Nucleic acid capture assay, a new method for direct quantitation of nucleic acids

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ABSTRACT

Technologies allowing direct detection of specific RNA/DNA sequences occasionally serve as an alternative to amplification methods for gene expression studies. In these direct methods the hybridization of probes takes place in complex mixtures, thus specificity and sensitivity still limit the use of current technologies. To address these challenges, we developed a new technique called the nucleic acid capture assay, involving a direct multi-capture system. This approach combines a 3’-ethylene glycol scaffolding with the incorporation of 2’-methoxy deoxyribonucleotides in the capture sequences. In our design, all nucleotides other than those complementary to the target mRNA have been replaced by an inert linker, resulting in significant reductions in non-specific binding. We also provide a versatile method to detect the presence of captured targets by using specific labeled probes with alkaline phosphatase-conjugated anti-label antibodies. This direct, flexible and reliable technique for gene expression analysis is well suited for high-throughput screening and has potential for DNA microarray applications.

INTRODUCTION

Conventional amplification methods for the detection and quantification of specific nucleic acid sequences are known to be extremely sensitive. These methods, however, require multiple steps that can generate false positives and affect their reproducibility. In addition, time-consuming and labor-intensive steps make these approaches unsuitable for high-throughput applications. As an alternative, sandwich hybridization approaches were investigated (1). First described by Dunn and Hassell (2), these methods used nucleic acid probes complementary to the DNA or RNA target to be identified and quantified, of which one type was attached to a solid support and the other labeled. Although limited interference from proteins or other biological contaminants allowed direct quantitation, the sandwich hybridization was relatively slow and inefficient (3,4). Furthermore, the original methods employed radioisotopic detection systems that limit probe shelf-life.

Progress in synthetic oligonucleotide synthesis, in conjunction with the development of branched oligodeoxyribonucleotides (5), revolutionized hybridization assay technology. Rapid nucleic acid hybridization assays were developed (6) by combining solution and sandwich hybridization with the use of branched DNA (bDNA) and enzyme-labeled probes. These methods, known as bDNA assays, rely on the solution-phase hybridization of two sets of target probes called capture and label extenders. Capture extenders hybridize to both the nucleic acid target and a DNA oligomer bound to a solid support. Label extenders bind to different sequences on the target molecule and the synthetic bDNA amplifier. Alkaline phosphatase-conjugated probes that hybridize with the amplifier mediate a chemiluminescent reaction, leading to the amplification and detection of the capture event. The bDNA assay technology has been used for the quantification of various nucleic acid targets in different types of samples (7–11) and generally allows quantitation of between 10⁴ and 10⁷ molecules (7–9). In some cases, using increased amplification, investigators were able to quantitate as few as 50–500 target molecules (10,11). Although bDNA technology provides sensitive hybridization assays with a wide dynamic range, precise and accurate quantitation, there are still major limitations preventing its broad and routine use in research laboratories. For example, bDNA assays necessitate the tedious task of synthesizing branched oligodeoxyribonucleotides and alkaline phosphatase-conjugated probes. Furthermore, they require multiple layers of probes to capture and signal the target molecule, which often triggers high background. Finally, although the bDNA technology format could be easily adapted to high-throughput screening, the assay costs limit such an application.

To overcome these limitations, we developed a new technology called the nucleic acid capture assay (NACA), which allows high-throughput direct quantification of mRNAs. Our approach combines a 3’-ethylene glycol scaffolding with the incorporation of 2’-methoxy deoxyribonucleotides in the capture sequences covalently attached to a solid support. In our design, all nucleotides other than those complementary to the target mRNA have been replaced by an
inert linker, which significantly reduces, if not eliminates, non-specific binding. We also provide an easy and versatile method to detect the capture of the nucleotided target of interest using specific probes labeled either with digoxigenin (DIG), fluorescein isothiocyanate (FITC) or biotin, combined with alkaline phosphatase-conjugated anti-DIG, anti-FITC antibodies or streptavidin, respectively, and a chemiluminescent substrate. Although the target molecule is directly captured onto the solid support and no branched oligodeoxynucleotide is used for detection, we could successfully quantitate the level of fetal hemoglobin mRNA (gamma hemoglobin, \( \gamma \)Hb) with greater sensitivity than the bDNA technology. In order to validate our technology with 'real world samples' we measured the expression of the human \( \gamma \)Hb gene in primary bone marrow cells and compared the NACA with quantitative RT–PCR, a well established and broadly used gene expression analysis method. Finally, we demonstrate that our method holds potential for improvements in the capture process for DNA array applications.

MATERIALS AND METHODS

All reagents were ordered from Sigma (St. Louis, MO, USA) unless indicated otherwise.

Branched DNA assay

Using ProbeDesigner software (12) (Chiron Diagnostic, East Walpole, MA, USA) a set of probes specific for the human fetal hemoglobin (\( \gamma \)Hb) was designed as follows. Capture extenders: 1. tggcggaaatgtgatcgaatattttcttgaaagaagat; 2. gcactctcggagttaacctttctcttgaaagaagat; 3. ttcctgcaagaggctctttcttgaaagaagat; 4. gctttcttcttgaaagaagat; 5. atttgattgcatagctaaatctttcttgaaagaagat; 6. tgttctttagctagattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
deoxynucleotide triphosphates (dNTP) and DIG-dUTPs in a template-independent reaction (16).

Quantitative RT–PCR
Total RNA was isolated from the NACA bone marrow cell lysate using the QiaShredder and Qiagen RNAeasy Mini Prep kits (Qiagen). Quantitative RT–PCR analysis for the human γHb gene was performed using an ABI PRISM 7700 sequence detection system and software (Perkin Elmer) as previously described (17) using the following primers: forward, GCCCTGTCCTCCAGATACCA; reverse, TTCCAGAATA- AAAGCCTATCCTTGA; and probe, TGAGCTCACTGCC- CATGATGCAGAG. The ribosomal protein L19 (RPL19) was chosen as internal standard. The relative expression level of γHb was computed with respect to the expression level of RPL19.

RESULTS AND DISCUSSION
Implementation of the NACA detection system
A set of capture, label and blocker sequences was designed to establish a bDNA assay for human fetal hemoglobin (γHb) (Fig. 1A). In the bDNA assay, synthetic γHb RNA hybridizes in solution to a set of specifically designed capture and label extenders. To reduce non-specific binding and to stabilize the target, blockers hybridizing specifically to the γHb RNA were also added. Capture of γHb RNA on a solid support subsequently results from the binding of capture extenders to DNA oligomers covalently bound to the solid support provided in the QuantiGene bDNA signal amplification kit (see Materials and Methods). The signaling of the captured γHb RNA is accomplished through a synthetic bDNA amplifier molecule containing 15 branches. This DNA structure hybridizes to a specific sequence present in all the label extenders. Several labels were investigated, including DIG, FITC and biotin. The captured γHb mRNA was successfully detected at similar efficiencies using DIG- or FITC-labeled oligonucleotide in conjunction with alkaline phosphatase conjugated to anti-DIG or anti-FITC, respectively (data not shown). Biotin-labeled oligonucleotide followed by alkaline phosphatase conjugated to streptavidin was similarly effective (data not shown). These detection

Figure 1. Comparison between the γHb bDNA assay and first generation NACA. The γHb bDNA assay (A) and the first generation NACA (B) differ in their detection strategies. The bDNA assay uses bDNA amplifier molecules that contain a maximum of 45 binding sites for alkaline phosphatase-conjugated probes. The label extenders contain a sequence specific for the γHb RNA target as well as an overhang sequence designed to bind the bDNA amplifier. Similarly, in the first generation NACA, the analyte-binding oligonucleotides contain a sequence specific for the γHb RNA target as well as an overhang sequence designed to bind the stem oligonucleotide. The labeled oligonucleotides, containing a maximum of 10 label molecules (DIG, FITC or biotin) bind to the stem oligonucleotide. Anti-label antibodies or streptavidin conjugated to alkaline phosphatase signal the capture event. Performances of the γHb bDNA assay (filled circles) and the NACA (open circles) with DIG-labeled oligonucleotides were compared using recombinant γHb RNA at concentrations ranging from 6.8 pg/ml to 5 ng/ml (C). While the bDNA assay generated a strong non-linear signal for the range of γHb RNA concentration tested, the first generation NACA generated a linear, albeit lower signal for the same range of concentrations (note the difference in RLU scales).
approaches show promise in multiplexing the analysis. Although the specific signal intensity was a hundred times lower in the \(g\)Hb NACA than in the bDNA assay (Fig. 1C), the background was also lower in the \(g\)Hb NACA, resulting in similar signal-to-noise ratios. As presented in Figure 1C, the \(g\)Hb NACA provides better sensitivity and a greater dynamic range than the bDNA assay. Later, we obtained similar results using a labeled oligonucleotide that directly hybridizes with the analyte-binding oligonucleotide (data not shown).

Direct binding of the capture sequences on the solid support

In the indirect capture system, the capture extenders hybridize freely to the target mRNA in solution before hybridizing to the DNA oligomer covalently attached to the solid support. Although this strategy increases the versatility of the technique, the introduction of an additional nucleic acid sequence could be a source of non-specific binding and decreased signal-to-noise ratio. To determine whether the indirect capture results in better performance than the direct capture approach, we covalently bound six different \(g\)Hb mRNA capture extenders directly to the solid support (Fig. 2B) and compared this format with the first version of the NACA using the DNA oligomer in conjunction with the same six different capture extenders (Fig. 2A). Direct binding of the six different capture extenders to the solid support resulted in a 2-fold reduction in the signal-to-noise ratio compared with the indirect format involving the DNA oligomer (Fig. 2H). To investigate any negative impacts that the reduction of different capture extenders would hold, we progressively withdrew different sequences during the coating process (Fig. 2B–G). The stepwise reduction in diversity of the different capture extenders resulted in a linear decrease in the assay sensitivity (Fig. 2H).

These results revealed fundamental concepts about the capture process that allowed us to improve the NACA technology. As described in Figure 2I, cooperativity, flexibility and stability are major parameters that influence the performance of capturing a specific nucleic acid sequence. Involvement of different capture extenders that recognize different regions on the target sequence increases the probability of multi-capture events that promote more stable and specific hybridizations.

The involvement of the DNA oligomer in the first version NACA results in higher flexibility and stability of the capture event. When a DNA oligomer is involved, different capture extenders freely hybridize to the target molecule in solution with few spatial constraints and subsequently bind to the DNA oligomer attached to the solid support. Since involvement of all capture extenders takes place, this format results in an optimal capture process. In addition, the complex formed by the target, the capture extender and the DNA oligomer covalently attached to the solid support remains thermodynamically stable. In the case where the capture extender binds directly to the solid support, the hybridization process is less flexible and the captured target molecule is submitted to more physical constraints, likely decreasing the hybridization stability. In this respect, the indirect capture performs better than the direct capture approach (Fig. 2H). However, we wanted to take advantage of the direct capture format to reduce non-specific hybridization. Because of this result, we decided to modify the capture extenders in order to compensate for the reduction in flexibility, stability and cooperativity resulting from the absence of the DNA oligomer.
Improvement of the NACA direct capture system

In the direct capture format of NACA (Fig. 3B), the sequence in the capture extender that previously hybridized to the DNA oligomer (Fig. 3A) becomes useless. Therefore, we synthesized a new set of capture extenders called modified capture polymers (see Materials and Methods), in which the nucleic acid sequences that do not directly hybridize with the Hb RNA were replaced by a 3'-ethylene glycol scaffolding (Fig. 3C). To maintain optimal spacing between the sequence that directly hybridizes to the target molecule and the solid support, the scaffolding was composed of four 18-O-dimethoxytritylhexaethyleneglycol,1[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite molecules. Being an inert carbon chain, this structure has the advantages of being uncharged and flexible. As presented in Figure 3D, the NACA sensitivity increased significantly when the modified capture polymers containing a 3'-ethylene glycol scaffolding (Fig. 3C) were directly attached to the solid support, compared with the direct format using regular capture extenders (Fig. 3B). This sensitivity increase is a result of a decreased background signal. The best sensitivity, however, was still obtained when an indirect capture strategy was used, as in the first generation NACA (Fig. 3A).

In the direct capture format, one of the theoretical effects of a reduction in flexibility is a decreased probability that capture events will involve all the different capture sequences on the solid support. Therefore, only a fraction of the different capture polymers will contribute to the capture of one target molecule, resulting in weak hybridization and lower assay sensitivity. To determine whether increasing the hybridization strength between the capture polymers and target sequences would compensate for the reduction in flexibility, stability and cooperativity in the direct capture format, 2'-O-methoxy-RNA (18) was incorporated into the 3'-ethylene glycol scaffolding capture polymers, in both the 3' and 5' regions that hybridize to the target molecule (Fig. 4C). As shown in Figure 4D, incorporation of 2'-O-methoxy-RNA in the 3'-ethylene glycol scaffolding capture polymers results in a significant increase in signal-to-noise ratio compared with the direct capture that uses capture polymers containing only the 3'-ethylene glycol scaffolding (Fig. 4B). Furthermore, the 2'-O-methoxy-RNA format also significantly improved the assay sensitivity compared with the original indirect capture format (Fig. 4A).

The final format of the NACA technology (Fig. 4E) includes several modifications required for an efficient direct capture system. Multiple modified capture polymers designed using an 'in-house' algorithm include 2'-methoxy deoxyribonucleotides as well as a 3'-ethylene glycol scaffolding. Moreover, because DNA oligomers are not involved in the direct capture format, there is no need for blocker oligonucleotides. Therefore, all the nucleic acid sequences involved actively contribute to the capture or signaling of the target molecule. As a result, non-specific hybridization is dramatically decreased. Contrary to the bDNA assay in which the capture and signaling events contribute equally to the overall efficiency of the technique, emphasis is given to the capture step in the NACA. Indeed, the use of short partially modified capture sequences contributes to increase the specificity and the hybridization strength of the capture event.

Fetal hemoglobin gene expression analysis in human bone marrow cells

To demonstrate the ability of the NACA technology to measure the expression of a specific gene directly in crude cell lysate, the expression of the human fetal hemoglobin gene was analyzed in primary bone marrow cells treated with hemin (Fig. 5). To validate the approach, the NACA data were compared with quantitative RT-PCR data generated after RNA purification and subsequent specific amplification (see
As shown in Figure 5A, the quantitative RT-PCR revealed a 10-fold increase in \( \gamma \text{Hb} \) gene expression in response to \textit{in vitro} treatment of the bone marrow cells with hemin as compared with the untreated control cells. Similar results were obtained with the NACA using the indirect (Fig. 5B) or direct modified capture (Fig. 5C) as described in Figures 1B and 4E, respectively. In addition to validating the NACA technology with ‘real world samples’, the agreement between the NACA and quantitative RT-PCR results demonstrates the experimental advantage of the NACA over conventional PCR-based approaches for this type of application. Notably, the human fetal hemoglobin gene expression was analyzed using the NACA directly from cell lysates without requiring a time-consuming RNA purification step or an expensive real-time PCR instrument.

**Application of the modified capture polymers to a DNA array format**

The cooperativity evidenced in the context of NACA also has applications for the field of DNA array technology. Indeed, the
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