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An exonuclease-amplification coupled capture technique improves detection of PCR product

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The polymerase chain reaction (PCR) assay is a powerful tool for diagnosis of infectious, genetic, and neoplastic diseases (1). However, standard methods of PCR product analysis-gel electrophoresis followed by ethidium bromide staining, and Southern blot hybridization to confirm product identity, remain tedious and time-consuming, preventing applied transfer of these procedures to clinical laboratories. Several solid-phase colorimetric assays that capture denatured PCR products on probes bound to nylon membranes (2, 3) or to microtiter plates (4, 5) offer improved convenience, but may lack optimal sensitivity due to the tendency of the denatured PCR product strands to reassociate and exclude oligonucleotide probes, and steric interference from the solid support that impedes hybridization. In some cases, colorimetric detection is improved by creating single-strand PCR products through asymmetric PCR that can associate with bound probes without interference (6). Unfortunately, asymmetric PCR is notoriously difficult to reproduce, and does not lend itself to automation.

To circumvent these problems, we describe a simple, highly specific, nonradioactive microtiter plate format for detection of PCR product that offers improved sensitivity over standard detection methods, and illustrate the application of this technique for detection of human parvovirus B19 DNA. The detection format, referred to as the Exonuclease-Amplification Coupled Capture Technique (EXACCT), enhances specific hybridization detection by converting PCR-amplified DNA into single strands. Amplified PCR products are incubated with the T-7 gene 6 exonuclease. This non-processive 5′ exonuclease digests double-stranded DNA to single strands; digestion of each strand is expected to proceed at equivalent rates and, as the enzyme is inactive against single-stranded DNA, the reaction terminates at the approximate midpoint of the PCR molecule, leaving two single-strand DNAs of approximately half original product length (7,8). After digestion, the PCR product is hybridized to two different detection oligonucleotide probes complementary to sequences near the 3′ end of the antisense fragment. One probe bears a biotin residue, the other digoxigenin. After hybridization, the PCR product-probe hybrids are captured in streptavidin-coated microtiter wells and detected with labeled anti-digoxigenin antibody. Digestion of one strand of the PCR product eliminates competition with complementary DNA and increases sensitivity of detection. Only molecules hybridized to both probes are detected by this format, giving a very high degree of specificity. The exonuclease is also relatively inexpensive, and active in standard 1×PCR reaction buffer when supplemented with dithiotreitol (DTT).

We adapted EXACCT for detection of a 284 bp PCR amplification product of a conserved sequence of the parvovirus B19 nonstructural gene (NSI) using primers (P1, P6) and amplification protocol previously described (9). After PCR and exonuclease treatment, biotin- (5′ CCATGTACAGGAAAAA-CAAACTTGG 3′) and digoxigenin- (5′ GCTATTATGTGAC-AAAGTGTTCCAG 3′) labeled probes were simultaneously hybridized to the single-stranded DNA. The efficiency of exonuclease conversion to single strands was assessed by non-denaturing Southern blot (Figure 1, Panels 1 and 2) that measured the quantity of single-strand DNA per sample via hybridization to a digoxigenin-labeled probe. Even small amounts of enzyme (0.04 U/μl) effectively converted PCR products into a detectable form, whereas higher amounts (0.4 to 4 U/μl) completely digested PCR products to single strand fragments. PCR products were captured on streptavidin-coated microtiter plates (Figure 1, Panel 3) after exonuclease digestion (Lanes C–E) with far greater efficiency than heat-denatured PCR product (Lane A), or asymmetric PCR product (Lane B).

Exonuclease-treated PCR products were detected with two logs greater sensitivity than standard gel analysis with ethidium bromide staining, and more than a log greater sensitivity than Southern blot analysis (data not shown). The sensitivity of a single PCR amplification (30 cycles total) followed by EXACCT was comparable to two sequential PCRs (60 cycles total) using nested amplification primers, detecting as few as 3 to 30 B19 genome copies (9). Analysis of PCR products by EXACCT required considerably less time or effort than Southern blot (< 3 h versus > 2 days for Southern analysis) and was more specific, since two separate probes must hybridize for detection.

A potential problem with EXACCT that could limit its application for detection of small PCR products is the restricted target sequence available for binding probes after enzyme digestion. We have resolved this problem by introducing several phosphorothioate analogs onto the 5′ end of the reverse primer (P6) using tetraethylthiruam disulfide (TETD) reagent (Applied Biosystems) during primer synthesis (10). This procedure protects the antisense strand from nuclease digestion (11), leaving a full length single-stranded target for hybridization (data not shown). EXACCT has greatly enhanced our ability to make sensitive, specific, and convenient diagnosis of parvovirus B19 infection, and should be readily adaptable to other PCR detection systems.

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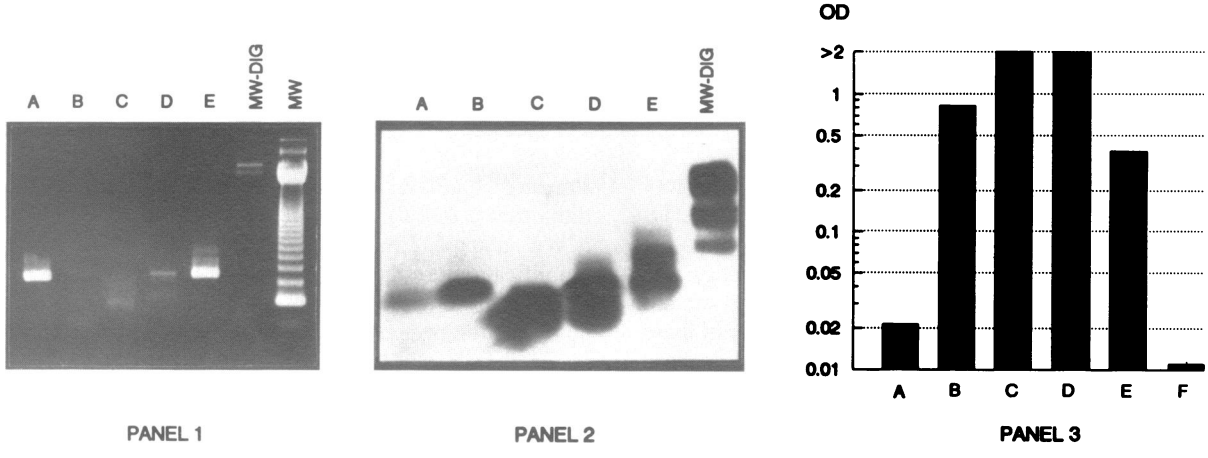

Figure 1. Conversion of PCR products to single strands and detection by EXACCT. Parvovirus B19 DNA was amplified using primers (P1, P6) and amplification conditions previously described (9). Three identical 10 μl reaction aliquots were supplemented with DTT to 1 mM and digested with different concentrations of T7 gene 6 exonuclease (United States Biochemical, Cleveland, OH) for 15 min at 37–C. The digested product was heated to 75–C for 15 min to denature the enzyme, and electrophoresed and transferred to a nylon membrane under nondenaturing conditions. **Panel 1**: Ethidium staining of the undigested 284 bp PCR product (Lane A) compared to product digested with 4 U/μl exonuclease (Lane C), 0.4 U/μl (Lane D), and 0.04 U/μl (Lane E). Lane B shows single-strand PCR product produced using reduced P1 and excess P6 in asymmetric PCR. MWM_{DIG} and MWM are 123 bp ladders with and without digoxigenin-labeling. **Panel 2**: Detection of single-strand PCR product via nondenaturing Southern analysis. The agarose gel shown in A was not exposed to alkali prior to transfer, so that only single-strand DNA would be efficiently detected by the digoxigenin-labeled oligonucleotide probe (see text for probe sequence). Undigested double-strand DNA (Lane A) is poorly detected compared to product treated with exonuclease (Lanes C–E) or amplified by asymmetric PCR (Lane B). **Panel 3**: Detection of PCR product by EXACCT in streptavidin-coated plates (Lanes C–E) compared to standard colorimetric detection of double-stranded PCR product (Lane A), and of single strand PCR product amplified by asymmetric PCR (Lane B). Twenty μl of digested PCR product was added to 200 μl of hybridization buffer (4×SSC, 20 mM Hepes, 2 mM EDTA, 0.15% $Tween_{20}$) containing 50 ng/ml each of biotin- and digoxigenin-labeled probes. Hybridization reactions were performed at 37°C for 1 h. Undigested double-stranded PCR product (Lane A) was heat-denatured at 95°C for 5 min prior to hybridization. Single-stranded PCR products produced by asymmetric PCR (Lane B) or by exonuclease digestion (Lanes C–E) were not heat-denatured. Lane F was an equal amount of control DNA amplified from an unrelated region of the B19 gene that was treated with exonuclease in parallel. After hybridization, 100 μl of each mixture were added to duplicate wells of a streptavidin coated Immulon II microtiter plate (Dynatech Laboratories, Chantilly, VA) prepared by coating with 200 ng of biotinylated BSA (Sigma Chemical Co., St Louis, MO) in 100 μl PBS/well overnight at 4°C, washed with PBS containing 0.15% $Tween_{20}$, and then saturated with 1000 ng of streptavidin (Sigma) in 100 μl of PBS with 0.5% gelatin/well for 30 min at room temperature with shaking. The plate was then washed and 100 μl/well of a 1:5000 dilution of peroxidase-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim, Indianapolis, IN) in hybridization buffer was added and incubated for 1 h at room temperature with shaking. After washing, 100 μl/well of a 3,3′,5,5′-tetramethyl-benzidine chromogen solution was added and incubated at room temperature. Color development was stopped after 15 min with 100 μl/well 2 M phosphoric acid and the plates read at 450 nm.