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Rare target enrichment for ultrasensitive PCR detection using cot–rehybridization and duplex-specific nuclease

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ABSTRACT

Nucleic acid detection by polymerase chain reaction (PCR) is invaluable for the detection of dilute and rare sequences, including pathogens and infrequent species in complex clinical and environmental backgrounds. The presence of excess complex background nucleic acid can reduce sensitivity and specificity. This is because mispriming can cause failure of the amplification reaction. Here we describe a new approach to ultrasensitive PCR detection, using enrichment of rare target nucleic acid from abundant background by combining the classic technique of cot–rehybridization to convert the abundant background to double-stranded form, with the use of a newly described, highly processive duplex-specific crab nuclease. We show that trace sequences in a vast excess of background DNA can be undetectable by PCR, independent of the amount of the mixture added to the PCR, and that these sequences can be made detectable by background suppression using this method.

Detection of rare nucleic acid sequences is essential to the discovery of single-copy genes or variants in large and complex genomes and to the detection of pathogens in clinical samples and low-abundance species in high-diversity metagenomic pools. Although whole-genome sequencing is an increasingly effective option, metagenomes can be very large and targets of interest can be sparse. For example, in agricultural soils the number of operational taxonomic units has been estimated to be as high as 3555 [1]. Estimates of abundance increase with sampling effort in high-diversity environments [2]. As a result, the low-abundance species may be masked by the high-abundance species [3]. In another domain, the U.S. Food and Drug Administration mandates that a single typical dose of 0.3 μg of a plasmid DNA vaccine must contain no more than 1 copy of a microbial genome for every 24,000 copies of a 5-kb plasmid [4]. Detection of low-abundance sequences against higher abundance backgrounds, therefore, is of widespread interest.

To allow the detection of rare sequences, target enrichment strategies such as hybridization capture on arrays [5–7] or in solution [8,9], as well as polymerase chain reaction (PCR)[2] amplification, have been applied. Recently, single-copy gene discovery and characterization of the highly repetitive genomes of plants have been achieved by integrating cloning and sequencing with selective removal of abundant repetitive sequences, taking advantage of the faster rehybridization of abundant sequences [10–12] based on classic methods first developed during the 1960s [13,14]. In so-called "cot analysis," the differential rates of reassociation of abundant and rare DNA sequences are used to characterize a sample's content of repetitive and unique sequences. The DNA of interest is fragmented to approximately 450 bp, heat-denatured, and then allowed to reassociate. More abundant sequences reassociate faster than rare sequences, and hydroxyapatite chromatography selectively removes the more highly renatured abundant double-stranded DNA (dsDNA), thereby enriching rare sequences. This method is particularly well-suited for the analysis of complex metagenomic samples in which both highly abundant and rare sequences may be present, for example, the genomes of common and rarer species.

As an alternative to hydroxyapatite chromatography, a recently described duplex-specific nuclease (DSN) isolated from the hepatopancreas of the Kamchatka crab (*Paralithodes camtschaticus*) is of interest because it specifically catalyzes the removal of dsDNA [15,16]. This enzyme is potentially very useful for the selective degradation of abundant species that reassociate more quickly to the nuclease-sensitive double-stranded form. Low-abundance sequences remain largely single-stranded and nuclease resistant and, thus, are enriched. Importantly, with its optimum

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[2] *Abbreviations used:* PCR, polymerase chain reaction; dsDNA, double-stranded DNA; DSN, duplex-specific nuclease; ssDNA, single-stranded DNA.

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activity at 65 °C, DSN is very compatible with cot–rehybridization, which usually is conducted at 60 to 65 °C to allow specific rehybridization.

This work describes the application of DSN removal of cot-rehybridized abundant species to enhance the PCR detection of rare target sequences present in a vastly more abundant background. We show that a rare sample target can be undetectable by PCR unless enriched in this manner. Simply increasing the amount of sample (and so template) added to the PCR does not solve the problem because the associated abundant background DNA poisons the PCR. Excess background DNA in PCR has also been referred to as "burden DNA," which can result in false priming or hindered diffusion of large polymerase and nucleic acid molecules [17]. This poisoning can be alleviated by dilution, but dilution sometimes brings the rare target concentration below the limit of PCR amplification. To allow sufficient enrichment of a rare target, nearly complete reassociation [13,14] of the background DNA is required to eliminate the poisoning effects of the abundant background.

Materials and methods

To demonstrate the improved detection of a rare target in an abundant DNA background using cot–rehybridization and selective elimination of renatured background DNA, we prepared a 410-bp fragment by amplification from a dengue virus complementary DNA (cDNA) clone. We used fragments of plasmid pUC18 to represent the abundant background and mixed the two species in various ratios as described below.

Preparation of abundant background sequence from pUC18 plasmid

Escherichia coli strain T1 harboring pUC18 plasmid (2686 bp) was cultured overnight at 37 °C in 2.5 L of Luria–Bertani broth. The plasmid was isolated using the Qiagen Plasmid Giga Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. A double restriction digestion with *Eco*RI and *Hin*dIII confirmed the plasmid's identity by the presence of characteristic 2630- and 56-bp fragments. Purified plasmid was fragmented to an average size of 450 to 500 bp (as determined by gel electrophoresis) by ultrasonication using an ultrasonic homogenizer (Biologics model 150V/T, Manassas, VA, USA) at 20% power output and 50% duty cycle for 6 min in an ice bath.

The melting temperature of the fragmented DNA was determined using SYBR Green I dsDNA-binding fluorescent dye in an Mx3005P Real Time PCR Thermocycler (Agilent Technologies, Santa Clara, CA, USA) and was observed to be 87.5 °C. Cot reassociation was carried out at 25 °C below the melting temperature [13].

Preparation of target sequence

A 410-bp sequence of a dengue virus (DENV-2 New Guinea C, M29095), cloned in the yeast–*E. coli* shuttle vector pRS424, was amplified using forward primer, 5′-cggattaagccatagtacg-3′ (the 5′ nucleotide hybridizes to nucleotide 10,311 of the virus genome), and reverse primer, 5′-aacctgttgattcaacagc-3′ [18]. The amplicon was purified using the QIAquick PCR Purification Kit (Qiagen) and was found to have a T_m of 84.4 °C.

Reassociation kinetics of background DNA fragments using hydroxyapatite chromatography

Background pUC18 DNA fragments were prepared at 100 μg/ml in 100 mM sodium phosphate buffer (pH 6.8), denatured at 100 °C for 5 min, and immediately transferred to a hybridization oven (Robbins Scientific Micro Hybridization Incubator, model 2000, San Diego, CA, USA) held at 62 °C. Reassociation was assayed at 30 s, 30 min, 3 h, and 24 h. Each of the following steps, including centrifugation and preheating, was performed at 62 °C:

1. *DNA sample application and binding:* A slurry of 0.46 g/ml Bio-Gel HTP Hydroxyapatite (Bio-Rad Laboratories, Hercules, CA, USA) was prepared in 100 mM sodium phosphate buffer (pH 6.8) in a 2 ml tube and preheated. For each time point, a 250-μl sample of reassociated DNA was applied to a new hydroxyapatite slurry tube and mixed end-over-end for 30 min.
2. *ssDNA elution:* The slurry was centrifuged at 3000 rpm (400*g*) for 3 min in a Spin-Fuge (Capsule HF120, TOMY Seiko, Tokyo, Japan). The supernatant was discarded, and the remaining hydroxyapatite was contacted with 0.25 ml of preheated 360 mM sodium phosphate. At the resulting final sodium phosphate concentration of 200 mM where the unreassociated single-stranded DNA (ssDNA) can be eluted, the slurry was mixed for 10 min and the supernatant was collected after centrifugation.
3. *Wash step 1:* The hydroxyapatite was washed with 0.5 ml of preheated 200 mM sodium phosphate buffer by mixing for 10 min, and any remaining ssDNA was collected after recentrifuging.
4. *dsDNA elution:* The dsDNA or reassociated DNA fraction was eluted by adding 0.5 ml of preheated 500 mM sodium phosphate (final concentration 350 mM) and mixing for 10 min, centrifuging, and collecting the supernatant.
5. *Wash step 2:* dsDNA elution was followed by a wash with 0.5 ml of 500 mM sodium phosphate for 10 min to collect any remaining dsDNA.
6. *Analysis:* The ssDNA and dsDNA fractions were quantified by absorbance at 260 nm, and the percentage of ssDNA was plotted against the cot value, to determine the rate of reassociation. The cot value is the product of the initial concentration (C_0 = 0.33 mM) of DNA in moles of nucleotides per liter and the duration (t) of reassociation in seconds. For a second-order reaction, the rate constant [k (M^{-1} s^{-1})] used to predict the duration of reassociation reactions can be determined from the inverse of $cot_{1/2}$ [14], which is the cot value at which 50% of the nucleic acid has reassociated to form double strands.

Detection limit of PCR amplification of target

A 2-fold dilution series of the target sequence ranging from 100 fg (240,000 copies) to 1.25 ag (average of 3 copies, subject to statistical fluctuations) per reaction was amplified. All PCRs were conducted in 25 μl aliquots containing 1 μl of template solution with the stated number of template copies, 12.5 μl of 2× Brilliant II SYBR Green Q-PCR Master Mix (Agilent Technologies), and 200 nM of each forward and reverse primer. After the initial activation of the polymerase (95 °C, 10 min), thermocycling was performed for 40 cycles of DNA denaturation (95 °C, 30 s), primer annealing (60 °C, 1 min), and primer extension (72 °C, 30 s). Controls omitting DNA template were included in each experiment. A Mx3005P Q-PCR system (Agilent Technologies) was used for thermocycling, and MxPro (version 3.04b) was used for data collection and analyses.

Effect of presence of abundant background on PCR amplification of rare target

To demonstrate the effect of excess background DNA on rare target amplification, we combined a 2.5×10^6-fold mass excess (a 380,000-fold molar excess) of pUC18 fragment background with the rare target at a concentration of 250 ng/μl and 100 fg/μl, respectively. Sequential samples (1 μl) of a series of 2-fold