

# Real-Time Monitoring *in Vitro* Transcription Using Molecular Beacons

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**A homogeneous fluorescence-based molecular beacon (MB) method has been developed for real-time monitoring of *in vitro* transcription reactions. MB probes are structured as target-specific antisense oligodeoxynucleotides containing a proximate fluorophore-quencher pair. Upon binding to its target sequence, the probe undergoes a structural rearrangement that separates the proximate pair, thus dequenching fluorescence. We demonstrate that this simple, inexpensive, rapid, and homogeneous fluorescence-based assay permits real-time monitoring of *in vitro* transcription and end-point measurement of RNA. The results from the RNA MB assay were comparable to those from other methods.** © 2001 Elsevier Science

**Key Words:** molecular beacon; RNA detection; RNA polymerase; *in vitro* transcription.

*In vitro* transcription assays are frequently used to dissect the complex steps of transcription. However, the traditional assays for detecting the transcribed RNAs require time-consuming separation steps such as gel electrophoresis and filtration. Recently, we reported a homogeneous scintillation proximity assay (SPA)<sup>2</sup> for RNA detection and used this SPA assay to probe RNA sites accessible to hybridization. While such a homogeneous assay has been utilized for high-throughput screens (HTS), the large quantities of radioisotopes used are still a hazard to researchers and the environment.

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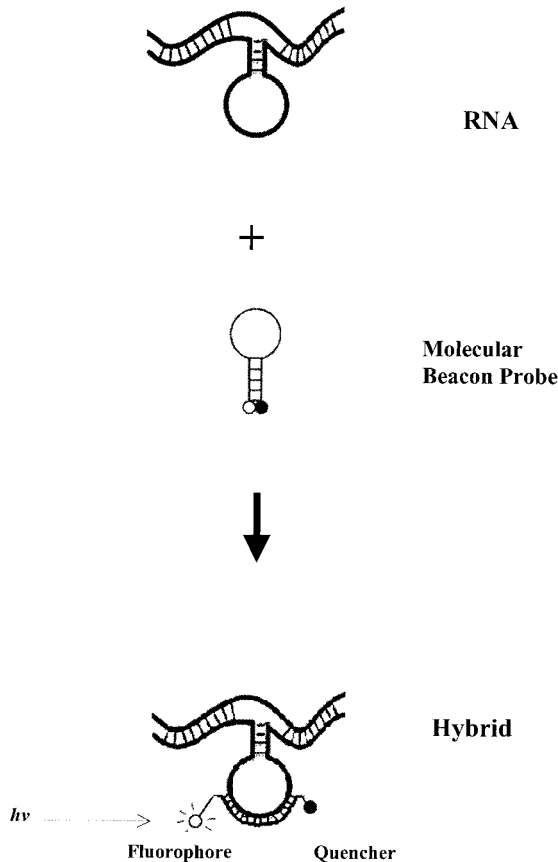
<sup>2</sup> Abbreviations used: SPA, scintillation proximity assay; ODN, oligodeoxynucleotide; HTS, high-throughput screening; MB, molecular beacon; NTP, nucleotide triphosphates; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide.

The molecular beacon (MB) concept (2) was employed to develop a nonradioactive assay for RNA detection. MB probes are single-stranded oligodeoxynucleotides (ODNs) with a stem-loop structure (see Fig. 1). The loop portion consists of the sequence complementary to the nucleic acid target. A fluorophore is covalently linked to one end of the stem and a quencher to the other end. The stem keeps the fluorophore and quencher in close proximity, and thus no fluorescence signal is detected. The emitted energy from the fluorophore is transferred to the quencher and released non-radiatively as heat (3). When the probe hybridizes to its target, the stronger and more rigid probe target double-helix forces the stem to unwind and results in the separation of the fluorophore and the quencher. Thus, the target-hybridized MB probe fluoresces upon excitation. Since free MB probe is internally quenched, no removal of the free probe is required and represents an ideal "gain in signal" assay with low backgrounds. This not only makes MB assays homogeneous, but also allows monitoring of a nucleic acid reaction progress in real time. There are currently no reported methods able to monitor *in vitro* transcription in real time. In this respect, MB technology is more attractive. This work demonstrates the use of MB technology to monitor the progress of *in vitro* transcription in real time resulting from RNA polymerase activity. Results from MB assays compare well with both SPA and filter-binding formats.

## MATERIALS AND METHODS

### Materials

Black 96-well Optiplates were purchased from Packard Instrument Co. (Meriden, CT). Ninety-six-well filter plates were purchased from Millipore Corp. (Bedford, MA). MB probes were synthesized by Research Genetics Inc. (Huntsville, AL). Other reagents were purchased from Sigma.



**FIG. 1.** Principle of RNA detection using MB probes. Free MB probes are unable to fluoresce since the stem keeps the fluorophore close to the quencher, causing the fluorescence to be quenched by energy transfer. When a probe forms a hybrid with its target RNA, the fluorophore is forced apart from the quencher, leading to increased fluorescence of the hybrid.

### Filter-Binding Assay

In a 96-well filter plate, 50  $\mu\text{l}$  of a typical *in vitro* transcription reaction was carried out in a pH 7.9 Tris buffer containing [ $^{33}\text{P}$ ]CTP (from NEN Life Science, Boston, MA), 10  $\mu\text{M}$  CTP, 500  $\mu\text{M}$  of other nucleotide triphosphates (NTPs, from Amersham), 5 mM  $\text{MgCl}_2$ , and 0.1 mg/ml BSA for 2 h at room temperature. The transcribed mRNA was precipitated with 7.5% TCA and filtered through GF filters. After being washed and dried, radioactive labeled mRNA was detected with a Packard TopCount in the presence of scintillation cocktail.

### RNA SPA

The RNA SPA assay was carried out as described by Liu *et al.* (1). Briefly, a typical *in vitro* transcription reaction of 15  $\mu\text{l}$  was carried out under the same conditions as the filter-binding assay above, except using [ $^3\text{H}$ ]CTP (from NEN Life Science) instead of [ $^{33}\text{P}$ ]CTP

and adding 40 nM antisense biotinylated ODNs to the reaction. The reactions were stopped with 65  $\mu\text{l}$  of a high salt buffer (pH 4) containing streptavidin-SPA polyvinyltoluene (PVT) beads (from Amersham-Pharmacia Biotech, Piscataway, NJ). The samples were counted with a Packard TopCount NXT microplate counter after incubation for at least 2 h.

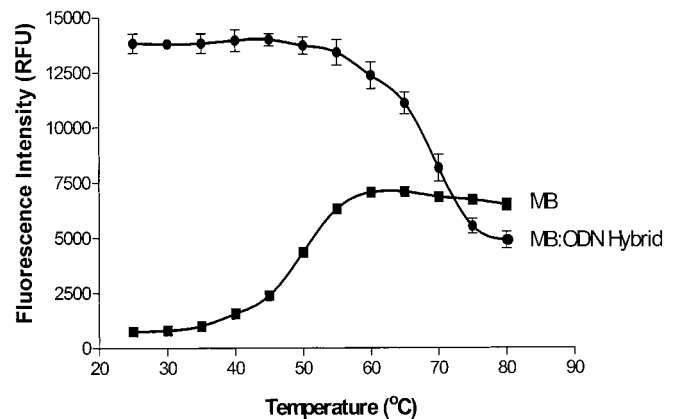
### Molecular Beacon

MB probes were designed based on the RNA SPA results (1). Active antisense ODNs were used as the loop sequences. The 5'-nucleotide paired stem (GAGGG . . . . . CCCTC) was labeled at the 5' and 3' ends with 5-iodoacetamidofluorescein and 4-(4'-dimethyl-aminophenylazo) benzoic acid (DABCYL), respectively.

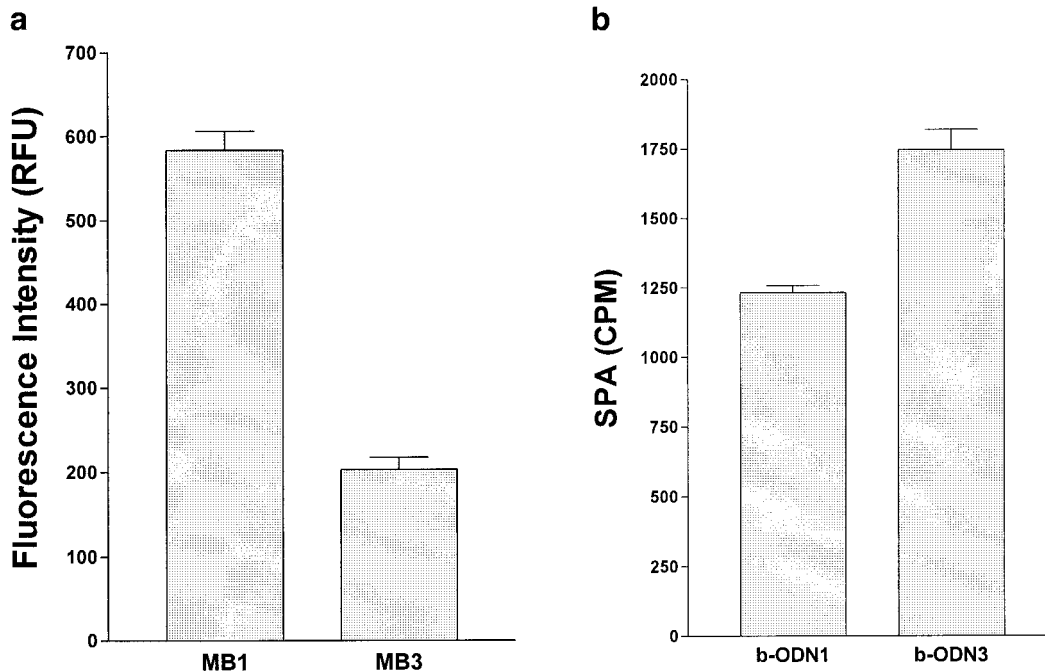
The sense ODNs were used to evaluate the MB probes. The fluorescence signal from 5 pmol MB probe in 50  $\mu\text{l}$  of 10 mM Tris-HCl buffer, pH 8.0, containing 3 mM  $\text{MgCl}_2$  was determined as a function of temperature in the absence or presence of a sense ODN (400 nM final concentration).

For real-time monitoring of reaction progress, a typical *in vitro* transcription was carried out in 96-well plates in a pH 7.9 Tris buffer containing 500  $\mu\text{M}$  NTP, 5 mM  $\text{MgCl}_2$ , and 0.1 mg/ml BSA for 2 h at room temperature. Since the antisense ODN did not interfere with *in vitro* transcription (1), the MB probe was included in the transcription reaction mixture. The reaction progress was monitored with a Cytofluor multiwell plate reader (Perseptive Biosystem) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

For application of MB probes to inhibition of RNA polymerase activity, the compounds were dissolved in



**FIG. 2.** Thermal profile of a MB probe and a hybrid with its target ODN. The fluorescence intensity of a solution containing a MB probe in the absence (■) or presence (●) of its target ODN was measured at different temperatures. The experiments were performed in independent triplicate samples. Similar results were obtained with another MB probe.



**FIG. 3.** Comparison of MB probes with their corresponding b-ODN in RNA detection. The fluorescence (a) or SPA (b) signals were measured after 3 h *in vitro* transcription in the presence of MB probes or b-ODNs, respectively. The assays were performed in triplicate at least.

DMSO. Reactions performed in the absence of enzyme were used as background controls and those in DMSO alone as positive controls.

## RESULTS AND DISCUSSION

### MB Probes

As illustrated in Fig. 1, for detection of RNA in solution at room temperature, the loop sequence of a MB probe must be complementary to an *accessible* site of the target RNA under nonstringent conditions. With RNA SPA, accessibility of a given RNA target has been successfully demonstrated (1). The active antisense sequences to these accessible sites of RNA were used as the loop structure of MB probes. The major nucleic acid sequence constraints for MB probe design have been described (2). A 30-mer MB stem-loop structure composed of a 5-mer complementary flanking sequence and a 20-mer intervening loop structure was designed and synthesized. Fluorescein, the fluorophore, and DABCYL, the quencher, were conjugated to the 5' and 3' ends, respectively. Figure 2 showed typical thermal profiles of a MB probe and a hybrid between a MB probe and its target ODN. In the absence of its target, the stem kept the fluorophore and quencher in close enough proximity for fluorescence resonance energy transfer (FRET) to occur so that very low fluorescent signal was observed. When temperature was increased, fluorescence intensity exhibited a sigmoidal profile, a characteristic of melting a nucleic acid helix.

The thermal denaturation profile indicated that this MB probe indeed formed a stem-loop structure to keep the fluorophore and quencher in close proximity at temperatures below 40°C. When the temperature reached its melting point, the conformation change from the double-helix to random-coil structures resulted in the separation of the fluorophore from the quencher, releasing internal proximity quenching. Fluorescence signals were then readily detectable upon excitation of fluorophore moiety of the MB probe.

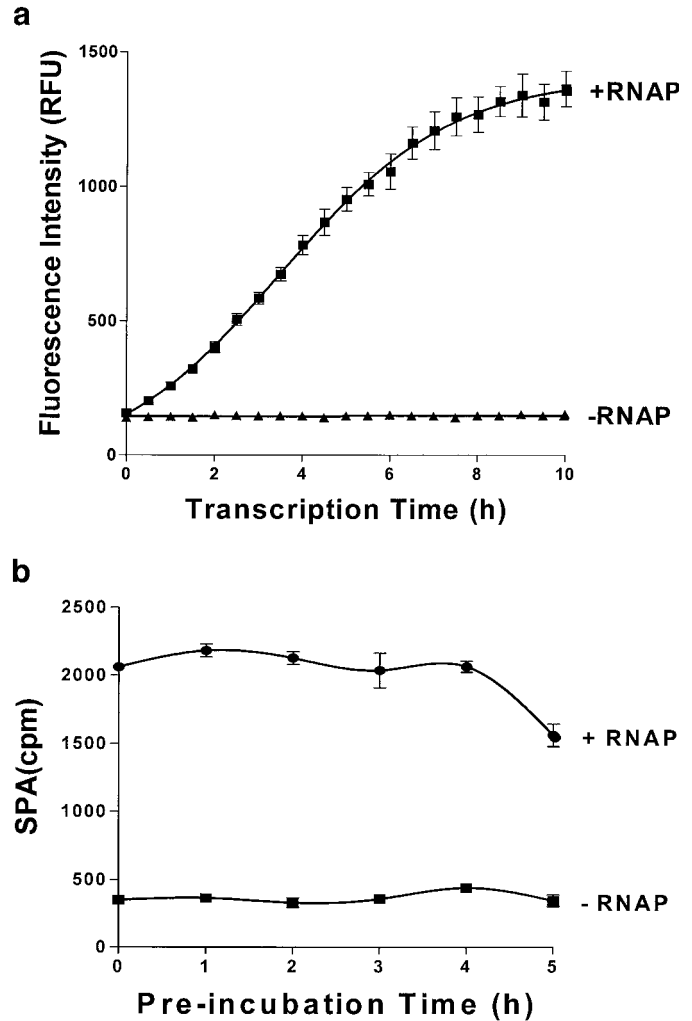
When the MB probe was incubated with its target sequence ODN in solution at room temperature, the loop sequence of the MB probe formed a longer and thus stronger and more stable double-helix with its target ODN. The rigid extended structure of this target-probe double-helix precludes the simultaneous existence of a stem structure of the probe oligonucleotide. The MB probe is forced open and adopts a more extended structure wherein the fluorophore is far from the quencher moiety and restores the fluorescence of the MB probe. This fluorescence was readily detected (Fig. 2). The higher melting temperature of the MB:ODN hybrid than the MB probe alone demonstrates that the MB probe indeed formed a stronger and more stable helix with its target ODN. The greater efficiency of fluorescence dequench of the MB:ODN hybrid (when temperature was lower than 50°C; Fig. 2) compared to the random-coil structures of the MB probe alone (when temperature was higher than 60°C; Fig. 2) is

demonstrated by the higher fluorescence signal of the MB:ODN. This is consistent with a greater proportion of conformations of the melted MB wherein the donor and quencher are proximate. It was noted that, at temperatures higher than 75°C, the fluorescence of MB in the presence of its target ODN was slightly lower than that of MB alone. This is most likely due to the quenching effect at high concentration of ODN (400 nM), since no difference of MB fluorescence was observed in the presence of a nontarget ODN under identical conditions (data not shown).

Specificity of MB probes in genotyping has been reported (4–6). We also observed that only the MB probes with a loop sequence corresponding to the active antisense sequence were able to monitor the progress of *in vitro* transcription or to determine target mRNA in the end-point assay. Interestingly, we observed that the fluorescence signal varied among the MB probes targeted to different accessible sites of the RNA. As shown in Fig. 3, MB3 gave a lower fluorescence signal than MB1, even though its corresponding active antisense ODN-3 gave a higher SPA signal in RNA detection. One possible explanation may be that the loop sequences of the MB probes have different degrees of base pairing with their RNA accessible sites. In RNA SPA, the proximity of radioactive RNA to SPA beads is the major determinant in signal output. The degree of base pairing between an ODN and its target might not affect the SPA signal significantly as long as hybridization of a biotinylated ODN (b-ODN) with its target sequence is strong enough to capture the RNA to SPA beads for detection. In the RNA MB method, however, the degree of base pairing of MB probes to its targets would impact the distance between the fluorophore and quencher with consequent effects on output fluorescence signal.

#### Monitoring Transcription Process in Real Time with MB

For real-time monitoring of RNA transcription, the MB probe needs to be present in the reaction mixture from the beginning of the reaction and must produce a detectable fluorescence signal concurrently with RNA transcription. We have demonstrated that an antisense ODN included in a transcription reaction did not interfere with RNA transcription (1). Real-time monitoring of RNA transcription reactions gave fluorescence plots with an initial lag phase, followed by a first-order increase (Fig. 4a). The initial lag may be due to assay sensitivity and kinetics of double-helix formation between the MB probe and its RNA target. The reaction remained linear for up to 6 h. After 6 h the reaction slowed, most likely due to enzyme instability. As shown in Fig. 4b, in the absence of NTPs and DNA template, RNA polymerase itself was only stable for 4 h

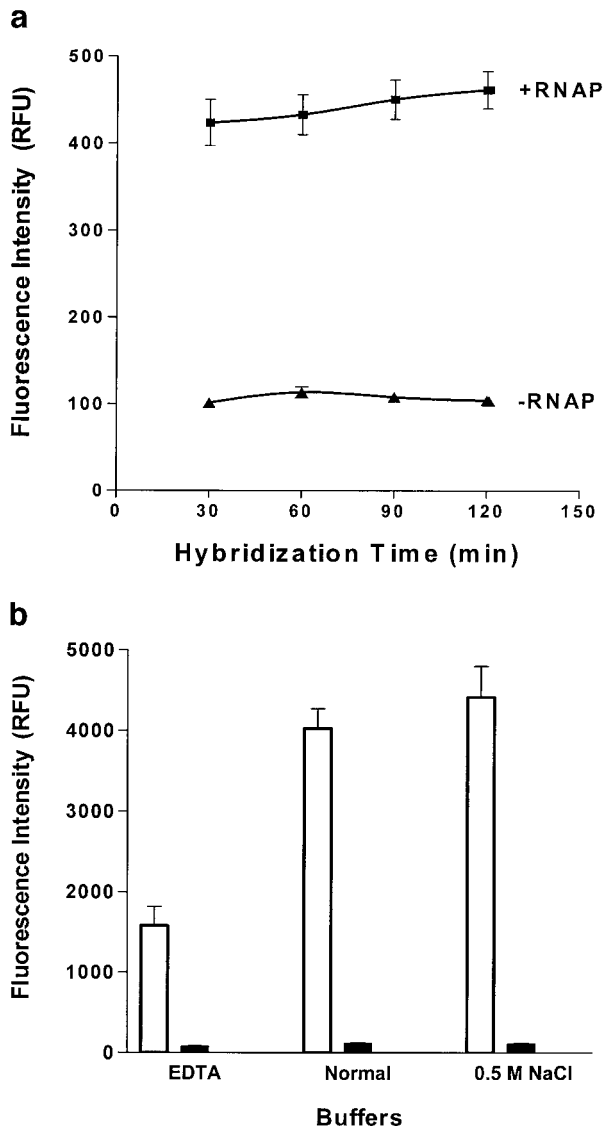


**FIG. 4.** Transcription process monitored with MB. (a) Fluorescence intensity was monitored with time from the beginning of the *in vitro* transcription reaction. (b) RNA polymerase was preincubated for different time periods at room temperature before initiating *in vitro* transcription. After 3-h reactions, the transcribed RNA was determined using RNA SPA (see Material and Methods). The experiments were performed in triplicate and repeated with identical results.

of preincubation in solution at room temperature. The linear phase of the transcription reaction was longer than 4 h, so the enzyme may be more stable when it forms a complex with the DNA template. The slower rate should not result from substrate depletion since NTPs were present at a very high concentration (500  $\mu$ M) in the reactions. This is another advantage over other radioactive methods which must use a limited amount substrate in order to provide high specific radioactivity for labeling.

#### Activity Assay of RNA Polymerase with MB and Filter-Binding

The homogeneous fluorescence-based MB assay detects the RNA product resulting from RNA polymerase



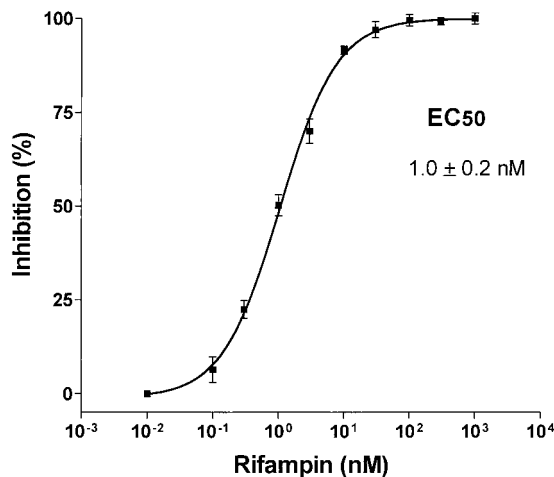
**FIG. 5.** End-point detection of RNA. (a) The *in vitro* transcription reactions were terminated with addition of 20 mM EDTA. The fluorescence intensity of the solutions was monitored after addition of a MB probe. (b) A MB probe was incubated in the transcription buffer (normal), the buffer plus 20 mM EDTA, or the buffer plus 0.5 M NaCl (final concentration) in the absence (solid bars) or presence (open bars) of its target ODN. The experiments were performed in triplicate.

action in real time. The speed of the MB method is another advantage over the RNA SPA or other traditional methods. All of these will facilitate fully automated HTS in drug discovery. Alternatively, this method is useful in end-point detection of RNA. As shown in Fig. 5a, after termination of RNA transcription with EDTA, RNA was readily detected using the MB probe. The fluorescence signal was stable for hours after the MB probe formed a hybrid with its target ODNs, which would allow large-scale batch processing

in HTS. Magnesium has a strong stabilizing effect on hybridization (2). Since  $Mg^{2+}$  (5 mM final concentration) was present in the RNA polymerase reactions, termination of the reaction with EDTA had a negative effect on subsequent hybridization between RNA and MB probes, as shown in Fig. 5b. Figure 5b also showed that termination with high salt increased the MB signal. While heating, followed by slow cooling or low pH, will terminate reactions for the end-point measurement of RNA polymerase activity with a MB probe, it is not practical for a scalable, automatable high-throughput microplate assay. Therefore, high salt was used to terminate the *in vitro* transcription reaction for the end-point measurement of RNA polymerase activity with a MB probe.

Therefore, the RNA MB method would be suitable for screening compounds which inhibit RNA polymerases. Rifampin, an inhibitor of RNA polymerase, was used to evaluate the RNA MB method in the enzyme assay. As shown in Fig. 6, the rifampin  $IC_{50}$  value obtained from the RNA MB was 1.0 nM, comparable with 0.9 nM from the RNA SPA and 1.5 nM from the filter-binding assay. The  $IC_{50}$  values of rifampin remained the same in the range of the reaction periods from 1 to 6 h, consistent with the linear range of the reaction determined in Fig. 5 and that fact that rifampin is a rapidly equilibrating inhibitor. Since the assay is also real time and therefore amenable to continuous assays, it could be further utilized in identifying and studying slow-binding inhibitors of transcription.

In conclusion, this paper presents a convenient and effective fluorescence-based method to detect RNA homogeneously and to monitor *in vitro* transcription in real time. This novel assay is based on the use of a MB



**FIG. 6.** Inhibition of RNA polymerase with rifampin. RNA polymerase activity was determined using the RNA MB method in the presence of rifampin.  $EC_{50}$  values are means  $\pm$  SE of three separate experiments (each in triplicate).

probe during a transcription reaction to generate a fluorescent signal for direct detection of targeted RNAs resulting from transcription processes. This represents the first reported method capable of monitoring *in vitro* transcription in real time. This MB assay could be further used to follow real-time induction of gene expression *in vitro*. Compared to other current methods of RNA detection, the reduction of handling steps in the MB method makes it easier to perform, saves time, and improves the precision and quality of data.

#### ACKNOWLEDGMENT

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#### REFERENCES

1. Liu, J., Feldman, P. A., Lippy, J. S., Bobkova, E., Kurilla, M. G., and Chung, T. D. Y. (2001) A scintillation proximity assay for RNA detection. *Anal. Biochem.* **289**, 239–245.
2. Tyagi, S., and Kramer, R. (1996) Molecular beacons: Probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14**, 303–308.
3. Stryer, L. (1978) *Annu. Rev. Biochem.* **47**, 819–847.
4. Tyagi, S., Bratu, D. P., and Kramer, F. R. (1988) Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.* **16**, 49–53.
5. Piatek, A. S., Tyagi, S., Pol, A. C., Telenti, A., Miller, L. P., Kramer, F. R., and Alland, D. (1998) Molecular beacon sequence analysis for detecting drug resistance in *Mycobacterium tuberculosis*. *Nat. Biotechnol.* **16**, 359–363.
6. Marras, S. A., Kramer, F. R., and Tyagi, S. (1999) Multiplex detection of single-nucleotide variations using molecular beacons. *Genet. Anal.* **14**, 151–156.