Use of High Specific Activity StarFire™ Oligonucleotide Probes to Visualize Low-Abundance Pre-mRNA Splicing Intermediates in *S. pombe*

M.A. Behlke, S.A. Dames, W.H. McDonald¹, K.L. Gould¹,
E.J. Devor and J.A. Walder

Integrated DNA Technologies, Coralville, IA and ¹Howard Hughes Medical Institute,
Vanderbilt University School of Medicine, Nashville, TN, USA

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ABSTRACT

An oligonucleotide labeling system was developed that can produce radiolabeled hybridization probes with tenfold or more higher specific activity than is obtained by traditional 5'-end-labeling with polynucleotide kinase. Yet the system is as rapid and simple as kinase labeling. The reaction uses the Klenow fragment of *E. coli* DNA polymerase to add α -³²P-dA residues to the 3'-end of an oligonucleotide in a primer-extension reaction. Unlike other methods of radioactive tailing (e.g., terminal transferase), a single species is produced of both known length and known specific activity. The reaction is efficient, and over 90% of probe molecules are routinely labeled. Using this method of labeling, an oligonucleotide was shown to be tenfold more sensitive in detecting target DNA sequences in a dot blot hybridization assay, compared to the same oligonucleotide labeled using polynucleotide kinase. Northern blots of *Schizosaccharomyces pombe* RNA were probed with an oligonucleotide specific for intron 1 of the *tf2d* gene, a TATA-box binding transcription factor. Kinase-labeled *tf2d* probe detected only unspliced RNA, while the same oligonucleotide labeled using the new method detected both unspliced *tf2d* RNA and rare pre-mRNA splicing intermediates.

INTRODUCTION

Oligodeoxynucleotides (ODNs) are commonly used as hybridization probes in molecular biology. Synthetic ODNs are inexpensive, readily available and can be made for any desired sequence. Unlike longer cloned DNA probes, ODN probes can differentiate between sequences having only a single base mismatch and so are uniquely suited to applications

where a high degree of specificity is needed.

However, one weakness in the use of ODNs as probes has been their relatively low sensitivity compared with longer probes. Cloned DNAs can be labeled to high specific activity using a variety of techniques (10), and ODNs are usually labeled with a single reporter group per molecule. The most common method of labeling oligonucleotides uses polynucleotide kinase (PNK) to transfer a single radioactive phosphate group from γ -³²P-ATP to the 5'-end of the oligonucleotide (10). The enzyme terminal deoxynucleotidyl-transferase (TdT) can be used to label an ODN to higher specific activity by adding a long homopolymeric tail to the 3'-end of the oligonucleotide (10). However, this method results in a heterogeneous reaction product ranging from unlabeled to hundred-fold labeled species. Also, the TdT-labeled probes with a tail that is longer than about 20 residues can result in high background and perform poorly when hybridized to complex nucleic acid samples.

Some schemes have been used to increase the specific activity and sensitivity of oligonucleotide probes without compromising their favorable hybridization properties. Ullrich et al. (13) and Studencki and Wallace (12) described methods in which two overlapping sequence-specific oligonucleotides are labeled in a primer extension reaction to a specific activity that can exceed 10^7 – 10^9 cpm/mg. While effective, these methods result in a double-stranded product, require two sequence-specific custom ODNs and use 2–4 radiolabeled substrate dNTPs, which makes such approaches more expensive and less convenient than labeling with PNK. ODNs having a dendrimeric structure at the 5'-end can be synthesized and la-

beled to high specific activity using PNK (11), yet these reagents are costly and not readily available.

We have developed a method of 3'-end-labeling for ODN probes to high specific activity by DNA polymerase, the StarFire[®] system, that requires only a single target-specific custom probe oligonucleotide, a universal template oligonucleotide, and a single radioactive dNTP substrate (α -³²P-dATP). The labeling reaction results in a single product of defined size that remains single stranded in the hybridizing domain. Optionally, the labeled probe can be purified using PAGE to obtain a pure species with precisely known specific activity. The method is compatible with non-isotopic labeling, and protocols have been developed for the incorporation of biotin-dA. Both DNA and RNA oligonucleotides can be labeled using this technique. Here, we show that this labeling method results in ODN probes with tenfold higher sensitivity in a dot blot hybridization assay when compared with PNK 5'-end-labeled ODN probes. We also demonstrate that probes labeled with this method allowed visualization of rare pre-mRNA splicing intermediates on Northern blots of *Schizosaccharomyces pombe* RNA that were not detectable using standard approaches.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were made using the phosphoramidite method on a DNA synthesizer (1) (model 394; PE Biosystems, Foster City, CA, USA). Phosphoramidites were obtained from PE Biosystems and Glen Research (Sterling, VA, USA). After deprotection, oligonucleotides were purified by PAGE using 15% acrylamide, 7 M urea and 1' TBE gels (10). Oligonucleotides were visualized by UV backshadowing, excised and eluted using the crush-and-soak technique. Purified oligonucleotides were desalted using a G25 NAP 10-column (Amersham Pharmacia Biotech, Piscataway, NJ, USA), lyophilized and quantified using UV spectrophotometry.

Probe ODN #R514 is a 36-base that contains a 30-base hybridization domain (50% GC with a predicted T_m of 64°C) specific for plasmid pGreen Lantern[®]-1 (Life Technologies, Rockville, MD, USA) at the 5'-end, and a 6-base template-binding domain at the 3'-end. Probe ODN #667113 is a 34-base that contains a 28-base hybridization domain (54% GC with a predicted T_m of 64°C) specific for yeast gene *tf2d* at the 5'-end and a 6-base template-binding domain at the 3'-end. ODN #R516 is a 16-base that contains a 10-base poly-dT extension domain at the 5'-end and a 6-base probe-binding domain at the 3'-end. It is the universal StarFire template. Probe ODNs R514 and 667113 end with a 3'-hydroxyl and can be extended by DNA polymerase. Template ODN R516 is 3'-end blocked (i.e., does not end in a 3'-hydroxyl) and therefore is not a substrate for DNA polymerase.

Probe #R514: 5'-CTTGATTAGGGTGATGGTTCACGT-AGTGGGxxxxxx-3'; Probe #667113: 5'-CCTTGTTCCCTATCCAAGAGGCACGACxxxxxx-3'; and Template #R516: 5'-TTTTTTTTTTTyyyyyy-3'.

The sequence "xxxxxx" represents the template-binding domain of the probe oligomers (identical for every probe), and the sequence "yyyyyy" represents the probe-binding domain of the template oligomer. The sequences xxxxxx and

yyyyyy are complementary. In this way, oligomer R516 can hybridize with any probe oligomer that bears the terminal xxxxxx sequence and so functions as a universal template in the context of the StarFire labeling system.

Oligonucleotide 5'-End Labeling

Probe oligonucleotides #R514 and #667113 were labeled by T4 PNK. Oligonucleotides were incubated in a 50- μ L reaction containing 2.5 pmol ODN (50 nM), 60 mCi γ -³²P-ATP (0.2 mM), 10 U T4 PNK (New England Biolabs, Beverly, MA, USA), 70 mM Tris, pH 7.6, 10 mM MgCl₂ and 5 mM DTT for 60 min at 37°C (1). The radionucleotide γ -³²P-ATP (6000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. The reaction was stopped with the addition of 10 μ L 100 mM EDTA, and the labeled ODN was separated from unincorporated nucleotides by two cycles of gel filtration through a MicroSpin[®] G25 column (Amersham Pharmacia Biotech). Radiolabel incorporation was measured on a Tricarb[®] 1900CA liquid scintillation counter (Packard Instrument, Meriden, CT, USA).

StarFire Oligonucleotide 3'-End Labeling

Klenow fragment (3'- $\rightarrow$ 5' exo⁻) of *E. coli* DNA polymerase was obtained from New England Biolabs. Probes (#R514 or #667113) and template (#R516) oligonucleotides were annealed in a 3 μ L reaction containing 0.5 pmol probe ODN, 12.5 pmol template ODN, 33 mM Tris, pH 7.5, 16.7 mM MgCl₂ and 25 mM DTT by incubating at 94°C for 1 min and cooling to room temperature. α -³²P-dATP and DNA polymerase were then added to the reaction, which resulted in a final labeling reaction volume of 10 μ L that consisted of 0.5 pmol probe ODN (50 nM), 12.5 pmol template ODN (1.25 mM), 5 U exo⁻ Klenow DNA polymerase, 60 mCi α -³²P-dATP (1 mM), 10 mM Tris, pH 7.5, 5 mM MgCl₂ and 7.5 mM DTT. The labeling reaction was incubated for 60 min at room temperature. The reaction was stopped with the addition of 40 μ L 10 mM EDTA, and the labeled ODN was separated from unincorporated nucleotides by two cycles of gel filtration through a MicroSpin G25 column. Radiolabel incorporation was measured on a Tricarb 1900CA liquid scintillation counter.

Dot Blot Hybridization

Plasmid pGreen Lantern-1 was diluted in water and denatured by heating at 94°C for 5 min. Aliquots of 100, 10, 1.0 and 0.1 μ mol of the plasmid target were spotted onto Hybond[®]-N Nylon membranes (Amersham Pharmacia Biotech). The filter was soaked in 0.5 N NaOH for 5 min, neutralized in 1 M, Tris, pH 7.5, 1.5 M NaCl for 5 min and baked at 80°C for 2 h.

Filters were prehybridized for 30 min in Perfecthyb[®] hybridization buffer (Sigma, St. Louis, MO, USA). Filters were hybridized for 3 h at 60°C in 10 mL Perfecthyb with 10⁷ cpm of either PNK 5'-end labeled or StarFire 3'-end labeled ODN probe #R514 (10⁶ cpm/mL). Filters were washed for 10 min, three times in 1' SSC (150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS at 37°C. Filters were exposed for 4 h to a Super Sensitive (Type ST) screen and visualized using a Cyclone[®] Phosphor Imaging System (both from Packard Instruments).

S. pombe wild-type and conditional RNA splicing mutant strain *prp2-1* (8) were grown at the permissive temperature (25°C) and shifted to the nonpermissive temperature (36°C) for 2 h. Total RNA was isolated as described by Moreno et al. (5), and 15 mg total RNA were separated using formaldehyde-agarose gel electrophoresis (1), transferred to Duralon-UV membranes by capillary action and UV-cross-linked by exposure to the Stratallinker UV source (both from Stratagene, La Jolla, CA, USA).

RESULTS AND DISCUSSION

with tenfold higher specific activity than can be obtained using PNK. The method requires synthesis of only a single custom oligonucleotide that contains a sequence-specific hybridization domain at the 5'-end and a template-binding domain at the 3'-end. The sequence-specific domain is usually 15–40 bases in length, is unique to each target and functions as the target-specific hybridization probe. The template-binding domain is a predefined element that is identical for every StarFire probe and directs association with a second “universal” ODN that serves as template in a primer-extension labeling reaction. In this way, the same template ODN can direct the labeling of any StarFire probe ODN. Binding domains of different length and chemical composition were tested. For use in commercial StarFire kits, a 6-base sequence was chosen that routinely results in greater than 90% probe labeling efficiency.

The end product of the StarFire reaction is a labeled ODN probe that remains single stranded in the 5¢ hybridization domain and is double stranded in the labeled 3¢ end. Unincorporated nucleotides are removed by gel filtration, and further purification is not necessary for most applications. However, if desired, the template oligonucleotide can be removed and the labeled oligonucleotide purified to homogeneity by PAGE because the labeled ODN is 10 bases longer than the starting substrate. Figure 1 summarizes the StarFire labeling tech-

Figure 1. The StarFire oligonucleotide labeling protocol. (A) Two oligonucleotides are used in the reaction—a target-specific probe oligonucleotide and a universal template oligonucleotide. The oligonucleotides are annealed (B), labeled in a primer extension reaction by DNA polymerase (C) and unincorporated nucleotides are removed by gel filtration (D).

nique, and additional information can be found at <http://www.idtdna.com/Starfire/StarfireProtocol.html>.

Oligonucleotide #R514, specific for plasmid pGreen Lantern-1 (bases 859–888), was labeled using both PNK and StarFire methods. Unincorporated nucleotides were removed by two cycles of gel filtration, and 1% of the reaction was tested for radiolabel incorporation by scintillation counting. Specific activity was calculated based on the mass of input probe and assumed that no material was lost in processing or purification. Because efficient probe recovery cannot be 100%, these calculations represent only a conservative estimate of probe specific activity. Kinase reactions resulted in probes having an average specific activity of 7.4×10^6 cpm/pmol. StarFire reactions resulted in probes having an average specific activity of 7.1×10^7 cpm/pmol or 4×10^9 cpm/mg. As predicted, StarFire labeling resulted in probes having about tenfold higher specific activity than kinase-labeled probes.

Dot blot hybridizations were done to quantitatively compare the sensitivity of a StarFire-labeled probe with a kinase-labeled probe. Duplicate dot blot filters were prepared with a serial dilution of plasmid pGreen Lantern-1 and were hybridized to 1×10^6 cpm/mL probe #514 labeled using either PNK or StarFire. In a 4-h exposure using a Cyclone phosphor imaging system, the limit of detection for the kinase-labeled probe was 10 amol target plasmid. Using identical hybridization, wash and exposure conditions, the StarFire-labeled probe detected 1 amol target (Figure 2). With longer exposures, the

StarFire probe could detect signal from as little as 100 zmol target. Thus, the StarFire probe performed as predicted in that a probe with a tenfold increase in specific activity showed a tenfold increase in sensitivity. We next examined the use of a StarFire-labeled ODN as a hybridization probe for RNA.

Short ODN probes offer certain advantages over long genomic DNA probes when studying RNA splicing mutants. For example, oligonucleotides can be designed specifically for pre-mRNA, mature mRNA or intermediate structures. ODN probes have been used to detect pre-mRNA species in yeast total RNA on Northern blots and to identify strains of yeast with splice-pathway mutations (3,4,6–8). However, pre-mRNA splicing intermediates are usually present in low abundance and are more easily detected with high specific activity, high sensitivity genomic DNA probes (labeled by random-priming or nick-translation methods) than with kinase end-labeled ODN probes.

The ideal tool to detect pre-mRNAs and other splicing intermediates would be a probe that combined the high sensitivity associated with longer genomic DNA fragments and yet retained the specificity of short ODNs. We tested whether the StarFire method would produce oligonucleotide probes having these favorable properties in a Northern blot assay for yeast *tf2d* pre-mRNA.

The *tf2d* gene encodes a TATA-box binding factor (2) that is expressed at relatively high levels. Oligonucleotide probes specific for *tf2d* have previously been used to assay pre-mRNA splicing defects in *S. pombe* (14). The *tf2d* precursor RNA (the exon + intron-containing species) is not easily detected in wild-type yeast by Northern blot analysis; however, *tf2d* pre-mRNA accumulates and can be more readily detected in certain pre-mRNA splicing mutants. Even in pre-mRNA splicing mutants, the utility of 5'-end-labeled ODN probes remains limited to transcripts that are relatively abundant, such as *tf2d*, and to those mutant strains that have a relatively profound splicing defect.

Northern blots were prepared using total RNA from wild-

Figure 3. Northern Blot demonstration of pre-mRNA splicing intermediates in *S. pombe*. Northern blots were prepared using total RNA from *S. pombe* wild-type (wt) and strain *prp2-1*, a conditional lethal mutant that accumulates pre-mRNA splice products at 36°C but proceeds with splicing normally at 25°C. Duplicate blots were probed with ODN #667113, specific for intron 1 of the *tf2d* gene. One blot (left) was hybridized to ODN #667113 end-labeled with PNK, and a second blot (right) was hybridized to the same ODN labeled with the StarFire method. Hybridization conditions, washes and exposures were otherwise identical.

type cells and the conditional lethal pre-mRNA splicing mutant strain *prp2-1*. Cells were grown at the permissive temperature (25°C) and shifted to the nonpermissive temperature (36°C) for 2 h. At 36°C, the *prp2-1* mutation blocks initiation of pre-mRNA splicing, and pre-mRNA accumulates (9,14). A 28-base sequence specific to intron 1 of the *tf2d* gene, (GenBank[®] accession no. X53415, bases 615–642) was synthesized (probe #667113) and labeled using both the StarFire and PNK methods. Duplicate blots were hybridized, washed and exposed in parallel (Figure 3). The kinase-labeled probe did not detect *tf2d* intron-containing transcripts in either wild-type or *prp2-1* cells that were grown at 25°C; at 36°C, unspliced *tf2d* transcript was detected in the *prp2-1* mutant.

Using the StarFire-labeled probe, *tf2d* precursor RNA was faintly detectable in both wild-type and *prp2-1* cells at 25°C and was easily seen in *prp2-1* cells grown at 36°C. Interestingly, two additional RNA species were detected using the StarFire probe in the *prp2-1* cells grown at 36°C, one migrating faster and one slower than *tf2d* pre-mRNA. These species presumably represent splicing intermediates of the *tf2d* transcript that have not been detected before. Since lariat structures retard the migration of RNAs, we predict that the slowest migrating intermediate represents a lariat-exon (E1 - I1+lariat - E2 - I2 - E3) structure. The more rapidly migrating species most likely represent the excised intron1+lariat structure. Neither of these species was detected using a PNK end-labeled oligonucleotide probe.

The StarFire method of labeling oligonucleotides provides a more sensitive approach for detecting pre-mRNA splicing defects in *S. pombe* than standard techniques. This added sensitivity should enable detection of more subtle pre-mRNA splicing defects and also allow a broader range of transcripts to be assayed. By designing oligonucleotide probes that recognize only precursor or mature forms, it will be possible to assay for splicing defects, even with transcripts in which the small size of the intron precludes separation of pre-mRNA and mRNA by gel electrophoresis. It should also be possible to design ODNs specific for the branched structure of the lariat intermediates to visualize these species.

The StarFire labeling system provides a tenfold increase in specific activity and sensitivity compared to PNK labeling but retains the low cost, specificity and ease-of-use of an oligonucleotide format. Because the sensitivity of StarFire-labeled oligonucleotides compares well to random-primed or nick-translated probes, they can be used in applications that require this degree of sensitivity. If greater than a tenfold increase in sensitivity is desired, longer labeled tails can be added. However, homopolymeric dA tails longer than 20 bases can lead to higher background hybridization in some settings. As an alternative to a longer tail, increased sensitivity can be obtained by using more than one probe for a given target. Non-isotopic labels such as biotin can also be incorporated using the standard StarFire kit by substituting the corresponding dNTP substrate for α -³²P-dATP in the primer-extension reaction.

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Address correspondence to Dr. Mark A. Behlke, Vice President, Molecular Genetics and Bioinformatics, Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241, USA. e-mail: mbehlke@idtdna.com