Molecular and Cellular Probes 29 (2015) 228–236

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Synthesis and use of universal sequence probes in fluorogenic multi-strand hybridisation complexes for economical nucleic acid testing

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ARTICLE INFO

Article history:
Received 19 February 2015
Received in revised form
6 May 2015
Accepted 12 May 2015
Available online 14 May 2015

Keywords:
Oligonucleotide probe synthesis
HyBeacon
Universal probes
Real-time PCR
Melting curve analysis

ABSTRACT

Analysis of nucleic acid amplification products has become the gold standard for applications such as pathogen detection and characterisation of single nucleotide polymorphisms and short tandem repeat sequences. The development of real-time PCR and melting curve analysis using fluorescent probes has simplified nucleic acid analyses. However, the cost of probe synthesis can be prohibitive when developing large panels of tests. We describe an economic two-stage method for probe synthesis, and a new method for nucleic acid sequence analysis which together considerably reduce costs. The analysis method utilises three-strand and four-strand hybridisation complexes for the detection and identification of nucleic acid target sequences by real-time PCR and fluorescence melting.

1. Introduction

Nucleic acid amplification has been a principal method for DNA diagnostics for a number of years, with the Polymerase Chain Reaction (PCR) widely accepted as the gold standard. The development of real-time PCR and fluorescent oligonucleotide probes such as TaqMan®, Molecular Beacons, Hybridisation Probes and Scorpion primers [1–5] allows homogenous analysis of amplified target sequences for applications such as pathogen detection, DNA quantification and Single Nucleotide Polymorphism (SNP) genotyping. These nucleic acid amplification testing (NAAT) methods have led to efficient, automated testing for a wide variety of targets through specialist real-time PCR instrumentation. Many such platforms use standard 96 or 384 well plates, meaning that large numbers of samples and many different targets can be simultaneously investigated, especially when tests are multiplexed using probes labelled with different fluorescent dyes.

More recently there has been a trend for NAAT to move out of the lab, and into the field with the development of point-of-care (POC) testing. Reductions in amplification times from both improved PCR enzymes and isothermal amplification techniques have meant that POC tests can achieve results within 30–60 min. Furthermore, advancements in the production of simpler, portable and less expensive equipment are opening up the possibility of providing NAAT using fluorescent probes to more remote locations and increasingly varied applications [6,7]. This has greatly increased the demand for inexpensive and reliable sequence-specific oligonucleotide probes. The development of multi-target tests, such as for a panel of SNPs, can require significant investment in probe synthesis. The cost of synthesising fluorescent DNA probes can be largely attributed to the fluorescent dyes and quencher molecules, many of which cost between $100 and $300 for each probe. The probe cost per test is extremely low for routine and high throughput applications where a single oligonucleotide synthesis batch can be used to test over five thousand samples. However, the investment in probe synthesis can be prohibitive if assays are required for applications with small numbers of samples, or for studies involving a large number of nucleic acid targets.

HyBeacon® probes are single-stranded oligonucleotides with one or more internal bases labelled with a fluorescent dye. They provide a simple fluorogenic method for the detection and

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http://dx.doi.org/10.1016/j.mcp.2015.05.007
0890-8508/

identification of nucleic acid sequence using real-time PCR and melting curve analysis [8,9]. HyBeacon probes are able to simultaneously detect multiple sequence variants and have been effectively used in multiplexes of up to six colours for applications such as pathogen detection, SNP genotyping and analysis of Short Tandem Repeats [10–12]. Production of HyBeacon probes can be achieved by the addition of dye-labelled phosphoramidites during solid-phase oligonucleotide synthesis or through post-synthetic addition of fluorophores [11]. Synthesis costs using these methods are typically between $250 and $500 per probe, which can be prohibitive when developing tests for a panel of SNPs such as for cytochrome P450 2D6 (CYP2D6) genotyping. We have therefore been exploring novel probe synthesis and analysis strategies, modelled on HyBeacons, to investigate whether major cost savings can be achieved whilst maintaining a high standard of analytical performance.

We now report that we have achieved this objective using two methods. The first is an economical two-stage solid-phase oligonucleotide synthesis process (Fig. 1), where an initial large-scale synthesis is used to assemble a universal probe sequence containing the expensive elements; the dye-labelled nucleotides and 3′-cap modifications. This is then followed by multiple small-scale syntheses to add target-specific sequences to the universal element. The universal element synthesised in the first stage can be used to produce many different probes for the detection of unrelated target sequences. The second method uses universal probes that do not hybridise to target sequences directly thereby allowing analysis of unrelated target sequences. Both methods utilise multi-strand hybridisation complexes for nucleic acid sequence analysis. These novel probing complexes were used to detect *Chlamydia trachomatis* target sequences and interrogate a number of SNPs in the CYP2D6 and VKORC1 (vitamin K epoxide reductase complex, subunit 1) genes using real-time PCR and melting curve analysis.

2. Materials and methods

2.1. Two-stage oligonucleotide probe synthesis

All oligonucleotides were synthesised by the solid-phase phosphoramidite method. Phosphoramidite monomers, solid supports and additional reagents were purchased from Link Technologies Ltd (Bellshill, UK), Sigma Aldrich (Dorset, UK) and Life Technologies (Paisley, UK).

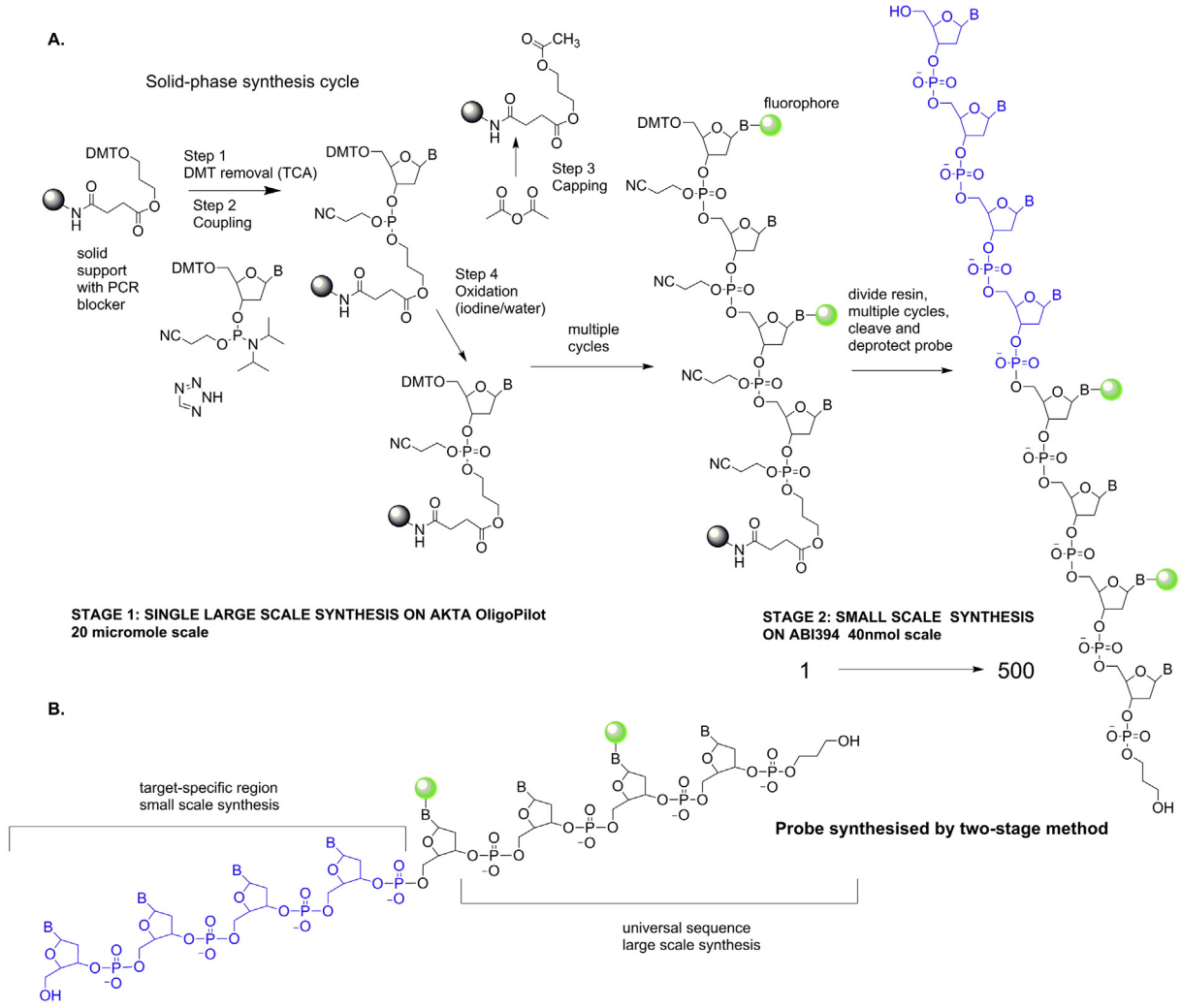

Fig. 1. A) Schematic representation of two-stage oligonucleotide synthesis. The first large scale stage comprises repeated cycles of detritylation, activation, coupling and oxidation to synthesise universal sequences containing internal dye-labelled bases and 3′-cap modifications. After division of resin, smaller scale synthesis is used to add unmodified target specific sequence onto the universal element. B) Representation of a probe synthesised using the two-stage method with universal (UE) and target specific (TE) sequence elements indicated. The structure does not present the recommended lengths of UE and TE sequences or the ideal placement of fluorophores relative to the junction.