reMelting curve analysis as a tool for enrichment monitoring in the SELEX process†

Jeroen Vanbrabant,*[a] Karen Leirs,[b] Katrijn Vanschoenbeek,[a] Jeroen Lammertyn[b] and Luc Michiels*[a]

Current aptamer selection procedures enable limited control and transparency on how the DNA selection pool is evolving. Affinity tests and binding analyses are not always informative. Here we show that real-time PCR provides a valuable tool for the follow-up of aptamer selection. Limited time, work and amount of amplified ssDNA make this an interesting instrument to set-up a SELEX design and monitor the enrichment of oligonucleotides. reMelting Curve Analysis (rMCA) after reannealing under stringent conditions provides information about enrichment, compared to a random library. Monitoring the SELEX process and optimising conditions by means of the proposed methods can increase the selection efficiency in a controlled way. rMCA is applied in enrichment simulations and three different selection procedures. Our results imply that rMCA can be used for different SELEX designs and different targets. SELEX pool diversity analysis by rMCA has been proven to be a useful, reproducible tool to detect and evaluate enrichment of specific binding aptamers while the selection procedure is being performed.

Introduction

Different iterative steps are involved in the selection of aptamers by means of SELEX (Systematic Evolution of Ligands by Exponential Enrichment): incubation with a target or blocked surface in binding buffer, washing with wash buffer, elution by denaturation and amplification of the remaining ssDNA pool.[1] Conditions of both positive and negative selections are essential for efficient selection of aptamers. Although the selection conditions are critical, they are to a great extent documented trial and error. Only after a number of SELEX rounds (*i.e.* 6–15) the selected DNA pool is sequenced and checked for enrichment of target-specific oligonucleotides.[1] Assessing the effect of separate selections under different binding and washing conditions on the DNA pool provides valuable information to increase selection efficiency. Monitoring of the progression is critical for the success of an *in vitro* selection experiment and the characteristics of the selected aptamers. This allows early intervention and adjustment of the selection pressure and stringency to achieve the desired activities of the selected aptamers.[2] To date, both direct measurement of SELEX progression, in terms of affinity of the ssDNA pool for the target, and indirect measurements have been addressed.

Measuring the amount of target-binding nucleotides in selection pools offers a way of monitoring enrichment directly by assessing average affinity of the SELEX pool for the target. For example, ELISA-like assays enable comparison of different consecutive ssDNA selection pools. A fraction is labeled with a fluorophore or enzyme and incubated on a target-coated substrate. Binding is then visualized and quantified by fluorescence or chemiluminescence on a membrane by blotting[3] or on magnetic beads to perform flow cytometry for fluorescence acquisition.[4,5] Other studies use radiolabeling to visualize and quantify the bound DNA.[6] Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) has proven to be a good way to control and compare SELEX fractions for target binding and even determine affinity constants (K_D) of the SELEX pools.[7] Another way of direct screening for affinity is the HAPIscreen (High throughput APtamer Identification screen) methodology.[8] Target and candidate fractions are coated on beads, which results in chemiluminescence when the beads come in proximity *i.e.* when there is interaction between them. As such, an arbitrary score for affinity is determined and only fractions with a high score are evaluated further.

These described assays suffer from several limitations and drawbacks. They are time- and material-consuming and do not always offer the required resolution or sensitivity for concise SELEX monitoring. One needs a very sensitive and good assay to see the difference in binding capacity of consecutive SELEX pools, especially in the onset of selection, when there are still a lot of random, non-specific oligonucleotides present in the eluted fractions. These assays are also target and platform dependent and not applicable for all SELEX targets. For example, small

[a]BIOMED, University of Hasselt, Diepenbeek, 3590, Belgium. E-mail: 1jeroenvanbrabant@gmail.com; luc.michiels@uhasselt.be

[b]BIOSYST-MEBIOS, University of Leuven, Leuven, 3001, Belgium

molecules often lack functional groups that are required for attachment of the target to the carriers needed in these assays. When they have a functional group, there is the risk of using a functional group the aptamer recognizes for binding. NECEEM measurements in solution yet suffer from other limitations. Not all targets are suited for analysis by capillary electrophoresis. For example, when a small target has a low pI and the charge to size ratio of the target is highly similar to that of the ssDNA, it is difficult to distinguish the complex peak from the peak with free ssDNA.[9,10] Moreover, these assays all involve labeling the ssDNA oligonucleotides with fluorophores, biotin or other functional groups. This labeling can change the characteristics, structure and hence binding properties of the ssDNA pools and decreases the power of these assays to assess enrichment and evolution in the selection process in a concise way. As stated by Rowe et al.,[11] complications may arise with the use of labels, either by altering the protein chemistry or from interactions between tags and the target aptamers, which can lead to either high background noise or a decrease in aptamer specificity. Since direct monitoring of affinity is often limited by the need for labels, signal enhancers or other functional groups, monitoring of progression needs to be done in other ways. As label-free and radio-labeled affinity assessments need specialised instrumentation, methods that indirectly measure selection progression in the SELEX pools have been developed.

Monitoring the *in vitro* selection process can be performed in terms of ssDNA quantities that are eluted during different SELEX rounds. Niazi et al.[12] performed seven selection rounds until the ssDNA quantity (measured by spectrophotometry) in the eluted pools was 90% of the ssDNA quantity that was added to the target. A more straightforward way of monitoring is assessing sequence diversity of the pools and the most informative but elaborate technique to do this is sequencing individual clones. However, this is expensive and one does not know how many clones to sequence. Furthermore, it is not applicable to the more random stages of the procedure. Müller et al.[2] proposed a method based on sequence diversity that enables monitoring of a SELEX procedure by employing denaturing high-performance liquid chromatography (dHPLC). The technique is useful for both analysis of the selection progression and separation of distinct sequences. Charlton and Smith[13] reasoned that the sequence complexity of an ssDNA pool can be quantified by observing the renaturation rate of its double-stranded PCR product by performing C_0t analysis. By deriving an equation for reannealing at a distinct temperature, the number of individual sequences in the pool can be determined. C_0t analysis was initially developed to measure the nucleotide complexity of genomes.[14] In addition, Amplicot analysis measures 'diversity', defined as the number of different full-length sequences in a PCR product. The annealing rates of the samples decrease as the diversity of their templates increases.[15] However, this analysis appears impossible for libraries with diversities higher than 10^6. Remelting curves following an annealing step are observed to be a better characteristic indicator for the analysed diversity.[16] On an increase of diversity, a decrease in melting temperature was detected, denoting the amount of imperfectly formed heteroduplex. The applied annealing temperature determines the resolution in a given diversity range. Using DiStRO (Diversity Standard of Random Oligonucleotides), population dynamics of an aptamer selection against daunomycin were analysed and quantified.

Various attempts have been made to monitor the selection progress of target-specific ligands. Direct measurements of SELEX pool affinities for the target are prone to several limitations and need a target-specific approach. Therefore, indirect monitoring applies the characteristics of the DNA pool as such, ideally without the need for labeling and independent of the target characteristics. Diversity analysis of the recovered DNA pools is the most straightforward way to achieve this. In this study, melting curve analysis after a short reannealing step (rMCA) has been proven to be a useful tool for diversity analysis of a given SELEX pool and hence for enrichment monitoring, independent of extra DNA modifications, the SELEX target and design. The principle of rMCA monitoring is shown using an enrichment simulation procedure. Furthermore, rMCA is applied to different SELEX strategies (plate based, bead based and capillary electrophoresis based SELEX) using targets with ranging physical properties (C-Reactive Protein, 17β-estradiol and peptide X) to demonstrate the general applicability of the rMCA as a monitoring tool. The selection on peptide X is analysed with the proposed method on a different real-time PCR platform. Monitoring different approaches in different labs demonstrates the robustness, reproducibility and ease of use of this method. This proves complexity analysis of SELEX pools by rMCA to be very useful to increase selection efficiency by hands-on evaluation of the selection process steps and making the right decisions.

Experimental section

Enrichment simulation in random starting libraries

All used ssDNA libraries, primers and oligonucleotides were synthesised by Integrated DNA Technologies (IDT, Haasrode, Belgium). Two different starting libraries are spiked with increasing concentrations of aptamer sequence, from 0% enrichment to 100% enrichment. The random library used for CE-SELEX is spiked with IgE aptamer, D 17.4.[17] The random library used for CRP selection is artificially enriched with either one single oligonucleotide or a homogeneous mix of ten different oligonucleotides, extended with the primer binding sites of the corresponding applied random libraries to allow amplification. The analysis is performed on the Lightcycler® 1.5 carousel (Roche, Merelbeke, Belgium) with 20 μL reaction mixtures containing: 1× SYBR Mastermix (Roche), 100 nM of each primer, extra 2 mM MgCl$_2$. 100 pM (for one oligonucleotide or the mix of ten) and 10 pM (for IgE aptamer) of enriched library is added. The PCR protocol consists of 5 minutes of denaturation, followed by 35 cycles of 5 s at 95 °C, 3 s at primer annealing temperature and 4 s at 72 °C for elongation. After amplification, a first melting curve analysis is performed after the final elongation at 72 °C plus a second remelting analysis after a short reannealing phase. Different reannealing temperatures and times are tested for rMCA. Reannealing is tested for 30 s, 1 and 2 minutes at 65 °C, 70 °C and 75 °C. The melting rates for both melting curve analyses to 95 °C are set at 0.1 °C s^{-1}.

Plate SELEX for CRP aptamers

A 40nt (nucleotides) library is constructed, flanked by 18nt primer binding regions: GCACCAGCATATTCGATT-40N-GGCTAGTAGGTGCATCAG. The primers used for amplification are F: GCACCAGCATATTCGATT and R: CTGATGCACCTAC TAGCC. CRP (BBI Solutions, Cardiff, UK) is covalently coupled to NucleoLink™ Plates (Life Technologies, Ghent, Belgium) by following the instructions provided by the manufacturer. These plates are deactivated with 100 mM ethanolamine. Plates with only ethanolamine are used, since negative selections are as important as positive ones. Each selection cycle consists of a negative selection step, a positive selection step and another negative selection step prior to PCR amplification, to avoid amplification of non-specific binders for CRP. By-product formation by excessive PCR amplifications is avoided by performing only 15 amplification cycles per round and PCR-product clean-up by 4% agarose gel-electrophoresis separation and extraction of the 76bp. band. During positive selections, elution is performed in four separate fractions. The buffer for incubation is 10 mM Hepes + 5 mM $CaCl_2$, pH 7.4; washing buffer is $1\times$ PBS, pH 7.4 and elution buffer is $1\times$ TE-buffer + 3 M urea, pH 8 at 80 °C. Before negative selection steps, sonicated salmon sperm ssDNA (Life Technologies) is added to a $50\times$ excess to avoid losing CRP binding oligonucleotides by non-specific DNA absorption in negative selections. After each selection step, a fraction of the elutions is analysed separately by real-time PCR on the Lightcycler® (Roche) for quantitative analysis, but also to assess genetic diversity of the pool by means of rMCA. PCR mix and amplification protocol are the same as mentioned for the enrichment simulation, with a primer annealing temperature of 58 °C. Reannealing is set at 1 min at 70 °C.

Bead SELEX for 17β-estradriol aptamers

A random 40nt library is constructed, flanked by 21nt primer-binding regions: GTCACCGTACTCAGCCTCTCA-40N-GATG TATTGCGAGAGTTTGGC. The primers used for amplification are F: GTCACCGTACTCAGCCTCTCA and R: GCCAAACTCTCG CAATACATC. Estradiol (E2) Sepharose® 6B affinity chromatography beads (Polysciences Inc., Eppelheim, Germany) are used for positive selections and nortestosterone Sepharose® 6B beads (Polysciences Inc.) are used for counter selections. All beads are blocked extra with a mix of 1% BSA (Sigma, Diegem, Belgium), 1% marvel (Sigma), 4% synthetic blocker NB3025 (NOF corporation, Tokyo, Japan) and an excess of sonicated ssDNA from salmon sperm (Life Technologies). Each selection cycle consists of a counter selection step, a positive selection step and another counter selection step prior to PCR amplification and amplicon clean-up. This is done to avoid amplification of non-specific binders for E2 and to direct aptamer binding to a specific epitope of E2. Buffer for incubation is 10 mM Hepes, pH 7.4; washing buffer is $1\times$ TBS, pH 7.4 and elution buffer is $1\times$ TE-buffer + 3 M urea, pH 8 at 80 °C. Before counter selection steps, sonicated salmon sperm ssDNA (Life Technologies) is added to a $50\times$ excess to avoid losing E2 binding oligonucleotides by non-specific DNA absorption in counter selections. SELEX fractions are amplified and analysed

as described above, with a primer annealing temperature of 56 °C. Reannealing was set at 1 min at 72 °C.

CE-SELEX for peptide X aptamers

A random 40nt library is constructed, flanked by 20nt primer regions: TCGCACATTCCGCTTCTACC-40N-CGTAAGTCCGTGT GTGCGAA. The primers used for amplification are F: TCGCA CATTCCGCTTCTACC and R: TTCGCACACACGGACTTACG. The peptide X is synthesised by Eurogentec (Seraing, Belgium). Selection is performed using a P/ACE-MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) with an uncoated fused silica capillary (length: 40.2 cm, outer diameter: 375 μm, inner diameter: 50 μm). The 488 nm line of a 3 mW argon-ion laser is used for excitation of the fluorescently labeled DNA. The emission is measured using a 520 ± 10 nm band pass filter. Running buffer is TGK (tris glycine potassium phosphate) buffer (25 mM tris(hydroxyamino)methane, 192 mM glycine and 5 mM K_2HPO_4, pH 8.4). Samples are incubated in tris magnesium buffer solution (10 mM tris(hydroxyamino) methane and 1 mM $MgCl_2$, pH 7.2). Separation is performed at 15 kV under normal polarity. Free DNA sequences will move slower through the column so only complex DNA pools are collected to use for the following SELEX round. After each selection step, a fraction of the pool is amplified by real-time PCR (Rotor-Gene Q, Qiagen, Venlo, Netherlands) followed by rMCA. A 30 μL reaction mix is prepared containing $1\times$ AccuMelt™ HRM SuperMix (Quanta Biosciences, Gaithersburg, USA), 67 nM of each primer and equal fractions SELEX pools. Before amplification, a denaturation step at 95 °C is performed for 10 minutes. This is followed by a two-step amplification process (5 s at 95 °C and 30 s at 60 °C) of 35 cycles. The amplification products are melted from 60 °C to 95 °C by increasing the temperature. Reannealing is allowed at 70 °C for 1 minute followed by a second melting analysis from 70 °C till 95 °C at 0.5 °C s^{-1}.

Results and discussion

Melting curve analysis after short reannealing (rMCA)

Annealing and melting kinetics of DNA is extensively studied since C_0t analysis,[14] from gene analysis[18] and genome sequencing[19] to analysis of selection libraries. Schütze et al.[16] created a diversity calibration standard (DiStRO) for the evaluation of DNA pools by analysing remelting curves after an annealing step of 180 minutes. Remelting analysis is applied and optimised here for monitoring diversity changes during the SELEX procedure, regardless of design and target properties. In a standard melting curve analysis, all high-copy pools are slowly denatured directly after the last elongation phase of amplification, containing in theory mostly a homoduplex. In this way, MCA analyses melting temperature in terms of internal structures and the stability of the homoduplexes. In rMCA, a short reannealing step at high temperature is introduced after total denaturation. In this stringent reannealing phase, hetero- and homoduplexes are formed and their proportions completely depend on the sequence diversity. reMelting curve analysis is

then performed as before and analyses melting temperatures in terms of hetero-*versus* homoduplexes. As the diversity of SELEX pools decreases due to enrichment for target-binding ssDNA species, more homoduplexes will be formed in the reannealing phase and one can expect a shift in the obtained rMCA melting temperatures. Consequently, rMCA will provide crucial information on enrichment in terms of diversity.

rMCA analysis of simulated enrichment

Simulation of enrichment in different libraries is performed to demonstrate the broad applicability of rMCA, studying amplification and melting behavior of complex DNA pools with different diversities.

As shown in Fig. 1, all three simulations show a similar trend in both amplification (Fig. 1 up) and melting data (Fig. 1 low). Where 100% enriched fractions show the normal exponential amplification curves, 100% random libraries show a drop in fluorescence after reaching a maximum. This occurs after 12 amplification cycles for one oligo (Fig. 1 left), 15 for the oligo mix (Fig. 1 middle) and 20 cycles for D 17.4 (Fig. 1 right). The amplification curves change along with increasing enrichment of spiked oligonucleotide(s). When diversity decreases, the drop in fluorescence is less pronounced and disappears under more enriched conditions. This can be explained by the same principle as the rMCA. At this drop, there is no more amplification (limitation of primers or dNTP) and fluorescence acquisition at 72 °C is performed after a short reannealing phase instead of an elongation phase. Random pools contain more unstable heteroduplexes at this temperature and fluorescence drops.

Remelting data show an evolution from a gradual melting behavior of 100% random library (broad melting zone from 70–75 °C) to a melting peak at the expected melting temperature of the spiked oligonucleotide or the average melting temperature of the oligo mix, which is reflected in a broader melting peak. This proves there is indeed a shift from a lower, broad remelting peak to a more distinct, higher remelting temperature as diversity decreases and enrichment occurs. Melting

peaks at 72 °C gradually disappear and evolve to the (average) melting temperature of the more stable duplexes of the enriched oligonucleotide(s). The speed and form of this shift differs, which makes the sensitivity of this method dependent on the library and enriched oligonucleotide(s). In Fig. 1, 2% enrichment of one oligonucleotide has a distinct effect on the remelting peaks in column 1, whereas almost no effect in column 3 with D 17.4 enrichment. Nevertheless, there is a clear general effect of higher remelting temperatures as the diversity decreases, with an enrichment of 10% clearly distinguishable from the random DNA pool in the three cases. Blank amplifications (gray curves) show a rise in fluorescence after 30 amplification cycles and a melting peak which correlates with the formation and disruption of primer dimers after iterative heating and cooling of the mix.

Simulation also shows that reannealing occurs very fast: reannealing for 30 s gives the same empirical results as for 2 min (ESI, Fig. S1†). Shorter reannealing times increase stringency and reflect the differences in enrichment better whereas longer reannealing results in sharper melting peaks. Changing the reannealing temperature changes the window and resolution of rMCA. Reannealing at 70 °C allows formation of heteroduplexes and hence, there is a broad melting zone starting from this reannealing point under poorly enriched conditions. Reannealing at a higher temperature allows one to zoom in on enriched fractions. When reannealing at 75 °C, the melting peak of the random library at 72–74 °C will not be formed and remelting starts at 75 °C (Fig. 2).

The fluorescence drop in the amplification curves of highly diverse SELEX pools indicates there is already heteroduplex formation before the amplification cycles are finished. This means that the first MCA is not performed after final elongation, but after a short reannealing step at primer annealing temperature and elongation temperature. Comparison between MCA and rMCA results indeed shows similarities but the rMCA data after reannealing at 70 °C display the increasing enrichment more accurately.

Since this is a simulated enrichment experiment, all pools endured the same amount of controlled amplification cycles. The effect of changing remelting temperatures of the DNA pools can only be attributed to the simulated changed composition of the DNA pools and not to PCR artifacts and by-product
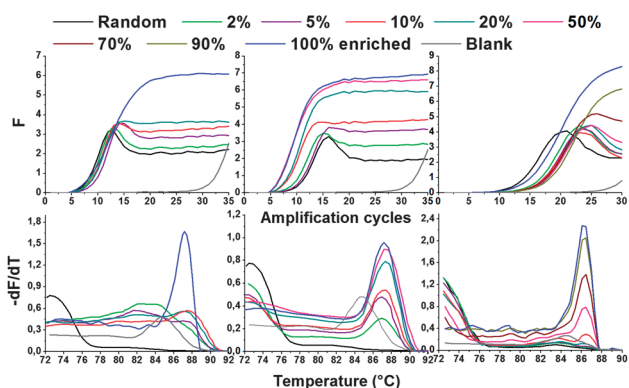

Fig. 1 Amplification data (up) and rMCA (down) of three enrichment simulations with random library and 2–20 & 100% enrichment of one oligonucleotide (left); random library and 2–50 & 100% enrichment of oligo mix (middle) and random library with 2–100% enrichment of D 17.4 IgE aptamer (right) after 1 min reannealing at 70 °C.
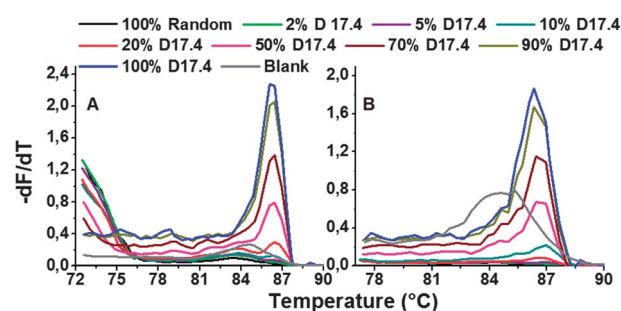

Fig. 2 rMCA of simulated 2–100% enrichment of D 17.4 in random library analysed after 1 min at 70 °C reannealing (A) and 1 min at 75 °C reannealing (B).

formation due to excessive PCR cycling. This shows the rMCA method to be specific for changing DNA pool compositions and diversities.

rMCA of different SELEX procedures – general applicability

rMCA is applied successfully in three different selection procedures for different targets. It is demonstrated to be applicable for different SELEX designs (plate based, bead based and CE-SELEX) and targets of various physical properties: a macroprotein (CRP, 115 kDa, pI 5.3–7.4),[20] a steroid (17β-estradiol, 272 Da, pK_a 10.7)[21] and a peptide (X, 1.6 kDa, pI 11).

In the plate SELEX protocol for CRP, four elution fractions are collected in the positive selections which are separately analysed by rMCA. Before round 10, all four fractions show similar melting peaks. After round 10, there is a clear differentiation in DNA composition of separate fractions, indicated by different melting peaks (ESI, Fig. S2†). At this point, the selection procedure is continued in parallel with these four DNA fractions separately. As all elutions are analysed separately, it is possible to monitor the whole SELEX procedure in detail, from round 1 to round 16 (Fig. 3.1). This shows the progress of selection and shifts of the average remelting temperature of the analysed ssDNA pools as enrichment takes place. rMCA data of fraction 3 are shown in Fig. 3.1 and illustrate clear evolution from a random DNA pool with a melting temperature of 72 °C (Fig. 3.1A) to a more enriched one with a higher average melting temperature of 84 °C (Fig. 3.1H). Sequencing analysis of the 4 fractions after 16 rounds of selection confirms enrichment for 4 oligonucleotides, occurring in all fractions. Fraction 3 is especially enriched for one oligonucleotide (ESI, Table S1 & S2†). Affinity analysis of selected aptamers by means of SPR (Biacore™ T200; Biacore™, Uppsala, Sweden) is performed by multi-cycle kinetic analysis on CRP coated CM4-chips and shows nanomolar affinities for aptamer A1 (K_D = 19 nM) (ESI, Fig. S3†) and A4 (K_D = 13 nM) (ESI, Fig. S4†).

The selection of ssDNA aptamers for 17β-estradiol is performed on sepharose beads. Fractions of the remaining DNA pools after each positive selection are analysed with rMCA, after an amplification step of 35 cycles and a reannealing phase of 1 min at 72 °C. After 8 rounds, the melting peak of the DNA pool is evolved from a broad random peak at 73 °C (Fig. 3.2A) to two specific peaks at 84 and 87 °C (Fig. 3.2H) and a low random part. Sequencing of the pool after selection round 8 confirms enrichment of oligonucleotides and specific motifs within the oligonucleotides. In both selections, the melting peaks shift gradually from a broad early-melting zone to higher melting temperatures and the proportion of early melting random DNA gradually decreases as enrichment proceeds.

CE-SELEX on peptide X is challenging because of the lack of a ssDNA-X complex peak in the CE separation. rMCA is performed on a different real-time PCR device, the Rotor-Gene Q (Qiagen) to test the general applicability of this diversity analysis method. rMCA after 1 min reannealing at 70 °C indicates that there is still a large fraction of random DNA present, illustrated by the large melting zone at low temperatures; 70–74 °C. This analysis is also performed on the Lightcycler® to compare the results. In Fig. 3.3,
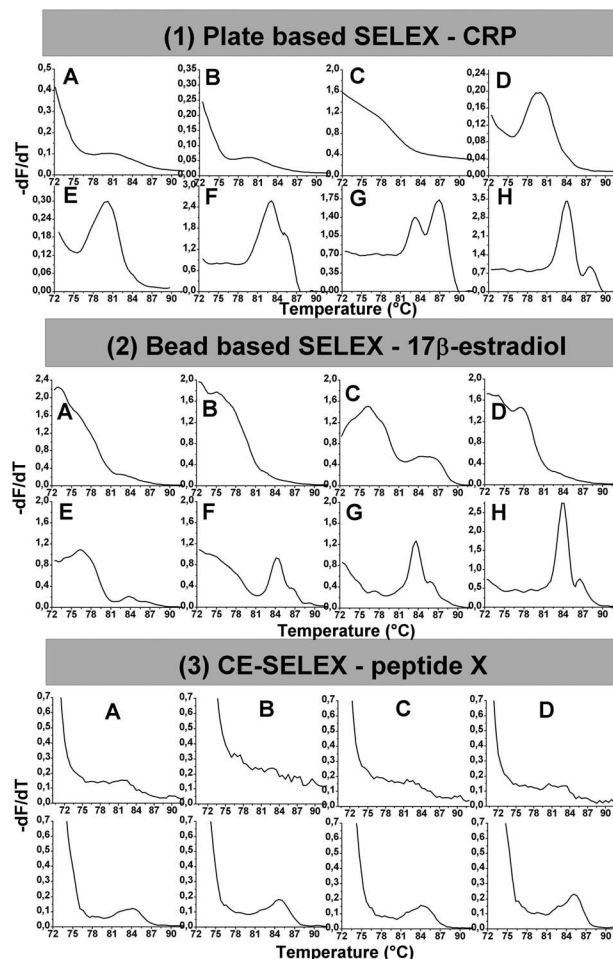

Fig. 3 (1) rMCA of plate based SELEX rounds for CRP; random library (A); round 1 (B); round 3 (C); round 7 (D); round 9 (E); round 12 (F); round 15 (G) and round 16 (H). (2) rMCA of bead based SELEX round for 17β-estradiol; round 1 (A); round 2 (B); round 3 (C); round 4 (D); round 5 (E); round 6 (F); round 7 (G) and round 8 (H). (3) rMCA of CE-SELEX rounds for peptide X on 2 devices; up: analysis on Rotor Gene-Q; down: analysis on Lightcycler®; round 1 (A); round 4 (B); round 6 (C); round 11 (D).

both rMCAs are compared. In both analyses, the large remelting zone which correlates with random DNA (72–75 °C) remains throughout the whole selection procedure.

Nevertheless, library evolution and changes in DNA composition are clear when one focuses on higher temperatures, correlating for enriched oligonucleotides. rMCA with a higher reannealing temperature of 75 °C (Fig. 4), changing the rMCA window, elucidates changes in the DNA pool better and indicates that enrichment occurs at a slow rate. In the onset of the procedure, rMCA only results in a broad melting zone. When the procedure continues, after round 4, there is a growing remelting peak appearing at higher temperatures. Since no discrete ssDNA-X complex peak can be discriminated, the collection window is chosen to be sufficiently broad, reducing separation efficiency of specific and non-specific oligonucleotides.

Although rMCA on the Rotor-Gene Q and the Lightcycler® give the same empirical results, the Lightcycler® and the SYBR
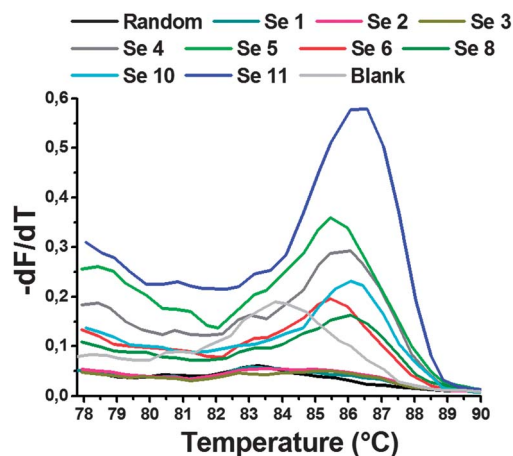
Fig. 4 rMCA of consecutive CE-SELEX rounds for peptide X after 1 min reannealing at 75 °C.

Mix appear more sensitive in fluorescence acquisition in the remelting phase. In the Rotor Gene Q the temperature transition rate needs to be increased from 0.1 °C s^{-1} to 0.5 °C s^{-1} to produce conclusive data. rMCA enables monitoring of the SELEX process regardless of target properties. When direct monitoring of affinity of the SELEX pools for the target is not possible, as demonstrated above or due to lack of extra functional groups for labeling or attachment, rMCA provides an interesting tool for arbitrary detection of enrichment in the analysed DNA pools. As it is clear from the enrichment simulation and three SELEX analyses, the observed shifts in remelting temperature of the pools are different since they are dependent of the random library and enriched oligonucleotides. Moreover, the eventual remelting temperature depends on the melting temperature of the enriched dsDNA sequences which are different for each selection or target and gives no extra information on enrichment. Although this method is not generally quantifiable for enrichment, it allows real time monitoring of the ssDNA pool diversity while the selection is performed and detection of sufficient enrichment in the pool to start sequencing, as the random remelting peak gradually disappears in favor of a higher, distinct DNA pool remelting temperature. In the CRP SELEX, the random remelting peak completely disappears and only one sequence is highly enriched. For the bead-SELEX, there still is a low random remelting peak and sequencing analysis shows a level of randomness, as binding motifs are highly enriched, and other parts of the sequences are still random. For the CE-SELEX, no enrichment is observed after sequencing analysis, which is illustrated by the random remelting peak that remains throughout the SELEX procedure.

Optimising and monitoring the SELEX progress – SELEX design and contamination detection

As mentioned in the selection protocol for CRP aptamers, sequential elutions are analysed separately by rMCA. After SELEX round 10, the selection procedure is continued in parallel with these four DNA fractions separately, because rMCA illustrated different DNA compositions (ESI, Fig. S2†). These differences can be explained by selection stringency differences: the oligonucleotides that are eluted in fraction 4 have survived the three previous elutions. After 16 rounds of parallel selection, both amplification and remelting data indicate enrichment in the four fractions. Moreover, these fractions still differ from each other, demonstrated by both the amplification curves and rMCA data (Fig. 5). The melting peaks of rMCA (Fig. 5B) have shifted from 72 °C to higher temperatures in all of them. The position and form of these peaks differ for each fraction. Fraction 3 forms a clearly distinct peak at 84 °C, whereas fraction 1 and 2 form broader peaks from 80–86 °C. The difference is reflected in the amplification curves as well (Fig. 5A), with a drop in fluorescence after 4 amplification cycles for fraction 1 and 2 and the absence of this drop for fraction 3. Fraction 4 demonstrates an intermediate enrichment and an intermediate drop. Sequencing results reflect these differences, with one aptamer highly enriched in fraction 3, whereas the other fractions contain an enrichment of multiple sequences (ESI, Table S1†). Following enrichment in different elutions results in selection of CRP aptamers in four fractions of different stringencies, which each have their own aptamers enriched (Fig. 5). The presented method can be used to optimise conditions for selection steps. It may be useful to assess the effect of negative or stringent positive selection steps on the DNA library before starting the SELEX procedure. Both quantitative and qualitative analysis of DNA pools provide valuable information to take into account when setting up a SELEX procedure. Moreover, in the onset of selection, rMCA detects possible contaminations very quickly as they appear as a large, narrow melting peak in an otherwise broad melting zone.

Importance of DNA quantity for rMCA

Monitoring the ssDNA aptamer selection procedure for CRP by real-time PCR shows that the eluted fractions contain low amounts of DNA. When performing the remelting analysis on low copy number samples, the remelting data shows a peak at the same position as the blank sample, indicating that primer dimers are melted here. When analysing the same sample with a pre-amplification step, the remelting data change to a melting peak at 72 °C, corresponding to the melting peak of the random library (ESI, Fig. S5†).
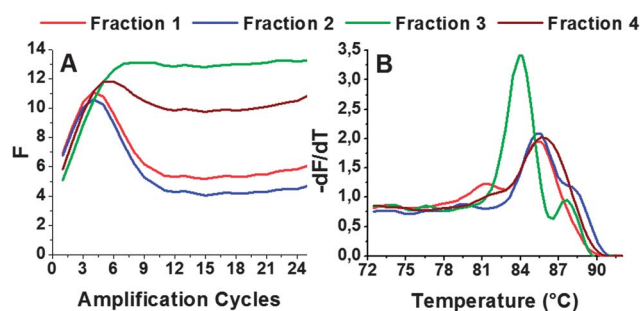

Fig. 5 Amplification curve of 4 SELEX fractions after SELEX round 16 (A) and rMCA data of 4 SELEX fractions after SELEX round 16 (B).

Future prospects

Expansion of the repertoire of rMCA will further demonstrate the power of this remelting method for assessing enrichment in various SELEX designs, on different real-time PCR platforms and for different targets and starting libraries. Effects of the primer binding regions are to be determined. As these regions are equal for all sequences in the pool, they may reduce rMCA resolution capacity. Studying the effect of primer removal on the outcomes and resolution of diversity analysis by rMCA can result in more sensitive analysis or provide more insights on the role of these identical regions.

Assessment of SELEX pool affinities for the target in the CE-SELEX has interesting potential for rMCA validation. A higher SELEX pool remelting temperature correlated to a higher overall SELEX pool affinity for the target, clearly indicates lost diversity and enrichment of oligonucleotides in the pool that has higher affinities for their selection target than other sequences.

Conclusions

rMCA enables monitoring of the SELEX process while the selection is going on, regardless of target properties and SELEX design. When direct monitoring of affinity of the selection pools for the target is not possible, rMCA provides an interesting monitoring tool. The method can be used to detect and judge enrichment for target binding sequences during the SELEX process, but can also be used in decision making to optimise selection conditions. rMCA does not require a lot of input material, no supplementary target is needed and no extra labeling and incubation steps are involved. Real time SELEX monitoring by rMCA makes use of the characteristics of the selected ssDNA as such in terms of amplification and denaturation kinetics and is therefore independent of the SELEX approach, properties of targets and different real-time PCR devices, as demonstrated in this work.

Acknowledgements

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