Homogeneous Real-Time Detection of Single-Nucleotide Polymorphisms by Strand Displacement Amplification on the BD ProbeTec ET System

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Background: The BD ProbeTec™ ET System is based on isothermal strand displacement amplification (SDA) of target nucleic acid coupled with homogeneous real-time detection using fluorescent probes. We have developed a novel, rapid method using this platform that incorporates a universal detection format for identification of single-nucleotide polymorphisms (SNPs) and other genotypic variations.

Method: The system uses a common pair of fluorescent Detector Probes in conjunction with unlabeled allele-specific Adapter Primers and a universal buffer chemistry to permit analysis of multiple SNP loci under generic assay conditions. We used Detector Probes labeled with different dyes to facilitate differentiation of two alternative alleles in a single reaction with no postamplification manipulation. We analyzed six SNPs within the human β2-adrenergic receptor (β2AR) gene, using whole blood, buccal swabs, and urine samples, and compared results with those obtained by DNA sequencing.

Results: Unprocessed whole blood was successfully genotyped with as little as 0.1–1 μL of sample per reaction. All six β2AR assays were able to accommodate ≥20 μL of unprocessed whole blood. For the 14 individuals tested, genotypes determined with the six β2AR assays agreed with DNA sequencing results.

Conclusion: SDA-based allelic differentiation on the BD ProbeTec ET System can detect SNPs rapidly, using whole blood, buccal swabs, or urine.

Single-nucleotide polymorphisms (SNPs) are the most common source of human genetic variation, occurring in human populations with a mean frequency of ~1 per 1000 bp (1, 2). These small changes in nucleotide sequence have great potential for identification of disease genes or markers for genetic disorders, predicting drug metabolism and therapeutic response, and in the study of population genetics. Such analyses require fast, reliable, and economical methods for detection of SNPs and other genetic variations. Although several high-throughput platforms exist for such applications in a research environment, there is a growing need for systems that are sufficiently robust and easy to use for routine testing in the clinical laboratory.

Apart from direct sequencing of PCR products, a variety of molecular methods are currently used for scoring or genotyping SNPs. Virtually all of these methods require PCR amplification of a target fragment bearing the SNP of interest before actual detection of the variant nucleotide. Such methods include hybridization of target to allele-specific oligonucleotides on DNA chip arrays (3) or other solid-phase formats [e.g., dot blots (4)], oligonucleotide ligation assays (5), allele-specific PCR (6), primer extension reactions or minisequencing (7), molecular beacons (8), 5′-nuclease assays (9), Invader technol-
ogy (10), and mass spectrometry (11). Several homogeneous, fluorescence-based methods have been reported that enable detection of SNPs during PCR (8, 9, 12, 13). By combining the processes of amplification and detection into a single step, these methods are amenable to higher throughput and are less prone to contamination than methods that involve manipulation of amplicons after the completion of the amplification reaction.

The BD ProbeTec™ ET System (BD Diagnostic Systems) is a real-time amplification and detection platform for the detection of Chlamydia trachomatis and Neisseria gonorrhoeae in the clinical laboratory. The system is based on isothermal strand displacement amplification (SDA) (14, 15) of target nucleic acids in sealed microwells, coupled with homogeneous real-time detection by fluorescent probes (16, 17). Here we describe the use of the BD ProbeTec ET System for the detection of multiple SNPs with a single pair of universal fluorescent energy transfer detector probes. The dual-dye capabilities of the instrument permit differentiation at any given locus of two alternative alleles in a single reaction without postamplification manipulation.

Materials and Methods

PRINCIPLES OF SDA-BASED SNP ANALYSIS

The principles of SDA for qualitative detection of nucleic acids have been described previously (14–17). Fig. 1 illustrates the homogeneous SDA-based universal fluorescent detection format for differentiation of multiple SNPs with use of a single pair of labeled detector probes. The system is based on allele-specific extension of an unlabeled Adapter Primer that hybridizes to the target sequence downstream of an SDA Primer. The Adapter Primer comprises a 3′ target-specific region and 5′ generic/universal tail sequence. In the present study, each SDA reaction contained a pair of Adapter Primers, each of which was specific for one of two allelic variants (i.e., either allele A or allele B of a given nucleotide locus). The 5′ tail sequences of the two Adapter Primers are different, permitting differentiation of alternative alleles through comparison of the fluorescent signals obtained in each of the two optical channels of the BD ProbeTec ET instrument. Importantly, the same pair of adapter tail sequences and generic fluorescent Detector Probes can be used for differentiation of multiple allelic pairs, thereby appreciably reducing the cost of assay development.

PRIMERS AND PROBES

Six polymorphisms within the human β2-adrenergic receptor (β2AR) gene were chosen as targets to investigate the capabilities of SDA and the BD ProbeTec ET system for analysis of SNPs. Reactions contained two each of the following oligonucleotides: SDA Primers, Bumper Primers, Adapter Primers, and universal fluorescence Detector Probes. The target-specific regions of the primers and adapters were based on the published sequence of the β2AR gene [GenBank accession no. M15169 (18)] and were synthesized by Integrated DNA Technologies, Inc. A complete listing of the primers and probes used in genotyping the six targeted SNPs of the β2AR locus is given in Table 1 in the Data Supplement that accompanies the online version of this article at http://wwwclinchem.org/content/vol49/issue10/.

CLONING OF CONTROL TARGET SEQUENCES

Control sequences containing either allele A or allele B of each of the six targeted β2AR SNPs [−654, −367, −47, +46, +491, and +523] (18)] were prepared by PCR amplification of pooled human placental DNA (Sigma). PCR fragments of 1.5 kb spanning the six SNP loci were cloned into the Escherichia coli plasmid vector pUC19. DNA sequencing of purified plasmid DNA was performed to verify the genotype of each cloned target.

SPECIMENS

Whole blood, buccal swabs, and urine samples were collected with informed consent from healthy volunteers at BD Diagnostic Systems (Sparks MD), according to protocols approved by the company’s Institutional Review Board.

Blood Samples were collected in PPT™ plasma preparation tubes containing dipotassium EDTA as the anticoagulant (BD Vacutainer Systems). After collection, specimens were stored at −20 °C before testing. For analysis of unprocessed blood, samples were thawed, and the desired volume of specimen was mixed directly with SDA buffer, heated for 5 min in a boiling water bath to lyse the cells and denature the DNA, cooled, and centrifuged at 10 000 g to pellet cellular debris. The blood–buffer supernatant was then added directly to priming microwells, and the standard SDA assay protocol was followed. For processed blood, 200 μL of thawed sample was extracted with use of a QIAamp® DNA Blood Mini Kit (QIAGEN Inc.) according to the manufacturer’s instructions. An aliquot of purified DNA was mixed with SDA buffer, boiled for 5 min to denature the DNA, cooled, and assayed.

Buccal specimens were collected using BD BBL CultureSwab™ EZ polyurethane swabs (BD Diagnostic Systems). Three swabs were collected from each individual at each collection time point. Swabs were stored frozen at −20 °C until analysis, at which time two swabs were thawed and expressed into 1 mL of SDA assay buffer. Tubes containing expressed swab material were heated for 5 min in a boiling water bath to denature the DNA, cooled, and centrifuged 1 min at 10 000 g to pellet cellular debris. Samples were then added directly to the SDA priming microwells and assayed.

Urine specimens were collected in sterile, plastic, preservative-free specimen collection cups that were stored at 4 °C for up to 3 days or frozen at −20 °C until analysis. A 2-mL portion of each urine sample was centrifuged at 600 g for 10 min. The supernatant was then decanted, and the concentrated urine was resuspended in 0.5–1.0 mL
Samples were then boiled for 5 min to denature the target nucleic acid and assayed according to standard procedures.

**Genotyping with the BD ProbeTec ET system**

The workflow used in the present study for genotyping on the BD ProbeTec ET System is summarized in Fig. 2. The assay reagents include a Priming Microwell containing SDA Primers, Bumper Primers, Adapter Primers, and nucleotides; and an Amplification Microwell containing the amplification enzymes. The same Amplification Microwell formulation and Universal Buffer were used for each of the six β2AR assays. For two of the assays that targeted particularly GC-rich regions of the β2AR gene (Δ47 and +46 loci; depending on the assay) of universal SDA assay buffer. Samples were then boiled for 5 min to denature the target nucleic acid and assayed according to standard procedures.
Specimens were processed as described above. Denatured target nucleic acid in SDA buffer was dispensed into the Priming Microwells and incubated at room temperature for 5 min. The Priming Microwells were then placed on a special heating block set at 72 °C; at the same time, the Amplification Microwells were prewarmed on an adjacent block at 54 °C. The two heating blocks comprise part of the standard hardware for the BD ProbeTec ET System. After a 10-min incubation, the samples were transferred from the Priming to the Amplification Microwells, which were then permanently sealed and loaded in the BD ProbeTec ET reader set at the standard operating temperature of 52 °C. The final reagent concentrations in each well were as follows (140-μL volume): 24.5 mM potassium phosphate (pH 7.6); 90.5 mM Bicine; 54.3 mM KOH; 70 mL/L dimethyl sulfoxide; 70 mL/L glycerol; 5 mM magnesium acetate; 100 mg/L bovine serum albumin; 100–500 nM SDA Primers; 50 nM Bumper Primers; 100–400 nM Adapter Primers; 150–500 nM fluorescent Detector Probes; 0.1 mM each of dATP, dGTP, and dTTP; 0.5 mM 2'-deoxycytidine 5’-O-(1-thiotriphosphate) S-iso-mer; ~0.8 U/μL of Bst DNA polymerase; and ~2.6 U/μL of BsoDI restriction enzyme (NEB). For the −47 and +46 assays located in particularly GC-rich regions of the β₂AR gene, additional glycerol and dimethyl sulfoxide were included in the Priming Microwell to bring the final concentrations to 140 mL/L and 125 mL/L, respectively. Data were collected over a period of 60 min and downloaded from the instrument for analysis.

**DATA ANALYSIS**

We have developed SNP analysis software to analyze BD ProbeTec ET data and generate genotyping results. The instrument measures output from both optical channels at discrete intervals (passes) throughout the course of the reaction. Raw optical data are processed by dark correction and dynamic normalization using internal fluorescence calibrators, followed by step detection/repair and smoothing with running mean and median filters to remove periodic noise. The background fluorescence is then calculated over a predetermined number of readings from early in the course of amplification and subtracted from all subsequent data points. The maximum density (ρ-Max) algorithm is applied to the background-corrected data to determine the ratio (r) of fluorescent signals from the two Detector Probes at each pass during the reaction according to the equation: $r = \ln(\text{ROX}/\text{FAM})$, where ROX and FAM correspond to the signals in relative fluorescence units (RFU) obtained from the rhodamine- and fluorescein-labeled probes, respectively. A maximum density function is then used to determine the most likely value of $r$ for the sample, and this number is output as the $\rho$-Max score. For the β₂AR assays, $\rho$-Max values greater than +1 corresponded to high ROX signals and were considered indicative of a homozygous allele A genotype. Similarly, $\rho$-Max values less than −1 corresponded to high FAM fluorescence and a homozygous allele B geno-
type. $p$-Max values between −1 and +1 indicated the detection of similar fluorescence intensities in the two optical channels and a heterozygous allele A/B genotype.

**Sequence Analysis**

The results of SDA-based genotyping of processed and unprocessed blood, urine, and buccal swabs were compared with those obtained by DNA sequencing of PCR products obtained from amplification of purified DNA from blood. DNA sequencing was performed in a blinded fashion without reference to the SDA genotyping results (Commonwealth Biotechnologies, Inc.). Heterozygous genotypes were confirmed by visual analysis of the sequencing electropherograms.

**Results**

**Differentiation of homozygous and heterozygous alleles**

Fig. 3 shows representative amplification curves generated with the SDA assay for the −47 β2AR SNP locus, the amplicon for which has a G+C content of 86%. The assays were performed with 3 μL of whole, unprocessed blood added directly to the reaction mixture. SDA results were in complete agreement with DNA sequencing of PCR products. The two optical channels of the BD ProbeTec ET instrument were used to detect the two alternative alleles that are possible at the −47 locus. The ROX-labeled Detector Probe corresponded to allele A (C nucleotide at position −47), whereas the FAM-labeled probe corresponded to allele B (T nucleotide at position −47). Data were analyzed using the $p$-Max algorithm as described in the Materials and Methods.

For specimen B (Fig. 3), there was a strong ROX signal and corresponding weak FAM fluorescence with a $p$-Max score of +2.70, indicating the presence of the homozygous C/C allelic pair. In contrast, for specimen E, there was a strong FAM signal with little increase in ROX fluorescence over the course of the reaction. The $p$-Max score for this sample was −2.33, indicating the presence of the homozygous T/T allelic pair. With specimen G, there were approximately equivalent increases in both FAM and ROX fluorescence during the course of the reaction, yielding a $p$-Max score of +0.05. This value lies between −1 and +1, thereby indicating that the sample was heterozygous at the −47 position (C/T allelic pair). The control reaction containing no target DNA yielded only background signal in both optical channels. In this case, because neither optic yielded a detectable signal, no $p$-Max score was calculated and the sample was therefore recorded as indeterminate.

![Fig. 3. Genotyping of human blood specimens by the BD ProbeTec ET System.](image-url)
ACCURACY OF ALLELIC DIFFERENTIATION

The accuracy of SDA-based SNP analysis relative to DNA sequencing was demonstrated by genotyping the six targeted $\beta_2$AR loci ($-654$, $-367$, $-46$, $+46$, $+491$, and $+523$) in 19 different blood specimens from 14 healthy volunteers and by analysis of cloned target nucleic acid. Blood specimens were genotyped either by directly adding whole blood to SDA reactions or by analysis of purified genomic DNA prepared as described in the Materials and Methods. We used 3 $\mu$L of whole blood or 100 ng of purified genomic DNA in each SDA reaction. In parallel with analysis of processed and unprocessed blood, two plasmid clones of the $\beta_2$AR gene were genotyped with use of 0.45 pg of purified plasmid DNA ($1 \times 10^5$ copies) per reaction. A single genotyping reaction for each SNP locus was performed with each sample. A total of 7 different genotypes [haplotype pairs (18)] was identified among the 14 individuals tested. For all six SNP loci and for all samples, the SDA-based genotyping results were in complete concordance with those obtained by DNA sequencing (data not shown).

REPRODUCIBILITY OF ALLELIC DIFFERENTIATION

The reproducibility of SDA for differentiation of allelic variants was demonstrated with the $-654$ $\beta_2$AR SNP assay. Various amounts of processed and unprocessed blood were analyzed from individuals who had previously been genotyped by DNA sequence analysis. Between 0.1 and 3 $\mu$L of whole blood or 2–105 ng of purified genomic DNA was used for genotyping in each SDA reaction.

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![Image of cluster analysis](image-url)

Fig. 4. Cluster analysis of data obtained with the $-654$ $\beta_2$AR assay.

A and C, relative fluorescent signals emitted by the FAM- and ROX-labeled detector probes. Ellipses correspond to 95% probability limits based on the bivariate gaussian distribution. Genotyping reactions contained 0.1–3 $\mu$L of whole blood (A) or 2–105 ng of purified genomic DNA (C) as target. B and D, $p$-Max analysis of fluorescent signals shown in panels A and C, respectively.
reaction. Three replicates were run at each target concentration for a total of 15 data points with each sample type plus negative controls. Fig. 4 shows the clustering of signals within the three possible genotypes at the −654 locus. All SDA-based genotyping results agreed with the corresponding DNA sequence analysis. For both unprocessed blood and purified DNA, there was a clear distinction in terms of the relative FAM and ROX signals between the homozygous samples B and E and the heterozygous sample G. Regardless of the quantity of sample analyzed or the purity of the target DNA, results clustered into three distinct groups, representing the homozygous and heterozygous genotypes and in agreement with DNA sequence analysis. This clustering of data was particularly evident when the ρ-Max algorithm was been applied (Fig. 4, B and D). All homozygous samples yielded ρ-Max scores that were either greater than +1 or less than −1 depending on the allele. In contrast, all heterozygous samples yielded ρ-Max scores between −1 and +1.

SAMPLE VOLUME
All six assays yielded the correct genotyping results as determined by comparison with DNA sequence analysis with 1–20 μL of blood per reaction. Four assays (−654, −367, +491, and +523) gave positive results with 0.3 μL of blood, and two (−654 and +491) also yielded results with as little as 0.1 μL of blood per reaction (Table 2 in the online Data Supplement). With purified genomic DNA extracted from blood, correct results were obtained from the six assays when we used 3.5–35 ng (980–9800 genomic-equivalents) per reaction.

ANALYSIS OF MATCHED BLOOD, BUCCAL SWABS, AND URINE
Because sample types such as hair, buccal swabs, or fingernails may be appropriate for widespread genotyping applications (19), we performed a series of experiments to demonstrate the ability to genotype specimens other than blood. Ten matched blood, buccal, and urine specimens from different donors were analyzed in the −654, −367, +491, and +523 β2AR SNP assays (Fig. 5). With the exception of specimens from donor G, for each SNP locus, homozygous samples yielded ρ-Max scores either greater than +1 or less than −1 depending on the allele, whereas heterozygous samples yielded values between −1 and +1. Matched samples from the same individual yielded identical β2AR genotypes that were in complete concordance with DNA sequence analysis.

Initial testing of buccal swab specimens from donor G incorrectly identified the individual as heterozygous at the −367 locus (ρ-Max score = +1.1). This was related to an abnormally low yield of DNA from the swab specimens from this donor. Repeat testing of additional buccal swabs from the same donor yielded the correct genotyping result. Similarly, the indeterminate results obtained with urine from donors E (+491 and +523 assays) and L (+523 assay) and from buccal swabs from donor I (+523 assay) were associated with low target loads present in these samples. These data emphasize that control over

Fig. 5. Use of alternative specimen types from the same individual for genotyping with the BD ProbeTec ET System.

SDA-based SNP analysis was conducted on four loci within the human β2AR gene using matched blood, buccal swabs, and urine specimens from 10 individuals (A–L). Dashed lines represent the −1 and +1 ρ-Max cutoff values segregating homozygous and heterozygous genotypes. ρ-Max greater than +1 indicates homozygous allele A; ρ-Max less than −1 indicates homozygous allele B; ρ-Max between −1 and +1 indicates heterozygous alleles A/B. Solid lines represent the mean ρ-Max score obtained for each individual across the different sample types. Specimens that yielded indeterminate results are not shown. Indeterminate results were obtained with the following samples: locus +491, donor L, urine; locus +523, donors E and L, urine; donor I, buccal swabs.
input target DNA concentration and the establishment of appropriate $p$-Max cutoffs are required to ensure accurate reporting of correct genotypes and to identify inadequate specimens.

**Discussion**

In the present study we developed a novel SDA-based method for the detection of SNPs with the BD ProbeTec ET System. The method permits the use of a single pair of universal fluorescently labeled probes for the detection of multiple polymorphisms, thereby reducing the cost and complexity of assay development. The utility of this method was demonstrated by the development of six individual assays for polymorphisms within the human $\beta_2$AR gene (18). Through the use of two fluorescent probes labeled with different fluorophores, each assay enables the differentiation of two alternative alleles at the targeted SNP locus. Importantly, all six assays described here were optimized to work with the same universal buffer, further streamlining assay set-up and workflow. The adapter-mediated universal detection system we have described therefore provides a simple, rapid, sensitive, and specific method for SNP analysis and haplotyping. Furthermore, through the appropriate location and design of Adapter and SDA Primer sequences, the principles of SNP analysis described here are also amenable to the differentiation of other nucleotide acid sequence variations, such as insertions, deletions, and splice variations.

One of the objectives of this study was to develop a set of generic amplification conditions that would permit the differentiation of alternative alleles at multiple SNP loci without the need for reoptimization of the assay buffer. This was a particular challenge for the six $\beta_2$AR assays, the target regions for which ranged in G+C content from 53% to 86%. Reports of PCR-based SNP analysis indicate difficulty in genotyping sequences with $>$60% G+C content (20). Although the SDA assays we developed were able to differentiate polymorphisms within highly G+C-rich regions of DNA, we did observe differences in analytical sensitivity among the six assays that correlated broadly with G+C content. Nevertheless, we were successful in obtaining the correct genotypes with all six assays, using as little as 1 $\mu$L of unprocessed blood per reaction. We also observed that all of the $\beta_2$AR assays were tolerant to $\geq 20$ $\mu$L of unprocessed blood (equivalent to $>14\%$ of total reaction volume). The system provides an efficient method for direct determination of unphased genotypes from a variety of sample types, including blood, buccal swabs, and urine. However, we observed that care is necessary in validating genotyping protocols for use with different specimen types that may yield widely varying quantities of nucleic acid. Such fluctuations in target load can impact amplification efficiency, and the establishment of appropriate algorithm parameters to safeguard against incorrect assignment of genotypes and identify inadequate specimens is an important aspect of assay development.

The assay format we have described here uses a final reaction volume of 140 $\mu$L, although this may readily be adjusted for specific applications. Using the standard BD ProbeTec ET microwell format and assay workflow, we have demonstrated the ability to genotype specimens in a 50-$\mu$L reaction volume containing 10% whole blood by volume. The ability to circumvent time-consuming and costly sample processing represents a major advance in the field of genetic analysis. The blood samples tested in the present study were collected with dipotassium EDTA as the anticoagulant, although we have also demonstrated the compatibility of the $\beta_2$AR assays with unprocessed blood containing heparin or citrate as the anticoagulant (data not shown).

The method we describe here provides differentiation between single nucleotide variants through the positioning of the diagnostic nucleotide one base from the 3’ end of the Adapter Primer. This differs from typical allele-specific PCR systems, in which high selectivity is accomplished only when the diagnostic nucleotide is positioned at the very 3’end of the SNP-specific primer (21, 22). For highly G+C-rich sequences, in which primer-primer interactions are common, artificially created mismatches in the SDA primers or toward the 3’ terminus of the adapter primer may be introduced to improve amplification efficiency and/or differentiation. For example, the −654 $\beta_2$AR Adapter Primers include intentional mismatches 3 bases from the 3’ terminus to improve allelic differentiation (Table 1 in the online Data Supplement).

The novel genotyping procedure we have described here involves isothermal amplification without the need for temperature cycling. For the purposes of this study, we used an amplification time of 60 min, although, depending on the target concentration, there is no significant change in $p$-Max score after the first 20–30 min of incubation (data not shown). With suitable control over the input of target DNA, the amplification and detection time could therefore be reduced substantially to enhance throughput. With a 30-min amplification reaction as many as 960 specimens may be processed and genotyped in one 8-h shift on a single BD ProbeTec ET instrument.

In conclusion, the BD ProbeTec ET System permits rapid genotype analysis using common fluorescent detector probes and a streamlined workflow with a wide range of specimen types and sample volumes.

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**References**


