

Produce High Yields of a High Affinity 2'-F-RNA Aptamer Using the DuraScribe® T7 Transcription Kit

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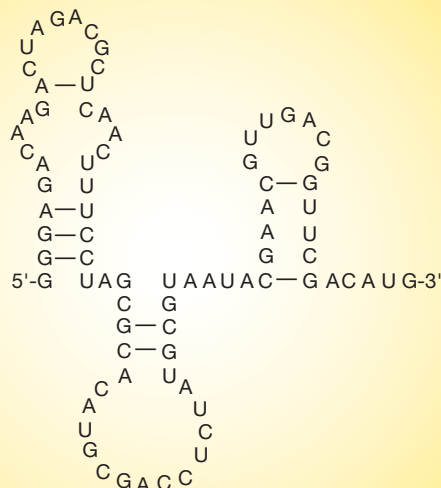
Introduction

Current proteomics initiatives are focused on the production of high affinity ligands or probes that specifically target proteins. Particularly versatile probes with immense potential for use as affinity molecules are aptamers.¹ Aptamers are short single-stranded nucleic acid molecules (<100 bases) that have been selected from random pools based on their ability to bind other molecules. These high affinity molecules bind to proteins and allow detection of bound proteins in microarrays, or capture protein complexes for functional identification.² They can serve as probes or therapeutics by altering biological activity and inhibiting critical interactions by blocking access to active sites and interaction surfaces.¹ Traditionally, antibodies serve this function, but difficulties in selecting and producing antibodies in a high-throughput manner have sent researchers looking for alternatives. The most promising alternatives are aptamers, which are beginning to emerge as a class of molecules that rival antibodies in both therapeutic and diagnostic applications.³

Modification of pyrimidine nucleotides in RNA by substitution with fluoro (F) functional groups at the 2' position is sufficient to protect an RNA sequence from degradation by nucleases.⁴ It has been shown that 2'-F-RNA ligands have high affinities (0.3-3 pM), bioactivities (K_i approximately 34 pM), and have extreme thermostabilities and specificities.²

Here we demonstrate the high yield synthesis of a biologically active 2'-F-RNA aptamer for streptavidin using the DuraScribe®* T7 Transcription Kit and a sequence selected and characterized by Tahiri-Alaoui *et al.*⁵ The DuraScribe T7 RNA Polymerase provided in the kit, recognizes the same T7 transcription promoters as standard T7 RNA Polymerase, but is about 100-fold more active in the incorporation of 2'-fluorine-pyrimidines.⁶ Using this mutant T7 RNA Polymerase in the DuraScribe T7 Enzyme Mix, which is formulated to utilize very high concentrations of nucleotides, produces the highest possible yields of 2'-fluoro-substituted RNA or DuraScript® RNA.

FIG 1. Secondary structure of the streptavidin-binding 2'-F-RNA aptamer suggested by Tahiri-Alaoui *et al.*⁵



Methods

Transcription template construction

The double-stranded transcription template was constructed by the overlap and extension of two oligodeoxynucleotides. The oligo containing the T7 RNA polymerase promoter sequence is 5'-TAA TAC GAC TCA CTA TA-3'. The streptavidin-aptamer oligonucleotide overlaps the T7 promoter oligonucleotide by 8 bases (underlined) 5'-CAT GTC GAA CCG TCA ACG TTC GT**A** TTA CGC ATA GAG GTC GCA TGT GCG CTA GGA AAG TTG AGC GTC TAG TCT TGT CTC CCT ATA GTG A-3'. It was designed to produce domains I and II of the streptavidin-specific aptamer selected by Tahiri-Alaoui *et al.*⁵ A mutant, non-binding version of the aptamer was produced from a similar oligonucleotide containing a single base change, A to G (indicated in bold red) in the above sequence. An excess of T7 promoter oligo (75 picomoles) was annealed to 50 picomoles of streptavidin-aptamer primer in 5 mM Tris-HCl (pH 8.0) and 5 mM NaCl at 75°C for 2 minutes and cooled to room temperature. The overlapping single-stranded oligos were extended to form a blunt double-stranded transcription template with 20 units of Exo-Minus Klenow DNA Polymerase (EPICENTRE Biotechnologies) using standard conditions. The product was cloned into the vector pUC19, then plasmids were prepared, purified, and linearized with *Acc65 I*.

In vitro transcription

One microgram of linearized template was used in a standard 20 µl DuraScribe T7 Transcription reaction where the canonical CTP and UTP were replaced with 2'-F-dCTP and 2'-F-dUTP, respectively. Reactions were incubated at 40°C for 4 hours. The DuraScript RNA was precipitated and spin column purified, and yields were determined by spectrophotometry.

Gel mobility shift assays

The DuraScript RNA streptavidin-aptamer and the mutant non-binding aptamer were dephosphorylated, then end-labeled using T4 Polynucleotide Kinase (EPICENTRE) and γ -³²P-ATP under standard conditions. Each aptamer probe (10,000 cpm Cerenkov) was incubated in a binding reaction with 20 mM HEPES-NaOH, 100 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 1 µg tRNA, and increasing amounts of streptavidin for 30 minutes at room temperature. Bound and free aptamers were then resolved on an 8% polyacrylamide native gel; the gel was then dried and exposed to film.

Results

Efficient in vitro transcription of 2'-F-RNA aptamers with DuraScribe T7 Transcription Kit

A double-stranded DNA transcription template containing the T7 promoter sequence and 80 bases of a streptavidin-specific aptamer sequence was produced. The product was cloned,

purified, and restricted to produce a linear transcription template 2.8 kb in length, which produces a run-off transcript of 86 bases (80 bases are aptamer sequence and 6 bases at the 3'-end are vector sequence). DuraScribe® T7 *in vitro* transcription reactions containing 1 µg (0.54 pmoles) of linear template produced 12.9 µg (~ 460 pmoles) of DuraScript® RNA wild-type streptavidin-aptamer, and 15.9 µg (~ 560 pmoles) of the mutant aptamer, which does not bind streptavidin. FIG 1 shows the secondary structure of the DuraScript RNA aptamer, with binding domains I and II of the previously described streptavidin-aptamer.⁵ The *in vitro* transcription reactions resulted in specific transcripts of the expected size as determined by denaturing polyacrylamide gel electrophoresis (FIG 2).

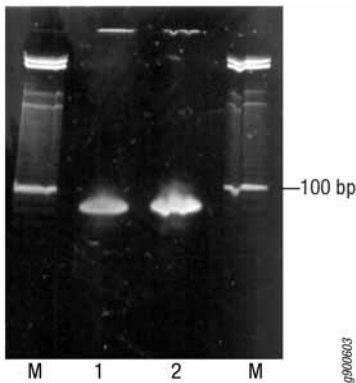


FIG 2. Uniform, full-length aptamer transcripts are produced with the DuraScribe® T7 Transcription Kit. DuraScript® streptavidin-aptamer (Lane 1) and mutant DuraScript aptamer (Lane 2) were analyzed by denaturing polyacrylamide gel electrophoresis; Lane M, 10 bp DNA ladder.

Specific binding of DuraScript RNA aptamer to streptavidin

A native gel mobility shift assay was used to detect the specific binding of the DuraScript aptamer to increasing amounts of streptavidin (FIG 3). The ³²P-labeled DuraScript RNA aptamer is smaller and therefore moves further in the gel when no streptavidin is present ("free" aptamer), but forms a slower migrating complex in the presence of 5 - 20 nM streptavidin ("bound" aptamer). No aptamer-streptavidin complex was formed when a mutant aptamer, containing a single-base change, was used in a similar binding reaction (FIG 4). The specificity of the binding was confirmed by adding excess wild-type unlabeled-streptavidin aptamer to the binding reaction. The unlabeled aptamer successfully competed with the labeled aptamer for the strep-

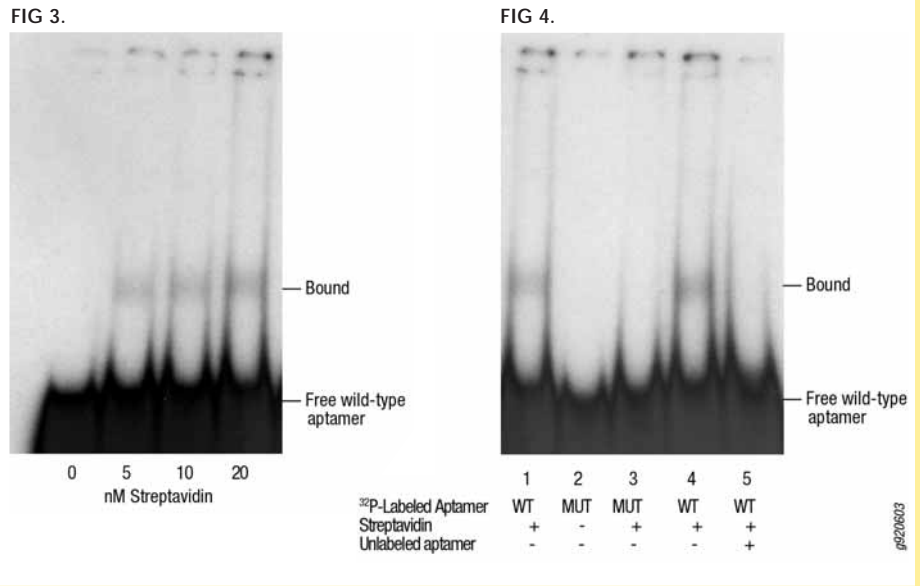


FIG 3. DuraScript® RNA aptamer complexes with streptavidin in a native gel mobility shift assay. Increasing amounts of streptavidin were incubated with 10,000 cpm of ³²P-labeled DuraScript 2'-F-RNA aptamer. The free aptamer migrates faster in the native 8% polyacrylamide gel than the protein-bound aptamer seen with 5 to 20 nM streptavidin in the binding reaction.

FIG 4. The DuraScript® 2'-F-RNA aptamer binds with high specificity to streptavidin. A native gel mobility shift assay for streptavidin binding was performed using both the DuraScript RNA aptamer and an aptamer containing a single-base mutation which disrupts binding, or using excess unlabeled-DuraScript RNA aptamer to effectively compete for the streptavidin binding activity. Lane 1, wild-type aptamer plus 20 nM streptavidin; Lane 2, free mutant aptamer; Lane 3, mutant aptamer plus 20 nM streptavidin; Lane 4, wild-type aptamer plus 20 nM streptavidin; and Lane 5, wild-type aptamer plus 20 nM streptavidin and excess unlabeled wild-type aptamer.

tavidin, resulting in the loss of the bound complex (FIG 4).

Conclusion

Unlike antibodies or canonical DNA or RNA aptamers, DuraScript RNA aptamers are resistant to both ribonuclease and deoxyribonuclease degradation,^{7,8} making them ideal for both therapeutic and diagnostic applications. High yields of DuraScript RNA aptamer can be produced quickly and affordably by *in vitro* transcription with the DuraScribe T7 RNA Polymerase, which efficiently accepts non-canonical nucleotides as substrates.

References

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www.EpiBio.com/durascribe.asp

DuraScribe® T7 Transcription Kit

DS010910	10 Reactions
DS010925	25 Reactions

Contents: DuraScribe® T7 Enzyme Mix, DuraScribe® T7 10X Reaction Buffer, ATP, GTP, 2'-F-dCTP and 2'-F-dUTP, DNase I, DTT, DuraScript® RNA Control Template, and Sterile RNase-Free Water.

T4 Polynucleotide Kinase, Cloned

P0505H	500 Units
P0501K	1,500 Units
P0503K	3,000 Units

Includes 10X Reaction Buffer without ATP. ATP is available separately.

Exo-Minus Klenow DNA Polymerase

KL04011K	1,000 Units
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Now available at concentrations of 20 U/µl and 50 U/µl.

*The use of DuraScribe T7 Transcription Kit to synthesize nucleic acids with non-canonical bases or for partial ribosubstitution is covered by U.S. patents 5,849,546; 6,107,037 and other patents issued or pending. These products are accompanied by a limited non-exclusive license for the purchaser to use the purchased product(s) solely for life science research. Contact EPICENTRE concerning licenses for other uses.