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Real-Time Quantification of RNA Polymerase Activity using a “Broken Beacon”

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Abstract

A novel assay was developed for real-time monitoring of RNA synthesis using a hybridization-based method. In this work, a “broken beacon” in which the fluor and quencher were located on separate but complementary oligonucleotides was used to quantify the amount of RNA production by T7 polymerase. The relative lengths of the fluor-oligo and quencher-oligo, as well as their relative concentrations, were optimized. The experimentally determined limit-of-detection was ~45 nM. The new assay was compared to the “gold-standard” radiolabel (³²P]NTP incorporation) assay for RNA quantification. While the broken beacon assay exhibited a higher limit of detection, it provided an accurate measure of RNA production rates. However, the broken beacon assay provided the significant analytical advantages of i) a real-time and continuous measurement, ii) no requirement for the use of radiolabels or gel-based analysis, and iii) substantial time and labor savings.

Keywords

RNA AMPLIFICATION; REAL-TIME; RNA POLYMERASE; FLUORESCENCE; DISPLACEMENT PROBES; DETECTION

Introduction

Due to important applications in diagnostics and drug screening, many techniques have been used for in-situ quantification of DNA during enzymatic synthesis, e.g., “real-time” polymerase chain reaction (RT-PCR). The most common approach is based on indirect quantification of DNA by fluorescence monitoring during amplification. For example, a simple but useful method relies on the incorporation of an intercalating dye, such as SYBR® green (Invitrogen, Carlsbad, California), into double-strand DNA (dsDNA) during its production. Upon intercalation the fluorescence quantum yield of the dye increases substantially, and the resulting increase in fluorescence is proportional to the concentration of dsDNA. However, since intercalating dyes bind nonspecifically to DNA, amplification of mutations and contaminants can be problematic [1]. As a result, sequence-specific labeled probes are more prevalent in real-time detection.

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Figure 1 contains a graphical representation of several sequence specific monitoring approaches. The so-called “adjacent” hybridization probes are readily used in conjunction with commercially-available RT-PCR instruments [2]. In this case, as shown in Figure 1A, two oligonucleotide-based probes are used, one contains a fluorescence donor and the other contains a fluorescence acceptor. Upon hybridization of both probes to the target sequence, the two dyes are positioned in close spatial proximity and fluorescence resonance energy transfer (FRET) can occur. Under appropriate conditions, the donor molecule can excite at a wavelength that does not excite the acceptor probe. The donor, when within the Förster distance, can non-radiatively transfer its energy to the acceptor, which will subsequently emit a Stokes-shifted fluorescence photon. The advantage of this method is due to the strict spatial limitation for observing FRET. In this case, non-bound probes in solution exhibit only a minimal background so that lower detection limits can be achieved [1,3–5].

TaqMan® (Roche Molecular Systems, Alameda, CA) probes also use FRET to monitor real-time production of DNA. The probe contains two molecules, a fluorescence reporter and quencher, located on opposite ends of a short (20–25 nt) oligonucleotide (see Figure 1B). Once the probe oligonucleotide is hybridized to the amplicon, the exonuclease activity of the Taq DNA polymerase cleaves the TaqMan® probe thereby separating the reporter dye from the quencher molecule. The result is an increase in fluorescence with an increase in the amount of DNA produced. The TaqMan® probes are now widely used for the detection of DNA amplification during PCR [1, 3–4, 6]. However, this approach can not be easily used with RNA polymerases since the product is single-stranded RNA.

Another DNA quantitation method using hybridization probes involves “molecular beacons”. These single-stranded DNA molecules have a secondary stem-loop structure with complementary ends that anneal to one another. Opposite ends are labeled with a fluorophore and quencher, respectively. As shown in Figure 1C, the loop structure is complementary to the target so that when the beacon binds to the product, the fluorophore is separated from the quencher, and fluorescence increases in proportion to the amount of DNA produced [1, 3, 6–9].

Clearly, real-time DNA detection has greatly benefited from these techniques, however there has been relatively little effort dedicated to methods for monitoring RNA synthesis *in situ*. Nucleic acid sequence-based amplification (NASBA) synthesizes RNA in an isothermal manner. Molecular beacons are the primary detection system used in conjunction with NASBA for real-time examination with typical detection limits in the low nM range [7,10]. While molecular beacons have been used to detect RNA production *in situ*, they tend to be at least a factor of 4–10 more expensive than simple fluor-labeled oligos and typically require sophisticated software packages for appropriate design [11].

In this study, an inexpensive real-time assay for RNA was developed and found to provide the advantages of specificity and low background from unbound probes. As shown in Figure 2, the oligo probe was designed with a fluorophore on the 3' end. Solution-based fluorescence background is minimized by hybridization to a complementary oligo with a quencher on the 5' end. In the presence of a longer target sequence, hybridization of the fluor-oligo to the target is thermodynamically favored. Thus, in the presence of ssRNA, the quencher is displaced and fluorescence increases in proportion to the amount of RNA produced. Although “displacement hybridization” has been used in the detection of DNA, as far as we are aware this is the first reported use for real-time quantification of RNA during RNA synthesis [12–15]. These probes, herein referred to as “broken beacons,” are significantly less expensive than true molecular beacons or common FRET probes.

Materials and Methods

Reagents and Instruments

Unless otherwise stated, all experiments were conducted in 20 μ L solution volume in a 384-well, clear-bottomed plate (Corning, Corning, NY) and measurements were made with a FLUOstar Optima (BMG Labtech, Offenburg, Germany), using 530 nm excitation and emission monitoring at 570 nm. All oligo sequences used are presented in Table 1.

Quencher Studies

All samples contained 200 nM fluor-oligo, 4X SSPE (SSPE contains NaCl, NaH_2PO_4 , and EDTA), 2.5X Denhardt's Reagent, 30% formamide, and 400 nM quencher-oligo. The length of the quencher-oligo was varied (6, 8, 10, 12, 14, 16, 18, and 20 nucleotides). Each sample fluor-quencher oligo pair was allowed to incubate at least 3 h prior to use. DNA target concentrations of 0, 10, 25, 50, 100, and 200 nM were analyzed for each quencher-oligo length. The DNA target is a synthetic mimic of the RNA target produced by the T7 polymerase.

For the melting temperature studies, samples contained 4X SSPE, 2.5X Denhardt's Reagent, 30% formamide, 100 nM fluor-oligo, and 200 nM quencher-oligo. Each sample 20 μ L volume was prepared in a 96-well plate with an optically clear film (Applied Biosystems, Foster City, CA). Using a Stratagene MX 3005P (La Jolla, CA) fluorescence (Cy3 wavelengths) was monitored as the plate temperature was increased one degree per minute from 25°C to 80°C. To determine the melting temperature of each duplex, the fluorescence intensity was plotted as a function of temperature for all quencher-oligo lengths and T_m was taken as the midpoint of the rise curve.

The Promega Biomath Calculator (<http://www.promega.com/biomath/calcl1.htm>) was used for melting temperature calculations. The melting temperature of the duplex probe set was evaluated for each quencher-oligo length, (6 – 20 nt). The “salt-adjusted” calculation took into account 640 mM Na^+ from 4X SSPE. A substrate concentration of 100 nM was used for the “base-stacking” calculation.

Hybridization Kinetics

Samples were prepared containing 4X SSPE, 2.5X Denhardt's Reagent, 30% formamide, 1 μ M fluor-oligo, and 2 μ M quencher-oligo (14 nt) in a total volume of 20 μ L with final DNA target concentrations of 0, 50, 100, 250 and 500 nM. A humidified environment was maintained by placing deionized water around the wells on the 384-well plate and covering with a lid. One measurement was taken before the DNA target was spiked into each well, and measurements were taken for 90 minutes at 37°C. Measurements were read from the bottom of the plate at a gain of 2600.

Limit-of-Detection

All samples contained 1X T7 polymerase reaction buffer (40 mM Tris-HCl, pH 8.0, 8 mM MgCl_2 , 2 mM spermidine-(HCl)₃, 25 mM NaCl), 1 mM NTPs, 5 mM DTT, 1 U/ μ L T7 RNA polymerase and prehybridized duplex of 1 μ M fluor-oligo and 2 μ M quencher-oligo (14 nt). Final concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 800, and 1000 nM DNA target were added to a final volume of 20 μ L of the above mixture. The reaction was incubated at 37°C for 1 hour in the scanner using a clear-bottomed plate with a lid and deionized water around the outside of the wells. Five consecutive measurements were taken from the bottom after 1 hour at a gain of 2560 and averaged for every concentration of DNA target. The averages of the blanks were subtracted from each signal and the trendline was forced through the origin.

Kinetics of RNA Production

The same assay conditions as above were used, except that assays contained the partially duplex T7 DNA substrate at either 420 nM or 42 nM. Measurements were taken at 37°C at 5 minute intervals for 2 hours at a gain of 2400. In order to quantify the results, samples were run with known concentrations of DNA target added to the reaction assay at concentrations of 0.5 μ M, 1 μ M, and 2 μ M.

T7 RNA Polymerase Assay Using [α - 32 P]NTPs

T7 RNA polymerase assays were carried out in a total volume of 60 μ L containing 42 nM or 420 nM template DNA, 1X T7 polymerase reaction buffer, 5 mM DTT, 0.187 mM each of ATP, GTP, UTP and CTP, and 15 μ Ci [α - 32 P] GTP (3000 Ci/mmol). T7 polymerase was diluted into the reaction mixture to a concentration of 1 unit/ μ L. Aliquots (5 μ L) were removed after 0.5, 1, 2, 4, 8, 15, 30, 60, 90, and 120 minutes at 37°C and the reaction stopped by adding 5 μ L of 90% formamide gel loading buffer. Quenched samples were incubated for 2 minutes at 95°C and 5 μ L aliquots were analyzed by denaturing polyacrylamide gel electrophoresis (7M urea, 6% polyacrylamide). After electrophoresis, the amount of product was quantified by PhosphorImagery (Molecular Dynamics), and values corrected for the fact that each product contained 17 guanosines.

The limit of detection for radiolabeling was determined in 20 μ L assays containing 20 units T7 RNA polymerase, 420 nM template DNA, 1X T7 polymerase reaction buffer, 5 mM DTT, 0.175mM each of ATP, GTP, UTP and CTP, and either 9 μ Ci or 3 μ Ci [α - 32 P] GTP (3000 Ci/mmol, final specific activities of 5700 and 1900 cpm pmol GTP⁻¹, respectively). After 45 minutes at 37°C, 10 μ L were quenched by adding 10 μ L of 90% formamide gel loading buffer. Serial dilutions were made from this stopped reaction and incubated for 2 minutes at 95°C. Aliquots (5 μ L) were analyzed by denaturing polyacrylamide gel electrophoresis (7 M urea, 6% polyacrylamide). After electrophoresis, gels were exposed for both 15 minutes and 24 hours, and gel band intensity of the elongated product was quantified by PhosphorImagery.

Results and Discussion

Optimization of Relative Probe Lengths

As a first step in designing an optimal fluor-quencher oligonucleotide combination, the melting temperature (T_m) for a range of fluor-quencher oligonucleotide (nt) lengths was determined experimentally and the results compared to calculated values. The requirement for monitoring in situ RNA production kinetics is that the T_m of the probe be higher than the operational temperature of 37 °C but optimized for rapid annealing to the target. The fluor-oligo length was fixed at 22 nt and the quencher-oligo was varied between 6 and 20 nt. Figure 3 shows the melting curves for quencher-oligos 12–20 nt hybridized to the 22 nt fluor-oligo. The melting temperature for shorter lengths could not be determined. As summarized in Table 2, for this simple system the measured T_m values exhibit the same trend as calculated values, with melting temperature increasing with quencher length. Based on these results, quencher-oligo lengths ≥ 12 nt were found to meet the minimal requirement for the particular fluor-oligo probe used in this study.

The second step in optimizing the broken beacon was determination of the limit-of-detection (LOD) as a function of quencher-oligo for lengths ≥ 12 nt. The LOD, which was defined by a measured signal-to-noise ratio of 3, was first determined in the absence of polymerase using a 22 nt fluor-oligo probe. In this, and all remaining preliminary studies, a 51 nt ssDNA target strand was employed as an inexpensive substitute for a ssRNA target. The quencher-oligo length was varied from 12 nt to 20 nt, at 2 nt steps. In a preliminary study (data not shown), it was determined that a fluor-oligo to quencher-oligo concentration ratio of 1:2 was optimal for

minimizing background fluorescence while maintaining the fluorescence dependence on target concentration, consistent with the findings of Li et al. [14].

The LOD study results are displayed in Figure 4. The average signal of the blank was subtracted from the mean fluorescence signal for each sample and plotted for every quencher length, Figure 4A. The linear regression line was forced through the origin. The LOD was determined, from the slope of the regression line, as the concentration that resulted in a signal 3x the noise. Noise was measured as the standard deviation in the blank measurement. The LOD was plotted as a function of quencher length, Figure 4B.

The sensitivity of this assay, in the absence of polymerase, was clearly dependent on the quencher length associated with the fluor-oligo. Although quencher-oligo lengths of 12 and 14 nucleotides exhibited similar slopes, the background for the 12 nt quencher-oligo was higher than that for 14 nt quencher-oligo, resulting in two different LODs: 11.5 ± 0.1 nM or 230 ± 5 fmol, and 1.2 ± 0.1 nM or 24 ± 1 fmol, respectively. As can be seen in Figure 4B, the best LOD was obtained with a quencher-oligo length of 14 nt.

These general trends in sensitivity may be understood by considering that the degree of quenching depends on the thermodynamics of the equilibrium between the hybridized and unhybridized states between the fluor-oligo (F) and the quencher-oligo (Q):

A commonly used measure of the likelihood of hybridization is duplex melting temperature, T_m . The FQ duplex T_m is lower at shorter quencher lengths. A lower T_m generally means that the equilibrium shifts in favor of an unhybridized state, which in turn would result in an increased background and poorer LOD. However, quencher-oligo lengths longer than 14 nt exhibited slightly reduced concentration sensitivity, as evidenced by the slope of the regressions shown in Figure 4A and slightly higher LOD's shown in 4B. To understand this trend it is important to also consider the equilibrium condition in the presence of a target (T):

The competition between the target and the quencher-oligo is an important aspect of this displacement-based hybridization method. Since the thermodynamics of the displacement of Q from F are highly dependent on the length of the duplex formed (as demonstrated above), shorter quencher-oligos are displaced more easily in the presence of target. At longer quencher-oligo lengths, such as 20 nt, displacement by the target does not occur as readily due to less accessibility along F. Ultimately, the sensitivity and LOD are determined by a trade-off between displacement and background fluorescence. For the system studied here, the optimal condition was obtained with a Q length of 14 nt, which was used in all subsequent studies.

Hybridization Kinetics

The kinetics of the broken beacon method were evaluated to determine whether this system could be practically utilized in a real-time assay. The pre-hybridized 22 nt FQ duplex was incubated with varying concentrations of ssDNA target at 37 °C for 90 min. From the kinetics assay, the fluorescence intensity at each time point was plotted as a function of DNA target concentration (Figure 5).

The use of broken beacons for real-time applications depends on the equilibrium between F and T (see Eqn. 2) being reached within a reasonable amount of time. The equilibrium was determined by the plateau region of an exponential rise to a maximum fit via non-linear regression to the raw data. As observed in Figure 5, equilibrium was achieved within ~20 min

over an 10-fold change in target concentration. Since production of RNA by T7 RNA polymerase is typically monitored over a 2 hr time period, the broken beacon assay was deemed viable.

Limit of Detection with Respect to DNA Target in the Presence of Polymerase

As an additional control study, the LOD for the broken beacon was determined in the presence of T7 RNA polymerase and appropriate reaction buffers. In this study, varying amounts of DNA target were added to the reaction mixture in the absence of template to prevent RNA synthesis. The fluorescence was measured multiple times after 1 hour and averaged in order to construct a standard curve, Figure 6. The LOD for the 51 nt ssDNA target was found to be 44 ± 2 nM, which corresponds to 880 ± 40 fmol in the 20 μ L reaction volume.

The detection limit for the broken beacon in the presence of T7 RNA polymerase was found to be nearly a factor of 40 higher than the LOD determined in the absence of the enzyme, which is likely due to a combination of less optimal hybridization conditions and the complex nature of the reaction mixture. However, a LOD in the nM range is sufficient for most RNA polymerase reactions. Based on these preliminary studies, a direct relationship between fluorescence from the broken beacon and target concentration was established.

For comparison to a well-established RNA quantification method detection limits using [α - 32 P]NTPs and T7 RNA polymerase were determined. In this assay, the products were subjected to gel electrophoresis and quantified by phosphorimagery and cross-section analysis. Setting the limit of detection as the peak height of the product being 3x the standard deviation of the background, the radioactive assay could detect as little as 0.33 fmol of RNA. Reducing the exposure time to just 10 min slightly reduced the sensitivity of the assay (LOD = 0.98 fmol), and increasing the radioactivity 3-fold marginally increased the sensitivity of the assay (LOD = 0.25 fmol and 0.56 fmol for 24 hr and 10 min exposures, respectively). Thus, the radiolabeling assay, which required several hours of additional time due to the gel electrophoresis and phosphorimagery steps, exhibited a substantially lower limit of detection (~2700x).

Two alternative assays are filter binding assays and scintillation proximity assays (SPA). Filter binding assays typically involve either [32 P]NTPs (for RNA polymerases) or [32 P]dNTPs (for DNA polymerases), stopping the reaction, and capturing the products on an appropriate filter. SPAs typically involve capturing and then quantifying the amount of [3 H](d)NTP incorporated into products using an appropriate SPA bead [10]. Unlike the radioactive assay describe above, these assays avoid the time consuming gel electrophoresis step. A filter binding assay using [32 P]NTPs can approximate the sensitivity of the gel-based assay described above and, theoretically, the sensitivity of an SPA can approach that of the [32 P]NTP polymerization assay. However, this would require the use of undiluted [3 H]NTPs due to the much lower specific activity of 3 H relative to 32 P, a rather expensive proposition. In practice, the [3 H]NTPs are diluted with NTPs to keep the cost reasonable and minimize radioactive waste, but at the cost of a corresponding decrease in sensitivity. Unlike the real-time broken beacon assay described herein, filter binding assays and SPAs are single time-point assays and require additional sample manipulation after initiating the reaction. More significantly, SPAs and filter binding assays result in the production of significant amounts of radioactive waste that must be disposed of properly.

Kinetics of RNA Production

RNA synthesis by the T7 RNA polymerase in real-time was evaluated using the broken-beacon assay and two different template concentrations (Figure 7A). In order to relate fluorescent counts to the concentration of RNA produced by the polymerase, a range of known ssDNA target concentrations for each time point were employed to construct a standard reference

curve. The standard reference curve was subsequently used to determine the concentrations of RNA synthesized versus time for each template concentration based on the assumption of equivalent hybridization. The calculated standard curve for RNA synthesis is displayed in Figure 7B. The measured rates for the production of RNA for two different concentrations of template are summarized in Table 3. Note that even in the absence of template there is a slight rise in fluorescence. This rise in the negative control is attributed to slow dissociation of the pre-hybridized fluor-quencher oligo duplex due to the non-optimal hybridization conditions necessary for the reaction. However, the negative control experiment is simple to run and is quantitatively distinct from the rates measured in the presence of template.

In order to evaluate the broken beacon assay performance, the RNA production rate was also evaluated using the standard radiolabel/gel analysis method (as described in the Materials and Methods section). Rates were measured as phosphorimager units per unit time, and this value was converted into pmol RNA per minute. The rates of RNA synthesis using template concentrations of 420 nM and 42 nM are given in Table 3. Within a 95% confidence level, the results are the same as the broken-beacon assay.

Summary

Based on the similarity in the independently measured production rates, the broken beacon method is deemed a reliable substitute for radiolabel/gel analysis. The tremendous advantages of this simple, inexpensive, and homogeneous assay is that it provides a real-time *in situ* measurement of RNA production and eliminates the need for radiolabels. Furthermore, this broken-beacon assay should be applicable to both DNA-dependent RNA polymerases, such as the T7 RNA polymerase used in this study, and RNA-dependent RNA polymerases (e.g. Dengue fever RNA polymerase).

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Figure 1. Schematics of fluorescence techniques that monitor real-time nucleic acid synthesis. (A) Adjacent hybridization probes, where D is the donor fluorophore and A is the acceptor fluorophore. (B) TaqMan® probe system, where R is the reporter dye and Q is the quencher dye. (C) Molecular beacon approach, where F is the fluorophore.

Figure 2. Schematic of broken beacon system, where F illustrates the fluorescent probe and Q, the quenching probe. The fluorescent probe and the quenching probe are prehybridized, allowing for a low background. When in the presence of target, the amplicon displaces the quenching probe, resulting in increased fluorescence.

Figure 3.
Melting curves for broken beacons in which the quencher-oligo was varied. The y-axis is proportional to fluorescence intensity.

Figure 4.

(A) Standard curve for the fluorescence of TAMRA versus the concentration of added target for quenchers lengths 12 through 20 nucleotides (labeled Q12–Q20) in the absence of polymerase. The results were background corrected. (B) From the standard curves, the limits of detection were calculated and converted to fmol for each quencher length. The line represents best fit with $r^2=0.9937$.

Figure 5.

Kinetics of displacement hybridization as a function of target concentration. The fluor-oligo concentration was 1 μ M and the quencher-oligo concentration was 2 μ M. The solid lines represent nonlinear regression to the data but are intended only to guide the eye.

Figure 6.

Standard curve for ssDNA target in the presence of polymerase. All samples contained 1 μ M fluorescent probes and 2 μ M quencher-oligos (14 nt). The signal intensity for each point was background corrected. The solid line is a linear regression to the data, $R^2 = 0.9823$. Error bars, which are smaller than the symbol size, represent $\pm \sigma$.

Figure 7.

(**A**) Fluorescence counts as a function of incubation time for the production of RNA by T7 RNA polymerase for two different concentrations of template. The fluor-oligo concentration was 1 μM and the quencher-oligo concentration was 2 μM . (**B**) RNA concentration (determined by conversion using the calibration curve shown in Figure 6) as a function of incubation time. The solid lines are linear regression over the linear region of the kinetics curve ($R^2 = 0.9960$ for 420 nM template and 0.9949 for 42 nM template).

Table 1**Oligonucleotides used in the Broken Beacon Study**

Name	Sequence (5'-3')	Length, nt
Fluorescent Probe, HPLC purified (Sigma-Genosys, Woodlands, TX)	GAGCGAGGACTGCAGCGTAGACG-TAMRA	23
Quencher Probe, BHQ-2 (Biosearch Technologies, Novato, CA)	BHQ-2 CGTCTACGCTGC BHQ-2 CGTCTACGCTGCAGTCCTCG	12 to 20
DNA Target (Qiagen, Valencia, CA)	ATCGTCTACGCTGCAGTCCTCGCTCACTGGGCACGGTGAGCGTGAACACAA	51
T7 RNA polymerase Template (Sigma-Genosys, Woodlands, TX)	TTGTGTTACGCTCACCGTGCCAGTGAGCGAGGACTGCAGCGTAGACGATCCCTATAGTGAGTCGTATTAGAATTC	77
T7 RNA polymerase Substrate (Sigma-Genosys, Woodlands, TX)	GAATTCTAATACGACTCACTATAGGGATCGTCT	33
RNA Target synthesized by T7 polymerase	GGGAUCGUCUACGCUGCAGUCCUCGCUCACUGGGCACGGUGAGCGUGAACACAA	54

^aThe underlined bases are the T7 promoter region.

Table 2Measured versus Calculated T_m Values

Quencher Length	Measured T_m	Calculated T_m	Calculated "salt-adjusted" T_m	Calculated "base-stacking" T_m
6	N/A	18	-14	0
8	N/A	26	20	28
10	N/A	32	35	43
12	45	40	49	54
14	52	43	56	59
16	54	49	62	64
18	58	53	66	68
20	61	58	71	72

^b All T_m values are in °C

Table 3

Rates of RNA production by T7 RNA polymerase.

Method of Detection	Concentration of Template	
	42 nM	420 nM
Broken Beacon	0.47 ± 0.03 pmol RNA/min	1.37 ± 0.18 pmol RNA/min
Radiolabeling	0.51 ± 0.03 pmol RNA/min	1.03 ± 0.09 pmol RNA/min

^cThe error is represented by $\pm 3\sigma$.