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REVIEW

Methods developed for SELEX

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Abstract SELEX (systematic evolution of ligands by exponential enrichment) is a process that involves the progressive purification from a combinatorial library of nucleic acid ligands with a high affinity for a particular target by repeated rounds of partitioning and amplification. With the development of aptamer technology over the last decade, various modified SELEX processes have arisen that allow various aptamers to be developed against a wide variety of molecules, irrespective of the target size. In the present review, the separation methods used in such SELEX processes are reviewed.

Keywords Aptamer · DNA · RNA · SELEX · Separation

Introduction

SELEX (systematic evolution of ligands by exponential enrichment) has proven to be an excellent tool for finding nucleotide molecules that have a high affinity for a particular target from a random pool under specific conditions. It involves three processes, namely: selection of ligand sequences that bind to a target; partitioning of aptamers from non-aptamers via affinity methods; and amplification of bound aptamers. In the primary step, which involves designing the pool for conventional SELEX, four factors are involved, namely: type of randomization; the length of the random sequence region; the chemistry of the pool; and the utility of the constant regions [1]. The most successful aptamers selected by SELEX represent 1 in 10^9 to 1 in 10^{13} of the molecules in the starting library [2]. During the selection process, the nucleic acid pools are treated with the target molecule under appropriate buffer and at a temperature that depends on the requirements. Usually the selection process starts with a low ratio of nucleic acid to protein in order to check whether all of the molecules bind with the target [1]. After binding, partitioning of the RNA/DNA aptamer–target complex from nonspecific molecules can be achieved by various partitioning techniques (Fig. 1) and bound species are regenerated by enzymatic amplification processes. The amplified molecules are used in the next round of selection processes. Initial rounds of selection need long incubation times and less stringent conditions, whereas later cycles usually involve stringent conditions, such as changing the buffer conditions, reaction volume and time of incubation. In addition, the availability of nonspecific competitors in the reaction mixture also aids the selection of high-affinity molecules. Moreover, the presence of monovalent cations in the buffer reduces the nonspecific binding drastically, and for some targets divalent cations are also necessary to make the specific complex. It is generally recommended that pre-negative selection is performed in the absence of target to eliminate nonspecific binders. To complete entire selection process, 12 cycles are usually required, after that the selected molecules can be cloned into an appropriate vector and sequenced.

In the past, in vitro selection experiments have been used to study the functional and structural aspects of nucleic acids and they have become an important tool for the study of molecular recognition, molecular biology and molecular evolution [3]. In vitro selection procedures for DNA and

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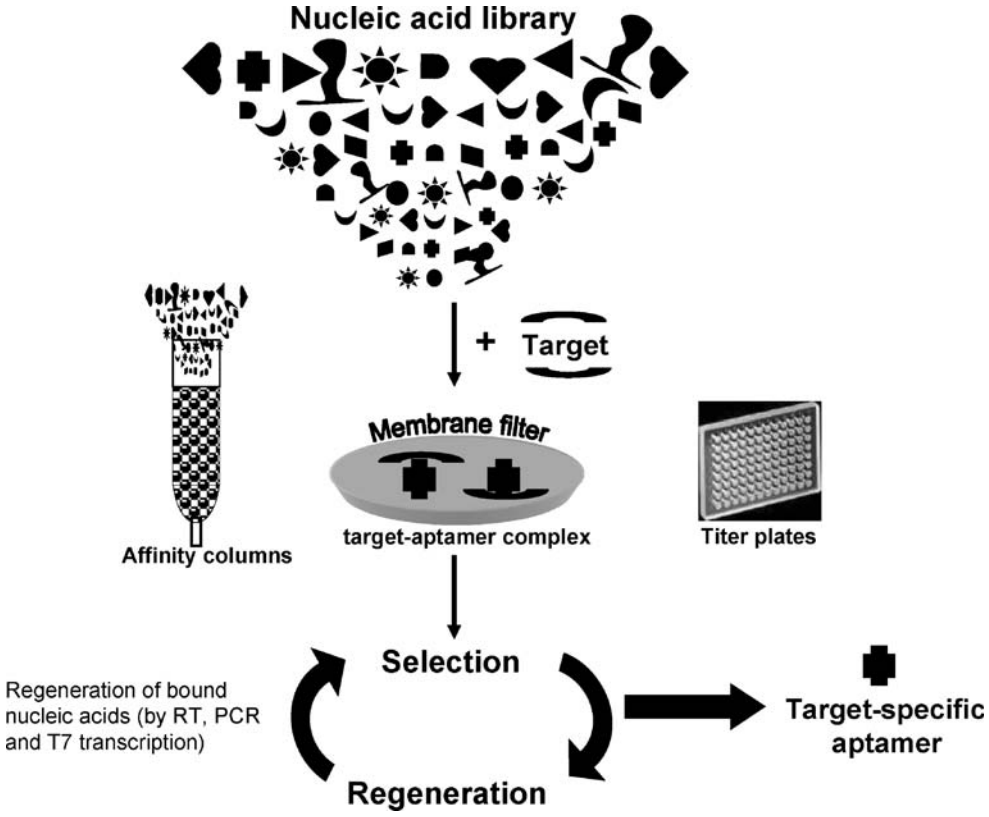

Fig. 1 In vitro selection scheme. Binders for specific targets were separated from a huge randomized library by a separating tool appropriate to the target. The bound molecules are regenerated by amplification processes for the next selection cycle

RNA ligands can be used to rapidly finding the residues of target molecules that cannot be changed without altering the functionality of the target [4]. Aptamers have been selected against a wide range of targets using in vitro selection procedures, and these have been reviewed previously [3, 5–14]. Aptamers are often considered to be artificial antibodies in immunodiagnostics and the treatment of diseases [15–17]. Due to the easy and quick preparation of aptamers, their cost-effectiveness, their small size and their versatility, they have become useful tools for the validation of intracellular and extracellular targets.

A general approach to the selection of aptamers from a completely random nucleic acid library was initially introduced by two teams [5, 6], based on general nucleic acid and protein separation techniques, especially those for RNA and RNA–protein complexes. Before their work, in vitro selection experiments were initiated by Kramer et al. [18], who created the basic concept for SELEX [3, 19]. Following Kramer et al. [18], in vitro selection experiments combined with in vitro amplification were performed by Kinzler and Vogelstein [20], who selected DNA sequences that can bind with transcription factor TFIIIA [4]. In order to adapt SELEX into an appropriate tool for selecting high-affinity ligands, several researchers [21–25] have developed various selection methods. One of the most critical steps in SELEX is the separation of the bound species from molecules not bound to the target. Any method that can separate the RNA, DNA or peptide sequences from the complex can be adopted for this enrichment process. In the present review, various separation methods used in SELEX processes are reviewed (Tables 1 and 2).

Nitrocellulose membrane filtration

A simple, rapid and quick separation method was developed to separate protein from RNA molecules on nitrocellulose membrane by Pristoupil and Kramlova [26] under neutral pH using a mixture of chicken leukaemic myeloblast and RNA that had been developed to study RNA–protein interactions. Later Carey et al. [27] also performed retention assays on nitrocellulose membrane to determine the binding events between phage R17 coat protein and a synthetic 21-nucleotide RNA fragment in a simple bimolecular reaction. This method appeared to be a simple and rapid method for the separation of free RNA from its complexes with protein. Using a similar strategy on nitrocellulose membrane, in vitro selection of RNA molecules that bind to specific ligands was achieved by some of the pioneers of this field, Tuerk and Gold [6]. In their SELEX process, 110-nucleotide DNA templates were synthesized for in vitro transcription. On each template, a region of eight bases that interact with the T4 DNA polymerase was randomized, and then gp43 was used to select the high-affinity species. After creating the complex, they separated out the complex on nitrocellulose mem-

Table 1 Examples of selected RNA aptamers obtained using nitrocellulose membrane filtration for partition

Target	Year	Reference
T4 DNA polymerase	1990	[6]
HIV-1 Rev	1991	[72]
Reverse transcriptase (HIV-1)	1992	[73]
Bacteriophage R17 coat protein	1992	[74]
Rev-binding element of HIV-1	1993	[75]
Reverse transcriptase (avian myeloblastosis and Moloney murine leukemia)	1994	[76]
Protein kinase C βII	1994	[77]
Human α-thrombin	1994	[78]
Vascular endothelial growth factor (VEGF)	1994	[79]
HIV-1 Rev	1995	[49]
HIV-1 Integrase	1995	[80]
Rous sarcoma virus (RSV)	1995	[81]
Human IgE	1996	[82]
Elongation factor SelB	1997	[83]
Keratinolytic growth factor	1997	[84]
Human rIFN-γ protein	1997	[85]
Nucleo capsid (NC) protein of HIV-1	1997	[86]
NS3 of hepatitis C virus	1997	[21]
NS3 of hepatitis C virus	1997	[87]
Coat protein of alfalfa mosaic virus	1997	[88]
HIV-1 gag polyprotein	1997	[37]
Human activated protein (APC)	1998	[30]
HIV-1 Tat	1998	[22]
Vascular endothelial growth factor (VEGF)	1998	[89]
Human nonpancreatic secretory phospholipase A_2	1998	[90]
Rex fusion protein	1999	[91]
Ribosome-inactivating protein, Pepocin	2000	[92]
Ricin A chain	2000	[93]
Human factor VIIa	2000	[94]
HIV-1 Tat	2000	[95]
Bacteriophage MS2 coat protein	2000	[96]
Hepatitis C virus nonstructural protein 3 protease	2000	[31]
Drosophila transformer 2 (Tra2)	2001	[97]
Human coagulation factor IXa	2002	[98]
Human epidermal growth factor receptor-3	2003	[99]
Angiopoietin-2	2003	[100]
HCV NS3 helicase domain	2004	[101]
Histidine utilizing protein (HutP)	2004	[102]
HA of B/Johannesburg	2005	[32]
	2006	[14]
HA of A/Panama	2006	[13]
Mouse prion protein	2006	[103]

brane. The nitrocellulose membrane was washed thoroughly and the bound species were amplified as double-stranded DNA via reverse transcription (RT) and by polymerase chain reaction (PCR) for the subsequent reaction, in vitro transcription. The RNA molecules obtained from in vitro transcription had a higher affinity for the target than the original pool molecules. Multiple selection rounds were carried out until enriched molecules with high affinity were obtained. They selected two kinds of RNA sequences: one that was exactly the same as the original sequence (bearing the same eight bases), and another that had four bases similar to the wild type but the remaining four bases were different. However, both of the selected RNA sequences had the same affinity to the target. This strategy paved the way for many researchers to use the same principle to select the aptamer against small- to macro-sized molecules via various separation methods. Since Tuerk and Gold's first attempt [6], several other aptamers have been selected rapidly using this filter binding process and it is still considered to be an efficient method of separation (Table 1). Obviously, most of the aptamers selected using nitrocellulose membrane are RNA aptamers, indicating the suitability of nitrocellulose membrane for efficiently separating RNA from its protein complex (Table 1). However, a few DNA aptamers have been selected using nitrocellulose membrane (for example, the DNA aptamer against *E. coli* RecA protein [28]). In addition, the RNA aptamer is usually preferred to select against various targets due to its complex structure, which carries diverse functions [29]. At different stages during the development of aptamer technology, researchers modified the separation methods used depending on their target.

Based on the above strategy, but using 120-nucleotide contiguous random sequences, an RNA aptamer was selected against human activated protein C (APC) to inhibit its protease function using nitrocellulose membrane [30]. Similarly, RNA aptamers were selected against Tat protein of HIV-1 [22] and hepatitis C virus nonstructural protein 3 protease from a completely random pool of RNA [31]. Recently, we also selected RNA aptamers against influenza A/Panama/2007/1999 (H3N2) and B/Johannesburg/05/1999 using a N74 random pool by the membrane filtration method mentioned above, along with other affinity processes. We used covalent-binding microwell titer plates (Xenobind), which have surfaces that interact with molecules, to achieve an affinity process [13, 14, 32]. Even though several researchers have succeeded in selecting aptamers against various targets using membrane filters (Table 1), these have limitations for small molecules and peptides. Moreover, selection processes using nitrocellulose membranes usually require up to 12 selection cycles.

Using affinity surfaces

At a more fundamental level, using macro- and small molecules bound to specially prepared surfaces can facilitate studies of their interactions with other molecules. Affinity surfaces that allow proteins and small molecules to bind with them will have affinities with RNA or DNA,

Table 2 Selected aptamers obtained with different separation methods

Target	Separation method	Aptamer	Year	Reference
Lysozyme	Affinity beads	RNA	2001	[23]
Biotoxin		DNA	2002	[10]
Streptavidin		DNA	2005	[39]
HSV-1 US11		RNA	2005	[38]
Human oncostatin M	Titer plate	RNA	2000	[34]
B/Johannesburg influenza virus		RNA	2005	[32]
			2006	[14]
A/Panama influenza virus		RNA	2006	[13]
Ribosomal protein L22	GST fusion	RNA	1995	[7]
Prion		RNA	1997	[35]
Nucleocapsid of HIV-1		RNA	2000	[36]
Zinc	HiTrap Chelating Sepharose	RNA	1995	[40]
Tachykinin substance P	SP Sepharose	RNA	1995	[41]
N-methylmesoporphyrin IX	Ligands (oxirane acrylic)	RNA	1996	[42]
Sulforhodamine B	Agarose	RNA	1998	[44]
Cellobiose	Cellulose	DNA	1998	[43]
Spores of *B. antracis*	Ligands (5′-amino 6-carbon)	DNA	1999	[45]
A site of 16S rRNA	Thioprophyl Sepharose B	RNA	2000	[46]
Phenylalanyl	Ligands (biotin)	RNA	2001	[47]
Neutrophil elastase	Gel mobility shift	RNA	1995	[50]
RNA virus ScVL1 Integration		RNA	1997	[51]
Host factor (IHF)		DNA	1999	[52]
Hepatitis B virus RNA encapsidation	Antibody-based	RNA	1995	[54]
NF-κB P50		DNA	2003	[56]
Prohead	Centrifugation	RNA	1998	[9]
African trypanosome		RNA	1999	[58]
Prion		RNA	2003	[57]
A/Panama influenza virus	Surface plasmon resonance	RNA	2005	[25]
YPEN-1 rat endothelial cells	Flow cytometry	DNA	2001	[64]
NF-κB P50		DNA	2003	[65]
IgE	Capillary electrophoresis	DNA	2004	[24]
Neuropeptide Y		DNA	2005	[66]
MutS protein		DNA	2005	[67]

and can therefore assist in the discovery of new lead compounds and advance the understanding of RNA or DNA recognition. To this end, the assembly of an immobilized RNA–ligand complex was demonstrated and applied to the discovery of new RNA binders using the SELEX process by Ellington and Szostak [5], one of the teams that initiated SELEX. They used small ligands as targets in the selection process, especially six types of organic dyes (Cibacron Blue 3GA, Reactive Red 120, Reactive Yellow 86, Reactive Brown 10, Reactive Green 19 and Reactive Blue 4). These molecules have hydrogen-bond donor and acceptor groups as well as planar surfaces for interactions. Since these dyes have the ability to bind with agarose beads by cross-linking, they are suitable for selecting RNA ligands. For the selection process, they made 100 nts random sequences with flanked primer regions on both the 5′ and the 3′ ends and a T7 promoter region to get RNA molecules. In this SELEX, they separated the complex on the column, and bound RNAs were eluted with high salt solution after washing. They followed similar strategies for amplification and selection to those described above. After five selection cycles, the recovered molecules are cloned and sequenced. The selected molecules showed over 50% binding efficiency, whereas the original pool exhibited only 0.1%. The cumulative amplification factors after five selection cycles are approximately 3×10^8 for Reactive Green 19 and 5×10^{10} for Reactive Brown 10. Based on this affinity system, several aptamers associated with different affinity processes have been selected by researchers. The use of functionalized magnetic adsorbent particles with the a magnetic separation system has also been considered to be a useful tool for the separation of protein and nucleic acids. Using magnetic beads, an RNA aptamer was selected against Panama influenza virus [33]. Initially the whole virus was coated onto the epoxy affinity dynabeads and the remaining sites were blocked with BSA (3% stock solution). The beads were then washed with binding buffer using a

magnetic support to hold them, before the beads were used for the entire selection process (Fig. 2). The RNA pool was denatured at 90 °C for 2 min and allowed to cool at room temperature for 10 min to allow the different conformers to reach equilibrium. Then, the pool RNA and tRNA (competitor) along with the BSA-coated bead in binding buffer were incubated for 10 min at room temperature to remove the BSA binders. The unbound RNA was collected with the help of magnet and applied onto beads coated with the same subtype A/Aichi virus as the counter-selection to remove molecules specific to A/Aichi for typing purposes. The reaction mixture was incubated for a further 10 min. The unbound molecules were again collected and incubated with beads coated with the target A/Panama virus (Fig. 2). After this incubation, the beads were washed three times with 300 μl of binding buffer. Bound RNAs were recovered with a hot 7M urea solution after 20 min of incubation. Bound molecules were precipitated by ethanol and regenerated for the next round of selection by RT, PCR, and in vitro transcription processes. This selection process was continued for ten cycles. Eventually the selected aptamer could distinguish the A/Panama virus from A/Aichi even though these viruses belong to the same subtype. Similarly, an A/Aichi-specific aptamer can also be selected for typing purposes by counter-selection with the A/Panama virus. This type of magnetic bead separation system bypasses many intermediate steps like centrifugation, filtration and other separation systems used to purify molecules. Similary, affinity titer plates with covalent cross-linking surfaces can also be used to select aptamers against various targets [13, 14, 32]. Many kinds of titer plates with different affinity surfaces are currently available for proteins with ligands such as His-tag, GST, biotin and so on. To generate an RNA aptamer against human oncostatin M, a multifunctional member of the interleukin-6 cytokine family, a titer plate was used as the affinity surface to attach the target protein [34].

Using affinity tags

There are currently several ways to immobilize proteins in order to allow the interactions of specific ligands to be analyzed. Tags fused to the N-termini or C-termini of proteins have been developed to improve the affinity and detection, to address the cellular compartment and to improve expression. Using ribosomal protein L22 fused with glutathione S-transferase (GST) as the target, high-affinity RNA molecules were selected by SELEX. After the complex was prepared, the bound RNA molecules were separated using affinity beads for the ligand, GST. After four rounds of selection, the selected RNA molecules matched the original sequences expressed in the Epstein–Barr virus that can bind L22 under native conditions. The selected nucleotides were highly conserved at three positions within the stem-loop region, and these were identical

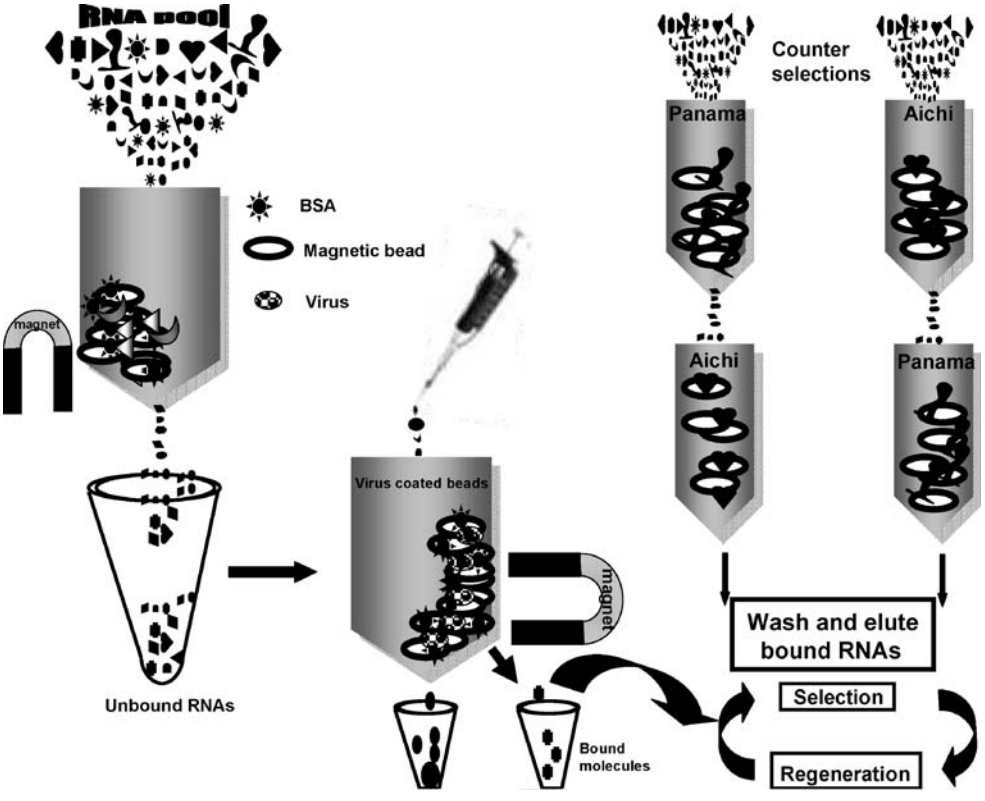

Fig. 2 Aptamer selection using magnetic beads. The pool RNA and tRNA (competitor) are incubated with BSA-coated beads to remove the BSA binders. The unbound RNAs are collected with the help of a magnet and applied onto beads coated with the subtype A/Aichi virus used as the counter-selection in order to remove molecules specific to A/Aichi for typing purposes. The unbound molecules are then collected and incubated with beads coated with the target A/Panama virus. Bound RNAs were recovered, precipitated by ethanol, and regenerated

to the original sequences [7]. Similarly, an RNA aptamer was selected against the recombinant syrian golden hamster prion protein rPrP23-231 fused with the ligand GST. They found that the aptamers may fold into G-quartet-containing structural elements and that substituting all G residues in the G-quartet will destroy the affinity between the aptamer and the target [35]. Using Glutathione Sepharose 4B to separate the protein aptamer complex, an RNA aptamer was also selected against the GST-fused nucleocapsid protein of HIV-1 [36]. Lochrie et al. [37] also used this ligand (GST) on their target protein (gap polyprotein from HIV-1), although they used nitrocellulose filtration to separate the complex from the free RNA molecules.

Cox and Ellington [23] have used biotinylated lysozyme bound to beads with streptavidin-derivitized surfaces to select aptamers in their automated workstation. After the formation of the complex, the beads were transferred to a multiscreen vacuum manifold containing a Millipore HV (PVDF) filter plate. Free RNA species were separated from the bound RNA associated with the lysozyme by vacuum. The beads were washed thoroughly with the selection buffer and the bound species were eluted by increasing the temperature to 98 °C for 3 min. The RNA bound to the washed beads was used as the template for amplification. Similarly, very recently Bryant et al. [38] have selected an RNA aptamer specific for herpes simplex virus-1 US11 using streptavidin-coated magnetic beads. Bruno and Kiel [10] have also used magnetic beads for the selection of an aptamer against biotoxin. They conjugated biotoxins to tosyl-activated magnetic beads to facilitate selection, and they carried out the PCR amplification directly on the surfaces of the beads after complex had formed. Stoltenburg et al. [39] have also used streptavidin-coated magnetic beads to develop a method of selecting aptamers for their targets. Using these kinds of tags usually reduces the number of selection cycles required compared to the membrane filtration method.

Using column matrices or ligands

Although several gel format options are available to fit the requirements of each particular experimental system, the fundamentals of the assays are similar for different separation mechanisms such as ion exchange and affinity. Using HiTrap chelating sepharose, the affinity column was charged with Zn ions and used to select the RNA aptamer against Zn. In this selection process, the column was charged with $ZnCl_2$ solution, and then the RNA pool (containing randomized 50 nts) was passed through the column. The column was washed with buffer and the bound RNA molecules were eluted by buffer containing EDTA. After seven rounds of selection, the selected pool had 73% affinity for Zn [40].

The selection of RNA ligands for tachykinin substance P, a decapeptide that plays vital roles as a neurotransmitter and neuromodulator in the central and peripheral nervous systems of mammals, was achieved by SELEX. The RNA–target complex was separated in this selection system using a SP-Sepharose column. The bound RNA molecules were recovered by passing through buffer containing 100 mM dithiothreitol (DTT), which breaks the linker disulfide bond, releasing the peptide from the matrix. The RNAs were extracted by phenol followed by ethanol precipitation [41]. Similary, DNA aptamer that binds to the cofactor *N*-methylmesoporphyrin IX (NMM) was obtained by Li et al. [42]. Preparation of NMM selection columns was achieved by derivatizing oxirane-acrylic beads with *N*-methylmesoporphyrin IX. The beads can bind NMM after 48 hrs of incubation in the dark. After washing, the beads were treated with 4% mercaptoethanol to inactivate any unreacted epoxy groups. The DNA molecules were pretreated on a negative column which contained oxirane–acrylic beads inactivated with mercaptoethanol. After passing through this column, the molecules were passed through the NMM columns and washed with binding buffer. The bound species were then eluted with TE buffer [10 mM Tris (pH 7.4); 10 mM EDTA] and used for the next round of selection.

High-affinity DNA aptamers against cellobiose have also been identified. Using a DNA pool with a 40 nts randomized region, the complex was made with the target and passed through the cellulose column to remove the unbound molecules. To separate the bound molecules, two kinds of elution systems were followed: either EDTA or formamide. The molecules from the two elution methods are combined in the first round of selection and kept separate in other selection rounds. After 14 selection rounds, the selected DNA molecules could differentiate the target cellobiose from other sugars [43]. Another aptamer, which recognizes the fluorophore sulforhodamine B, was also selected by SELEX using a similar strategy. An RNA pool of sequences with a 72 nts randomized region was used to select the aptamer against this small molecule. Seven rounds of selection were needed to select the apamer that binds to sulforhodamine agarose. The column was equilibrated with tRNA to reduce background selection. In addition, the RNA pool was pretreated with an adipic acid dihydrazide column to remove nonspecific binders. Samples obtained from the precolumn were passed through sulforhodamine (SR) agarose and washed thoroughly with binding buffer. The bound species were eluted with binding buffer containing 3 mM SR [44].

A DNA aptamer against the nonpathogenic Sterene strain *Bacillus anthracis* was selected by Bruno and Kiel

[45]. A template with a 5′-amino-6-carbon linker was used to conjugate with tosyl-activated magnetic beads. After the complex was prepared on the beads in the affinity process, the unbound DNA molecules were washed and the bound molecules were collected by heating the beads. The heat-eluted molecules were amplified for the next round of selection. An aptamer–magnetic bead–electrochemiluminescence (AM–ECL) sandwich assay scheme was formulated to detect the anthrax spores.

The RNA binding to the A-site of the 16S rRNA decoding region was selected using a thioprophyl sepharose 6B solid support in order to study RNA–RNA recognition. After making the complex with the 5′-thiol-C6-linked A-site of the 16S RNA on the thioprophyl sepharose 6B column, the unbound molecules were washed out. Bound RNA molecules were eluted with DTT-containing buffer and amplified [46]. Since many column matrices that have affinities for protein are available, they are often used in SELEX processes. A pool of RNA containing 70 nts randomized regions were incorporated onto the 5′-end of a tRNA sequence in order to find a precursor tRNA with aminoacylation activity that is evolved in vitro. Initially the RNA and the target phenylalanyl substrate (Biotin–Phe–CME) were mixed and the complex was added to streptavidin–agarose. Unbound RNAs were washed out and resin-bound RNAs were eluted by heating [47].

Cross-linking

UV cross-linking has proven to be a useful tool for stabilizing RNA and protein complexes, and the complex obtained is resistant to heat, detergent and alkali [48]. This method is faster and minimizes the nonspecific interactions. Using UV cross-linking, high-affinity RNA-ligands have been selected against human immunodeficiency virus type 1 Rev [49]. For this selection, they substituted the photoreactive chromophore 5-iodouracil into the RNA in the pool and irradiated the RNA with monochromatic UV light in the presence of the target Rev in order to make the complex. This complex was further partitioned on nitrocellulose membrane and washed to remove free molecules. The other steps were the same as for the other, more common, selection strategies.

Using gel electrophoresis

In all of the above methods, the resulting target–nucleic acid complexes are easily isolated from the sample solution by mechanical or magnetic means and the bound nucleic acids are released by simple chemical treatment. Electrophoretic separation is an effective alternative to these separation tools for nucleic acids and proteins. In gel electrophoresis, the different compounds within the gel migrate to the poles due to an electric field and are separated based on their different speeds, which depend on their individual sizes and charges. Using this strategy, Smith et al. [50] described a new approach to drug discovery that involves joining the fields of medicinal and combinatorial chemistry in order to obtain the in vitro selection of RNA-based irreversible inhibitors of human neutrophil elastase. For this selection, the valyl phosphonate moiety (valP) was conjugated to the 5′ linker of the DNA splint oligonucleotide, which in turn was annealed with the SELEX RNA to get the stable DNA:valP. After making the complex (RNA:DNA:valP:hNE), it was separated from the uncomplexed molecules using polyacrylamide gel electrophoresis. The RNA was recovered from the gel by a crush-and-soak method and then RT and PCR amplifications were performed. In this case, selection resulted in a 20-fold increase in activity towards the target.

In vitro selection was carried out using gel-shift with designed oligonucleotides in order to analyze packaging and in vivo replication in the RNA virus ScVL1. The selected sequences were conserved in four residues which proved to aid tight binding, and these sequences were found in the single stranded region [51]. Similarly, an aptamer against integration host factor (IHF) of bacterial protein was selected which can bind several sequences of DNA. The sequence requirement of the 5′ binding domain of IHF was determined. The DNA–target complex was separated by SELEX with native polyacrylamide gels. After resolving the complex on the gel, it was stained with ethidium bromide and visualized under UV. The DNA from the shifted band was eluted by a crush-and-soak method and used for amplification [52].

Antibody-based

Interactions between proteins constitute one of the most important of all signal transduction events and they can help to elucidate the functions of individual proteins. Using protein interactions, an immunoprecipitation procedure has been developed in which peptides or proteins that react specifically with an antibody and this is used to selectively purify an antigen that is in a complex mixture. A method of in vitro selection based on immunoprecipitation of the DNA–protein complex was introduced by Pollock and Treisman [53]. Using this approach, an RNA aptamer was selected against an encapsidation component of the hepatitis B virus (HBV). Selection of encapsidation-competent individuals from pools of randomized ε-sequences in transfected cells was carried out in vivo, followed by amplification processes performed in vitro. They conducted

this selection procedure to find the requirements of primary sequences in HBV RNA encapsidation signal. They used 10 μg of their pools with randomized bulge or loop sequences of ε-sequences and 5 μg of encapsidation-deficient helper construct pCH-3142 per 10 cm plate and then cotransfected into huh7 cells using calcium phosphate, as in the standard procedure. After three days of transfection, the total RNA and the encapsidated RNA from the immunoprecipitated particles were treated with proteinase K followed by phenol extraction. The RNA was amplified by in vitro RT followed by PCR using the corresponding regions (3122–3129) of HBV and regions from the intron ε-*lacZ* construct. Using their selection procedure, they revealed the importance of the 5′-proximal bulge and 3′-bulge positions [54]. Using immunoprecipitation, an RNA aptamer was also selected against the U1-snRNP-A protein [55].

Yang et al. [56] have identified the oligonucleoside phosphorodithioate (ODN) aptamers from a bead-bound ODN library by immunofluorescence assay. An aptamer was selected based on five steps. In the first step, the bead was bound with ODN sequences; in the second step, the beads were incubated with NF-κB p50 protein for 2 h; the beads were then washed and incubated again with anti p-50 antibody as a third step. In the fourth step, the complex was again incubated with Alexa fluor 488-labeled goat anti-rabbit IgG after washing. In the final step, the mixture was washed thoroughly and viewed under a microscope to find the labeled beads. The labeled beads were recovered based on observation and the nucleotides were amplified after the proteins were removed via urea.

Centrifugation

Sedimentation refers to the process by which particles in a fluid settle to the bottom under the influence of gravity. Using this strategy, it is possible to alter the composition of the buffer to force the precipitation of a portion of proteins. To find sequences necessary for prohead binding, DNA-gp3 packaging, and to find the active sequences of prohead RNA (pRNA), the pRNA sequences are randomized and the RNA variants undergo multiple rounds of selection using a centrifugation method. For this selection, a 62-base proheading domain was randomized. pRNA-free proheads were produced by *E. coli* with IPTG induction. After making the complex, the RNA molecules bound to proheads were separated from unbound molecules by centrifugation using a 5–20% sucrose gradient in a Beckman rotor at 35,000 rpm for 30 min at 4 °C. The band with prohead was isolated and diluted with H_2O and the RNA molecules were recovered [9]. An RNA aptamer against disease-associated prion protein from scrapie-associated fibrils (SAF) was also selected using centrifugation. Before each round of selection, the SAF from a hamster brain was sonicated with three pulses and incubated with the RNA pool for 4 h at room temperature in the presence of reaction buffer containing magnesium ions. To partition the complex from free molecules, the reaction mixture was centrifuged at 25,000×g for 1 h at 10 °C. The unbound RNA was removed from the supernatant. To remove nonspecific bound RNA molecules, the pellet containing RNA:SAF was washed three times with binding buffer, and a similar centrifugation was used. The bound RNA molecules were converted into cDNA for the next round of selection [57]. Similarly, an RNA aptamer against African trypanosomes was selected using centrifugation as the separation tool by Homann and Göringer [58].

Surface plasmon resonance

In general, the methods available for separation include affinity processes, membrane filtration, and panning, all of which require multiple selection cycles to enrich a nucleic acid pool from a library. These methods do not provide binding efficiency information and tedious off-line evaluations are required after every few selection cycles. A selection method based on surface plasmon resonance (SPR) can overcome these problems, as it is extremely useful for analyzing biochemical interactions (Fig. 3). SPR spectroscopy is an evanescent wave biosensor technology that can be used to monitor the interactions of two or more molecules in real time. The technique can be used to measure interactions of various biomolecules, including peptides, proteins, nucleic acids, carbohydrates and phospholipid vesicles, and it allows the real-time monitoring of complexes [59, 60]. Using this method, Misono and Kumar [25] identified the conserved regions of the RNA ligand binding to the human influenza A/Panama/2007/1999 (H3N2) virus. To select the RNA aptamer, they injected the RNA pool into the flow cell of a CM4 chip coated with target virus for 2 min. After that, the binding buffer was injected into the cell at a flow rate of 20 μl/min. During this period, the buffer was allowed to flow into the fraction tray instead of the waste bottle. The collected fractions were ethanol-precipitated to recover the bound RNAs and amplified further for the next cycle. Initial SPR studies suggested its potential for the panning and enrichment of binding species from a library [61–63], and it has several advantages, such as the repeated use of an immobilized target and the identification of binding species fractions. Moreover, the great advantage of SPR is that it provides information about the target binding abilities of molecules before they are selected.

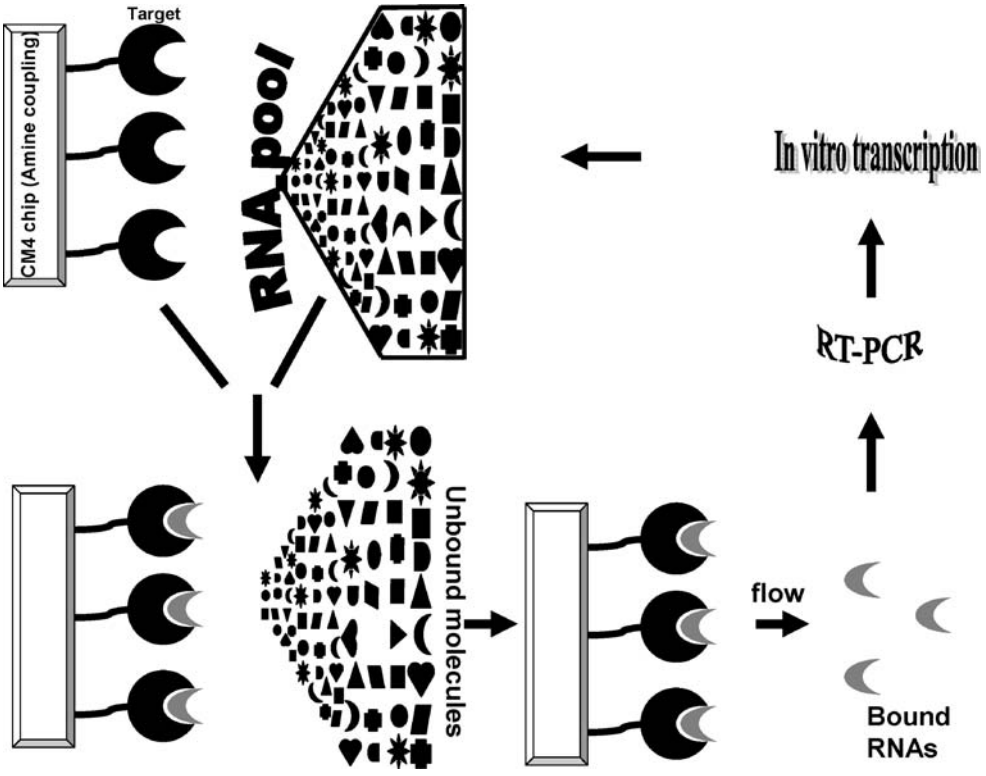

Fig. 3 Aptamer selection by surface plasmon resonance (SPR). The RNA pool was injected into the flow cell of a CM4 chip coated with target for 2 min. The binding buffer was injected into the cell at a flow rate of 20 μl/min and allowed to flow into the fraction tray instead of the waste bottle. The collected fractions were ethanol-precipitated to recover the bound RNAs and amplified further for the next cycle

Flow cytometry

Another strategy for finding the selected aptamer is based on detecting its fluoroscence via flow cytometry [64]. To generate DNA aptamers against YPEN-1 rat endothelial cells, instead of radiolabeled detection, they were labeled with FITC, a fluorescence reporter molecule. The selection was performed with a 60 nts randomized region. The counter-selection was carried out against N9 microglial cells. The enrichment was monitored by flow cytometry by incubating the pools with YPEN-1 endothelial cells. The molecular target protein with aptamer was isolated from endothelial cells by DNA affinity purification using a ligand-mediated magnetic system. A similar selection procedure was followed to select the aptamer towards NF-κB p50 protein by flow cytometry with two-color analysis on the bead-bound molecules [56]. Using fluorescence detection, they picked the beads with the complex and amplified it.

Capillary electrophoresis

Capillary electrophoresis (CE) has several appealing advantages over the other analytical separation methods discussed above, such as speed, resolution capacity, and minimal sample dilution. In addition, when used in SELEX processes, CE requires fewer cycles than other separation methods. Affinity capillary electrophoresis is used to manipulate and optimize the selectivity, and it has the ability to extract quantitative information about interacting systems [65]. A typical selection procedure usually involves ~12 selection rounds to obtain high-affinity aptamers. But the high-affinity aptamer against IgE was selected using CE with four selection rounds in a process named CE-SELEX [24]. CE-SELEX involves the selection of binding species based on a mobility shift due to complex formation. For this selection, the authors used a poly(vinyl alcohol)-coated capillary 40.2 cm long and with an inner diameter of 50 μm for separation. The sample was applied to the capillary at 5 psi for 5 s and monitored under UV detection at 254 nm. After the nonspecific species had migrated out, the CE fractions containing specific DNA sequences were collected. The collected DNA sequences were amplified by PCR and used for the next round of selection. Using a similar strategy, an ssDNA sequence was selected against a small peptide, neuropeptide Y [66]. This selection proved that CE-SELEX is also suitable for smaller targets. Recently Drabovich et al. [67] have introduced the equilibrium capillary electrophoresis of equilibrium mixtures, and they selected the aptamer against MutS protein with three rounds of selection processes.

Very recently, a non-SELEX selection process for aptamers was also introduced by Berezovski et al. [15]. This non-SELEX strategy involves repeated partitioning steps without amplification. They used nonequilibrium capillary electrophoresis of equilibrium mixtures for partitioning, and they took 1 h to complete the selection

processes, which increased the selected molecule affinity by four orders of magnitude. In their study, they used h-Ras protein as the target to find the molecules from a DNA library of 39 random sequences labeled with 6-carboxy-fluorescein at the 5′ end. The main advantage of this method was that the selection processes could be completed within one hour instead of several days or weeks.

Automated selection

Generally speaking, the in vitro selection systems in practice (Tables 1 and 2) are repetitive, time-consuming and not applicable for high-throughput selections. To overcome these limitations, in the past few years, various attempts at automating in vitro selections have been made [23, 68, 69]. Automated systems are formulated to provide for high flexibility and versatility in terms of choice of buffers, reagents and stringent conditions in selections performed with the methods described above. In general, it is necessary to spend time generating the appropriate aptamers from a starting pool of aptamers. Automated systems can handle multiple targets efficiently since they can be processed in parallel. Moreover, selection cycles can be conducted without any direct intervention steps. Cox and Ellington [23] generated automated workstations to select anti-protein aptamers, and demonstrated the automated selection of anti-lysosome that functions as an efficient inhibitor of cell lysis. To improve them further, Cox et al. [70] have developed an automated aptamer selection procedure with a protein translational system. They attempted to select aptamers against protein targets generated by in vitro transcription and translation from individual genes. For specific immobilization they also used in vitro biotinylation. To prove the applicability of this method, they selected aptamers against translated human U1A, a component of the nuclear spliceosome. Based on this high-throughput generation, aptamers against the translation products of the target genes within an average bacterial genome can be found within a period of months. Eulberg et al. [69] also selected the RNA aptamers against the mirror image configuration (D-peptide) of substance P, the so-called Spiegelmer, using an automated system. Very recently, a microfludic SELEX prototype was described as being an automated microfluidic, microline-based assembly with Labview-controlled actuatable valves and a PCR machine to verify the selection and synthesis of anti-lysozyme aptamer [71]. According to their view, this system is relatively inexpensive and useful for the continued "morphing" of macro→meso→microfabricated structure and applicable for more rapid selection. The rapid selection of aptamer ligands by this automated system and its options for controlling experimental settings should provide many opportunities in several fields.

Advances in aptamer technology involve the selection of active sequences and stabilization by chemical modification in order to enhance the nuclease resistance of the resulting aptamers. Modifications can be incorporated to the exact position determined by the user, or it can be introduced during the selection process in order to increase the half-lives of the selected molecules. To detect the binding events between the aptamer and the target, modulating aptamers have been designed that yield fluorescence signal upon binding. Aptamers capable of binding and discriminating among structurally related small molecules can be concocted in the laboratory. Taken together, the selection methods, the aptamer detection systems with modulations, and the ability to make them stable will be useful for monitoring specific analytes (under native conditions) for imaging and diagnostic purposes. Considering these developments in aptamer research, aptamers will be used commercially in both diagnostic and therapeutic applications. Aptamers will also be used as molecular recognition elements in analytical systems for applications other than medical diagnostics. Based on the separation methods developed over the last decade, many applications are making use of aptamer technology for rapid screening. Rapid improvements in selection methods have allowed many researchers to isolate high-affinity aptamers against an analyte of choice, probably much more easily than screening for monoclonal antibodies. Due to the successful commercialization of aptamers as drugs, many aptamers are now emerging for use against small- to macro-sized molecules.

References

1. Marshall KA, Ellington AD (2000) Methods Enzymol 318:193–214
2. Gold L (1995) J Biol Chem 270:13581–13584
3. Uphoff KW, Bell SD, Ellington AD (1996) Curr Opin Struct Biol 6:281–288
4. Famulok M, Szostak JW (1992) Angew Chem Int Ed Engl 31:979–988
5. Ellington AD, Szostak JW (1990) Nature 346:818–822
6. Tuerk C, Gold L (1990) Science 249:505–510
7. Dobbelstein M, Shenk T (1995) J Virol 69:8027–8034
8. Conrad RC, Giver L, Tian Y, Ellington AD (1996) Methods Enzymol 267:336–367
9. Zhang F, Anderson D (1998) J Biol Chem 273:2947–2953
10. Bruno JG, Kiel JL (2002) Biotechniques 32:178–180
11. Nimjee SM, Rusconi CP, Sullenger BA (2005) Ann Rev Med 56:555–583
12. Pestourie C, Tavitian B, Duconge F (2005) Biochimie 87:921–930
13. Gopinath SCB, Misono T, Kawasaki K, Mizuno T, Imai M, Odagiri T, Kumar PKR (2006) J Gen Virol 87:479–487

14. Gopinath SCB, Yuriko S, Kazunori K, Kumar PKR (2006) J Biochem 139:837–846
15. Berezovski M, Musheev M, Drabovich A, Krylov S (2006) J Am Chem Soc 128:1410–1411
16. Ng EW, Shima DT, Calias P, Cunningham ET, Guyer DR Jr, Adamis PA (2006) Nature Rev 5:123–132
17. Gopinath SCB, Misono T, Kumar PKR (2006) Crit Rev Anal Chem (in press)
18. Kramer FR, Mills DR, Cole PE, Nishihara T, Spiegelman S (1974) J Mol Biol 89:719–736
19. Ellington AD (1994) Curr Biol 4:427–429
20. Kinzler KW, Vogelstein B (1989) Nucleic Acids Res 17:3645–3653
21. Kumar PKR, Machida K, Urvil PT, Kakiuchi N, Vishnuvardhan D, Shimotohno K, Taira K, Nishikawa S (1997) Virol 237:270–282
22. Yamamoto R, Murakami K, Taira K, Kumar PKR (1998) Gene Ther Mol Biol 1:451–466
23. Cox JC, Ellington AD (2001) Bioorg Med Chem 9:2525–2531
24. Mendonsa SD, Bowser MT (2004) Anal Chem 76:5387–5392
25. Misono TS, Kumar PKR (2005) Anal Biochem 342:312–317
26. Pristoupil TI, Kramlova M (1968) J Chromatogr 32:769–770
27. Carey J, Cameron V, de Haseth PL, Uhlenbeck OC (1983) Biochem 22:2601–2610
28. Tracy RB, Kowalczykowshi C (1996) Genes Dev 10:1890–1903
29. Guo P (2005) J Nanosci Nanotechnol 5:1964–1982
30. Gal SW, Amontov S, Urvil PT, Vishnuvardhan D, Nishikawa F, Kumar PKR, Nishikawa S (1998) Eur J Biochem 252:553–562
31. Fukuda K, Vishnuvardhan D, Sekiya S, Hwang J, Kakiuchi N, Taira K, Shimotohno K, Kumar PKR, Nishikawa S (2000) Eur J Biochem 267:3685–3694
32. Gopinath SCB, Kawasaki K, Kumar PKR (2005) Nucleic Acids Symp Ser 49:85
33. Kumar PKR, Gopinath SCB, Misono T, Kawasaki K (2004) Japanese patent JP2004-293679
34. Rhodes A, Deakin A, Spaull J, Coomber B, Aitken A, Life P, Rees S (2000) J Biol Chem 275:28555–28561
35. Weiss S, Proske D, Neumann M, Groschup MH, Kretzschmar HA, Famulok M, Winnacker E (1997) J Virol 71:8790–8797
36. Kim SJ, Kim MY, Lee JH, You JC, Jeong S (2002) Biochem Biophy Res Comm 291:925–931
37. Lochrie MA, Waugh S, Pratt DG Jr, Clever J, Parslow G, Polisky B (1997) Nucleic Acids Res 24:2902–2910
38. Bryant KF, Cox JC, Wang H, Hogle JM, Ellington AD, Coen DM (2005) Nucleic Acids Res 33:6090–6100
39. Stoltenburg R, Reinemann C, Strehlitz B (2005) Anal Bioanal Chem 383:83–91
40. Ciesiolka J, Gorski J, Yarus M (1995) RNA 1:538–550
41. Nieuwlandt D, Wecker M, Gold L (1995) Biochem 34:5651–5659
42. Li Y, Geyer R, Sen D (1996) Biochem 35:6911–6922
43. Yang A, Goldstein IJ, Mei H, Engelke DR (1998) Proc Natl Acad Sci USA 95:5462–5467
44. Holeman LA, Robinson SL, Szostak JW, Wilson C (1998) Fold Des 3:423–431
45. Bruno JG, Kiel JL (1999) Biosens Bioelectron 14:457–464
46. Tok JB, Cho J, Rando RR (2000) Nuleic Acids Res 28:2902–2910
47. Saito H, Kourouklis D, Suga H (2001) EMBO J 20:1797–1806
48. Pellé R, Murphy N (1993) Nucleic Acid Res 21:2453–2458
49. Jensen KB, Atkinson BL, Willis MC, Koch TH, Gold L (1995) Proc Natl Acad Sci USA 92:12220–12223
50. Smith D, Kirschenheuter GP, Charlton J, Guidot DM, Repine JE (1995) Chem Biol 2:741–750
51. Yao W, Adelman K, Bruenn JA (1997) J Virol 71:2157–2162
52. Goodman SD, Veltenm NJ, Gao Q, Robinson S, Segall AM (1999) J Bateriol 181:3246–3255
53. Pollock R, Treisman R (1990) Nucleic Acids Res 18:6197–6204
54. Rieger A, Nassal M (1995) Nucleic Acids Res 23:3909–3915
55. Tsai DE, Harper DS, Keene JD (1991) Nucleic Acids Res 19:4931–4936
56. Yang X, Li X, Prow TW, Reece LM, Bassett SE, Luxon BA, Herzog NK, Aronson J, Shope RE, Leary JF, Gonestein DG (2003) Nucleic Acids Res 31:e54
57. Rhie A, Kirby L, Sayer N, Wellesley R, Disterer P, Sylvester I, Gill A, Hope J, Williams J, Tahiri-Alaoui A (2003) J Biol Chem 278:39697–39705
58. Homann M, Göringer U (1999) Nucleic Acids Res 27:2006–2014
59. Park S, Myszka DG, Yu M, Litter SJ, Laird-Offringa IA (2000) Mol Cell Biol 20:4765–4772
60. Katsamba PS, Myszka DG, Laird-Offringa IA (2001) J Biol Chem 276:21476–21481
61. Schier R, Marks JD (1996) Hum Antibod Hybridomas 7:97–105
62. Khati M, Schuman M, Ibrahim J, Sattentau Q, Gordon S, James W (2003) J Virol 77:12692–12698
63. Pileuf F, Andreola M, Dausse E, Michel J, Moreau S, Yamada H, Gaidamkov SA, Crouch RJ, Toulme J, Cazenave C (2003) Nucleic Acids Res 31:5776–5788
64. Blank M, Weinschenk T, Priemer M, Schluesener H (2001) J Biol Chem 276:16464–16468
65. Schou C, Heegaard NH (2006) Electrophoresis 27:44–59
66. Mendonsa SD, Bowser MT (2005) J Am Chem Soc 127:9382–9383
67. Drabovich A, Berezovski M, Krylov SN (2005) J Am Chem Soc 127:11224–11225
68. Cox JC, Rudolph P, Ellington AD (1998) Biotechnol Prog 14:845–850
69. Eulberg D, Buchner K, Maasch C, Klussmann S (2005) Nucleic Acids Res 33:e45
70. Cox JC, Hayhurst A, Hesselberth J, Bayer TS, Georgiou G, Ellington AD (2002) Nucleic Acids Res 30:e108
71. Hybarger G, Bynum J, Williams RF, Valdes JJ, Chambers JP (2006) Anal Bioanal Chem 384:191–198
72. Bartel DP, Zapp ML, Green MR, Szostak JW (1991) Cell 67:529–536
73. Tuerk C, Macdougal S, Gold L (1992) Proc Natl Acad Sci USA 89:6988–6992
74. Schneider D, Tuerk C, Gold L (1992) J Mol Biol 228:862–869
75. Giver L, Bartel D, Zapp M, Pawul A, Green M, Ellington AD (1993) Nucleic Acids Res 21:5509–5516
76. Chen H, Gold L (1994) Biochem 33:8746–8756
77. Conrad R, Keranen LM, Ellington AD, Newton AC (1994) J Biol Chem 269:32051–32054
78. Kubik MF, Stephens AW, Schneider D, Marlar R, Tasset D (1994) Nucleic Acids Res 22:2619–2626
79. Jellink D, Green LS, Bell C, Janjić N (1994) Biochem 33:10450–10456
80. Allen P, Worland S, Gold L (1995) Virol 209:327–336
81. Pan W, Craven RC, Qiu D, Wilson CB, Wills JW, Golovine S, Wang J (1995) Proc Natl Acad Sci USA 92:11509–11513
82. Wiegand TW, Williams BP, Dreskin SC, Jouvin M, Kinet J, Tasset D (1996) J Immunol 157:221–230
83. Klug SJ, Hüttenhofer A, Kromayer M, Famulok M (1997) Proc Natl Acad Sci USA 94:6676–6681
84. Pagratis NC, Bell C, Chang Y, Jennings S, Fitzwater T, Jellinek D, Dang C (1997) Nature Biotechnol 15:68–73
85. Kubik MF, Bell C, Fitzwater T, Watson SR, Tasset DM (1997) J Immunol 159:259–267

86. Berglund JA, Charpentier B, Rosbash M (1997) Nucleic Acids Res 25:1042–1049
87. Urvil PT, Kakiuchi N, Zhou D, Shimotohno K, Kumar PKR, Nishikawa S (1997) Eur J Biochem 248:130–138
88. Houser-Scott F, Ansel-Mckinney P, Cai J, Gehrke L (1997) J Virol 71:2310–2319
89. Ruckman J, Green LS, Beeson J, Waugh S, Gillette WL, Henninger DD, Claesson-Welsh L, Janjić N (1998) J Biol Chem 273:2056–20567
90. Bridonneau P, Chang Y, O'Connell D, Gill SC, Snyder DW, Johnson L, Goodson T, Herron DK, Parma DH (1998) J Med Chem 41:778–786
91. Baskerville S, Zapp M, Ellington AD (1999) J Virol 73:4962–4971
92. Hirao I, Madin K, Endo Y, Yokoyama S, Ellington AD (2000) J Biol Chem 275:4943–4948
93. Hesselberth JR, Miller D, Robertus J, Ellington AD (2000) J Biol Chem 275:4937–4942
94. Rusconi CP, Yeh A, Lyerly HK, Lawson JH, Sullenger BA (2000) Thromb Haemost 84:841–848
95. Kawakami J, Imanaka H, Yokota Y, Sugimoto N (2000) J Inorg Biochem 82:197–206
96. Shtatland T, Gill SC, Javornik BE, Johansson HE, Singer BS, Uhlenbeck OC, Zichi DA, Gold L (2000) Nucleic Acids Res 28: e93
97. Brunel C, Ehresmann B, Ehresmann C, McKeown M (2001) Bioorganic Med Chem 9:2533–2541
98. Rusconi CP, Scardino E, Layzer J, Pitoc GA, Ortel TL, Monroe D, Sullenger BA (2002) Nature 419:90–94
99. Chen CB, Chernis GA, Hoang VQ, Landgraf R (2003) Proc Natl Acad Sci USA 100:9226–9231
100. White RR, Shan S, Rusconi CP, Shetty G, Dewhirst MW, Kontos KD, Sullenger BA (2003) Proc Natl Acad Sci USA 100:5028–5033
101. Nishikawa F, Funaji K, Fukuda K, Nishikawa S (2004) Oligonucleotides 14:114–129
102. Kumarevel TS, Gopinath SCB, Nishikawa S, Mizuno H, Kumar PKR (2004) Nucleic Acids Res 32:3904–3912
103. Sekiya S, Noda K, Nishikawa F, Yokoyama T, Kumar PKR, Nishikawa S (2006) J Biochem 139:383–390