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Novel, Ultrasensitive, Q-Beta Replicase-Amplified Hybridization Assay for Detection of *Chlamydia trachomatis*

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A sensitive, nonisotopic hybridization assay termed "dual capture" is described. The assay rapidly and specifically detects very low levels of target nucleic acids and organisms. The assay is based on the principles of sandwich hybridization, reversible target capture, and Q-Beta replicase amplification. The assay can be completed in less than 4 h, and in the described model format, it detects *Chlamydia trachomatis* rRNA or rDNA. Up to 96 samples can be analyzed simultaneously. The assay employs two types of probes: a test-specific capture probe, which mediates the cycling of the target probe complex on and off derivatized magnetic beads, and a replicatable RNA detector molecule containing a sequence complementary to and adjacent to the capture probe site on the target. Following reversible target capture, detection of the signal is accomplished by replication of the detector molecule by Q-Beta replicase in the presence of propidium iodide. A specific assay signal can be detected from as few as 1,000 molecules above the background. In a limited study of 94 urogenital samples the assay detected five of the six culture-positive samples and did not detect the *C. trachomatis* target in 85 of the 88 culture-negative samples.

A large variety of nucleic acid probe-based hybridization assays for the detection of infectious agents have been described (17). However, a number of practical considerations have delayed their widespread introduction in clinical testing laboratories. In particular, it is well recognized that very high sensitivity is required for the detection of clinically important pathogens directly from specimens. This prompted the intensive development of amplified nucleic acid assays including PCR, ligase chain reaction, transcription amplification system, self-sustained sequence replication, nucleic acid sequence-based amplification, strand displacement amplification, repair chain reaction (15), branched DNA signal amplification, and Q-Beta replicase probe amplification.

Here we describe a novel nonisotopic amplified nucleic acid assay for the detection of *Chlamydia trachomatis* that uses sandwich hybridization, reversible target capture, and Q-Beta replicase amplification. The concept of sandwich hybridization was introduced by Ranki et al. (13). The basic method involves the hybridization of two probes to the target nucleic acid, at least one of which must hybridize specifically. A number of variations on this basic theme have been developed, but in general, such single-capture methods are limited to a sensitivity of about 10^6 molecules by the nonspecific binding of the unhybridized reporter probe to the solid support. Recognizing not only that nonspecific binding is a function of the chemical nature of the solid support but also that it is driven by the concentration of labeled probe, Morrissey et al. (9) developed a method of noise reduction termed reversible target capture (RTC). RTC involves the iterative capture and elution (cycling) of probe-target hybridization complexes on solid supports. It is capable of reducing the amount of unhybridized reporter probe between 10^3- and 10^4-fold during each cycle. We report on the basic assay methodology and its performance in the detection of *C. trachomatis*.

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Q-Beta replicase is an RNA-dependent RNA polymerase derived from the bacteriophage Q-Beta (4). The enzyme is capable of replicating a limited family of RNA molecules, a number of which have been engineered to contain probe sequences (2, 5, 6). Q-Beta replicase copies the template RNA, producing a complementary product strand in as little as 12 s (4, 8, 10). Since the daughter strands are also templates for the enzyme, RNA production proceeds exponentially. A single probe molecule can yield an easily detectable amount of product RNA in a 15-min amplification reaction. Thus, in principle, an extremely small number of target molecules can be detected in such an assay.

This report describes an assay format, termed "dual capture," which is a nonisotopic version of the RTC assay in which the isotopically labeled probes have been replaced with novel RNA probes that are replicatable by Q-Beta replicase (5, 6). The assay is highly sensitive and specific and can detect the presence of *C. trachomatis* nucleic acid in patient samples.

MATERIALS AND METHODS

Paramagnetic particles. Oligo(dT)-derivatized ferromagnetic particles (Advanced Magnetics, Inc., Cambridge, Mass.) were prepared as described previously (9). The particles were stored as 0.25% (wt/vol) suspensions and had a binding capacity of 300 pmol of $d(A)_{50}$ per mg. Immediately prior to the experiment the dT particles were separated from the buffer and were made up to 0.12% in fresh bead blocking buffer (100 mM Tris-HCl [pH 7.8], 20 mM EDTA, 4% bovine serum albumin [BSA], 0.5% Sarkosyl, 0.01% antifoam). Paramagnetic streptavidin-coated particles (Promega, Inc., Madison, Wis.) were similarly exchanged into bead blocking buffer and were used at 0.04%.

Target nucleic acid. Purified *C. trachomatis* rRNA and *C. trachomatis* elementary bodies were prepared from infected McCoy cell cultures as described previously (1a). *Chlamydia psittaci* 16S rRNA was transcribed from a pGEM4 vector containing a PCR rDNA sequence from positions 8 to 1525. The identity of the cloned insert was verified by sequence

analysis. The transcribed rRNA was quantitated by UV absorbance and was run on denaturing polyacrylamide gels to verify its integrity. *C. trachomatis* rDNA target was derived from an M13 clone which contains a partial rRNA operon from the chromosome of *C. trachomatis*, including the entire 16S gene, 661 bases of the 23S gene, as well as small amounts of flanking and intervening sequences. The single-stranded M13 DNA was digested with *Hha*I according to the manufacturer's recommendations (New England Biolabs). The probe target region is contained within a single *Hha*I fragment of approximately 800 bases. All M13 manipulations were done as described by Sambrook et al. (14).

Preparation of patient specimens. Coded samples were submitted to our laboratory from the University of Massachusetts Medical Center as described previously (1a). For culture, a 0.3-ml aliquot of sample in 2-SP was cultured in shell vials, and the shell vials were incubated for 3 days and stained with a fluorescein-conjugated monoclonal antibody as described previously (1a). For the dual-capture assay, a 0.5-ml portion of the sample was pelleted by centrifugation in a microcentrifuge at 13,000 × *g* for 10 min. The supernatant was removed and the pellet was suspended in 0.21 ml of 1% Nonidet P-40. A 0.1-ml aliquot was mixed with 0.4 ml of sample processing buffer (100 mM Tris-HCl [pH 7.8], 20 mM EDTA, 8 M GuHCl, 0.6% CTAB), and the mixture was vortexed for 30 s. Duplicate samples were tested. Samples were considered positive when signals were detected in both samples.

Probes. The probe sequences are given below. Single-stranded capture probes 3018 and 781 were prepared by using β-cyanoethyl phosphoramidite chemistry on a 380-B synthesizer (Applied Biosystems, Foster City, Calif.). The sequences of probes 3018 and 781 are 5′-biotin-NH_2-CCTTTAACGT TACTCGGATGCCCAAA and 5′-CTTTAACGTTACTCG GATGCCCAAATACGCCACAT-$(dA)_{150}$, respectively. Both of these probes were modified at the 5′ ends to contain a primary amine by the addition of an aminopropyl-modified cytosine phosphoramidite. Deprotection of the phosphates and nucleotide bases was accomplished by standard methods, and the crude oligonucleotide mixtures were purified by reverse-phase high-pressure liquid chromatography. The amino-modified oligonucleotide was labeled with biotin by using FluoReporter biotin labeling kit F-2610 (Molecular Probes, Eugene, Oreg.). A tail of about 150 deoxyadenosine residues was added to the 3′ end of the capture probe by using terminal deoxynucleotidyl transferase (9).

A Q-Beta replicatable RNA reporter probe containing a probe sequence complementary to the *Chlamydia* target was transcribed from a recombinant plasmid with T7 RNA polymerase (2). This RNA transcript is referred to as C29 and is used in the examples described below as an MDV reporter probe. MDV is an abbreviation for midivariant RNA, which is the most extensively studied nonviral substrate for Q-Beta replicase into which probe sequences have been cloned (6). Briefly, C29 is a recombinant MDV molecule containing a probe sequence designated 1126 (5′-AGGCCTTTACCCCAC CAACTAGCTGATATCACATA).

This sequence was inserted at the unique *Mlu*I and *Nhe*I site located between nucleotides 63 and 64 of the plus strand. In addition, it is flanked by 10-nucleotide spacer elements which improve the replication properties of the recombinant probe (2). Purified plasmid DNA containing the C29 sequence cloned downstream of a T7 promoter was cut with *Sma*I at the 3′ end of the MDV sequence and was transcribed into RNA with T7 RNA polymerase in the presence of a trace amount of [^{32}P]UTP. RNA transcripts were quantitated by the percent incorporation of counts as measured by retention on DE81 ion-exchange filters (Whatman). The transcripts were checked for size and integrity by denaturing polyacrylamide gel electrophoresis. The RNA substrates were diluted and tested in duplicate for replication efficiency (2).

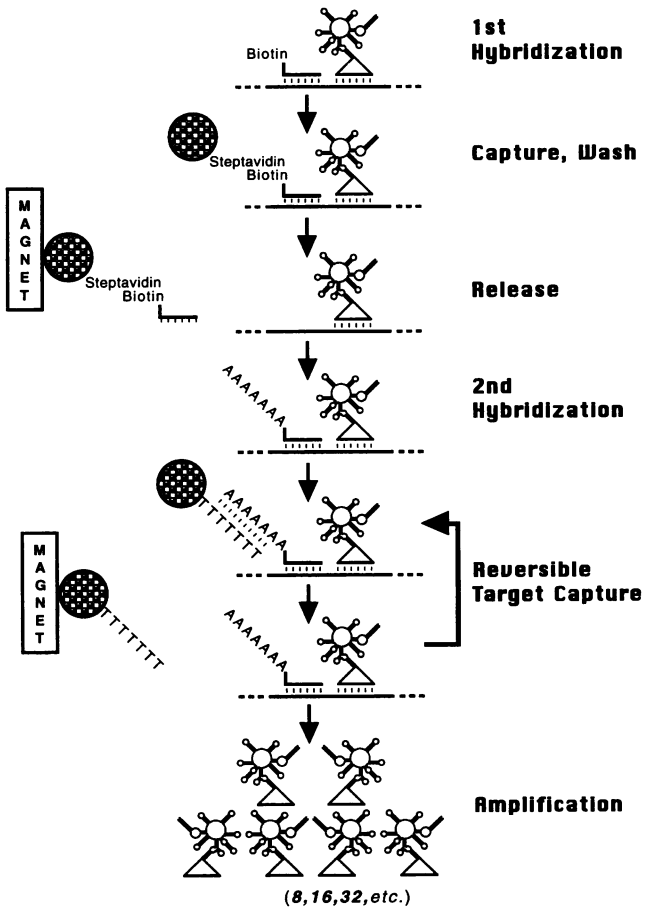
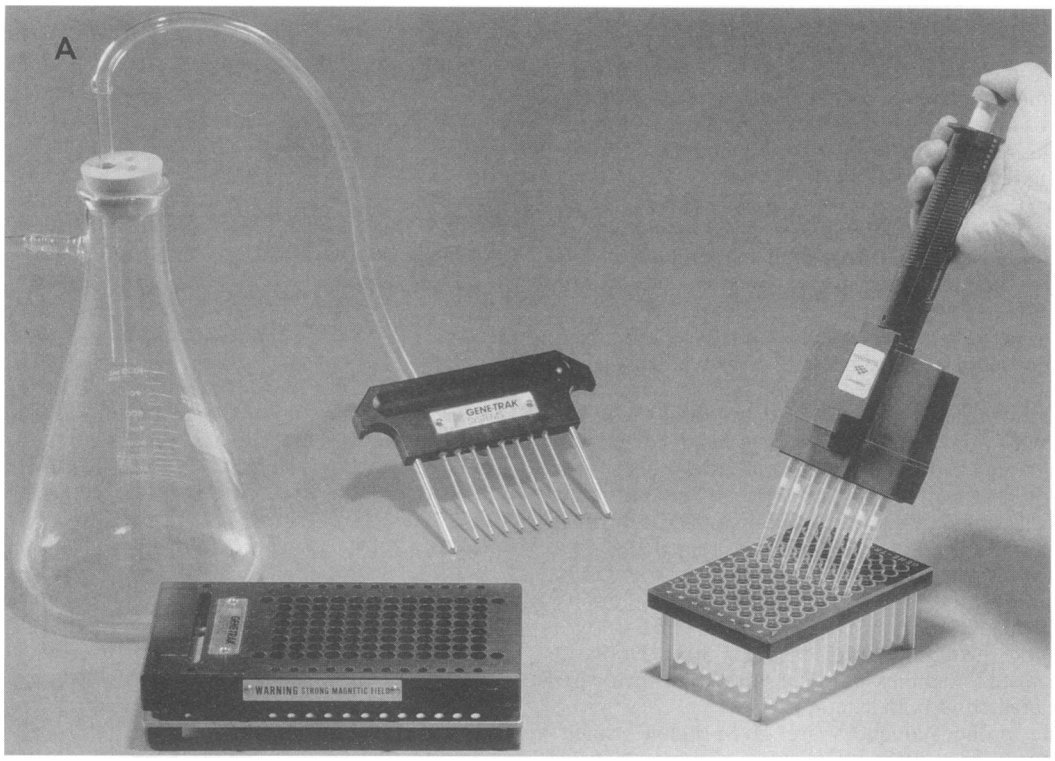


FIG. 1. Dual-capture assay format.

Dual-capture RTC format. A schematic representation of the dual-capture RTC format is given in Fig. 1, and details of the assay are provided below. The hybridization and reversible target capture reactions were carried out with a tube rack, magnetic separator block, and aspirator head specially designed to hold 96 1-ml tubes (Micronic B.V., Lelystad, The Netherlands), as shown in Fig. 2. Hybridization and reversible target capture reactions were conducted at 37°C by keeping the magnetic separator and tubes partially submerged in a circulating water bath. In all, four sets of racked tubes were required for all steps of the assay up to the amplification step. The reaction mixtures were assembled in the first set of tubes, and at every cycle of the assay the reaction mixture was transferred to another rack of clean tubes. This configuration was compatible with a microtiter plate format, enabling us to perform manipulations with multichannel pipettors.

The samples containing various amounts of purified 16S rRNA, 16S rDNA, elementary bodies, or human specimans were diluted in sample processing buffer to a final concentration of 6.4 M GuHCl. Capture probes and MDV probes were diluted with probe dilution buffer to a final concentration of 300 ng/ml each. Hybridization reaction mixtures (100 μl) were prepared by adding 67 μl of diluted target to 33 μl of the



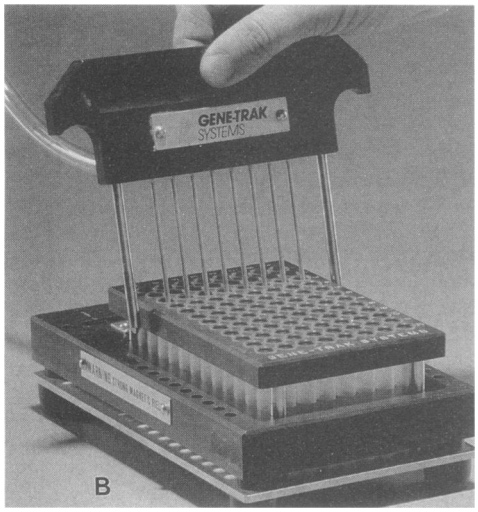


FIG. 2. Hardware used in reversible target capture assay. (A) The set consists of three components: a test tube rack that holds 96 1-ml tubes with the same configuration and spacing as those of microtiter plates, a magnetic separator block which holds the tube rack, and an eight-channel aspirator head which is hooked up to a collection flask on standard laboratory vacuum lines. Alternately, the liquid can be removed and transferred to another rack of tubes by using any number of standard multichannel microtiter pipettors. (B) The rack fits into the magnetic separator block in which magnetic beads are rapidly pulled to the sides of the tube, allowing removal of liquid by the eight-channel aspirator head.

capture probe-C29 RNA mixture to yield a final concentration of 100 ng/ml for both capture and detector probes.

Following the 30-min hybridization at 37°C, 100 μl of a suspension of 0.04% streptavidin-derivatized paramagnetic particles in bead blocking buffer was added to each of the tubes. After mixing, the tubes were incubated at 37°C for 5 min to capture the ternary hybrids (capture probe-target-detector probe). The particles containing the captured hybrids were collected onto the sides of the tubes by using a specially designed magnetic separator for 2 min, and the supernatants were removed by aspiration. C29 detector probe that had not bound to the target was removed by washing the magnetic particles twice with 200 μl of low-salt buffer (100 mM Tris [pH 8.1], 20 mM EDTA, 25 mM NaCl, 0.2% Sarkosyl, 0.05% BSA, and 0.05% Bronopol). Washing was accomplished by vortexing the tubes for 30 s and separating the particles from the buffer as described above.

The target-detector probe hybrid was released from the initial solid support by adding 100 μl of GuSCN release buffer (240 mM Tris-HCl [pH 7.8], 60 mM EDTA, 3 M GuSCN, 0.6% Sarkosyl) to all tubes, mixing, and incubating the tubes for 5 min at 37°C. The biotinylated capture probe dissociates from the target and remains bound to the streptavidin particles under these conditions. After the 5-min incubation the particles were collected onto the sides of the tubes by using the magnetic separator, and the supernatants containing the released reporter probe-target hybrids were transferred to a clean set of tubes.

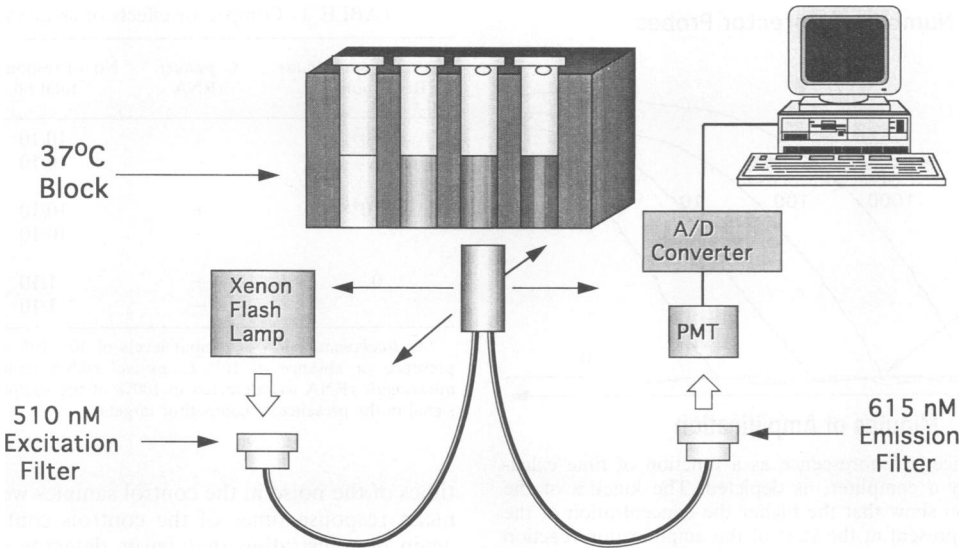


FIG. 3. Principal features of the kinetic fluorescence reader depict amplification reactions incubating in a 37°C heated block; the xenon flash lamp source transmits a 510-nm-excitation wavelength to the reaction tubes via a fiber optic line. The increase in fluorescence is monitored by a sensor mounted on an XY table, which enables it to read all 96 positions every 40 s. An outgoing fiber optic line transmits the measured fluorescence to a photomultiplier tube (PMT) via a 615-nm-emission-wavelength filter. The signal is translated by an analog-to-digital (A/D) converter into a computer, which calculates the time at which the fluorescence rises above the baseline.

To all of the tubes, 50 μl of 300 ng of dA-tailed capture probe 781 per ml of probe dilution buffer was added to yield a final concentration of 100 ng/ml. The tubes were vortexed for 30 s and then incubated for 30 min at 37°C to allow the second (dA-tailed) capture probe 781 to hybridize to the target-detector probe complex. To all of the tubes, 250 μl of 0.05% dT14 particles in the bead blocking buffer was added. The ternary complexes were captured onto the dT beads by incubating them for 5 min at 37°C. Particles were washed three times with 200 μl of high-salt wash buffer (100 mM Tris [pH 8.1], 20 mM EDTA, 300 mM NaCl, 0.5% Sarkosyl, 0.5% BSA, 0.5% Bronopol) and ternary complexes were released by using 100 μl of low-salt buffer per tube. The low-salt buffer disrupts the dA-dT hybrids made between the dA tail of the second capture probe and the oligo(dT) on the solid support, thereby releasing the intact capture probe-target-detector probe ternary hybrid complex into the solution.

Each of the released supernatants was transferred to a clean set of tubes as described above, and 100 μl of a suspension of fresh 0.12% oligo(dT) particles in bead blocking buffer and 200 μl of GuHCl dilution buffer (100 mM Tris [pH 7.5], 20 mM EDTA, 8 M GuHCl) were added. The complexes were recaptured onto the particles as described above. The beads were washed once in 200 μl of high-salt buffer. The ternary complexes were released by using 100 μl of the low-salt buffer, and the supernatant was transferred to clean tubes as described above. To each of the released supernatants, 100 μl of a suspension of 0.12% oligo(dT) particles in bead blocking buffer and 200 μl of GuHCl dilution buffer were added. The complexes were captured onto this final (fourth) set of particles as described above. The beads were washed twice in a 200 μl of preamplification wash buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 300 mM KCl, 0.5% Nonidet P-40). The target MDV RNA complexes were released by using 150 μl of amplification release buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% Nonidet P-40) and were again transferred to clean tubes as described above.

Amplification with Q-Beta replicase. A 100-μl aliquot of released hybrid complex was added to an equal volume of the Q-Beta replicase buffer (220 mM Tris [pH 7.8]; 40 mM $MgCl_2$; 1.2 mM [each] GTP, ATP, CTP, and UTP; 2.0 μg of propidium iodide per ml; 25% glycerol containing about 110 μg of Q-Beta replicase per ml) in a closed tube. The contents of the reaction tubes were mixed and placed in the Gene-Trak kinetic fluorescence reader, which contains a 37 ± 0.25°C preheated block. This instrument monitors the production of RNA in as many as 96 Q-Beta replicase amplification reactions. The increase in fluorescence is due to the binding of the dye propidium iodide to the product RNA. The salient features of the reader are depicted in Fig. 3. A 510-nm-wavelength light from a xenon flash lamp is transmitted to the reaction via a fiber optic line. Fluorescence is received through another fiber optic line into a 615-nm emission filter. The sensor, containing both fiber optic lines, is fixed to a tram on an XY table, which enables it to read all 96 positions through the bottom of the heater block. Fluorescence was measured in each reaction tube every 40 s during a 30-min time period. The signal is received by a photomultiplier tube, which relays it to an analog-to-digital converter.

The information was received, analyzed with a proprietary software package, and plotted by an International Business Machines 386 computer. A depiction of the plotted data is shown in Fig. 4. Figure 4 represents an amplification of diluted C29 detector probe in the amplification release buffer. A 50-μl aliquot containing 10-fold dilutions of C29 molecules was added to an equal volume of the Q-Beta replicase buffer. The software calculates a parameter termed "response time" (3). This was defined as the time required to reach an arbitrary (fixed) level of RNA in each reaction tube. In the present case, the response time corresponds approximately to the time at which the fluorescence signal rises just above the baseline fluorescence because of the free propidium iodide in the reaction mixture. The response time is inversely proportional to the logarithm of the number of detector molecules present at the start of the amplification reaction.

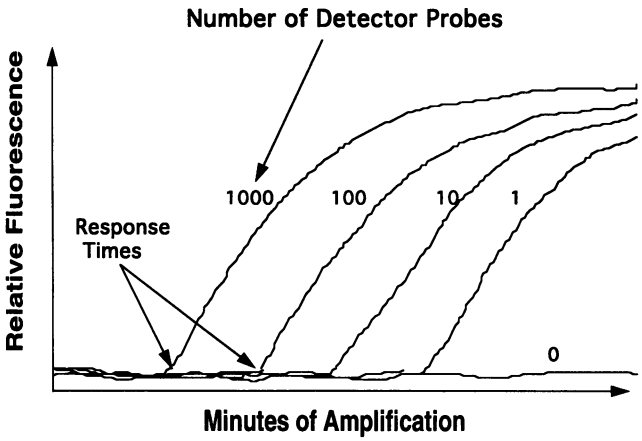


FIG. 4. Appearance of fluorescence as a function of time calculated and plotted by a computer, as depicted. The kinetics of the amplification reaction show that the higher the concentration of the C29 detector probe present at the start of the amplification reaction the shorter the time needed to produce a level of fluorescence above the baseline of unbound propidium iodide.

RESULTS

Detection of *C. trachomatis* rRNA or rDNA targets. The dual-capture assay was performed as described in Materials and Methods by using various concentrations of purified *C. trachomatis* rRNA or synthetic DNA targets (Fig. 5). As in the case of the reporter probe amplification, there is an inverse logarithmic relationship between the target concentration and the mean response time (signal). The zero-target (negative) controls show that the signal from RNA or DNA targets is statistically less than those from reactions containing 10^3 targets. Specifically, 2 of 12 zero-target controls yielded signals, whereas 100% of the samples containing 10^3 targets yielded signals. At higher target concentrations, the response times of replicate samples were faster than those of the zero-target samples which responded. In addition, the mean response

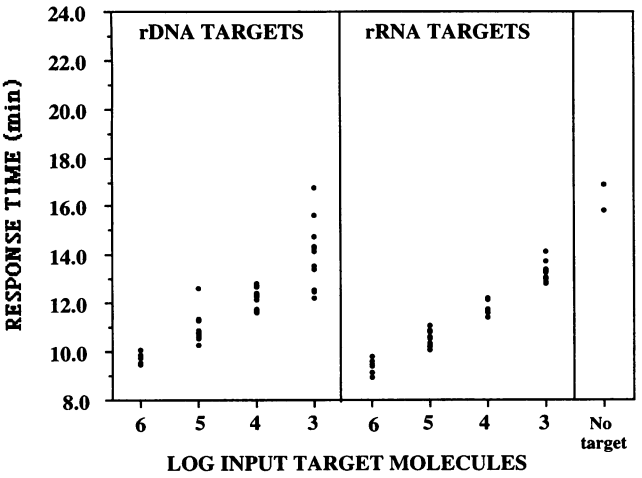


FIG. 5. Dose response of *chlamydia* RNA and DNA targets in an amplified assay. The RNA target was rRNA purified from *C. trachomatis* elementary bodies, and the DNA target was an *Hha*I digest of a single-stranded M13 recombinant clone containing a partial rRNA operon, including the entire 16S gene. The sample size for each target level was 12, and 2 of the zero-target reaction mixtures gave a signal.

TABLE 1. Competitor effects of assay signal and noise[a]

No. of *C. trachomatis* rRNA molecules	*C. psittaci* rRNA	No. of responders/ total no.	Mean ± SD response time (min)
10^5	+	10/10	13.60 ± 1.10
	−	10/10	13.32 ± 1.42
10^4	+	10/10	15.11 ± 2.02
	−	10/10	15.19 ± 1.29
0	+	1/10	19.87
	−	1/10	17.60

[a] *C. trachomatis* rRNA at input levels of 10^5, 10^4, and 0 was tested in the presence or absence of 10^{11} *C. psittaci* rRNA molecules. In all cases *C. trachomatis* rRNA was detected in 100% of the samples, with no decrease in signal in the presence of competitor targets.

times of the noise in the control samples were slower than the mean response times of the controls containing 10^3 targets, again demonstrating that fewer detector probes were being amplified in the negative controls. The false-positive rate was typically between 0 and 10%. When the final elution reactions of false-positive samples were diluted 10-fold, no signal was observed by amplification, indicating that the detector probe was at the level of a single molecule (unpublished data). From studies in which reaction mixtures containing no added detector probe were tested along with reaction mixtures containing detector probe, this variability was traced to the impact of exogenous contamination and the limitations of an open system. The sensitivity of the amplified assay format was between 10^2 and 10^3 input targets. By using a detector probe which can replicate at the single-molecule level, this translates to a 0.1 to 1% efficiency of target hybridization, capture, and amplification. Isotopic studies which have used ^{32}P-labeled target transcripts or detector probes have corroborated this efficiency (data not shown).

Assay specificity. The specificity of the *C. trachomatis* assay was examined by testing samples containing 10^{11} *C. psittaci* 16S rRNA molecules in the absence and presence of various levels of *C. trachomatis* rRNA. The results of the study (Table 1) show both the qualitative data, i.e., the number of reactions in which amplification was detected, and the mean response time of positive reactions, or the quantitative data. In reaction mixtures containing *C. trachomatis* rRNA, 10 of 10 reactions were positive and in only negative control reaction mixtures 1 of 10 reactions was positive. Statistical analysis of the signal demonstrated no difference in response times in the presence or absence of competitor RNA. Furthermore, no signal was detected in reaction mixtures containing *C. psittaci* rRNA.

Detection of *Chlamydia* elementary bodies. The sensitivity of the assay was tested by using a dilution series of elementary bodies. Quantitation of elementary bodies was performed by using direct immunofluorescence as described earlier (1a). Elementary bodies were added to the sample processing buffer, and the mixture was vortexed for 30 s prior to the assay. Purified *C. trachomatis* RNA was tested in parallel. Signal was detected at all dilutions of elementary bodies down to 10 in all 12 replicate samples. In the reaction mixtures with one elementary body, 10 of 12 reactions gave a signal, which is consistent with Poisson failure (Fig. 6). In the control reactions, 2 of 12 reaction mixtures not containing RNA and all reaction mixtures containing RNA gave a signal. Comparison of the response times between elementary body and RNA doses indicated that there were between 10^3 and 10^4 RNA molecules per elementary body.

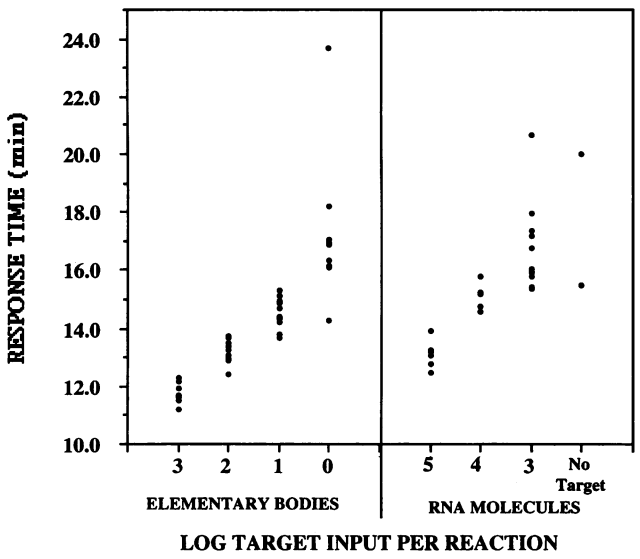


FIG. 6. Elementary bodies were harvested from infected McCoy cells and were quantitated by direct fluorescent-antibody staining. The elementary bodies were resuspended in sample processing buffer containing 8.0 M GuHCl and tested in replicates of 12 at each input level. Controls containing 10^5 (n = 6), 10^4 (n = 6), 10^3 (n = 12), and 0 (n = 12) RNA molecules in the sample processing buffer were run in parallel.

Detection of *C. trachomatis* in patient samples. Ninety-four urogenital samples containing six culture-positive samples were tested in duplicate. Five of these culture-positive samples were positive by Q-Beta amplification. For culture 300 μl of sample was inoculated onto the cells, while 50 μl of sample was used in duplicate for the dual-capture assay. Of the 88 culture-negative samples, the assay was concordant with 85 samples. However, three culture-negative samples were positive by our assay (Table 2).

DISCUSSION

The dual-capture assay format incorporates the background reduction principles of reversible target capture (9). However, there is one significant conceptual modification in this chemistry which permits the background to be driven down even further. The term dual capture incorporates an additional hybridization and capture step early in RTC that uses a ligand-antiligand capture pair distinct from that which is used later in the assay to cycle target-probe complexes on and off magnetic particles.

The following are two key design elements of the dual-capture chemistry. (i) The hybrid between the first capture probe and the target sequence is weaker than the hybrid between the reporter probe and its target sequence, and (ii) there is a lack of cross-affinity of the first capture probe ligand to the second antiligand solid support. The first design element allows for a second round of specific hybridization to be imposed against closely matched competitors, while the second design element effectively excludes the nonspecific first capture probe-detector probe complex from subsequent rounds of capture. With the reduction in the background signal, the ultimate assay sensitivity is determined by the cumulative efficiencies of the hybridization capture and release steps. In the format described here, the conditions for hybridization capture and release have been optimized from physical measurements of the stabilities of the individual hybrid bonds.

TABLE 2. Comparison of Q-Beta amplification with cell culture for the detection of *C. trachomatis* in patient specimens[a]

Dual-capture assay result	No. of specimens with the following culture result:	
	+	−
+	5	3
−	1	85

[a] A 300-μl aliquot of the specimen was inoculated onto HeLa 229 cells seeded on coverslips for culture and stained after 3 days. The equivalent of 50 μl of specimen was tested in duplicate by the dual-capture assay. Specimens which gave signals in both replicates were considered positive.

The application of two independent and successive hybridizations with distinct ligand and antiligand capture pairs is a highly selective process in which nonspecific hybrids and nonspecifically bound probe complexes are effectively eliminated. With respect to nonspecific hybrids, close competitor target sequences are subjected to two successive hybridizations against the same probe sequences. The discrimination of a specific capture probe against closely related nontarget sequences is the product of the discrimination factor obtained for each hybridization event. This multiplicative factor translates to the detection of 10^3 *C. trachomatis* rRNA targets in the presence of a 10^8-fold excess of *C. psittaci* rRNA molecules.

In the case of nonspecifically bound probe complexes, the elimination of nonspecifically bound probe complexes results from both RTC and the use of two distinct ligand-antiligand pairs. RTC eliminates the vast majority of the nonspecifically bound reporter probe through the process of multiple and sequential cycles of specific capture of target complexes, washes, and target elution from the magnetic beads. However, under assay conditions of high capture and reporter probe concentrations, the high degree of sensitivity afforded by the Q-Beta amplification step allows detection of the formation of a very low level of nonspecific capture probe-detector probe complexes. A similar observation was reported by Van Ness and Chen (16), who used oligonucleotide probes in chaotropic hybridization mixtures. Although the efficiency of this formation is extremely low (on the order of 1 in 10^8 detector probes), this type of noise subsequently cycles on and off the beads when a single ligand-antiligand pair is used (unpublished data). The presence of such complexes is below the sensitivity of standard isotopic methods. The application of two different capture ligand sets greatly reduces this carry through, because any capture probe-detector probe complex which is formed and captured via the first ligand-antiligand pair is unable to bind to the second set of beads containing a different antiligand. Pritchard and Stefano (11, 12) previously reported on the use of Q-Beta amplification and reversible target capture for the detection of human immunodeficiency virus type 1 *pol* region RNA. In those earlier studies, the technology was limited by the background signal, which corresponded to about 100 probe molecules which carry through the assay. Additional cycles of capture and release did not reduce this level of noise. It is precisely this type of background which is eliminated by the dual-capture format. However, an additional cycle of capture and release is still required to eliminate all detector probes bound nonspecifically to the solid support (9).

In the dual-capture format, noise has been reduced by more than 11 log units. This conclusion is based on the frequency with which 6.5×10^{10} molecules of input detector probe are replicated by Q-Beta replicase in replicate samples containing *C. psittaci* rRNA or no targets. From the data presented in Fig.

5 and 6, the false-positive rates from 33 negative controls was 15.2% (5 of 33). By Poisson distribution, in which the probability of a negative reaction is 84.8%, the mean number of MDV detector molecules per sample remaining at the end in the assay is 0.16. This noise reduction efficiency is a conservative estimate and is not solely based on statistical predictions but is made with the understanding of the limitations of performing an amplification assay in an open system. It is understood and documented that many of these ordinary manual manipulations such as pipetting and aspiration in the presence of high initial concentrations of reporter probe molecules create aerosols which can cross-contaminate the samples. Our unpublished observations as well as similar technical hurdles faced by PCR amplification point to the limitations of performing amplification in an open system. When the final elution reaction mixtures of false-positive samples are diluted 10-fold, no signal is observed in the presence of Q-Beta replicase, indicating that the levels of the MDV probe are at the level of a single molecule. The solution to this problem lies in the present efforts aimed at developing a fully automated closed system. In the meantime, precautions are taken when practicing the open manual assay. Reagents are manufactured in isolated facilities and are brought to the laboratory in sealed single-use packages. The laboratory is segregated into areas of high and low MDV levels in which laminar flow hoods are used during the latter stages of the assay. Liquid and solid wastes are discarded every day, and physical and chemical cleaning of working surfaces is regularly practiced.

A new amplified nucleic acid hybridization assay format, termed dual capture, has been developed. The assay uses two powerful and complementing technologies: Q-Beta amplification and dual capture-RTC. The assay can detect RNA or DNA targets by using the same assay chemistry. Competitor signal from *C. psittaci* was not detected by using RNA equivalent to 10^8 elementary bodies. The sensitivity of this amplified assay is 10^3 16S rRNA or rDNA molecules, which corresponds approximately to one chlamydial elementary body. The assay's high degree of sensitivity is afforded by the C29 detector molecule, which in the described format can be detected at the level of a single molecule.

In a limited study of 94 patient samples, the dual-capture assay detected 5 of 6 culture positive samples. When the signals from these five positive samples were compared with those from parallel rRNA controls, these samples contained *C. trachomatis* rRNAs which were consistent with levels of between 10^4 and 10^6 rRNA molecules per reaction mixture. As for the one false-negative sample, we attribute this difference to the sixfold difference in sample utilization between culture and the assay and not the inhibition of amplification. Our studies have shown that the signal is not affected by the presence of inhibitors in clinical matrices such as cervical mucus and blood (unpublished data). Additionally, PCR analysis of this sample was also negative (1). The assay did not detect *C. trachomatis* target in 85 of 88 culture-negative samples. It is well known that some serovars of *C. trachomatis* are much less infective than others. Comparison of the response times in these three culture-negative samples with those of parallel rRNA controls indicated rRNA levels of between 10^4 and 10^5 molecules per reaction mixture. The presence of *C. trachomatis* nucleic acid in these three samples was confirmed by PCR analysis (1).

The sensitivity of the assay described here should be sufficient for the detection of infectious organisms such as bacteria and fungi in clinical specimens. The dual-capture assay format is equally sensitive when the probe sets are modified to detect other organisms, such as *Mycobacterium tuberculosis*, and cytomegalovirus (unpublished data). For many of these pathogens, positive identification can take weeks and is often difficult and expensive. The successful development of the dual-capture assay technology is directed toward addressing these needs, and the dual-capture assay offers a rapid and sensitive alternative to traditional identification methodologies.

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