Direct Detection of a Specific Cellular mRNA on Functionalized Microplate

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Systems design for nucleic acid detection and quantification, such as the bDNA assay, transcription-based amplification, rolling circle system, real-time PCR, and DNA microarrays, has long been an interest of research.[1] Gene-expression profiling and mRNA analysis in functional genomics have revealed insights into biological systems and disease mechanisms at a molecular level.[2–4] Extremely low quantities of cellular RNAs can be detected and analyzed by real-time PCR,[4c] and up to tens of thousands of genes can be monitored simultaneously by using DNA microarray technologies, including both cDNA and oligonucleotide arrays.[1c,e] Instead of direct analysis of mRNA, real-time PCR and DNA microarray technologies analyze mRNA expression indirectly by reverse transcription, DNA polymerization, transcription, and fluorophore labeling and detection. Development of a direct, simple, rapid, accurate, and sensitive system for RNA (particularly individual mRNA) detection and quantification is a big challenge. Unfortunately, few general strategies can meet these requirements, particularly if the RNA is significantly degraded. We report here the development of a simple and direct system for rapid detection and quantification of mRNAs in a total mRNA or total RNA sample that will meet such needs.

This novel system based on the RNA 3′-labeling approach,[5] in which a targeted RNA is extended and labeled by using labeled-dNTPs and DNA polymerase on a DNA template. Our previous success in the liquid phase[6,7] has encouraged us to explore RNA detection and quantification in the solid phase (such as on a microplate and microchip) by immobilization of the template. Though mRNA with 3′-poly(A) can be labeled and detected in a total RNA sample by using a poly(T) template,[6] labeling and detection of a specific mRNA transcript is a challenge due to the 3′-common sequences, such as the 3′-untranslated region (3′-UTR) and 3′-poly(A) tail in eukaryotic organisms. In order to perform detection and quantification for a specific mRNA in the solid phase, its 3′-region needs to be removed so as to expose its unique internal sequence for selective labeling and detection. Unlike DNA restriction endonucleases, an RNA endonuclease capable of selectively cutting RNA is not readily available. Fortunately, RNase H can be used as an "RNA endonuclease" in the presence of a DNA guiding sequence, as RNase H is capable of cutting RNA in an RNA/DNA duplex.[8] In addition, RNase H does not digest an RNA/RNA duplex, including an RNA/2′-Me-RNA duplex.[8] Therefore,

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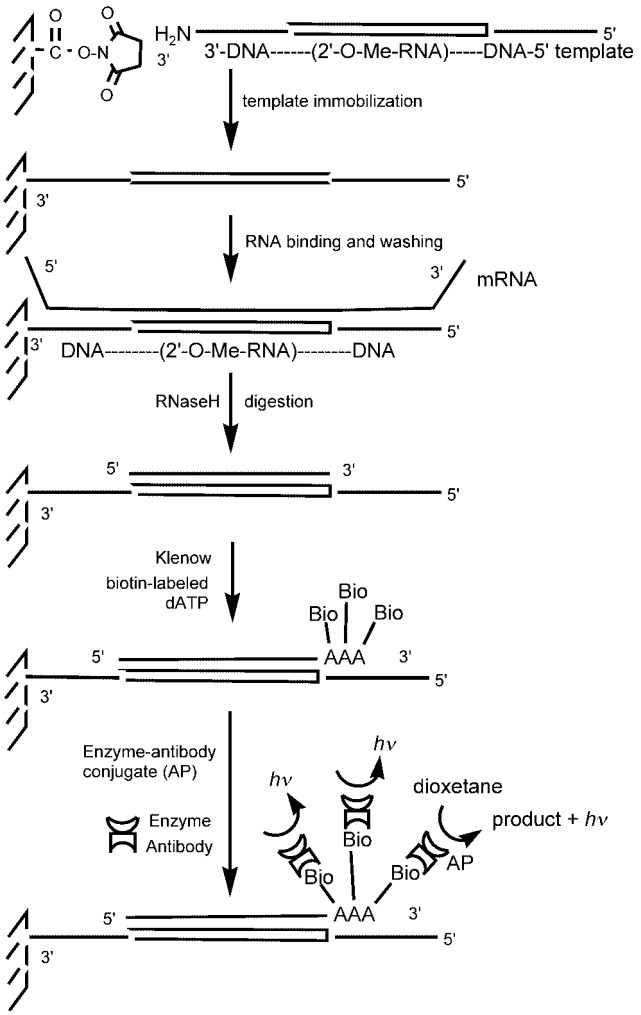
we designed a 5′-DNA-(2′-Me-RNA)-3′ hybrid template in which the DNA and RNA sequences serve as a guiding sequence and a protecting sequence, respectively. Furthermore, the 5′-DNA sequence also serves as the template for Klenow extension.

Immobilization of the 3′-terminus of the DNA–RNA hybrid template on a microplate allows immediate Klenow labeling following RNase H digestion of the mRNA 3′-region. As the undigested 5′-region of mRNA might interfere with the solid surface and the enzymatic reactivities, we later designed a 5′-DNA-(2′-Me-RNA)-DNA-3′ template. The 3′-DNA sequence can also guide RNase H to cut off the RNA 5′-region (Scheme 1).



Scheme 1. *Schematic flow chart of specific RNA detection on a microplate.*

The double digestion of the RNA target leaves a short RNA fragment hybridized to its template in the solid phase for detection and quantitation. This template design allows detection of partially degraded mRNAs in real-life samples. In addition, to avoid interference of the microplate surface with enzymatic activities, the template needs to be sufficiently long. Our experiments showed that the 3′-DNA sequence (10 nucleotides) allowed effective removal of the RNA 5′-region by RNase H.

The hybrid template can be immobilized through a 3′-NH_2 group on a microplate by *N*-hydroxylsuccinimide (NHS) displacement.[9] Incubation of a mixed RNA population on the functionalized microplate results in hybridization of a specific RNA to the template and the unbound RNAs are washed away. Hapten labels (such as biotin) are introduced via the Klenow extension following RNase H digestion of the bound RNA. The enzyme-binder conjugate (for example, antibiotin antibody–alkaline phosphate (AP) conjugate) specifically binds to the immobilized RNA target through binding of the hapten label. The immobilized enzyme catalyzes a chemiluminescence reaction in the presence of substrates (e.g., dioxetane substrate);[10] this allows detection of the specific RNA. Unlike DNA microchip and real-time PCR technologies, the signal here is amplified by the enzyme-catalyzed substrate turnover.[11]

Incorporation of multiple labels, such as biotinated dATPs, can further enhance the signal. Therefore, RNA24.1 (5′-AUGUGGAUUGGCGAUAAAAAACAA-3′, a section of the lacZ mRNA sequence) was chosen as the target RNA, and DNA35.1 (5′-d(GTTGTTTTTT)-2′-Me-RNA(AUCGCCAAUCCACAU)-d(CTGTGAAAGA)-NH_2-3′) was designed as the template for RNA 24.1 and the double digestion template for lacZ mRNA. As expected, in the absence of substrate RNA24.1, template, enzyme, or label, there was no chemiluminescent signal (wells 1–4, Figure 1 A). The signal was observed when all reagents were properly used

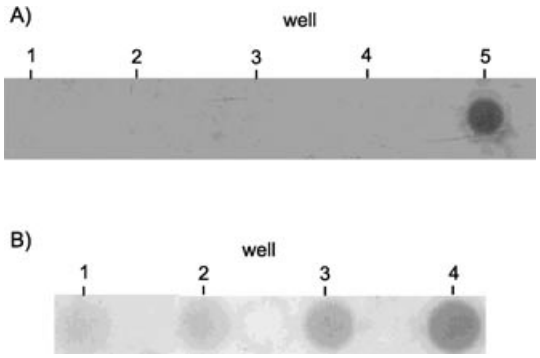


Figure 1. *A) Enzymatic detection of RNA on a 96-well microplate. Target RNA24.1 (1 pmol) and template DNA35.1 (100 pmol). The experiments were conducted with RNase H digestion and Klenow extension followed by incubation with the antibody–AP conjugate, and the film was exposed on the microplate for 1 h after the addition of the dioxetane substrate. Well 1, no RNA24.1; well 2, no DNA35.1; well 3, no biotin-dATP; well 4, no Klenow; well 5, the positive experiment with all reagents; B) Detection sensitivity studies (5 h exposure). Well 1 no RNA24.1; well 2,* 1×10^{-15} *mol; well 3,* 1×10^{-14} *mol; well 4,* 1×10^{-13} *mol.*

(well 5, Figure 1 A), indicating the presence and detection of the target RNA24.1. By varying the RNA quantity, it was shown that the RNA-detection sensitivity can reach as high as 1 fmol (10^{-15} mol) of RNA (Figure 1 B). Due to a longer exposure (5 h), the background was also observed. Naturally, a shorter exposure time reduced the background (Figure 1 A). The signal-to-noise ratio and sensitivity can be significantly increased by using smaller microwell-plates or microchips. As the background is generated by nonspecific binding of the conjugate, we washed the plates extensively to remove such conjugates. Other approaches, such as protein blocking and chemical coat-

ing,[12] can also prevent nonspecific sticking of the enzyme conjugate.

To investigate detection specificity, two yeast mRNA samples were prepared. One sample contained lacZ mRNA isolated from a yeast strain (CWXY2) containing galactose-inducible lacZ-expressing plasmids (PEG202/Ras, PJG4-5/Raf, pCWX24),[7] and the other was isolated from glucose-repressed cells that contained no lacZ mRNA.[13] The experimental results showed that galactose-induced lacZ mRNA generated a strong signal (well 1, Figure 2), while glucose-repressed lacZ mRNA only generated a signal at the background level (wells 2 and 3, Figure 2). As yeast contains thousands of mRNAs,[14] these experimental results showed that lacZ mRNA can be selectively labeled and detected on the microplate in the presence of many other mRNAs. In addition, comparison of wells 2 and 4, in which RNA24.1 was added to glucose-repressed mRNA, revealed that detection of the specific RNA was not interrupted by any other yeast mRNAs. Well 5 was the positive control with RNA24.1.

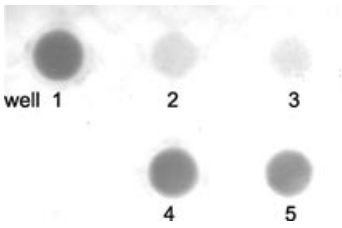


Figure 2. *Selective detection of lacZ mRNA on a microplate. Total mRNA and RNA24.1 for each experiment were 0.1 μg and 10 fmol, respectively (6 h exposure). Well 1, galactose-induced mRNA; well 2, glucose-repressed mRNA; well 3, no RNA (negative control); well 4, glucose-repressed mRNA and RNA24.1; well 5, RNA24.1 (positive control).*

In conclusion, we have demonstrated for the first time a novel system for RNA-specific detection on a microplate by immobilizing the hybrid templates and using an enzyme label (e.g., AP). Unlike DNA microarray and real-time PCR,[15] this approach is direct, simple, cost-effective, and rapid without reverse transcription, PCR, transcription, laser excitation, or fluorescence detection. This method is exquisitely selective as only lacZ mRNA was specifically detected among all of the mRNA molecules, and the sensitivity is high, at the femtomole level. The detection sensitivity can be further increased if a smaller plate or a microchip is used (work in progress). Experimental time and steps will be further saved by using microchips. Background reduction can also increase the detection sensitivity.[12] Since a short portion of a mRNA molecule is needed for the detection, this RNA-detection approach is suitable for analysis of environmental samples, in which mRNAs are partially degraded. This novel strategy has great potential in developing methodologies for rapid on-site detection of bacteria and viruses by identification of their signature RNAs. This strategy is being applied to the development of RNA microarray technology by systematic template immobilization on microchips. This will allow for the rapid detection of pathogens and diseases in an emergency, for point-of-care diagnosis, and for direct gene-expression profiling.

Experimental Section

Immobilization of the RNA–DNA hybrid template containing a 3′-NH_2 group on the DNA-binding plate: Coupling buffer (10 μL, Na_2HPO_4 (50 mM), EDTA (10 mM), pH 9.0), RNase-free water (89 μL), and the 3′-NH_2-template (1 μL, 0.1–0.6 mM) were added to the DNA-binding 96-well plate (Corning), and the plate was incubated for 1 h at 37 °C. Each well was then washed three times with post-coupling washing buffer (250 μL, NaCl (150 mM), maleate (100 mM), pH 7.5) to remove the nonimmobilized templates.

RNA binding and washing: After addition of 5X SSC buffer (50 μL, NaCl (3.0 M), sodium citrate (3.0 M), pH 7.0) to each well, RNA samples (1 μL each) were added to the wells. The plate was then incubated at room temperature for 30 min. Subsequently, the unbound RNAs were removed by washing each well three times with 2X SSC buffer (250 μL, NaCl (1.2 M), sodium citrate (0.12 M), pH 7.0).

RNase H digestion: After addition of RNase H buffer (50 μL, Tris-HCl (50 mM) pH 7.5; KCl (40 mM), $MgCl_2$ (6 mM), 1,4-dithio-DL-threitol (DTT; 1 mM), BSA (0.1 mg mL^{-1})) to each well, RNase H (1.0 μL, 0.2 u μL^{-1}) was added to each well, followed by 30 minutes' incubation at 37 °C.

Klenow extension: After draining the RNase H solution from each well, Klenow buffer (50 μL, Tris-Cl (10 mM), pH 7.5;, $MgCl_2$ (5 mM), DTT (7.5 mM)) was added to each well, followed by addition of the Klenow fragment (1 μL, 1 u μL^{-1}) and Biotin-7-dATP (1 μL, 1 mM). The plate was incubated for 1 h at 37 °C. Subsequently, the unincorporated biotin-dATP was removed from each well by washing twice with blocking buffer (250 μL, 1X, Sigma). Moreover, blocking buffer (250 μL, 5X, Sigma) was used to wash each well.

Enzyme binding and chemiluminescence detection: After the polymerase extension, blocking buffer (100 μL, 1X, Sigma) was added to each well, followed by addition of the antibiotin–AP conjugate (1 μL, 300-fold-diluted conjugate with blocking buffer (1X, Sigma)). The plate was then incubated for 20 min at room temperature. After the incubation, each well was washed four times with washing buffer (250 μL, 1X, Sigma) and once with alkaline phosphatase buffer (250 μL, 1X, Sigma). Finally, the CDP™ substrate (90 μL, Sigma) and alkaline phosphatase buffer (10 μL, 10X, Sigma) were added to each well. Film was exposed on the transparent bottom of the DNA-binding plate to record the chemiluminescence emitted. Chemiluminescence may also be recorded by luminometer microplate reader.

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