A Transcriptionally Amplified DNA Probe Assay with Ligatable Probes and Immunochemical Detection

William R. Carpenter,¹,⁴ Ted E. Schutzbank,¹,² Vincent J. Tevere,¹ Kenneth R. Tociyloki,¹ Nanibushan Dattagupta,¹,³ and Kwok K. Yeung¹

Transcriptionally amplified DNA probes are valuable tools in the development of sensitive nucleic acid-based diagnostic assays. Here we describe a model assay using a novel oligonucleotide hairpin probe that encodes a T7 RNA polymerase promoter. The hairpin probe and an adjacent hybridizing biotinylated capture probe were hybridized to target DNA and the duplex was captured onto streptavidin-coated magnetic particles. After ligation of the immobilized probes, which served to maintain specificity, the hairpin probe was transcribed by T7 RNA polymerase. The amplified RNA probe was hybridized to the capture probe and bound to the streptavidin-coated magnetic particles. The immobilized heteroduplex was detected with an antibody–alkaline phosphatase conjugate specific for DNA:RNA hybrids, and the chemiluminescent substrate adamantyl-1,2-dioxetane phenyl phosphate. Ten attomoles of target DNA could be detected in a background of 5 μg of unrelated DNA. The chemiluminescent immunonassay was as sensitive as radioactive detection of specific product after gel electrophoresis.

Indexing Terms: streptavidin–biotin • antibody to DNA:RNA • chemiluminescence • RNA polymerase

The application of DNA probe diagnostics to clinical assays requires in vitro amplification of nucleic acid sequences for ultimate sensitivity. The best-known amplification process is the polymerase chain reaction (PCR). Amplification schemes based on the use of RNA polymerases (2–4) and Qβ replicase (5) to produce RNA copies of the target sequence have also been described.

Bacteriophage RNA polymerases are useful in DNA probe assays because they transcribe very efficiently from a promoter site of defined sequence (6). Partially single-stranded DNA templates can be transcribed (7), allowing ligation of synthetic promoters to the target sequence of interest (8). Ligatable probes also improve specificity (9) for detection of single base mutations (10).

Routine use of DNA probes in clinical laboratories requires substitution of the traditional isotopic labels with nonradioactive methods and simplification of hybridization procedures. Many direct and indirect labels have been described (11); the most common is biotin (12). However, detection of hybridized probe with monoclonal antibody (mAb) to DNA:RNA hybrid (13) has some unique advantages. No labeling of the probe is required and, since the antibody does not react with single-stranded RNA or DNA, nonspecific signal is minimized. Enzyme conjugates of this antibody have been used in both direct (14, 15) and PCR-amplified (16) assays. The use of chemiluminescence instead of colorimetric substrates should significantly improve the sensitivity of DNA probe assays. Magnetic particles have simplified the manipulations involved in DNA hybridization (17) and provide more rapid binding kinetics than traditional membranes (18).

Here we describe a model system that combines the technologies described above into a prototype DNA probe assay for Chlamydia trachomatis major outer membrane protein (MOMP) gene sequences.

Materials and Methods

T4 polynucleotide kinase (EC 2.7.1.75), T7 RNA polymerase (230 000 U/mg; EC 2.7.7.6), and RNA-Guard were from Pharmacia (Piscataway, NJ); T4 DNA ligase (EC 6.5.1) was from Bethesda Research Laboratories (Gaithersburg, MD). Streptavidin-conjugated magnetic particles (Dynabeads M-280) were purchased from Dynal (Great Neck, NY). The Fab fragment of a mouse mAb directed against DNA:RNA hybrids, conjugated to alkaline phosphatase, was provided by R. Carrico (Ames Division, Miles Laboratories, Inc., Elkhart, IN). The chemiluminescent substrate adamantyl-1,2-dioxetane phenyl phosphate (AMPPD) and Sapphire enhancer were obtained from Tropix (Bedford, MA).

Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method on a Pharmacia Gene Assembler Plus DNA synthesizer. Genomic C. trachomatis DNA was prepared from inclusion bodies in HeLa cell cultures by detergent lysis, proteinase K digestion, and ethanol precipitation (19). The 1275–1655 base region of the C. trachomatis L2 serovar MOMP gene (20) was amplified by PCR, with complementary primers 5'-GATTACCATGAATGGCAGC and 5'-GAAGCGGAATTGTCATT and inserted into the pCR-2000 TA cloning vector (In Vitrogen Corp., San Diego, CA).

The oligonucleotide probes are shown in Figure 1. The 69-base hairpin probe contains both a double-stranded region that encodes the T7 RNA polymerase promoter

¹ Miles Inc., Diagnostics Division, 511 Benedict Ave., Tarrytown, NY 10591.
² Current address: Children's Hospital, 111 Michigan Ave., NW, Washington, DC 20010.
³ Current address: Gen-Probe, 9880 Campus Point Drive, San Diego, CA 92121.
⁴ Author for correspondence. Fax (914) 524-2458.

Nonstandard abbreviations: AMPPD, adamantyl-1,2-dioxetane phenyl phosphate; PCR, polymerase chain reaction; MOMP, major outer membrane protein; SSC, saline–sodium citrate; and mAb, monoclonal antibody.

Received April 28, 1993; accepted June 14, 1993.
and a single-stranded region that is complementary to the C. trachomatis MOMP gene. The 39-base capture probe is also complementary to the MOMP gene and hybridizes directly to the hairpin probe. The capture probe was biotinylated at the 5' end with Biotin-ON™ (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions; the hairpin probe was phosphorylated with 5' Phosphate-ON (Clontech Laboratories). In some experiments, the hairpin probe was end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase (19).

Sandwich hybridization (21) was conducted as follows. Target DNA was denatured (98 °C, 5 min) and then annealed with hairpin and capture probes (10 min, 52 °C) in 6× saline–sodium citrate (SSC) (1× SSC is NaCl, 150 mmol/L, and sodium citrate, 15 mmol/L, pH 7) containing dextran sulfate (50 g/L) and salmon sperm DNA (0.5 g/L). Biotinylated hybridization complexes were captured in 200 μL of particle buffer (per liter, 100 mmol of sodium phosphate, pH 7, and 1 g of Tween 80), containing 200 μg of streptavidin-coated magnetic particles. After a 10-min incubation on a hematocrit mixer (Fisher Scientific, Pittsburgh, PA), particles were drawn to the side of the tube with a magnetic separation device (Dynal), and the supernate was removed. Particles were washed three times in particle buffer to remove unhybridized probe. Ligation of adjacently hybridized oligonucleotide probes was performed with 2 U of T4 DNA ligase in 10–20 μL of buffer (per liter, 50 mmol of Tris-HCl, pH 7.6; 10 mmol of MgCl2; 1 mmol of ATP; 1 mmol of dithiothreitol; and 50 g of polyethylene glycol-8000).

Transcriptional amplification reactions were performed in 25-μL volumes containing, per liter, 50 mg of bovine serum albumin; 40 mmol of Tris-HCl, pH 8; 10 mmol of MgCl2; 10 mmol of NaCl; 10 mmol of dithiothreitol; 1000 U of RNA-Guard; 0.5 mmol each of ATP, CTP, and UTP; 5 mmol of GTP; and 8 × 105 U of T7 RNA polymerase. Tween 80 (1 g/L) was also added to the reactions performed in the presence of magnetic particles. Radioactive labeling of transcribed RNA was accomplished by adding [α-32P]ATP (0.3 Ci/L). For autoradiography, samples were double-diluted with formamide (980 mL/L) containing bromophenol blue (0.5 g/L) and xylene cyanol (0.5 g/L), heated to 95 °C (2 min), and loaded onto an acrylamide gel (150 g/L) containing urea (7 mol/L). After electrophoresis (2 h, 25 V/cm), the gel was exposed to x-ray film while wet. Bands were excised and counted by Cerenkov radiation directly in the gel slice.

Amplified transcript was first diluted threefold in 30 μL of hybridization buffer [2× SSC containing, per liter, 25 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 2 mmol of EDTA] and 4 pmol of biotinylated capture probe was added. After denaturation (2 min, 98 °C) and hybridization (10 min, 57 °C), 200 μg of magnetic particles was added in 170 μL of sodium phosphate (0.1 mol/L, pH 7) containing sodium dodecyl sulfate (1 g/L), incubated, and washed three times with particle buffer as described above. For non-isotopic detection, particles carrying RNA:DNA hybrids were washed two additional times in antibody buffer (per liter, 0.1 mol of Tris-HCl, pH 7.4, 0.1 mol of NaCl, 1 g of bovine serum albumin, and 1 g of Tween 80), after which particles were resuspended in 250 μL of antibody buffer containing an alkaline phosphatase-labeled Fab' fragment of the anti-DNA:RNA mAb (0.05 mg/L). After incubation on a hematocrit mixer (15 min, 37 °C), particles were washed three times in antibody buffer. Finally, particles were resuspended in 0.5 mL of assay buffer containing diethylamine (0.1 mol/L, pH 9.5), MgCl2 (1 mmol/L), AMPPD (0.1785 g/L), and Sapphire enhancer (1 g/L). After 20 min at 37 °C, the enzyme-generated chemiluminescence of a 50-μL aliquot was measured in a Turner Designs (Mountain View, CA) Model 20 photometer, integrated over 10 s.

**Results**

A schematic for the immunochemical detection of transcriptionally amplified ligatable DNA probes is...
shown in Figure 2. Amplification is achieved by means of a novel, partially double-stranded hairpin probe encoding a T7 RNA polymerase promoter (Figure 1). Transcription from the T7 promoter generates multiple copies of RNA complementary to the single-stranded portion of the probe. The hairpin probe and a biotinylated capture probe are hybridized to target DNA and the duplex is captured on streptavidin-coated magnetic particles. The immobilized probes, which hybridize adjacent on the target DNA, are then ligated together. Transcription of the ligated probes by T7 RNA polymerase produces an extended transcript that can be generated only by accurate hybridization of both probes to their intended target. The extended transcript is specifically rehybridized to the capture probe and immobilized onto streptavidin-coated magnetic particles. The short transcript generated from nonspecifically bound hairpin probe does not hybridize to the capture probe. Bound heteroduplex is detected with an antibody–alkaline phosphatase conjugate specific for DNA:RNA hybrids and the chemiluminescent substrate AMPPD. The assay was optimized as described below.

The hairpin probe is transcribed efficiently by T7 RNA polymerase. RNA transcript continues to accumulate for 2 h after addition of polymerase. Under appropriate conditions, amplification factors in excess of 1000-fold can be achieved. A high concentration of polymerase (>2.75 x 10^6 U/L) and an increase in the concentration of GTP, the initiating base, from 0.5 to 5 mmol/L are required for this level of amplification (data not shown).

The hairpin probe can be used in a straightforward sandwich hybridization format to amplify the concentration of a specific target nucleic acid sequence. However, because the amplified sequences derive from the probe, rather than the target, it is impossible to discriminate between RNA produced from specifically hybridized probe and RNA from nonspecifically hybridized probe, or probe that is nonspecifically bound to the solid phase. To alleviate this problem, we designed a capture probe that hybridizes directly adjacent to the hairpin probe (bases 1417–1452), allowing the probes to be ligated into one contiguous sequence. Figure 3 demonstrates that ligation occurs only in the presence of target DNA containing sequences complementary to the probes. Genomic Chlamydia DNA and a plasmid harboring the Chlamydia MOMP gene promoted ligation of the hairpin and capture probes, whereas Escherichia coli DNA did not. Ligation was more efficient if the target DNA was first fragmented by restriction endonuclease digestion. Ligation in solution required as little as 50 mU of DNA ligase, but ligation could also be performed once the hybridized probes had been bound to magnetic particles if the ligase was increased to 2 mU (data not shown).

Transcription of the ligated hairpin probe produces an RNA transcript that is extended at its 3’ end by sequences complementary to the capture probe. The capture probe cannot be transcribed alone because it lacks a promoter. Therefore, the presence of amplified RNA complementary to the capture probe is a specific measure of the presence of the target DNA of interest. Rehybridization of amplified RNA to the capture probe allows the extended RNA to be bound to streptavidin-coated magnetic particles as a DNA:RNA hybrid. Only the extended transcript is captured; shorter RNAs, transcribed from unligated or nonspecifically bound hairpin, are not bound (Figure 4).

The DNA:RNA hybrids bound to magnetic particles are detected by a mAb specific for DNA:RNA heteroduplexes. Alkaline phosphatase conjugated to an Fab’ antibody fragment, together with the chemiluminescent
Fig. 4. Capture of extended transcript
5 and 10 fmol of MOMP plasmid, in a background of 5 μg of salmon sperm DNA, were used to initiate ligation of hairpin and capture probes. The extended hairpin was transcribed in the presence of [α-32P]ATP. A portion of the reaction was hybridized to capture probe, bound to streptavidin-coated magnetic particles, and washed. Equivalent amounts of captured and total RNA were subjected to electrophoresis and made visible by autoradiography.

![Image of autoradiography](image)

Fig. 5. Chemiluminescent detection of captured extended transcript
Ligation initiation, transcription, and capture of extended transcript were as described in Fig. 4. Hybridized RNA was detected by chemiluminescence (—△—, left ordinate) and by autoradiography (inset). Bands were excised from the gel and counted for radioactivity (—○—, right ordinate).

Discussion
The hairpin promoter probe is a novel means for achieving transcriptional amplification of DNA sequences. The hairpin promoter is simple to synthesize, spontaneously reforms after heat-denaturation, requires only one enzyme, and generates amplification of three orders of magnitude. Coupled with alkaline phosphatase-mediated chemiluminescent detection, the method is highly sensitive for target DNA. The clinical utility should therefore be similar to direct DNA probes that target ribosomal RNA (present at 10 000 copies per organism) without amplification (22). PCR-based assays are in principle more sensitive, because exponential product accumulation can generate amplification factors > 107-fold. However, this requires continuous thermal cycling, which is difficult to integrate into a fully automated method. Hairpin probe amplification proceeds isothermally.

Cycling amplification is also possible with the hairpin probe, if the RNA produced serves as a template for additional rounds of transcription (23). An alternative configuration of the hairpin, with a 3' rather than a 5' single-stranded region, would allow direct ligation of the probe to the analyte DNA and subsequent amplification of the target sequence rather than the probe sequence.

The anti-DNA:RNA mAb has been used to detect naturally occurring (14) and PCR-amplified nucleic acids (16). Here we extend this principle to transcriptional amplification and show that the anti-DNA:RNA mAb can detect DNA:RNA heteroduplexes as short as 40 nucleotides. Probes of this length can be synthesized chemically rather than by cloning techniques. The readout system we describe would be generally applicable to any amplification technique that generates RNA as an end product. Schemes such as self-sustained sequence replication (3) and nucleic acid sequence-based amplification (4), for which two complementary primers/probes are used, would probably not require a ligation step to maintain specificity.

Magnetic particles are a convenient solid phase for probe assays, simplifying many of the otherwise labor-intensive steps involved in nucleic acid hybridization. Coupled with immunochemical readout, magnetic particle assays could be adapted for routine use in clinical laboratories without substantial modification of existing instrumentation. A number of magnetic particle-based immunochemistry instruments are now commercially available and can provide a platform for the introduction of such assays.

CLINICAL CHEMISTRY, Vol. 39, No. 9, 1993 1937
We thank Linda Anderson for growing Chlamydia and Peter Rae and Donald Crothers for their intellectual contributions to this work.

References