

Nonradioactive fluorescence microtiter plate assay monitoring aptamer selections

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Since its discovery in 1990, aptamer technology (1,2) has emerged as a novel and increasingly important approach to evolve specifically binding nucleic acid molecules (3). As aptamers can be developed to a vast range of target molecules, their potential application is very diverse. They can be used as molecular recognition elements in analytical systems for detection, separation, or purification of target molecules (4–8). They also play an important role in medical therapy (9) and environmental analysis (10,11).

Aptamers are usually generated using SELEX (systematic evolution of ligands by exponential enrichment) technology (1,2). In this cyclic, repetitive process, a combinatorial library of synthetic nucleic acids containing approximately 10^{15} different molecules is used as a starting pool. In subsequent steps of target binding, removal of unbound sequences, elution of bound sequences and their amplification and purification, the nucleic acid pool is enriched with specifically binding sequences.

To monitor the progress of selection, nucleic acids are usually radioactively labeled. This is a rather sensitive method, which enables detection of trace amounts of nucleic acids. Disadvantages of monitoring enrichment radioactively are that appropriate safety precautions have to be taken and that it is rather costly.

In a modified selection process for DNA aptamers, named FluMag-SELEX, Stoltenburg and coworkers have inserted fluorescent labels into the DNA via PCR and used it for detecting enrichment (12). Fluorescence allows for a fast and nonradioactive monitoring of selection progress.

Since direct integration of fluorescent labels is expensive and might also interfere with the binding properties of the nucleic acid (13), we have developed an easy and sensitive analytical microtiter plate assay, the fluorescent dye-linked aptamer assay, that utilizes the advantages of fluorescence without being invasive.

For monitoring enrichment over the course of selection, we used the fluorescent dye OliGreen® (Invitrogen GmbH, Karlsruhe, Germany). This reagent is specific for single-stranded DNA and enables the quantification of as little as 100 pg/mL nucleic acid with a standard spectrofluorometer using fluorescein excitation and emission wavelengths (14). For the assay setup, the biotinylated target molecule is immobilized on a streptavidin-coated microtiter plate, incubated with the single-stranded DNA aptamer pool, and while nonbinding sequences are removed, binding sequences are detected (Figure 1). The unbound dye is basically nonfluorescent.

Binding assays were performed using streptavidin-coated 96-well microtiter plates (high-binding capacity, black, with SuperBlock blocking buffer; Perbio Science Deutschland GmbH, Bonn, Germany). Between incubation steps, which were done at room temperature and 100 rpm on an Unimax 1010 orbital shaker (Heidolph Instruments, Schwabach, Germany), wells were washed three times with 300 μ L of the same binding buffer as was used in aptamer selections.

According to the indicated binding capacity of the plate, 125 pmol biotinylated target molecule in a total volume of 100 μ L binding buffer were immobilized onto each well in

a 30-min incubation step. Wells were subsequently blocked with 200 μ L 52 μ M biotin (Sigma-Aldrich Chemie GmbH, Munich, Germany) in binding buffer for 30 min. In parallel, one well for each probe was blocked with biotin as a negative control. Prior to application to the binding assay, 200 ng single-stranded DNA in a total volume of 100 μ L binding buffer were heated to 90°C for 3 min and immediately chilled on ice for 5 min, followed by a short incubation at room temperature. The DNA was added to each well and allowed to incubate for 1 h. After washing, 150 μ L OliGreen, in a 1:200 working dilution in Tris-EDTA (TE) buffer, pH 7.6, were added automatically by a Fluoroskan Ascent™ device (Thermo Electron GmbH, Dreieich, Germany). After 10 min incubation, fluorescence was measured (excitation, 485 nm; emission, 527 nm).

To show the applicability of our assay, we monitored the enrichment of an example selection that comprised 10 rounds. For each round, two samples of 200 ng single-stranded DNA pool were heat-denatured as described. One sample was applied to the wells containing the immobilized target, the other sample was added to the blocked well, serving as a background control. After removing nonbinding

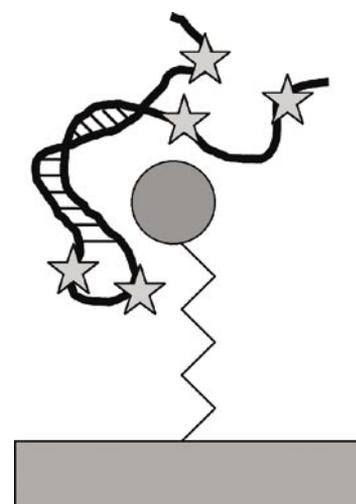


Figure 1. Assay setup. The target molecule (circle), which is biotinylated via a spacer-molecule, is immobilized on a streptavidin-coated microtiter plate (rectangle). The assembly is incubated with single-stranded DNA. After washing, binding DNA molecules remain on the target and can be detected using the single strand-specific fluorescent dye OliGreen (stars).

sequences, binders were detected with OliGreen. The absorption results were plotted as fluorescence versus selection round (Figure 2). Screening of single-stranded DNA from the different selection steps revealed an increasing enrichment of binding sequences, with the first enrichment monitored in round 6. Nonspecific binding to the microtiter plates was hardly observed, and background remained stable over the entire course of selection.

If a calibration curve is measured in parallel, the fluorescent dye-linked aptamer assay can also be used to quantitate the absolute enrichment in percent. Here, the amount of immobilized target molecule has to be verified to ensure that every DNA molecule can theoretically bind to one target molecule. Since as little as 2 ng DNA/100 μ L can be detected on a streptavidin microtiter plate (data not shown), any enrichment can theoretically be monitored.

Even though enrichment cannot be monitored directly during selection, the fluorescent dye-linked aptamer assay allows for a comparison of enrichment data because it provides equal conditions for samples of every selection step. With direct labeling of nucleic acids, the signal obtained for enrichment is strongly dependent on the ratio of nucleic acid to target used in the selection. Since this parameter is varied to increase stringency, determination of an absolute enrichment can be difficult when nucleic acid is applied in different excess.

Another considerable advantage over direct labeling is that not the totality of eluted nucleic acids is analyzed, which could also include nonspecific binders, but information on the actual binding properties of the aptamer pool are also obtained.

With the fluorescent dye-linked aptamer assay, we have established a rapid and sensitive method for analyzing the actual course of an aptamer selection. In addition, after sequences have been separated, this assay allows for analysis and comparison of the aptamer's binding quality. Through its ease of handling and possible parallelization, the assay facilitates and accelerates the usually very time-consuming

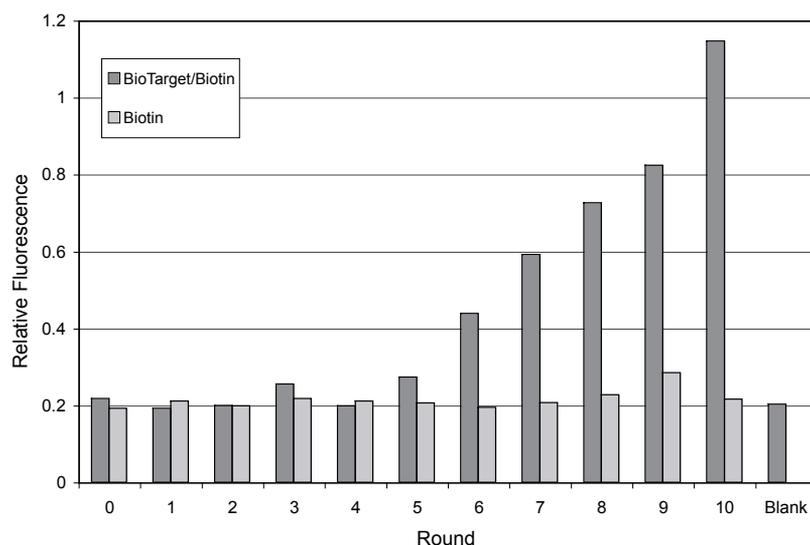


Figure 2. Monitoring the enrichment of binding DNA molecules over the course of a selection. Ten rounds of an example aptamer selection were analyzed using the fluorescent dye-linked aptamer assay. Single-stranded DNA pools from each of the rounds were applied onto two wells in parallel: one well containing the target molecule of the selection (dark gray) and the other well being the background control with no target immobilized (light gray). No binding was observed in rounds 0 (precursor pool) to 5. From round 6 onwards, a strong increase in binding sequences could be detected in wells containing the target molecule, whereas no binding was observed in the control wells. Experiments were carried out in three replicates, the target molecule used here was the anthracycline antibiotic daunomycin. A 40-nucleotide randomized library was used as a starting pool.

procedure of classifying the aptamer's binding properties.

This method is not only restricted to DNA selection. In case of RNA selection, RNA-specific fluorescent dyes like RiboGreen® (Invitrogen GmbH) (15) could be used alternatively, making the fluorescent dye-linked aptamer assay an extremely useful tool for the entire range of aptamer technology.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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