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Detection of *Cryptosporidium parvum* Using Oligonucleotide-Tagged Liposomes in a Competitive Assay Format

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To meet the technical challenge of accurately and rapidly detecting *Cryptosporidium parvum* oocysts in environmental water, the authors developed a single-use visual-strip assay. The first step in the overall assay procedure involves extracting *C. parvum*'s mRNA coding for heat-shock protein hsp70, followed by amplification using nucleic acid sequence-based amplification (NASBA) methodology as described previously (Baeumner, A. J.; Humiston, M.; Montagna, R. A.; Durst, R. A. *Anal. Chem.*, in press). Subsequently, generated amplicons are hybridized with dye-entrapping liposomes bearing DNA oligonucleotides (reporter probes) and biotin on their surface. The liposome−amplicon complex is then allowed to migrate upward on a nitrocellulose membrane strip. On the nitrocellulose strip, antisense-reporter probes are immobilized in a capture zone and antibiotin antibodies are immobilized in a second zone above the capture zone. Depending on the presence or absence of amplicon in the sample, the liposomes will bind to the capture zone, or they will be caught via their biotin tag in the second zone. Visual detection or gray-scale densitometry allows the quantification of liposomes that are present in either zone. The detection limit of the assay was determined to be 80 fmol amplicon/test. High accuracy and an internal assay control is established using this competitive format, because the presence or absence of liposomes can be quantified in the two capture zones.

No other species of *Cryptosporidium* has a more significant impact on the health of human beings than *C. parvum*.[2,3] Infections by this coccidian parasite can lead to life-threatening conditions in individuals with impaired immune systems, such as patients with acquired immune deficiency syndrome (AIDS).[4] It also causes acute gastrointestinal symptoms in healthy people. Between 1984 and 1996, massive outbreaks in the U.S.A., U.K., and Japan were provoked by the waterborne route of transmission of *C. parvum*.[5] Because standard disinfection procedures such as chlorination cannot effectively inactivate *C. parvum*'s oocysts,[6,7] it is important to develop sensitive tests for detecting *C. parvum* in drinking water.

A successful detection scheme requires collecting and concentrating the oocysts from environmental water samples, separating the oocysts from contaminating debris, and finally, detecting them. Our paper focuses on detecting viable *C. parvum* oocysts after having concentrated them. A procedure for rapidly and accurately detecting oocyst viability would enable researchers to assess (1) the risk posed by the detected oocysts and (2) the effectiveness of newly developed disinfection procedures.

The standard procedure for detecting *C. parvum* uses fluorescently labeled antibodies that stain the oocysts, which can thereupon be identified microscopically.[8] However, a study by Moore et al. demonstrated that some carbohydrate epitopes at the oocyst wall are labile after chlorine treatment and under oxidizing conditions similar to those used to eliminate bacteria found in drinking water.[9] Therefore, although the oocysts would still be infectious, they would not be detected by the use of antibodies toward these epitopes. A further drawback of detection using epifluorescence microscopy is that commercially available antibodies cross-react with organisms other than *C. parvum*.[10] Finally, the standard procedure does not permit researchers to determine the viability of oocysts.

Various detection methods that overcome the difficulties encountered with epifluorescence microscopy have been reported.[11−17] Slifko et al. developed a detection scheme that focuses on determining oocyst viability by specifically identifying

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(1) Baeumner, A. J.; Humiston, M.; Montagna, R. A.; Durst, R. A. *Anal. Chem.*, in press.

(2) Current, W. L. *Am. Soc. Microbiol. News* **1988**, *54*, 605−611.

(3) Current, W. L.; Garcia, L. S. *Clin. Microbiol. Rev.* **1991**, *4*, 325−358.

(4) Cook, G. C. *Q. J. Med.* **1987**, *65*, 967−983.

(5) Smith, H. V.; Rose, J. B. *Parasitol. Today* **1998**, *14*, 14−22.

(6) Campbell, I.; Tzipori, A. S.; Hutchinson, G.; Angus, K. W. *Vet. Rec.* **1982**, *111*, 414−415.

(7) Korich, D. G.; Mead, J. R.; Madore, M. S.; Sinclair, M. A.; Sterling, C. R. *Appl. Environ. Microbiol.* **1990**, *56*, 1423−1428.

(8) Fricker, C. R.; Crabb, J. H. *Adv. Parasitol.* **1998**, *40*, 241−278.

(9) Moore, A. G.; Vesey, G.; Champion, A.; Scandizzo, P.; Deere, D.; Veal, D.; Williams, K. L. *Int. J. Parasitol.* **1998**, *28* (8), 1205−1212.

(10) Graczyk, T. K.; Cranfield, M. R.; Fayer, R. *Am. J.Trop. Med. Hyg.* **1996**, *54*, 274−279.

(11) Slifko, T. R.; Friedman, D.; Rose, J. B.; Jakubowski, W. *Appl. Environ. Microbiol.* **1997**, *63* (9), 3669−3675.

(12) Slifko, T. R.; Friedman, D. E.; Rose, J. B.; Upton, S. J.; Jakubowski, W. *Water Sci. Technol.* **1997**, *35* (11−12), 363−368.

(13) Wagner-Wiening, Ch.; Kimmig, P. *Appl. Environ. Microbiol.* **1995**, *61* (12), 4514−4516.

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the reproductive stages of *C. parvum*.[11,12] Host cells are first infected with *C. parvum*; after 24 to 48 h, infective foci in the cell culture are identified intracellularly by labeling them with antibodies specific to the reproductive stages of the parasite. The method is very sensitive, but culturing the cells is time-consuming. Further, the method still requires the use of microscopy.

Other approaches rely on detecting DNA or RNA specific to *C. parvum*.[13−16] The methods that detect RNA instead of DNA offer two advantages. First, because of the rapid turnover and postmortem decay of cellular RNA, the presence of certain RNA molecules has been correlated with the viability of oocysts.[16] Second, in viable organisms, there are many more copies of RNA than of DNA, and therefore, the sensitivity of detection is increased. Vesey et al. developed a technique for detecting rRNA by in situ hybridization, using fluorescently labeled oligonucleotide probes.[17] In combination with immunofluorescence staining, the method enabled species-specific detection and assessment of oocyst viability that correlated with in vitro excystation. However, the method encounters problems when detection is attempted in environmental water concentrates that contain autofluorescent algae and mineral particles.

Other researchers demonstrated the successful use of polymerase chain reaction (PCR) protocols for amplifying species-specific gene fragments, thus enabling subsequent detection.[13,18] The method using reverse-transcription PCR (RT-PCR) in combination with gel electrophoresis was shown to detect one viable oocyst spiked into concentrates from creek- and river-water samples.[15]

Nucleic acid sequence-based amplification (NASBA) technique was previously utilized to amplify mRNA coding for the heat-shock protein (hsp70) produced by *C. parvum*.[1] NASBA is a continuous, isothermal process that had been employed for amplification of single-stranded RNA by others.[19−22] This amplification technique has an advantage over other RNA amplification techniques (e.g. RT-PCR), because it does not require thermal cycling and, therefore, no special equipment is needed.

In this study, the authors extend the earlier study and present a test-strip assay for the detection of amplicons produced by NASBA from *C. parvum* mRNA. The detection is based on a competitive binding assay and signal generation by liposomes. Dye-containing liposomes are tagged with biotin and oligonucleotides (reporter probes). These probes are complementary to a specific region in the amplicon sequence and to a synthetic oligonucleotide sequence (antisense-reporter probe) immobilized on a nitrocellulose membrane strip. In the first step, *C. parvum* amplicon is mixed with the probe-tagged liposomes. Thus, if the target sequence (amplicon) is present, liposomes will bind via the reporter probes to the target. Subsequently, the mixture is allowed to migrate along the membrane strip. If no target sequence is present, liposomes will bind (via the reporter probe) to the antisense-reporter probe immobilized in the first capture zone on the strip. However, if amplicon is present in the sample, the reporter probes on the liposomes will have bound to the amplicon prior to entering the strip and ,therefore, will not bind in the capture zone. The biotin that the liposomes also contain on their surfaces enables the binding of these liposomes to the second zone in which antibiotin antibodies are immobilized (Figure 1). Gray-scale densitometry is employed to quantify the amount of liposomes present in either zone.

The described assay format allows quantifying the presence (or absence) of liposomes in two zones (capture zone and antibiotin antibody zone); therefore, the results obtained from this assay are very accurate, and additionally, an internal assay control is established.

EXPERIMENTAL SECTION

Reagents. Common laboratory reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), Boehringer Mannheim (Indianapolis, IN), or Fisher Scientific (Pittsburgh, PA). Ficoll type 400, poly(vinylpyrrolidone) (PVP, 10 000 Da), and cholesterol were obtained from Sigma Chemical Co. Plastic-backed nitrocellulose sheets, 32 × 32 cm with 8-μm pore size, came from Sartorius Co. (Goettingen, Germany). Sulforhodamine B dye and N-(4-(*p*-maleimidylmethyl)-cyclohexane-1-carbonyl)-dipalmitoylphosphatidylethanolamine (MMCC−DPPE) and dipalmitoylphosphatidylethanolamine conjugated with biotin (DPPE−biotin) were obtained from Molecular Probes (Eugene, OR). The lipids dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Succinimidylacetylthioacetate (SATA) was purchased from Pierce (Rockford, IL). Nonfat dry milk (NFDM) was acquired locally (Geneva, NY). Polycarbonate syringe filters of 0.2-, 0.4-, and 3.0-μm pore size were purchased from Poretics (Livermore, CA). Antibiotin and streptavidin came from Rockland (Gilbertsville, PA). Test kits for nucleic acid sequence-based amplification (NASBA) were provided by Organon Teknika (Boxtel, The Netherlands).

Oligomers. Because DNA is more stable than the NASBA-generated amplicon RNA, experiments for optimizing the test were conducted using synthetic target DNA that has the same sequence as the amplicon. The 103-mer synthetic target DNA sequence (5′-aga agg acc agc atc ctt gag tac ttt ctc aac tgg agc taa agt tgc acg gaa gta atc agc gca gag ttc ttc gaa tct agc tct act gat ggc aac tga a-3′) and all of the other oligonucleotides used for this study were synthesized by the BioResource Center, Cornell University (Ithaca, NY). The reporter probe, a DNA 20-mer (5′-gtg caa ctt tag ctc cag tt-3′), complementary to a part of the amplicon, was modified using a C3 aminolinker at the 3′ end. An antisense sequence to the reporter probe was biotinylated at the 5′ end and used as the capture probe.

Encapsulant Preparation. A 150 mM sulforhodamine B solution was prepared in 0.02 M tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 7.4) containing a volume fraction of 0.01%

(14) Stinear, T.; Matusan, A.; Hines, K.; Sandery, M. *Appl. Environ. Microbiol.* **1996**, *62* (9), 3385−3390.
(15) Kaucner, Ch.; Stinear, T. *Appl. Environ. Microbiol.* **1998**, *64* (5), 1743−1749.
(16) Widmer, G.; Orbacz, E. A.; Tzipori, S. *Appl. Environ. Microbiol.* **1999**, *65* (4), 1584−1588.
(17) Vesey, G.; Ashbolt, N.; Fricker, E. J.; Deere, D.; Williams, K. L.; Veal, D. A.; Dorsch, M. *J. Appl. Microbiol.* **1998**, *85* (3), 429−440.
(18) Johnson, D. W.; Pieniazek, N. J.; Griffin, D. W.; Misener, L.; Rose, J. B. *Appl. Environ. Microbiol.* **1995**, *61* (11), 3849−3855.
(19) Kievits, T.; van Gemen, B.; van Strijp, D.; Schukkink, R.; Dircks, M.; Adriaanse, L.; Sooknanan, R.; Lens, P. J. *Virol. Methods* **1991**, *35*, 273−286.
(20) van Gemen, B.; van Beuningen, R.; Nabbe, A.; van Strijp, D.; Jurriaans, P.; Lens, P.; Kievits, T. *J. Virol. Methods* **1994**, *49*, 157−168.
(21) Malek, L.; Sooknanan, R.; Compton, J. *Methods Mol. Biol.* **1994**, *28*, 253−260.
(22) Heim, A.; Grumbach, I. A.; Zeuke, S.; Top, B. *Nucleic Acid Res.* **1998**, *26*, 2250−2251.

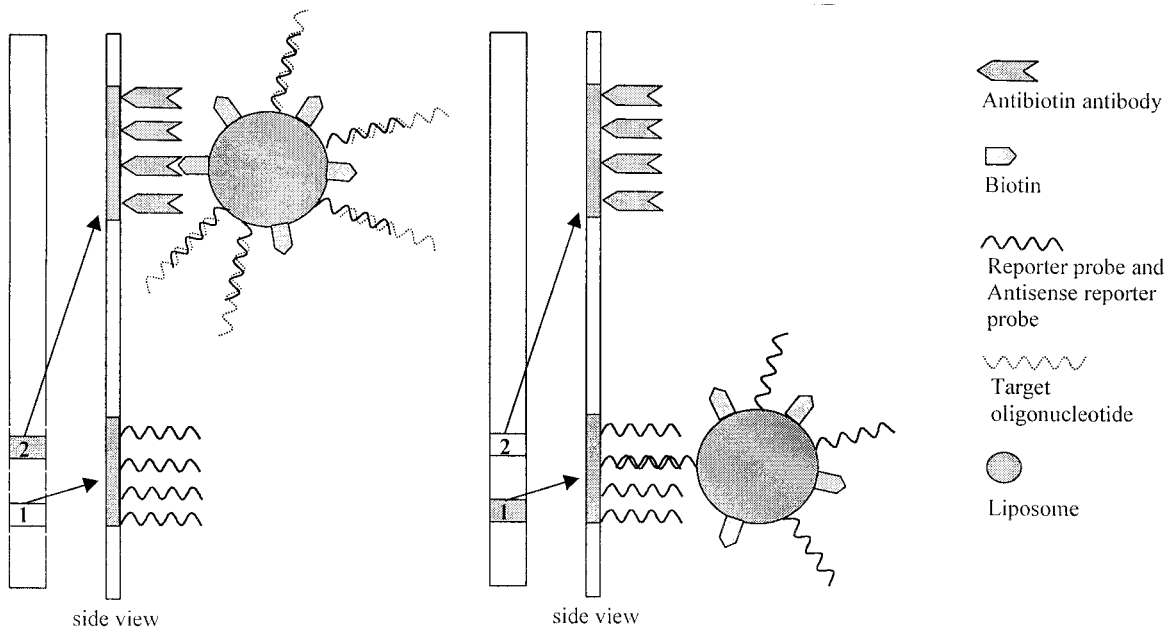

Figure 1. Competitive assay format on a test strip. The test strip has two zones: (1) an oligonucleotide zone consisting of immobilized probe that is complementary to the reporter probe, and (2) an antibiotin antibody zone. If the target RNA is present at the hybridization step, the liposomes bind to the antibiotin antibody zone. Otherwise, the liposomes bind to the oligonucleotide zone.

sodium azide. The final osmolality was 376 mOsmol/kg. To keep the liposomes intact, all of the other buffers used for liposome preparation were prepared with an osmolality of up to 50 to 100 mOsmol/kg higher than the encapsulant osmolality. Sucrose was used to adjust osmolalities.

Liposome Preparation. Liposomes were prepared using a modified version of the reverse-phase evaporation method described by Siebert and coauthors.[23] DPPC, DPPG, and cholesterol were dissolved in 8 mL of a solvent mixture consisting of chloroform, isopropyl ether, and methanol in a volume ratio of 6:6:1. DPPE−MMCC and DPPE−biotin were initially dissolved in a chloroform/methanol solution (volume ratio, 4:1), and an aliquot was added to the first lipid solution so that the final solution contained mole fractions of 3% and 0.2% of DPPE−MMCC and DPPE−biotin, respectively. While sonicating the suspension under a low stream of nitrogen at 45 °C, 2 mL of encapsulant was added. Using a vacuum rotary evaporator, the organic solvent was removed. The last two steps were repeated once. After the liposomes were formed, they were left for 10 min at 45 °C and, finally, forced twice through each of the 3.0-, 0.4-, and 0.2-μm pore-size polycarbonate syringe filters in series. Unencapsulated dye was separated from the liposomes by size-exclusion chromatography using Sephadex G-50-150.

When encapsulated in high concentrations, the fluorescence of sulforhodamine B undergoes self-quenching. Therefore, the encapsulation efficiency was determined by measuring the fluorescence intensity of liposome solutions before and after lysis at a wavelength of 596 nm (the excitation wavelength was 543 nm). Lysis was caused by adding 170 μL of 930 mM *n*-octyl-D-glucopyranoside to 3 mL of the liposome solution.

Conjugating Reporter Probe to Liposomes. The C3 amino-linker-modified reporter probe [105 mmol of reporter probe dissolved in 0.05 M phosphate buffer, pH 7.8, containing 1 mM ethylenediaminetetraacetate (EDTA)] was derivatized with an acetylthioacetate group by incubation with 315 mmol of freshly prepared succinimidylacetylthioacetate (SATA) dissolved in DMSO. After 90 min, the reaction was stopped using 41 μmol of Tris−HCl (0.5 M stock solution). Hydroxylamine hydrochloride was used to deacetylate the acetylthioacetate-reporter probe to yield the reactive sulfhydryl group. The reaction was allowed to proceed for 90 min, and the pH was brought down to 7.0 by adding 0.5 M KH_2PO_4. For conjugation, the maleimide-tagged liposomes were reacted with SH-reporter probe for 4 h at room temperature and then overnight at 4 °C. All unconjugated maleimide groups were capped with cysteine solution isotonic to the encapsulant. The liposomes were then purified on a Sepharose CL-4B column. The liposomes were kept in the dark at 4 °C in Tris−Cl buffer having an osmolality 100 mOsmol/kg higher than the osmolality of the encapsulant. These storage conditions prevent leakage of the dye from the liposomes.

Preparation of Test Strips. Nitrocellulose membranes were wetted for 20 min in 0.01 M K_2HPO_4/KH_2PO_4 buffer, pH 7.0, containing 0.15 M NaCl and a volume fraction of 10% methanol. They were then dried for 45 min under vacuum (15 psi) at 40 °C. The biotinylated antisense-reporter probe was incubated with streptavidin in a 4:1 molar ratio for 15 min. Using a thin-layer chromatography plate applicator (Camag Scientific Inc.; Wrightsville Beach, NC), the mixture was applied to the membrane 15 mm above the bottom edge. At 5 mm from the first band, a band of antibiotin was applied. The oligonucleotide was immobilized by baking at 55 °C for 60 min under vacuum (15 psi).

The coated nitrocellulose sheet was then immersed in the blocking agent [0.02 M Tris−HCl buffer, 0.15 M NaCl, pH 7.00, containing volume fractions of 5% PVP and 0.07% nonfat dry milk for 30 min and dried for 2 h at room temperature under vacuum. The membranes were stored in vacuum-sealed plastic

(23) Siebert, S. T. A.; Reeves, S. G.; Durst, R. A. *Anal. Chim. Acta* **1993**, *282*, 297−305.

Table 1. Optimizing the Experimental Conditions[a]

	SSC concn				incubation time, min					formamide volume fraction, %				temperature, °C					
	2×	3×	4×	5×	0	5	10	15	20	15	20	25	30	25	30	35	40	45	50
slope	−133	−173	−221	−174	−89	−149	−175	−232	−315	−173	−183	−88	−11	−42	−95	−98	−252	−180	−208

[a] Dose−response curves were generated from duplicate strips at target concentrations of 0, 100, and 400 fmol/test. The gray-scale density was measured in the oligonucleotide zone and plotted against the target concentration. By fitting the plots linearly, we obtained slopes that indicate which assay conditions increase the sensitivity of the assay (because the gray-scale density in the oligonucleotide zone is highest for control samples that do not contain analyte and decreases with increasing analyte concentration, more negative slopes indicate a higher sensitivity).

bags at 4 °C and were cut into strips immediately prior to use. Each membrane was cut into 4.5 mm × 8 cm strips, so that, because of the way the upper and lower bands were applied, each strip contained 10 pmol of streptavidin and 40 pmol of antisense-reporter probe in the first (oligonucleotide) zone, and 80 pmol of antibiotin in the second (antibiotin) zone.

The prepared test strips are stored in vacuum-sealed bags at 4 °C. If the test strips were stored under these conditions, the authors were able to use them for up to 6 months after preparation.

Assay Protocol. The hybridization was carried out in a 60-μL reaction mixture containing either synthetic target DNA or NASBA-generated amplicons and varying concentrations of formamide, standard sodium citrate (SSC) solution (a 20× SSC standard solution contains 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0), Ficoll type 400, and sucrose. After a 20-min incubation at 40 °C, the test strip was placed into the test tube. It should be noted that the liposomes bind in either of the two zones under nonequilibrium, but steady-state, conditions; therefore, the test strips were removed from the test solutions as soon as the liquid front reached the top edge of the strip.

Detection and Quantification. Gray-scale densitometry was performed using a scanner (Epson) and scan analysis software (Biosoft; Cambridge, U.K.). The red coloration is converted to gray-scale density, which can be quantified.

Nucleic Acid Sequence-Based Amplification (NASBA). The Extraction of nucleic acids from *C. parvum* and the amplification of the mRNA coding for the heat shock protein (hsp70) by NASBA was conducted as described previously.[1] In short, the mRNA production in oocysts was stimulated by heating the oocysts for 20 min at 42 °C. The oocysts' membranes were disrupted by incubation in lysis buffer (provided in Qiagen RNeasy kit and Organon Teknika Boom extraction kit). NASBA reactions were performed on either hsp70 mRNA isolated from *C. parvum* oocysts, H_2O (negative control), or mRNA from other microorganisms (for specificity testing) using the Organon Teknika NASBA kit.

RESULTS AND DISCUSSION

Characterization of Liposomes. For this study, sulforhodamine B (SRB) was incorporated into liposomes. Because the fluorescence of the dye is quenched when encapsulated in high concentrations, nonentrapped dye can be determined in a spectrofluorometer. Hence, intact liposome solutions show little fluorescence in contrast to lysed liposomes (free SRB in solution). An encapsulation efficiency of 3.3% was calculated after liposome preparation, assuming that the concentration of encapsulated SRB equaled the initial concentration of 150 mM.

Optimizing the Experimental Conditions. The detection of the *C. parvum*-specific sequence begins by hybridizing it to the reporter probes on the liposomes. The second hybridization takes place in the capture zone on the test strip. To avoid false positive results, the hybridization should be as specific as possible; therefore, the components of the hybridization mixture need to be optimized with respect to hybridization stringency and also liposome stability.

To determine the optimum stringency conditions, varying concentrations of standard sodium citrate (SSC) buffer and formamide were investigated. On the basis of these data, we then optimized temperature and incubation time. Results were generated by testing duplicate strips using concentrations of synthetic target DNA of 0, 100, and 400 fmol/test. The gray-scale density in the oligonucleotide zone was plotted against the target concentration, and the data were fitted linearly. Table 1 summarizes the slopes of the generated curves. The slopes of the curves provide adequate information for comparing the sensitivity of the test under varying test conditions. In this context, a more negatively sloped curve indicates a higher sensitivity.

The SSC concentration was optimized first. To provide an acceptable level of stringency, the experiment was initially conducted using a hybridization medium containing a volume fraction of 15% formamide and 15 min incubation at 40 °C. Although salt concentrations of 2× SSC in the hybridization solution are sufficient to provide osmotic conditions in which liposomes do not lose their integrity (at lower salt concentrations, the liposomes lose part of the entrapped dye) the optimum SSC concentration was found to be 4× SSC. SSC concentrations higher than 4× SSC led to increasing slopes (less negative slopes), indicating a lower sensitivity. The formamide concentration was optimized next. It is the second most important component, because it provides stringency but produces lysis of liposomes at higher concentrations. When used in hybridization mixtures containing 4× SSC, with a 15-min incubation time at 40 °C, increasing formamide concentrations up to a volume fraction of 20% in the test solution resulted in increased sensitivity. In the absence of formamide, hybridization at all of the target concentrations was very low. At volume fractions higher than 20%, the signal decreased. This was due to either liposome lysis (and thus, fewer liposomes available for signal generation) or too stringent conditions (and thus, fewer liposomes binding in the first capture zone). For the determination of the optimum assay temperature, the liposome-target mixtures were incubated at temperatures ranging from 25 to 50 °C. A temperature of 40 °C was found to be optimum (using 4× SSC and 20% formamide). In addition, it was observed that longer incubation times resulted in higher sensitivity. The longest incubation time investigated was 20 min. Because we wanted to keep testing times as short as possible, longer incubation times were not investigated. Additionally, using a

volume fraction of 0.2% Ficoll 400 and 0.125 M sucrose in the incubation mixture improved the sensitivity of the test further.

The hybridization steps are influenced by the underlying hybridization kinetics. Wolf et al. found that hybridization rates for DNA attached to latex particles approach those of DNA in solution.[24] Because we use reporter probes that are end-linked to liposomes that are similar in size to the particles used by Wolf et al., solution hybridization kinetics should apply to the first hybridization step. Second, according to Reinhartz et al., who developed a paper chromatography hybridization assay (PACHA), the hybridization on the nitrocellulose strip is controlled by the flow velocity and the volume of sample migrating across the oligonucleotide zone.[25] In our experiments, sample volume and flow velocity were held constant.

The assay format resembles a conventional competitive assay. Therefore, the tag density on the liposome surface and the amount of immobilized probes on the membrane were optimized in order to achieve maximum sensitivity. Initially, different amounts of reporter probe tags on the liposomes were investigated using liposomes with 0.1, 0.2, and 0.4% (mole fractions) tag. Tests with 0.4% tagged liposomes showed the highest resolution for analyte amounts between 0.1 pmol/test and 1 pmol/test; however, the highest sensitivity in the range between 0 pmol/test and 0.1 pmol/test and better reproducibility were achieved with 0.1% (mole fraction) tagged liposomes.

In initial experiments, direct immobilization of the antisense-reporter probe on the nitrocellulose strip was carried out. As a result of possible redissolving of probes during membrane preparation and assay incubation steps, irreproducible results were obtained (data not shown). Thus, the principle of probe immobilization via biotin−streptavidin, as shown earlier, was used.[26] The combination of 10 pmol of streptavidin and 40 pmol of biotinylated capture probe per strip gave the highest sensitivity. It was important to avoid biotinylated liposomes' binding to streptavidin in this capture zone. Thus, a high ratio of streptavidin and biotinylated probe (1:4, molar ratio) was used. Smaller and larger amounts of immobilized probe lowered the signal intensity in the oligonucleotide zone as well as the sensitivity of the test. Finally, the optimum amount of antibiotin antibody immobilized in the antibiotin zone was determined to be 80 pmol/strip.

Detection Limit. The detection limit was determined using the optimized assay described above. Amounts of synthetic target DNA ranging from 0 to 2.9 pmol/assay were tested. Dose−response curves at the oligonucleotide and antibiotin zones were obtained by gray-scale densitometry and are shown in Figures 2 and 3. Both zones provide a region in which the exact amount of target can be determined (80 fmol/test to 2.9 pmol/test). The detection limit (defined as the smallest concentration of analyte that can be statistically distinguished with 99% confidence from the measurement value of the blank control) is, under optimum conditions, 80 fmol/test at both zones. Control experiments with liposomes that lack the reporter-probe tag showed no observable binding at the oligonucleotide zone but strong binding at the antibiotin zone. The same result was obtained if the test strip was not coated with probe in the oligonucleotide zone.

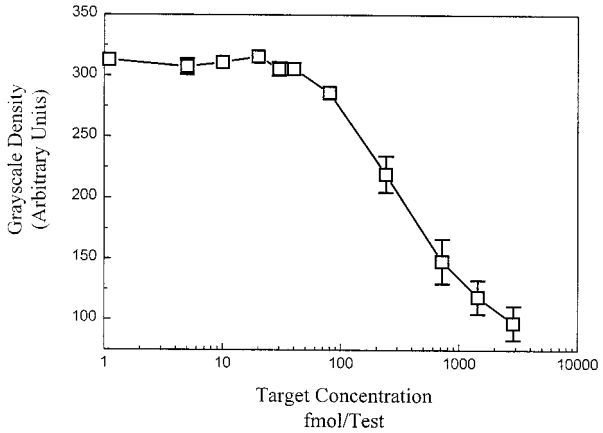

Figure 2. Dose−response curve for the synthetic target, measured at the oligonucleotide zone. Each point represents the mean of three measurements. Error bars represent ±1 standard deviation.

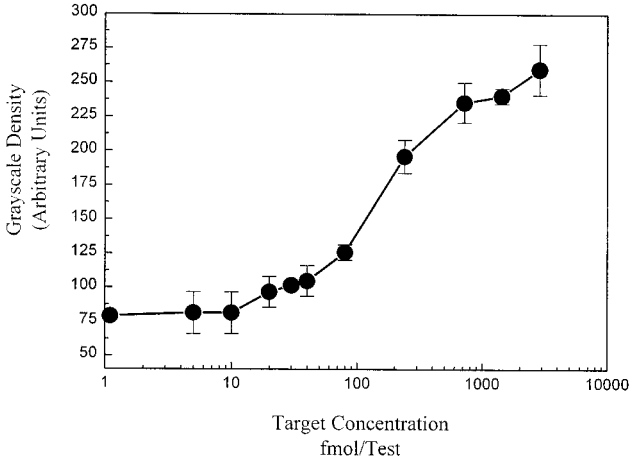

Figure 3. Dose−response curve for the synthetic target, measured at the antibiotin antibody zone. Each point represents the mean of three measurements. Error bars represent ±1 standard deviation.

The dose−response curves demonstrate that the test-strip assay is very sensitive. This high sensitivity is achieved for two reasons. First, the migration format brings the probes into close proximity with each other, thereby limiting the diffusional distances, that is, minimal bulk diffusion of the reacting species. Second, the use of liposomes as signal-reporter particles has advantages over the use of conventional reporter particles such as latex, gold, or a single fluorophore. Large numbers of dye molecules can be incorporated into the liposomes, thereby increasing the measured intensity of the signal. By using liposome-tagged analytes in a competitive flow injection immunoassay, Lee et al. demonstrated a 1000-fold increase in sensitivity when compared with a single fluorophore-tagged analyte.[27] Furthermore, the two-dimensional fluidic nature of the liposome membrane allows the probes on the liposome surface to diffuse within the membrane.[28] After one event of binding to an immobilized probe occurs, additional probes on the same liposome move into positions where additional binding can take place (e.g., biotin to

(24) Wolf, S. F.; Haines, L.; Fisch, J.; Kremsky, N.; Dougherty, J. P.; Jacobs, K. *Nucleic Acids Res.* **1987**, *15*, 2911−2926.
(25) Reinhartz, A.; Alajem, S.; Samson, A.; Herzberg, M. *Gene* **1993**, *136*, 221−226.
(26) Baeumner, A. J.; Siebert, S. T. A.; Montagna, R. A.; Durst, R. A. *Biosens. Bioelec.*, In preparation.
(27) Lee, M.; Durst, R. A.; Wong, R. B. *Anal. Chim. Acta* **1997**, *354*, 23−28.
(28) Laidler, K. J.; Meiser, J. H. *Physical Chemistry*; Benjamin/Cummings Publishing: Reading, MA, 1982; pp 826.

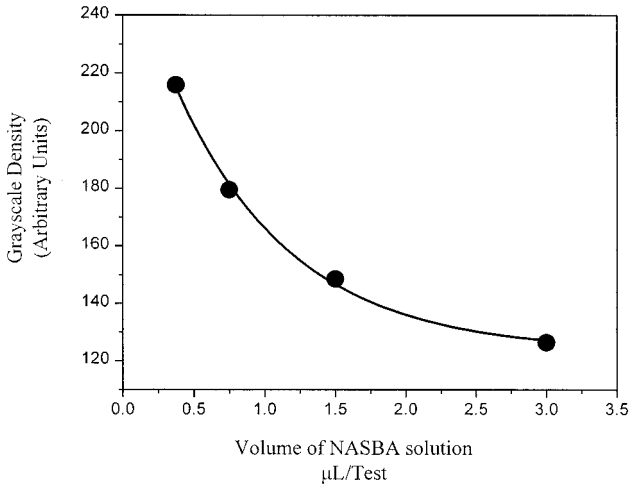

Figure 4. Testing different volumes of RNA amplified by NASBA. Water samples were spiked with 10 *C. parvum* oocysts. The RNA was extracted from the oocysts, and the amplicons are subsequently generated by amplification with NASBA methodology. The compatibility of the test strip assay and NASBA is tested by conducting the assay using different volumes of the NASBA solution containing the generated amplicon. The gray-scale density was measured in the oligonucleotide zone and plotted against the volume of NASBA solution.

antibiotin antibodies). Liposomes may also become deformed to bind to the immobilized oligonucleotide.

Although test strips using a sandwich assay provide higher sensitivities (~1 fmol/test, reported by Rule et al.[29]), the competitive assay format has advantages. It covers a wider range of detectable analyte concentrations, and it precludes the false negative results at high analyte concentration that can occur in sandwich assays due to overloading the capture and reporter probes, that is, the so-called "hook effect". In the test that Rule et al. reported, false negative results occurred at concentrations above 100 fmol/test. In addition, the second binding reaction of biotin to antibiotin antibodies can be used as an internal control. If no signal is obtained in either zone, it is clear that the assay was invalid and thus, no interpretation of data is possible.

Testing of Target RNA Generated by NASBA Methodology. The test strip is designed to detect amplicons generated by NASBA from extracts of *C. parvum*. To prove that NASBA-generated amplicons can be detected with the developed test strip, we conducted the following experiment. Water samples were spiked with 10 oocysts of *C. parvum*. The samples then underwent an extraction procedure so that the DNA and RNA was released from the oocysts. The mRNA coding for the heat-shock protein hsp70 was amplified by using primers developed by Baeumner et al..[1] After the NASBA reaction was completed, assays in which we tested volumes of this solution ranging from 0.375 to 3.0 μL per test were conducted. The measured oligonucleotide zone gray-scale density was plotted against the solution volume (Figure 4) and, by using the dose response curve, the gray-scale density was correlated to the amount of mRNA present in the sample. The measured gray-scale densities correlated to target concentrations that were in the dynamic range of the dose response curve. For example, in the specific case of 0.375 μL NASBA solution/test, the gray-scale density correlated to 204 fmol of synthetic target sequence. As measured in the antibiotin antibody zone, the estimated value of target was slightly higher. Any volumes of the NASBA mixture conducted with a negative control that did not contain oocysts gave signals that were always lower than the detection limit (in the oligonucleotide zone as well as in the antibiotin antibody zone).

Specificity of the Test. Signals lower than the detection limit (and, therefore, identified as negative samples) were obtained when tests were conducted using solutions of NASBA extracts of organisms other than *C. parvum* (*Giardia lamblia*, *Cyclospora*, *Listeria iranovii*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* culture no. a33, *E. coli* culture no. 43895), which was similar to results obtained earlier;[1] however, researchers should be aware of two recently published studies that report on *Cryptosporidium wrairi* and *Cryptosporidium meleagridis* having gene sequences that are identical (or to 98% matching) to the mRNA sequence utilized in this study.[30]

CONCLUSION

A test strip to be used for the rapid visual detection of amplified oligonucleotides has been developed. The assay was very sensitive and specifically detected RNA amplicons generated by NASBA from *C. parvum* hsp70 mRNA. Compatibility of the test strip assay with the NASBA amplification methodology was shown. The assay could be conducted in 30 min and is, therefore, much faster than oligonucleotide detection by Southern blotting or agarose gel staining. Oligonucleotides that are separated using agarose gels are commonly stained by chemicals such as ethidium bromide, which poses a potential health threat. Our method avoids using such chemicals. The method is suitable for large-scale screening and for batch processing analyses. Because the analyte concentration is directly related to the color intensity in the antibiotin zone, the unequivocal test result can easily be read. Because of the relative simplicity of the method, nontechnical personnel can easily perform the procedure.

The main purpose of this research was to demonstrate the applicability of a competitive assay format (using liposome amplification) for the detection and quantification of nucleic acid sequences. Although the test was developed for *C. parvum*, the principles are transferable to any other organism by changing the oligonucleotides used as the reporter probe and as the antisense to the reporter probe.

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(29) Rule, S. R.; Montagna, R. A.; Durst, R. A. *Clin. Chem.* **1996**, *42* (8), 1206−1209.

(30) Sulaiman, I. M.; Morgan, U. M.; Thompson, R. C. A.; Lal, A. A.; Xiao, L. *Appl. Environ. Microbiol.* **2000**, *66*, 2385−2391.