



Starfire™ System for High Efficiency 3' Oligonucleotide Labeling Using Polymerase Extension

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Introduction

Beginning with the classic methods of *in situ* hybridizations and the venerable Southern blot, labeling nucleic acids with a variety of reporting tags has led to an ongoing line of major advances in molecular biology [1–4]. Low specific activity *in vivo* labeling methods were superseded in the mid-1970s by a succession of *in vitro* enzymatic methods. Nick translation with DNase I followed by DNA polymerase-driven incorporation of radiolabeled deoxynucleotide triphosphates (dNTPs) increased specific activity more than 100-fold [5]. A few years later Feinberg and Vogelstein introduced random primer labeling [6, 7]. As the name suggests, random single-stranded primers from six to ten nucleotides long were used, in the presence of the Klenow fragment of DNA polymerase, to produce an additional ten-fold increase in specific activity. Moreover, though random priming was originally used with radiolabeled dNTPs, non-radioactive nucleotides like fluorescein-11-dUTP and biotin-14-dCTP could be incorporated as well.

While nick translation and random priming were opening new vistas for labeling large DNA fragments for use as reporters, these methods were inappropriate for efficiently labeling oligonucleotides. For these short, single-stranded DNAs the methods of choice were enzymatically-mediated end-labeling techniques. 3' end labeling reactions were catalyzed by terminal deoxynucleotidyl transferase (TDT) and 5' end labeling reactions were catalyzed by polynucleotide kinase (PNK) purified from T4 bacteriophage. These methods permitted placing a single label at one end of an oligonucleotide which, even at high labeling efficiencies, limited the obtainable specific activities. On the other hand,

these methods could also be used with non-radioactive labels with varying degrees of success [8].

What has always been needed is a method that labels oligonucleotides efficiently and results in substantially improved per molecule specific activities. It would also be ideal if this method could be adapted for use with non-radioactive tags as well. Such a method has been developed. Called Starfire, this method uses a DNA polymerase catalyzed primer extension reaction to generate oligonucleotide probes with ten-fold greater per molecule specific activity with radiolabels and can be adapted to incorporate non-radioactive tags [9].

Starfire DNA Polymerase Primer Extension

The very high per-molecule specific activities obtained for large nucleic acid probes via nick translation and random primer methods were simply a matter of incorporating more and more labeled dNTPs into each molecule. The Starfire method is a means of accomplishing the same thing with an oligonucleotide probe. The key, of course, is having a place to put the labels. This is accomplished by adding a single-stranded template to the 3' end of the oligonucleotide (Figure 1). The oligonucleotide probe sequence is extended with a 3' hexamer and a second, universal template oligonucleotide, composed of an oligo-dT₁₀ sequence with a 3' extension complementary to that on the probe, is introduced into an annealing reaction. The complementary hexamer sequence of the template is modified in such a way as to drive the bimolecular annealing reaction to a completion efficiency greater than 95%. The "tailed" oligonucleotide thus has a ready-made conformation ideal for a DNA polymerase extension reaction.



Figure 1. Schematic representation of a Starfire™ polymerase extension labeling reaction. 1. A target-specific oligonucleotide is synthesized with a 3' hexamer extension. 2. A 10-minute reaction is carried out between the extended target-specific oligonucleotide and the template oligonucleotide in which the complementary hexamers anneal. 3. Annealed duplexes are then

labeled with α -³²P-dATP in the presence of the Klenow fragment of DNA polymerase. 4. The template is removed (optional) by PAGE purification or thermal dissociation.

By incorporating a 3' "block" on the template molecule, the DNA polymerase extension reaction is forced to proceed only off the 3' end of the probe sequence. In the presence of the Klenow fragment of DNA polymerase, radiolabeled α -³²P-dATP is "filled in" on the oligo-dT template. This permits the addition of ten radiolabeled deoxynucleotides per molecule at very high efficiencies!

Dot blot hybridizations with Starfire-labeled oligonucleotides showed dramatic improvements in sensitivities. Detection of a target as rare as 1 attomole was routinely observed. In general, a ten-fold increase in sensitivity was achieved as a consequence of a ten-fold increase in per molecule specific activity.

Starfire Applications

Behlke et al. reported on the use of Starfire-labeled probes specific for the *Schizosaccharomyces pombe* transcription factor gene *tf2d* [9]. Compared to traditional kinase-labeled probes with the same target sequence, the Starfire-labeled probes were able to detect *tf2d* precursor RNA in both wild-type and *prp2-1* mutant *S. pombe*. In addition, two previously unknown *tf2d* splice intermediates were detected in the *prp2-1* splice initiation mutant. As these intermediates are present in very low copy numbers even in the *prp2-1* mutants their existence was not suspected until the significantly greater sensitivity of the Starfire-labeled probe revealed them.

Calcium/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) has been proposed as a candidate regulator of male germ cell differentiation. The CaMKIV/Gr gene encodes two different isoforms called α (short) and β (long) forms. These two forms are differentially expressed in mouse tissues. Testes express only the α -isoform whereas both isoforms are seen in neurons and T-lymphocytes. Detection of specific mRNA levels in mouse germ cell tissue using Starfire-labeled oligonucleotides was employed by Blaeser et al. to investigate the role of CaMKIV/Gr in spermatogenesis. CaMKIV/Gr transcripts were easily detected in wild-type mice but were completely lacking in CaMKIV/Gr deficient knock outs [10]. Since both the wild-type and the knock out lines exhibited normal spermatogenesis and fertility, Blaesner et al. concluded that CaMKIV/Gr is not essential in spermatogenesis.

The amazing and still developing story of small RNAs in eukaryotes now encompasses several classes of these molecules. One of these classes, the micro-RNA (miRNA), was first discovered and reported by Victor Ambros, Rosalind Lee, and colleagues [11]. The 22nt miRNAs *lin-4* and *let-7* of *Caenorhabditis elegans* were shown to be regulators controlling the timing of developmental events. Since then, numerous miRNAs have been identified and shown to have many diverse functions [12–14]. Recently, Sempere et al. profiled the expression of 119 miRNAs in human and mouse tissues using Starfire-

labeled oligonucleotide probes [15]. Many of these miRNAs were found to be specifically expressed or enriched in particular tissues. Of 13 miRNAs observed to be enriched or exclusively expressed in mouse and human brain tissues eight were also seen to be induced during differentiation of neurons derived from embryonic carcinoma (EC) cells treated with retinoic acid (RA). Their results suggest that these miRNAs are intimately involved in determining the specification and/or progression of neuronal fate in mammals [15]. This, of course, potentially infers a major tissue-specific regulatory role for many members of the rapidly growing family of miRNAs.

These, and other, studies using Starfire-labeled oligonucleotide probes attest to the enhanced sensitivities that can be achieved.

Starfire Oligonucleotide Labeling Kit

Starfire oligonucleotide probes are target- and application-specific custom syntheses that can be up to 40 nt in length. The Starfire modification consists solely of the 3' hexamer extension necessary for template annealing.

In addition to the user specified probe, the complete Starfire kit includes:

- Starfire Template oligonucleotide
- Universal labeling reagent (sufficient for 25 reactions)
- 10X Starfire™ buffer (100mM Tris pH 7.5, 50mM MgCl₂, 75mM DTT)
- Starfire Stop buffer (10mM EDTA)
- exo⁻ Klenow DNA polymerase

Additional reagents required but not supplied:

- [α -³²P]-dATP (10mCi/ml, 6000Ci/mmol)¹
- TE (10mM Tris pH 8.0, 0.1mM EDTA)²

Other necessary equipment and materials:

- Microcentrifuge and 1.5 mL microcentrifuge tubes
- Sephadex™ (G25 spin columns or gravity flow columns)
- Waterbath or heatblock (95°C)
- Pipettors with aerosol barrier tips
- PPE³

Starfire Radiolabeling Protocol

Oligonucleotide Resuspension and Stock Dilutions

Oligonucleotides, including Starfire probes, are provided lyophilized. Before using any dried oligonucleotide it is recommended that the tubes be briefly spun down. This will ensure that any of the dried material that may have become dislodged from the tubes during shipping will be at the bottom when the cap is removed. Following centrifugation:

1. Resuspend the Starfire TEMPLATE oligonucleotide in 25 μL 1x TE (final concentration will be 12.5 μM)
2. Resuspend the Starfire PROBE oligonucleotide to a storage stock concentration of 100 μM ⁴.
3. Dilute an aliquot of the stock probe 1:200 (v/v) in sterile, nuclease free water. This gives a final working concentration of 0.5 μM , or 0.5 picomoles/ μL .
4. All Starfire reagents should be stored at -20°C until needed.

Starfire Labeling Procedure

The Starfire labeling procedure follows the steps shown in Figure 1. Annealing the probe and template oligonucleotides requires ten minutes. Primer extension requires one to one and one-half hours. Removal of unincorporated radiolabels requires ten minutes. If the additional step of removing the template oligonucleotide from the labeled probe is desired, the time required will depend upon the method chosen for removal.

Annealing

1. Pipette 1 μL of the working stock (0.5 μM) Starfire probe into a 0.5 mL microcentrifuge tube.
2. Add 1 μL of the template oligonucleotide and 1 μL of 10X Starfire reaction buffer. Gently mix using a pipette (DO NOT VORTEX).
3. Place the reaction tube in a 95 $^{\circ}\text{C}$ heat block or water bath for 1 minute.
4. Remove the tube from the heat and allow the reaction to cool to room temperature (usually 5 minutes is sufficient). Briefly spin the reaction.

Labeling

1. Add 6 μL [α -32P]-dATP and 1 μL of exo^{-} Klenow DNA polymerase. Gently mix using pipette.
2. Incubate the labeling reaction at room temperature for 1 to 1½ hours.
3. Add 40 μL of Stop buffer.

Column Purification

1. Load entire labeling reaction onto a SephadexTM G25 column and spin for 3 minutes at 3,000 rpm.
2. Determine labeling efficiency by counting a 1 μL aliquot of the purified labeling reaction in a scintillation counter.

Additional Purification Option

The Starfire annealing reaction routinely proceeds at very high efficiency resulting in ~90% of the available probe molecules being annealed with the template

oligonucleotide. Klenow enzymatic extension is highly processive so that nearly 100% of the annealed templates will be completely filled in with radiolabels. The labeled probe will, therefore, constitute the greatest mass in the final reaction following removal of the unincorporated labels. If it is desirable to remove all but the labeled probes this can be accomplished via PAGE since the unlabeled species will be 10 nt **shorter** than the labeled species. PAGE purification will also remove the template oligonucleotide from the labeled probe. We have demonstrated, however, that the presence of the double-stranded region of the fully labeled probe (see Figure 1) does not interfere with the hybridization kinetics of the probe.

A Non-Radioactive Starfire Alternative

In some instances it may be preferable to use a non-radioactive probe instead of a radiolabeled probe. For such instances, non-radioactive ligands can be added to Starfire probes via derivatized dATP. Substitution of Biotin-14-dATP (Invitrogen Cat. No. 19524-016) is fully compatible with the Starfire labeling reaction. A ten-fold higher concentration of label (10 μ M) is recommended when using Biotin-14-dATP. The [α -³²P]-dATP is directly substituted from a 10 μ M stock solution of Biotin-14-dATP in the polymerase extension reaction.

Notes

¹Suppliers of [α -³²P]-dATP (10 mCi/mL, 6000Ci/mmol) are Amersham and New England Nuclear. When starting to use Starfire for the first time, it is useful to carry out test reactions with the materials supplied by each manufacturer to see which works best (RC Lee, personal communication).

²Purists would recommend making TE from scratch but IDT offers a nuclease-free buffer, IDTE (10 mM Tris, 0.1 mM EDTA, pH 8.0), that will save you time and money.

³When working with radioactive materials it is required that appropriate Personal Protective Equipment and adequate shielding be used at all times.

⁴The conversion, 10 μ L of water per nmole of oligonucleotide yields a 100 nM (100 pmole of oligonucleotide/ μ L) stock concentration.

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