80mer	80	$4^{80} = 1.5 \times 10^{48}$	$6{,}0 \times 10^{28}$	$6{,}63 \times 10^{30}$
Bank40*	40	$4^{40} = 1.2 \times 10^{24}$	$4{,}7 \times 10^{4}$	$2{,}60 \times 10^{6}$

*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 an agarose 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 1×10^{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 ~ 7×10^{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 the methodology 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

Legal Disclaimer:

The XELEX DNA Core Kit is a dedicated kit for easy and reproducible assembly of *in-vitro* aptamer selections, for particle-/ bead-free PCR (ePCR) as well as for PCR assays in emulsions (ePCR) assays and subsequent DNA purification thereof. In addition to particle-/ bead-free emulsion 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 as well as *in-vitro* aptamer selections and *in-vitro* evolution techniques are a broad field of ongoing research, development and innovation. New technologies continue being 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 XELEX DNA Core Kit does not include any licenses for technologies covered by third parties IP.

- ***XELEX – SELEX DNA Core Kit The XELEX Kit series is a modular kit system covering the entire SELEX work-flow. From random DNA library to 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 just a couple 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), sortase-mediated protein immobilization under physiological conditions (for sensitive target proteins) or for RNA aptamer selection.

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