Electronic Supplementary Information

Capture and Release (CaR): A simplified procedure for one-tube isolation and concentration of single-stranded DNA during SELEX.

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Experimental Section

Chemicals and reagents

Protease-free bovine serum albumin (BSA), biotin-labelled BSA, rabbit whole antiserum against streptavidin, and all general chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). HRP-labelled goat anti-rabbit antibodies were purchased from Dako (Hamburg, Germany). Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin) were purchased from Life Technologies (Karlsruhe, Germany). Recombinant human activated protein C (APC) was purchased from Eli Lilly (Giessen, Germany). Recombinant human activated FXIII A subunit (FXIIIAa) was purchased from Zedira (Darmstadt, Germany). The randomized ssDNA libraries IHT1 (5'- AAG CAG TGG TAA GTA GGT TGA - N_{40} - TCT CTT CGA GCA ATC CAC AC -3') and IHT2N (5'- GAT TGT TAC TGT CAC GAG GAT- N_{40} - ATA GCA CAT TAG TTC AGA TAC -3') were synthesized and PAGE-purified by Microsynth (Balgach, Switzerland). IHT1 and IHT2N-amplification primers (targeting the shown fixed sequences of the libraries in full length) and the below described capture molecules were synthesized and HPLC-purified by Eurogentec (Seraing, Belgium). The used BM chemiluminescence substrate was purchased from Roche (Mannheim, Germany). Aqua ad iniectabilia was purchased from Fesenius Kabi (Bad Homburg, Germany).

Prediction of DNA hybridization profiles and design of capture-molecules

The fraction of DNA duplexes (capture molecule - target ssDNA) over temperature ('melting curves') of a given sequence under given buffer conditions (concentration of capture-molecules, monovalent ions, and Mg^{2+} ions) was assessed using the 'DNA thermodynamics & hybridization' tool available on the 'biophysics' sub-domain of idtdna.com (http://biophysics.idtdna.com). This sub-domain runs stable and tested software to be included into the IDT SciTools collection (cf. to ref. #5 of the main manuscript). Details on applied formulas and calculations can be found at http://biophysics.idtdna.com/HelpMelt.html. The applied software returned the predicted fractions of duplex (0 to 1) over an integer temperature range of 0 to 100°C. No absolute accordance to real-world conditions was expected. However, obtained values were used as a basis for the design of the IHT1- and IHT2N-capture-molecules and to assess the chosen concentration of monovalent cations within the used washing buffer. Based on the returned data (melting profiles), the following sequences were chosen for the capture-molecules:

IHT1-capture-molecule: 5’-Biotin-GTG TGG ATT GC-3’

IHT2N-capture-molecule: 5’-Biotin-GTA TCT GAA CTA AT-3’

Temperatures at which 5%, 50%, or 95% of duplexes (capture molecule - target ssDNA) were predicted to be denatured were assessed from the returned data sets. For the IHT1-capture-molecule, the corresponding values for each step of CaR are shown in Scheme 1 of the main manuscript. Figure S1 shows the returned melting profiles at different buffer conditions and derived Tm-values for the IHT2N-capture-molecule.

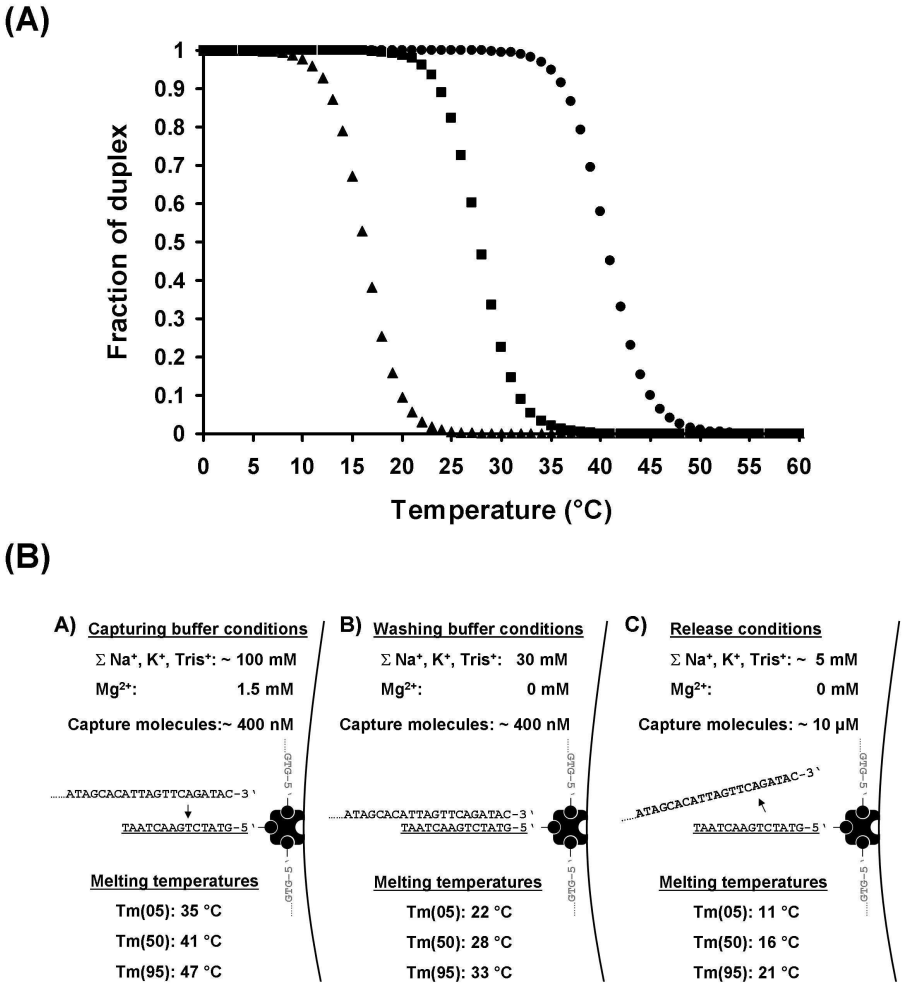


Fig. S1. (A) IHT2N-related melting curves (capture molecule - target ssDNA) as predicted by the ‘DNA thermodynamics & hybridization’ tool. Underlying buffer conditions: Capturing step: circles; Washing step: boxes; Release: triangles. (B) Details on buffer conditions and melting temperatures at which 5% [Tm(05)], 50% [Tm(50)], or 95% [Tm(95)] of captured ssDNA molecules are predicted to be released from the capture molecules.

Binding of capture-molecules to streptavidin-coated magnetic beads (SMB)

5'-biotinylated capture-molecules were bound to SMB as follows. Dynabeads M-280 Streptavidin (SMB) were washed 3x using B&W buffer (5 mM Tris-HCl, 1 M NaCl, 0.5 mM EDTA, pH 7.5) followed by incubation with B&W containing 1 µM of capture molecules (200 µl for each mg of SMB > 200 pmole of capture molecules / mg of SMB). The mixture was incubated at RT for 30 min under vigorous shaking to prevent beads from settling. After incubation, SMBs were washed 3x using B&W- (without EDTA). For storage, loaded SMB were suspended in 1 × PBS, 1 mg/ml BSA, 0.2 mg/ml NaN_3, pH 7.4 and stored at 4°C until used. Before use, the needed amount of stored (loaded) SMB was washed 3x using B&W-.

Assessment of binding and adverse release of capture molecules to/ from SMB using fluorescence measurements

In order to assess the amount of capture-molecules bound to or release from the SMB, 3'-FAM-labelled IHT1-capture-molecules (5'-biotinylated) were applied. After adding to B&W at 1 µM concentration, each 200 µl of this solution were incubated with 1 mg of SMB (washed 3x using B&W) for 30 min under vigorous shaking. Subsequently, beads were removed by magnetic force and the amount of FAM-labelled molecules remaining in the supernatant was determined by fluorescence measurements using black 96-well microtiter plates and a Synergy 2 microplate reader (Biotek, Bad Friedrichshall, Germany) and a λex 485 / λem 528 nm filter set.

To assess the temperature-dependent detachment of capture molecules from the SMBs, 3'-FAM-labelled IHT1-capture-molecules (5'-biotinylated) were bound to the SMB as described above. After incubation in purified water (1 mg loaded SMB / 20 µl) at different temperatures, the absolute amount of fluorescence in the supernatants was measured.

The FAM-labelled IHT1-capture-molecules could be detected down to sub-nanomolar concentrations in TE-buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.0). Thus, solutions to be tested were diluted in TE-buffer and the relative or absolute amounts of capture-molecules quantified by the standard-curve method.

Regarding determination of SMB-binding-capacity, merely 5% of the total fluorescence remained within the supernatant when using varying amounts of SMB around 1 mg, indicating efficient binding of capture-molecules when using 1 mg of SMB for immobilization of 200 pmole of (non-fluorescent) capture-molecules (Fig. S2). Results on the adverse release of capture-molecules from SMB are shown in Fig. 1D.

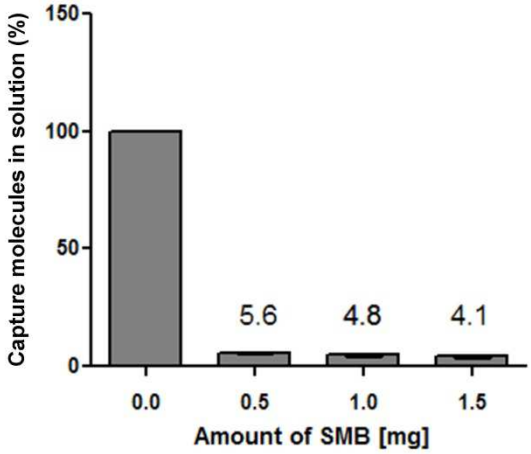


Fig. S2. Binding of 3'-fluorescently-labelled capture molecules to SMB introduced in different amounts. Values represent the relative amount of fluorescence that remained in solution after incubation.

Exponential amplification and preparation of asymmetric PCR mixtures for CaR

Initial exponential amplification of IHT1- or IHT2N-target-molecules was performed in a final volume of 100 µl using the following reaction mixtures and cycling conditions: 1 x PCR buffer (containing Tris-HCl, pH 8.7, KCl, and $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 200 µM each dNTP, 1 µM each forward- and reverse-primer, 1.25 U HotStarTaq *Plus* DNA polymerase, and 20 µl of the sample. Thermal cycling was done using the following profile: 95°C for 3 min followed by the indicated number of cycles of 95°C for 30 sec, 56°C (IHT) or 58°C (IHT2N) for 30 sec, and 72°C for 30 sec.

For the production of target single-strands, asymmetric PCR was applied using the reaction mixtures and conditions as described for the exponential amplification but without reverse primers. Ten µl of a 1 in 10 dilution of previously cycled exponential amplification mixtures were used as template. In general, before introduction to CaR, pooled asymmetric reaction mixtures were spiked with a final concentration of 100 mM NaCl (using a 5M stock solution).

Production of asymmetrically amplified IHT1-library for evaluation purposes

Asymmetrically amplified IHT1-library that was applied for evaluation purposes was prepared as follows. For initial exponential amplification, 10 µl of a 167 nM solution of the original IHT1-ssDNA-library (10^12 molecules) were added to the mastermix and amplified for 15 cycles. Subsequently, 50 cycles of asymmetric PCR were performed after introduction of 10 µl of 1 in 10 diluted exponential amplification products.

Assessment of quality and purity of ssDNA after asymmetric PCR/ CaR during basic assay evaluation

It should be noted that no special efforts were done to optimize the yield of ssDNA as produced by asymmetric PCR during this study which solely focused on the general functionality of the CaR procedure. Thus, total yields of isolated ssDNA may increase in case of further optimization of the asymmetric PCR procedure. In general, asymmetric amplification can also be run in one single reaction by using limiting amounts of reverse primer. Due to the lower effciency of amplification, however, more comprehensive optimization may be needed in order to find an appropriate balance between overall yield and the relative amount of generated DNA single-strands.

Single bands of dsDNA or ssDNA were observed when performing gel analysis of asymmetrically amplified IHT1-molecules (Fig. S3A, lane 1 and Fig. S4, lane 1). In order to assess the release of captured ssDNA at different elution temperatures, 500 µl of crude pooled asymmetric IHT1-reaction mixtures were added to 1 mg of SMB loaded with 200 pmole of IHT1-capture molecules (SMB+). After incubation for 30 min at RT, the SMB+ were washed 3x at RT using 1000 µl of 10 mM Tris-HCl, 20 mM NaCl, pH 7.6 and finally taken up in 20 µl of purified water pre-heated to designated temperature (RT, 37°C, 43°C, or 50°C). After 2 min of incubation in a water bath set to corresponding temperature, SMB+ were separated by magnetic force and supernatants collected. The final elution step was repeated 2x using new batches of water.

As shown in Fig.1A of the main manuscript, the majority of captured ssDNA was readily released from the IHT1-capture molecules at elevated temperatures during the first elution step. The quality of achieved ssDNA (see Fig. 1A for total yields) was determined by gel analysis (Fig. S3A) while yield and purity was assessed by triplicate UV-measurements (A260/A280 ratios) using a NanoDrop® ND-1000 UV/Vis-Spectrophotometer (Thermo Scientific) (Fig. S3B).

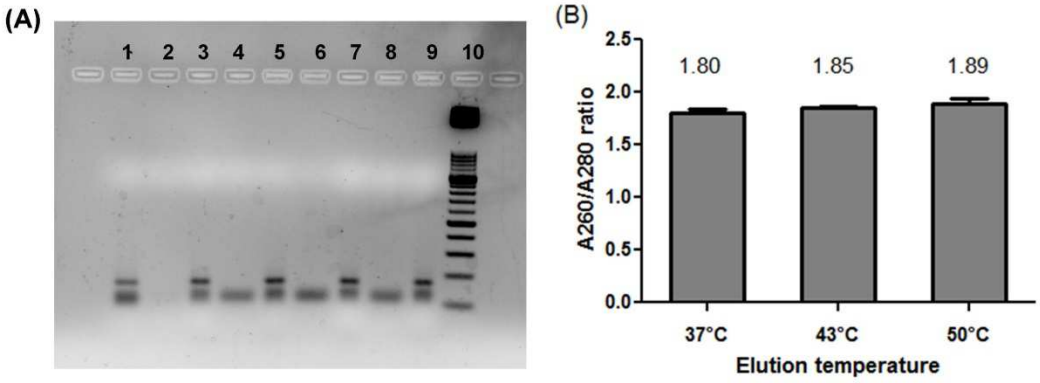


Fig. S3. (A) Agarose gel analysis (stained with ethidium bromide) of ssDNA yielded by CaR during the first elution at different temperatures. Lane 1: introduced crude asymmetric IHT1-PCR mixture. Lanes 2, 4, 6, and 8: 1 in 10 dilutions of ssDNA released from the IHT1-capture molecules at RT, 37°C, 43°C and 50°C, respectively. Lanes 3, 5, 7, and 9: corresponding supernatants (asymmetric PCR mixtures) after incubation with SMB+. Lane 10: 50 bp dsDNA ladder. (B) A260/A280 ratios of obtained ssDNA as determined by UV-measurements.

In order to provide further evidence for the proposed principle of CaR, underlying sequence-specificity was verified by the use of SMB loaded with IHT2N-capture molecules during CaR being performed with asymmetrically amplified IHT1-library. Besides SMB loaded with IHT1-capture molecules, also non-loaded beads that were passed through all incubation/ washing steps as described in '*Binding of capture-molecules to streptavidin-coated magnetic beads (SMB)'* (but in the absence of capture molecules), were run in parallel as controls. Again, 500 µl of crude pooled asymmetric PCR mixtures and 1 mg of SMB were used during each reaction.

As shown in Fig. S4, only the use of IHT1-capture molecules yielded detectable amounts of IHT1-ssDNA as determined by gel analysis (see Fig. 1B for obtained yield). The corresponding A260/A280 ratio was found to be 1.97 for the first elution.

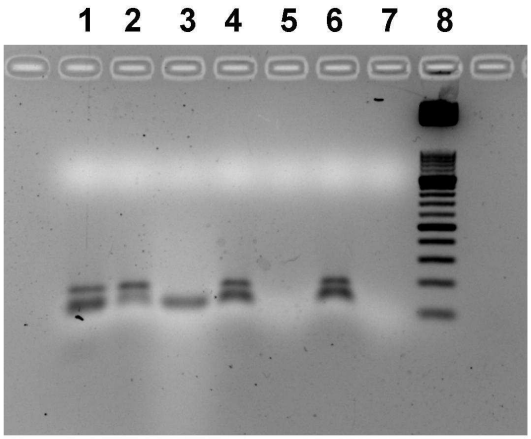


Fig. S4. Yield and quality of ssDNA isolated from IHT1 asymmetric amplification by CaR using non-loaded SMB or SMB loaded with specific (IHT1) or non-specific (IHT2N) capture molecules. Lane 1: introduced crude asymmetric IHT1-PCR mixture. Lanes 3, 5, and 7: 1 in 10 dilutions of ssDNA as obtained by the use of IHT1-capture molecules, IHT2N-capture molecules or non-loaded SMBs, respectively. Lanes 2, 4, and 6: corresponding supernatants (asymmetric PCR mixtures) after incubation with loaded or non-loaded SMB.

Quantification of streptavidin released from the SMB

Non-loaded SMB that were passed through all incubation / washing steps as described in '*Binding of capture-molecules to streptavidin-coated magnetic beads (SMB'*) (but in the absence of capture molecules), were applied to assess the potential adverse contamination with streptavidin at different incubation temperatures. Concentrations of streptavidin were measured by an immunoassay as follows. Primarily, Maxisorp microtiter modules were coated with 10 µg/ml BSA-Biotin (100 µl/well) in coating buffer (30 mM Na_2CO_3, 200 mM $NaHCO_3$ [pH 9.0]) overnight at 4°C followed by 3 times rinsing with 300 µl of washing buffer (1 x PBS [pH 7.4], 3 mM $MgCl_2$, 0.05 % Tween 20; general washing procedure using an automated plate washer [ELx50, Biotek, Bad Friedrichshall, Germany]). Remaining binding sites were blocked by incubation with blocking buffer (1xPBS [pH 7.4], 2 mg/ml BSA, 0.05 % Tween 20) for 2 h at RT. After incubation for 2h at RT, remains were aspirated and sealed modules stored at 4°C until used. To run the assay, standards or samples were diluted in washing buffer containing 1 mg/ml BSA (WB+) and 100 µl of the dilutions were added to the wells and incubated for 1h at RT. After washing, 100 µl of rabbit whole antiserum against streptavidin (diluted 1:2000 in WB+) were added to the wells and also incubated for 1h at RT. Subsequently, wells were washed and 100 µl of HRP-labelled goat anti-rabbit antibodies (diluted 1:2000 in WB+, yielding a final concentration of 0.125 µg/ml)

added and incubated for another hour at RT. Finally, after washing, bound HRP was detected using BM chemiluminescence substrate (100 µl well) and a Synergy 2 microplate reader (Biotek, Bad Friedrichshall, Germany). Applied streptavidin standard curves were prepared by half-logarithmic dilution series and showed a linear range from 530 down to 1.7 pM (31.6 down to 0.1 ng/ml). Original samples were diluted in WB+ to match the covered range and the absolute content of streptavidin calculated. The corresponding results are shown in Fig. 1C.

CE-SELEX against APC and FXIIIAa

CE-based separations were performed using a PA800 capillary electrophoresis system (Beckman Coulter, Krefeld, Germany) and 32 Karat software. A 60 cm long (50 cm to the detection window) uncoated fused silica capillary with an inner diameter of 50 µm (Beckman Coulter) was conditioned before the first use and rinsed between runs with an pressure of 50 psi (for 5 min each) with 100 mM HCl, 100 mM NaOH, destilled water, and selection / separation buffer (25 mM Tris-HCl, 30 mM NaCl, 1 mM KCl, 1 mM CaCl2 and 1 mM MgCl2, pH 8.3). Applied ssDNA-libraries (IHT1 or IHT2N) were added to 20 µl of selection buffer in a final concentration of 25 µM (500 pmole) for the first selection cycle and heated to 85°C for 5 min followed by snap cooling on ice. Subsequently, the target protein (either APC or FXIIIAa) was added and the mixture was incubated at RT for 30 min before hydrodynamic injection into the capillary (using 4 psi pressure for 5 second, thereby introducing ~ 40 nl of the sample into the capillary). Separation of protein from non-binding ssDNA-molecules took place under electroosmotic flow at a voltage of 25 kV. As determined during previous experiments, the collection window was set during the first 20 min of separation. Samples were collected into tubes containing 150 µl of separation buffer. Library molecules that remained in the capillary were flushed out by reverse rinsing. A total of 120 µl of the collected molecules (6 x 20µl) were exponentially amplified for 30 (IHT1) or 35 (IHT2N) cycles. Subsequently performed asymmetric PCR was routinely conducted for 50 cycles. In case of formation of unwanted by-products (as determined by gel-analysis), however, cycle numbers of asymmetric PCR were reduced to 30 - 45 in order to retain amplification specificity. Subsequently, single reactions were pooled and a total of 500 µl introduced to CaR for isolation of ssDNA. At this, captured ssDNA was eluted at 43°C. A constant amount of enriched library (0.5 µM; 20 pmole) was used during the following selection cycles. In contrast, the concentration of the target enzymes was gradually reduced from 1 µM during the first selection cycle to low nM-concentrations during the sixth (last) cycle.

Yield and purity of ssDNA as produced by asymmetric PCR/ CaR during SELEX

The following Tables S1-S3 show the yield and purity of ssDNA obtained from asymmetric PCR/ CaR during the process of SELEX as determined by UV-measurements.

Table S1. Yield and purity of ssDNA after each cycle of IHT1-based APC-SELEX.

	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	6th cycle
A260	1.48	1.05	1.022	0.645	1.121	0.811
A280	0.781	0.576	0.542	0.335	0.606	0.426
A260/A280	1.9	1.83	1.89	1.92	1.85	1.90
Conc. [µM]	1.95	1.39	1.34	0.85	1.48	1.07

Table S2. Yield and purity of ssDNA after each cycle of IHT2N-based APC-SELEX.

	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	6th cycle
A260	0.715	1.46	1.06	1.134	1.038	1.185
A280	0.408	0.74	0.535	0.577	0.522	0.631
A260/A280	1.75	1.96	1.98	1.96	1.99	1.88
Conc. [µM]	0.93	1.90	1.38	1.48	1.35	1.54

Table S3. Yield and purity of ssDNA after each cycle of IHT1-based FXIIIAa-SELEX.

	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	6th cycle
A260	0.469	1.048	1.353	1.235	1.130	1.719
A280	0.245	0.530	0.715	0.668	0.601	0.885
A260/A280	1.91	1.98	1.89	1.85	1.88	1.94
Conc. [µM]	0.62	1.38	1.78	1.63	1.49	2.26

Filter retention assay

The affinity of the single-stranded random pools, enriched libraries, and obtained single aptamer molecules was assessed by filter retention assay. Molecules (10 pmole) were radioactively 5′-phosphorylated using 20 U of T4 Polynucleotide Kinase (New England Biolabs, USA) in 70 mM Tris–HCl buffer [pH 7.6] containing 10 mM $MgCl_2$, 5 mM dithiothreitol, and 300 µM [γ-^{32}P]ATP (PerkinElmer, USA) and then purified using G-25 microspin columns (GE Healthcare, Munich, Germany). The integrity of the enriched libraries was qualitatively determined using 12% denaturing polyacrylamide gel electrophoresis.

To determine the dissociation constants, serially diluted APC or FXIIIAa (0-1 µM) were incubated with 0.5 nM ^{32}P-labeled ssDNA for 30 min at 37°C in PBS [pH 7.4] containing 1 mg/ml BSA, 10 µM tRNA, 1 mM $CaCl_2$ and 50 µM $MgCl_2$. After incubation, the reactions were passed through pre-equilibrated 0.45 µm nitrocellulose membranes followed by three washings using 150 µl of PBS [pH 7.4] containing 1 mM $CaCl_2$ and 50 µM $MgCl_2$ and then dried out. The retained radioactivity was quantified using a FUJIFILM FLA-3000 PhosphorImager equipped with AIDA Imagequant software (Fujifilm, Düsseldorf, Germany). Data were fitted by 4-parameter logistic curve fit presuming a 1:1 binding stoichiometry of ssDNA:target protein. K_d values were determined from at least two independent experiments.

Cloning and sequencing

The aptamer pool from the SELEX cycle that showed the highest apparent binding affinity was cloned into pGEM®-T vectors (Promega, Mannheim, Germany). For the IHT1- and IHT2N-based selections against APC, 19 and 32 colonies were sequenced, respectively. For the IHT1-FXIIIAa-selection, 32 colonies were sequenced. Sequencing was done using M13 primers and an ABI 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

Production of identified individual aptamers by asymmetric PCR/ CaR and determination of binding affinity

Identified single aptamers were produced from PCR-products using asymmetric/ CaR. Aptamers were radioactively labeled and tested for binding affinity as described above. The capture-efficiency and quality of the yielded ssDNA is demonstrated in Fig. S5. The results of the filter retention analysis are shown in Fig. S6.

As can be seen in Fig. S5, probably due their individual tertiary structures, single monoclonal ssDNA aptamers did not clearly separate from the corresponding double-stranded PCR products during electrophoresis. This was especially true for the FXIIIAa-aptamers and the aptamers #2 and #3 of the IHT2N-based selection against APC. In cases were distinct bands of ssDNA could be assessed, virtually complete capturing of ssDNA from the asymmetric PCR mixtures could be observed.

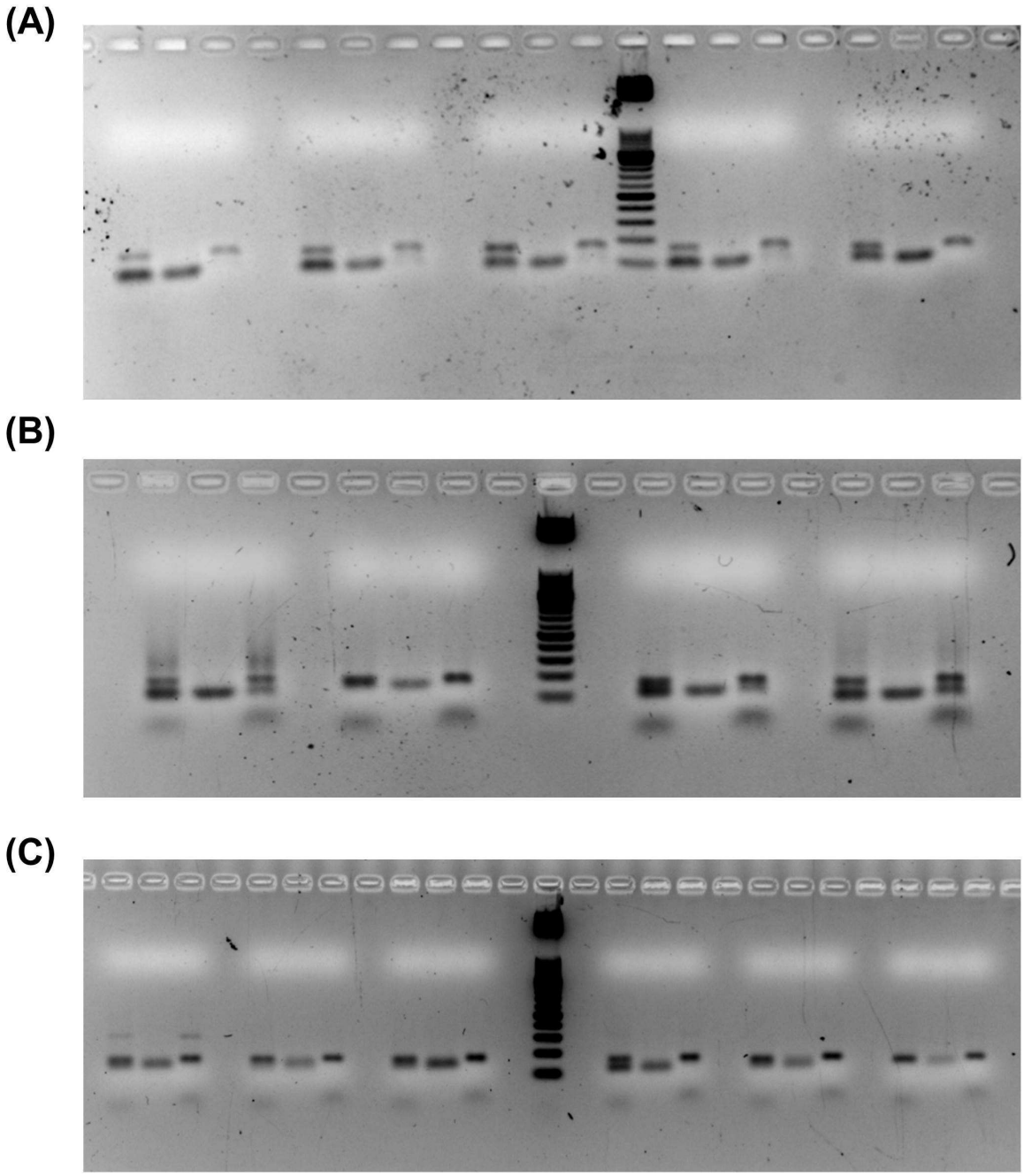
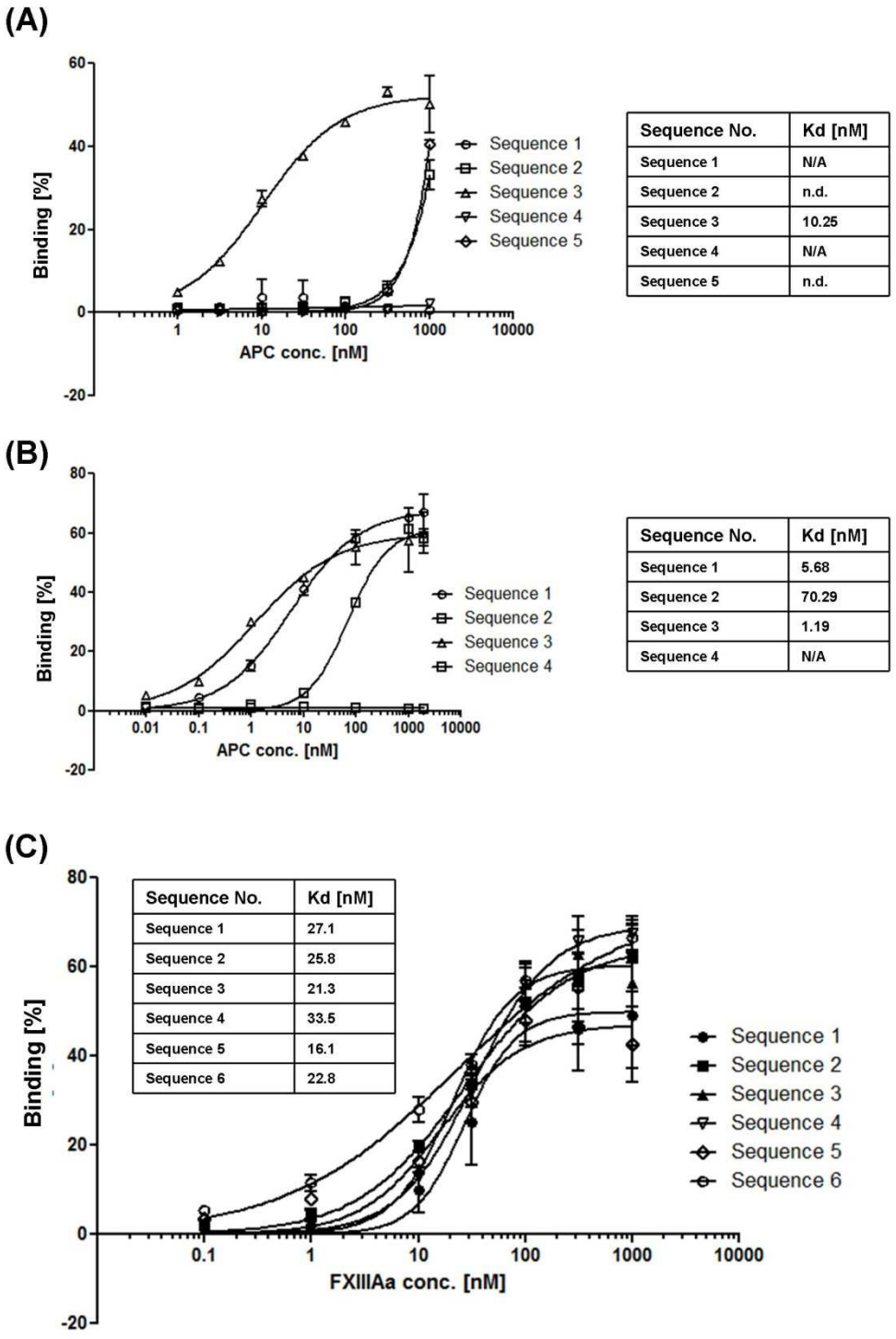


Fig. S5. Agarose gel analysis (stained with ethidium bromide) of asymmetric PCR mixtures, obtained monoclonal ssDNA (1:10 diluted) and supernatants after removal of loaded SMB (from left to right within each group) after introduction of identified single aptamer-clones to asymmetric PCR/ CaR. A, B: selection against APC using the IHT1 (A) and IHT2N library (B) respectively. C, selection against FXIIIAa using the IHT1 ssDNA library. A 50bp dsDNA-ladder was used in each gel.



Sequence No.	Kd [nM]
Sequence 1	N/A
Sequence 2	n.d.
Sequence 3	10.25
Sequence 4	N/A
Sequence 5	n.d.

Sequence No.	Kd [nM]
Sequence 1	5.68
Sequence 2	70.29
Sequence 3	1.19
Sequence 4	N/A

Sequence No.	Kd [nM]
Sequence 1	27.1
Sequence 2	25.8
Sequence 3	21.3
Sequence 4	33.5
Sequence 5	16.1
Sequence 6	22.8

Fig. S6. Filter retention analysis of individual aptamers. (A) IHT1-based APC-SELEX. (B) IHT2N-based APC-SELEX. (C) IHT2N-based FXIIIAa-SELEX. Shown K_D-values were determined by 4-parameter logistic curve fit; n.d., not determined; N/A, no binding observed up to 1 µM.

In silico folding predictions

The mfold web sever (M. Zuker. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res.2003, 31, 3406-15) as available at:

http://mfold.rna.albany.edu/?q=mfold/dna-folding-form

was used at default settings to predict the folding patterns of the identified single aptamers. Predicted foldings of the 4 efficiently binding APC-aptamers are shown in Fig. S7. As expected, the 3'-ends of the single sequences appear to be accessible to the corresponding capture molecules. The same was found for the FXIIIAa-binding aptamers (data not shown). However, since these predictions are limited to Watson-Crick base pairing in two-dimensional space, their validity is limited.

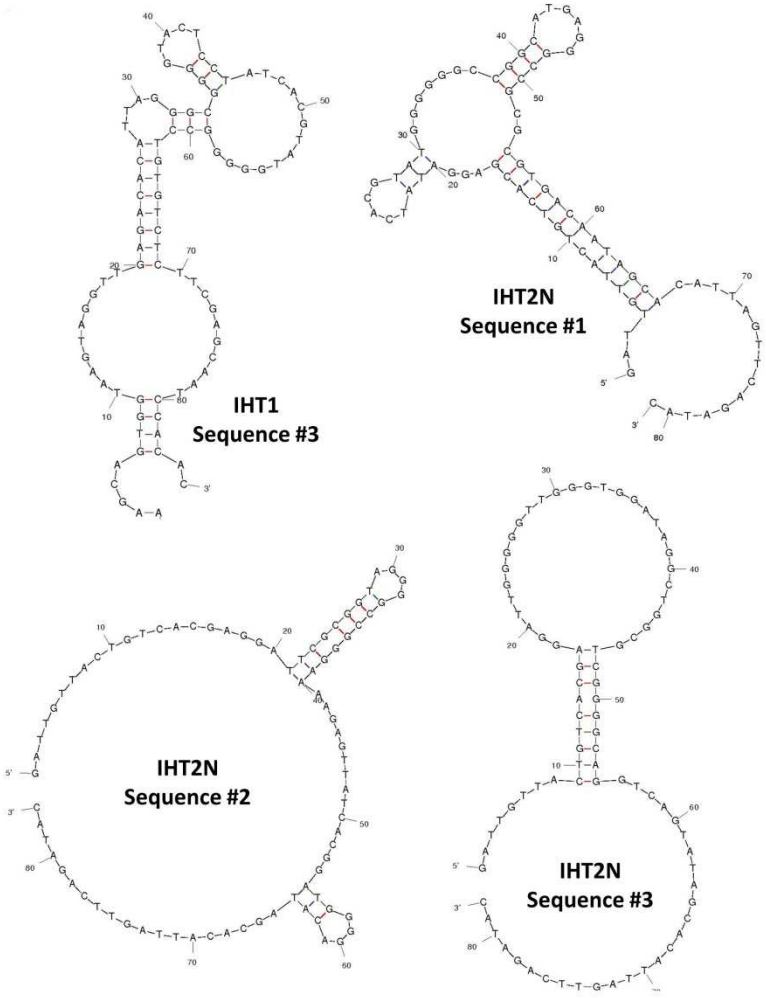


Fig. S7. Folding patterns of the identified APC-binding aptamers as determined by mfold.

Determination of the reusability of loaded SMB

Clone #1 identified during IHT1-based selection against APC was used to assess the reusability of SMB loaded with (IHT1) capture molecules (SMB+). After release of captured ssDNA by 3 consecutive incubations with a fresh batches of water, SMB+ were washed and again introduced to the CaR-procedure. In total, 4 cycles of capture and release were performed. As shown in Fig. S7, virtually complete capturing of ssDNA from the asymmetric PCR mixtures could be observed. As shown in Table S4, comparable yields and purities were found.

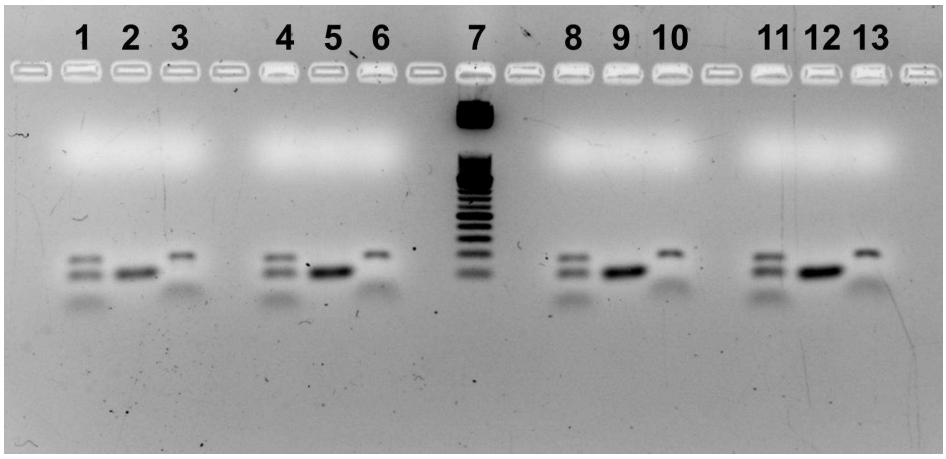


Fig. S8. Agarose gel analysis (stained with ethidium bromide) of obtained ssDNA by consecutive usage of SMB+ during CaR. Lanes 1, 4, 8, and 11: introduced crude asymmetric PCR mixture; lanes 2, 5, 9, 12: 1 in 10 dilution of yielded ssDNA when using SMB+ for the first, second, third and fourth time, respectively; lanes 3,6,10 and 13: supernatants after incubation of asymmetric PCR mixture with the SMB+ for the first, second, third and fourth time, respectively; lane 7: 50 bp dsDNA ladder.

Table S4. Yield and purity of ssDNA obtained after consecutive use of SMB+.

	1st use	2nd use	3rd use	4th use
A260	**0.432**	**0.513**	**0.524**	**0.508**
A280	**0.214**	**0.246**	**0.272**	**0.261**
A260/A280	**2.02**	**2.08**	**1.93**	**1.95**
Conc. [µM]	**0.58**	**0.68**	**0.69**	**0.67**