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ORIGINAL PAPER

Genotyping of single nucleotide polymorphisms by primer extension reaction and a dual-analyte bio/chemiluminometric assay

Jessica Konstantou · Penelope C. Ioannou · Theodore K. Christopoulos

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Abstract Primer extension reaction (PEXT) is the most widely used approach to genotyping of single nucleotide polymorphisms (SNP). It is based on the high accuracy of nucleotide incorporation by the DNA polymerase. We propose a dual-analyte bio/chemiluminometric method for the simultaneous detection of the PEXT reaction products of the normal and mutant allele in a high sample-throughput format. PCR-amplified DNA fragments that span the SNP of interest are subjected to two PEXT reactions using normal and mutant primers in the presence of digoxigenin-dUTP and biotin-dUTP. Both primers contain a $d(A)_{30}$ segment at the 5′-end but differ in the final nucleotide at the 3′-end. Under optimized conditions only the primer that is perfectly complementary with the interrogated DNA will be extended by DNA polymerase and lead to a digoxigenin- or biotin-labeled product. The products of the PEXT reactions are mixed, denatured, and captured in microtiter wells through hybridization with immobilized oligo(dT) strands. Detection is performed by adding a mixture of antidigoxigenin–alkaline phosphatase (ALP) conjugate and a streptavidin–aequorin conjugate. The flash-type bioluminescent reaction of aequorin is triggered by the addition of Ca^{2+}. ALP is then measured by adding the appropriate chemiluminogenic substrate. The method was evaluated by genotyping two SNPs of the human mannose-binding lectin gene (MBL2) and one SNP of the cytochrome P450 gene CYP2D6. Patient genotypes showed 100% concordance with direct DNA sequencing data.

Keywords Single nucleotide polymorphisms · Genotyping · (Bio)Chemiluminescence · High throughput · Dual analyte assay

J. Konstantou · P. C. Ioannou (✉)
Laboratory of Analytical Chemistry, Department of Chemistry, Athens University,
Athens 15771, Greece
e-mail: ioannou@chem.uoa.gr

T. K. Christopoulos
Department of Chemistry, University of Patras,
Patras 26500, Greece

T. K. Christopoulos
Foundation for Research and Technology Hellas, Institute of Chemical Engineering and High Temperature Chemical Processes (FORTH/ICE-HT),
Patras 26504, Greece

Introduction

Single nucleotide polymorphisms (SNP) constitute the most common form of human genetic variation. SNP may affect gene function by amino acid substitution, modification of gene expression, or alteration of gene splicing [1–3]. Current research efforts on SNP genotyping aim either at establishing association of various SNP with certain diseases or at the development of SNP genotyping methods that are suitable for the routine molecular diagnosis laboratory. DNA microarray technology plays an important role in genome-wide association studies because each chip enables parallel genotyping of thousands of SNP in a single sample. In a diagnostic setting, however, a small number of SNP markers for disease-related genes or drug-metabolizing enzymes will be genotyped per patient. Consequently, a high sample-throughput is much more useful than a high SNP throughput for the routine clinical laboratory.

SNP genotyping methods comprise isolation of genomic DNA, PCR amplification, a genotyping reaction, and detection of the products. Most genotyping reactions are based on one of the following principles: hybridization with allele-specific

oligonucleotide probes, oligonucleotide ligation reaction, primer extension (PEXT) reaction, and invasive cleavage. In a typical PEXT reaction, an allele-specific primer whose 3′-end is complementary to the nucleotide at the polymorphic site hybridizes with the interrogated DNA fragment. Because of the high accuracy of nucleotide incorporation by DNA polymerase, polymerization occurs only if the primer matches perfectly the target DNA. Two PEXT reactions are performed per mutation, one for the normal allele and the other for the mutant allele. PEXT reaction is the most widely used genotyping method because it is robust and can be easily optimized [4–6].

To facilitate automation and high sample-throughput we have developed a dual-analyte assay of the PEXT products. The assay exploits the different kinetics of light emission from aequorin and alkaline phosphatase (ALP) bio/chemiluminescent reactions. Indeed, the aequorin reaction has a decay half-life of about 1 s, whereas the emission of ALP-catalyzed chemiluminescent reaction lasts several minutes. The two reporters have already been used in quantitative PCR and oligonucleotide ligation reaction assays [7, 8]. As a model we chose the genotyping of two mutations of the mannose-binding lectin (MBL2) gene (−550 and −221) and one mutation of the cytochrome P450 gene CYP2D6 (CYP2D6*3). In recent years, there has been a strong interest in mannose-binding lectin because it is an important component of the innate immune system and numerous reports have shown that its deficiency leads to increased susceptibility to various infections and genetic disorders [9–12]. CYP2D6 is also important since it is involved in the metabolism of many commonly prescribed drugs [13, 14].

Bio- and chemiluminescence, the emission of light from chemically generated excited states, has found rapidly expanding applications in nucleic acid analysis, including hybridization assays for detection/determination of PCR products as well as SNP genotyping assays [15–17]. Because they do not require excitation light, bio/chemiluminometric methods offer higher detectability (lower background signal) and wider dynamic range than spectrophotometric and fluorometric ones. Bioluminescent proteins have already been introduced as reporters in SNP genotyping by primer extension. Firefly luciferase was used in the bioluminometric assay of pyrophosphate release in PEXT reaction [18, 19]. The photoprotein aequorin was used in a solid-phase assay for the extension product [20]. Horseradish peroxidase, in combination with a chemiluminogenic substrate, was employed for the genotyping of 15 HBB gene mutations based on the PEXT reaction [21]. Contrary to the above methods, which require that the primer extension products from normal and mutant alleles must be measured in separate reaction vessels (e.g., microtiter wells), the proposed method allows simultaneous detection of both products.

Experimental

Instrumentation

Flash- and glow-type (bio)chemiluminescence was measured by using the PhL microplate Luminometer/Photometer from Mediators (Vienna, Austria). Polymerase chain reactions (PCR) and primer extension reactions (PEXT) were carried out in a MJ Research PTC-150 thermal cycler (Watertown, MA). A digital camera, Kodak DC 120, and the Gel Analyzer software for DNA and protein documentation were purchased from Kodak (New York, NY). Hybridization assays were performed using the Titramax 1000 shaker/incubator from Heidolph (Kehlheim, Germany). The microtiter plate washer, model Wellwash 4, was from Labsystems (Milford, MA).

Materials and methods

Antidigoxigenin–alkaline phosphatase conjugate (anti-Dig–ALP) and Dig-11-dUTP were purchased from Roche (Mannheim, Germany). Bis(sulfosuccinimidyl)suberate (BS^3) was obtained from Pierce (Rockford, IL), and bovine serum albumin (BSA) was from Serva (Heidelberg, Germany). Opaque Microlite 2 polystyrene microtiter wells were supplied by Thermo Labsystems (Franklin, MA), and Sephadex G-25 Spin Pure columns were from CPG (Linkoln Park, NJ). Ultra-pure 2-deoxyribonucleotide 5-triphosphates (dNTPs) were purchased from HT Biotechnology (Cambridge, UK). Vent (exo-) DNA polymerase was from New England Biolabs (Beverly, MA), Φ×174 DNA/BsuRI marker was from MBI Fermentas (Vilnius, Lithuania), and biotin-11-dUTP was from Applichem (Darmstadt, Germany). Lumiphos was obtained from Aureon Biosystems (Vienna, Austria). The QIAmp DNA blood mini kit from Qiagen (Hilden, Germany) was used for the isolation of genomic DNA from whole blood. Streptavidin–aequorin conjugate (SA–Aeq) was synthesized and purified as described previously [22]. All other chemicals were purchased from Sigma (St. Louis, MO) or Fluka (Buchs, Switzerland). Oligonucleotides used as primers and probes in the course of this work were synthesized by the Research and Technology Institute (Irakleion, Crete, Greece) and Thermo Electron (Ulm, Germany). Oligonucleotide sequences are shown in Table 1.

The hybridization solution contained 10 mM Tris, 10 mM EGTA, 1 M KCI, 10 mM $MgCI_2$, 2 g L^{-1} BSA, 1 g L^{-1} NaN_3, pH 7.5, and 0.5 mL L^{-1} (0.05%) Tween-20 (polyoxyethylene (20) sorbitan monolaurate). The phosphate-buffered saline (PBS) was a 0.14 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 1.7 mM potassium phosphate solution, pH 7.4. The wash solution consisted of 50 mM Tris, pH 7.5, 0.15 M NaCl, 2 mM EGTA, and

Table 1 Oligonucleotides used in present study as primers for PCR and PEXT

Oligonucleotide	Name	Sequence (5′→3′)	Size
MBL2 PCR primers			
5′ primer (610-bp product)	U_{610}	CCTGCCAGAAAGTAGAGAGG	20-mer
3′ primer (610-bp product)	D_{610}	CTATATAGAAATAGATGACCCATC	24-mer
CYP2D6*3 PCR primers			
5′ primer (281-bp product)	–	GACCCAGCTGGATGAGCTG	19-mer
3′ primer (281-bp product)	–	GCCGAGAGCATACTCGGGAC	20-mer
MBL2 genotyping primers[a]			
3′-550 (normal)	N_{550}	$(A)_{27}$ATGCTTACCCAGGCAAGCCTGT*G*	50-mer
3′ -550 (mutant)	M_{550}	$(A)_{27}$ATGCTTACCCAGGCAAGCCTGT*C*	50-mer
3′ -221 (normal)	N_{221}	$(A)_{27}$GGAAGACTATAAACATGCTTTC*C*	50-mer
3′ -221 (mutant)	M_{221}	$(A)_{27}$GGAAGACTATAAACATGCTTTC*G*	50-mer
CYP2D6*3 genotyping primers[a]			
5′ CYP2D6*3 (normal)	$N_{CYP2D6*3}$	$(A)_{30}$TGAGCTGCTAACTGAGCAC*A*	50-mer
5′ CYP2D6*3 (mutant)	$M_{CYP2D6*3}$	$(A)_{30}$TGAGCTGCTAACTGAGCAC*G*	50-mer

[a] For convenience, we have named the wild-type nucleotide sequences as 'normal' or *N* and the variant nucleotide sequences as 'mutant' or *M*

1 mL L^{-1} Tween-20. The blocking solution contained 10 g L^{-1} BSA, 0.1 M maleic acid, 0.15 M NaCl, and 2 mM EGTA, pH 7.5. The light-triggering solution contained 25 mM $CaCl_2$ and 20 mM Tris, pH 7.5.

Conjugation of $(dT)_{30}$ oligodeoxynucleotide to albumin

The conjugation of BSA with the 5′-amino-modified $(dT)_{30}$ oligonucleotide was performed by using the homobifunctional cross-linking reagent BS^3 as described previously [23] and the conjugate was used directly without purification. Polystyrene microtiter wells were coated by physical adsorption for 2 h at 42 °C with 50 μL of 5 mg L^{-1} BSA–$(dT)_{30}$ conjugate diluted in PBS-5 mM EDTA.

Genomic DNA isolation from whole blood

Whole-blood specimens were obtained from the Medical School of the University of Crete and from St. Sophia Children Hospital, Athens, after informed consent. Genomic DNA was isolated from 200 μL of whole blood using the QIAmp DNA blood mini kit. Following extraction of genomic DNA (50–100 ng μL^{-1}), a segment in the cytochrome CYP2D6 gene flanking the CYP2D6*3 mutation and a segment in the promoter region of the MBL2 gene flanking two single nucleotide polymorphisms (SNP) at positions −550 and −221 mutations were amplified by PCR.

Polymerase chain reaction

PCR amplification of the cytochrome CYP2D6 gene was carried out in a final volume of 50 μL containing 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, pH 8.8, 2.5 mM $MgCl_2$, 0.5 pmol μL^{-1} of each forward and reverse primers (Table 1), 1.25 U of *Taq* DNA polymerase, 0.2 mM of each of the dNTPs, and 100 ng of genomic DNA. The cycling parameters for CYP2D6 gene were as follows: initial denaturation at 95 °C for 4 min, 35 cycles at 95 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. PCR amplification of the MBL2 gene was performed using 25 μL of the HotStar Taq Master Mix kit from Qiagen in a total reaction volume of 50 μL consisting of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.4 μM of each forward and reverse primers (Table 1), 2.5 U HotStar *Taq* DNA polymerase, 0.2 mM of each of the dNTPs, and 50–100 ng of genomic DNA. The cycling parameters were as follows: initial denaturation at 95 °C for 15 min, 32 cycles at 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 8 min. The amplification products were confirmed and quantified by agarose gel (1.7%) electrophoresis and ethidium bromide staining.

Primer extension reaction (PEXT)

PEXT reactions for CYP2D6*3 mutation were performed in a total volume of 20 μL containing 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, pH 8.8, 0.1 pmol of amplified DNA, 0.5 pmol of either normal (N) or mutant (M) primer, 0.25 U Vent (exo-) DNA polymerase, 2 mM $MgCl_2$, 2.5 μM of each dATP, dCTP, dGTP and either 1.75 μM dTTP/0.75 μM Dig-dUTP (PEXT reaction with N primer) or 1.25 μM dTTP/1.25 μM biotin-dUTP (PEXT reaction with M primer). PEXT reactions for −550 and −221 MBL2 gene polymorphisms were performed as above but using 2.75 mM $MgCl_2$. The thermal cycling conditions for CYP2D6*3 PEXT reaction were: denaturation at 95 °C for 5 min, followed by three cycles of 95 °C for 10 s, 65 °C for 10 s, and 72 °C for 30 s (total run time 7.5 min). For MBL2

PEXT reaction the conditions were: 95 °C for 5 min and three cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 15 s (total run time 7 min). Both PEXT programmes were followed by an additional denaturation step at 95 °C for 5 min and the products were placed immediately on ice for 2 min.

Dual-analyte (bio)chemiluminometric hybridization assay of PEXT reaction products

Polystyrene microtiter wells, coated with BSA–$(dT)_{30}$ conjugate, were washed three times with wash solution. A mixture of PEXT products (3 μL of each) was diluted to 50 μL with blocking solution and added into each well. The PEXT products were allowed to hybridize with immobilized oligo(dT) for 15 min at ambient temperature under gentle shaking. The wells were washed three times and 50 μL of a solution containing streptavidin–aequorin conjugate (11 nM with respect to aequorin) and 150 U L^{-1} antidigoxigenin–alkaline phosphatase conjugate diluted in hybridization buffer was added into each well. The reaction was allowed to proceed for 25 min at ambient temperature under gentle shaking and the excess of reagents was removed by washing the wells as above. The bioluminescence of the bound aequorin was measured by injecting 50 μL of triggering solution and integrating the signal over 3 s. The wells were then washed three times and the chemiluminescence of bound alkaline phosphatase was measured by adding 50 μL of lumiphos substrate, incubating at 37 °C for 30 min, and integrating the signal for 1 s.

Results and discussion

Assay principle

A schematic presentation of the proposed dual chemiluminometric hybridization assay for SNP genotyping by PEXT reaction is shown in Fig. 1. Genomic DNA, isolated from whole blood, is first subjected to polymerase chain reaction

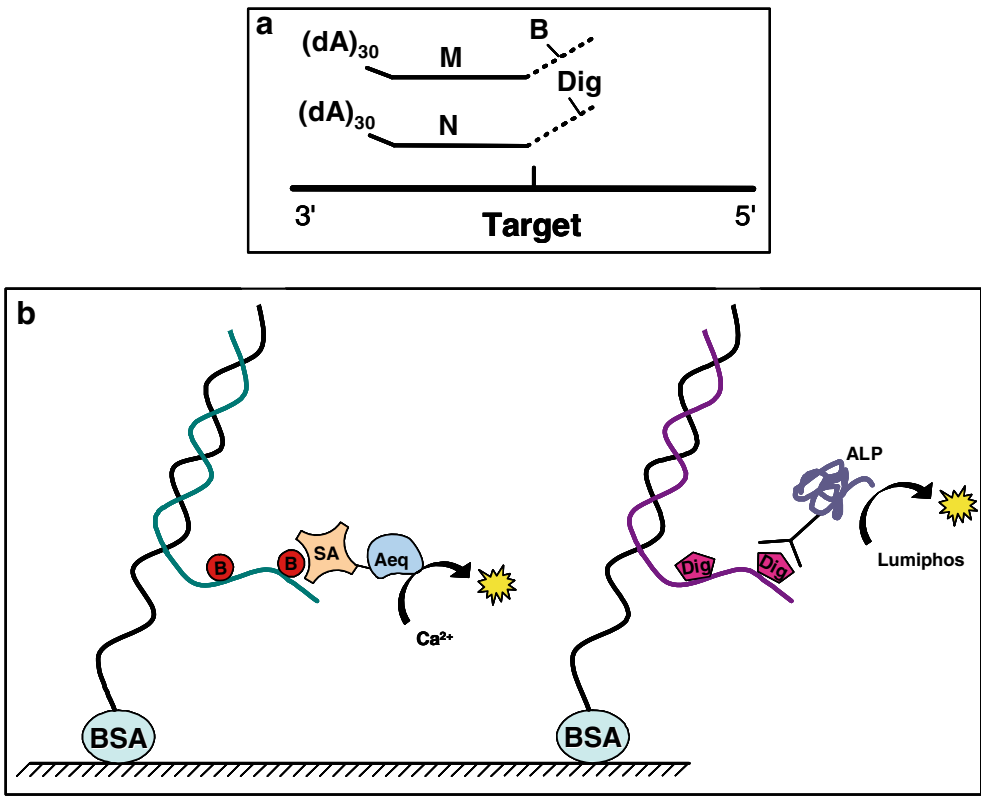

Fig. 1 Schematic illustration of SNP genotyping by primer extension (PEXT) reaction based on dual bio/chemiluminometric assay. **a** PCR-amplified DNA fragments that span the SNP of interest are subjected to two PEXT reactions using normal and mutant primers in the presence of Dig-dUTP and biotin-dUTP. Both primers contain a poly (dA) segment at the 5′-end but differ in the final nucleotide at the 3′-end. Under optimized conditions only the primer that is perfectly complementary with the target DNA will be extended by DNA polymerase and lead to a digoxigenin-labeled (Dig) or biotin-labeled (B) extension product. **b** The products of the PEXT reactions are mixed, denatured, and captured in microtiter wells through hybridization with immobilized oligo(dT) strands. The immobilized extension products are detected by adding a mixture of antidigoxigenin–alkaline phosphatase (anti-Dig–ALP) conjugate and a streptavidin-aequorin (SA–Aeq) conjugate. The flash-type bioluminescent reaction of aequorin is triggered by the addition of Ca^{2+}. Then ALP is measured by adding Lumiphos chemiluminogenic substrate

using primers flanking the polymorphic site. Amplified fragments with sizes of 281-bp (CYP2D6 gene) and 609-bp (MBL2 gene) were generated. Each PCR product served as a template for two separate PEXT reactions, one with a primer carrying at its 3′-end a nucleotide complementary to the normal allele (N primer) and a second reaction using a primer that carries, at its 3′-end, a nucleotide complementary to the mutant allele (M primer). Both N and M primers carry at their 5′-end a $d(A)_{30}$ tail. The PEXT reaction for the normal allele (N-PEXT) is carried out in the presence of a dNTP mixture containing Dig-dUTP, whereas the PEXT reaction for the mutant allele (M-PEXT) takes place in the presence dNTPs containing biotin-dUTP. When a perfect match occurs between primer and target sequence, the primer is extended by DNA polymerase and either digoxigenin (N-PEXT reaction) or biotin (M-PEXT reaction) is incorporated into the extended products. The products of the two PEXT reactions are mixed and the incorporation of modified dNTPs is detected by a dual (bio) chemiluminometric assay. To ensure separation of the extended primers from the target sequence (template), the reaction mixture is heated at 95 °C and placed immediately on ice. The mixture of N-PEXT and M-PEXT products is captured in microtiter wells through hybridization of the $d(A)_{30}$ tail of the extended primers to immobilized $d(T)_{30}$ oligonucleotides. Detection of captured products is performed by adding a solution containing antidigoxigenin–alkaline phosphatase and streptavidin–aequorin conjugate. The glow-type chemiluminescence of ALP signifies the presence of the normal allele, whereas the flash-type bioluminescence of AEQ denotes the presence of the mutant allele. The ALP/AEQ signal ratio gives the genotype of the allelic variant. It should be noted that the free (not extended) primer (either N or M) carrying a $d(A)_{30}$ tail is also hybridized with immobilized $d(T)_{30}$ strands but is not detected because it does not contain a modified dNTP.

In general, good discrimination between the two alleles is achieved when: (a) the PEXT reaction is optimized so that the primer is extended only if it is hybridized with a perfectly complementary DNA template, i.e., there is no extension of mismatched primer/template hybrids; (b) the determination of PEXT products by the dual (bio)chemiluminescence assay is closely related to the performance of the two separate hybridization assays.

Optimization studies

In order to assess the performance of the dual hybridization assay we tailed the two PEXT primers (N and M primer) with biotin-dUTP and Dig-dUTP, respectively, by using terminal deoxynucleotidyl transferase. The tailed primers were then tested, at various concentrations ranging from 4 to 1,000 pM, both by separate hybridization assays and the dual hybridization assay. The results are presented in Fig. 2 and show the close agreement between the two assay formats. The signal-to-background ratios at 4 pM were 8.5 and 13.5 for the aequorin and alkaline phosphatase assay, respectively. The dynamic range extends up to 1,000 pM.

To achieve the best discrimination between the two alleles (N-PEXT and M-PEXT reactions), we performed a series of optimization studies using, as a model, the genotyping of CYP2D6*3 mutation of cytochrome CYP2D6 gene. First, we optimized the Mg^{2+} concentration, considering its strong effect on the activity of DNA polymerase. A series of PEXT reactions were performed using a normal (N/N), a mutant (M/M), and a heterozygote (N/M) sample containing 100 fmol of amplified target DNA, 1 pmol of the allele-specific primer (N or M), and various Mg^{2+} concentrations in the range 1–3 mM. We observed that the ALP signal for N/N increases almost three times with the increase of Mg^{2+} concentration from 1 to 2 mM and then remains practically constant. The AEQ

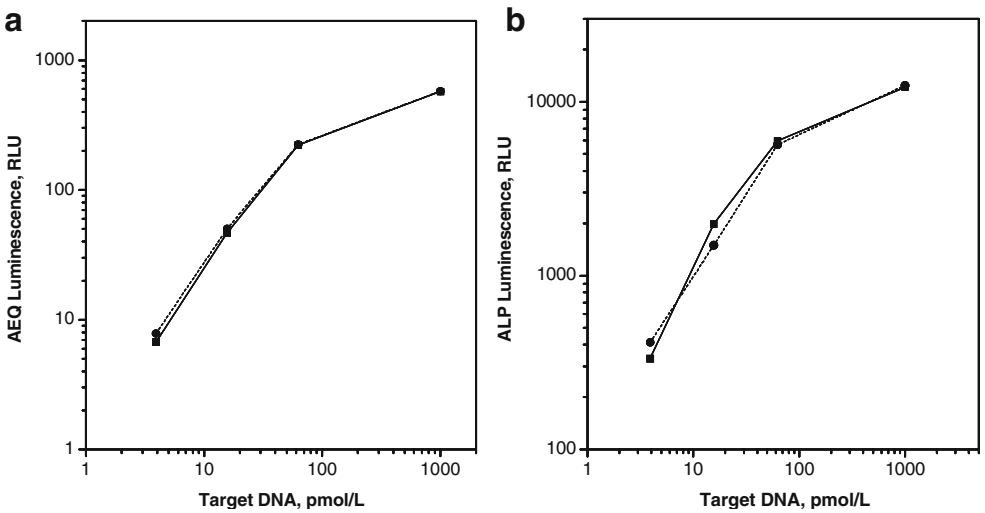

Fig. 2 Comparison between single- and dual-analyte (bio) chemiluminometric assay of primers extended with biotin-dUTP (**a**) and Dig-dUTP (**b**). The *solid* and *dashed lines* represent the single- and dual-analyte assays, respectively

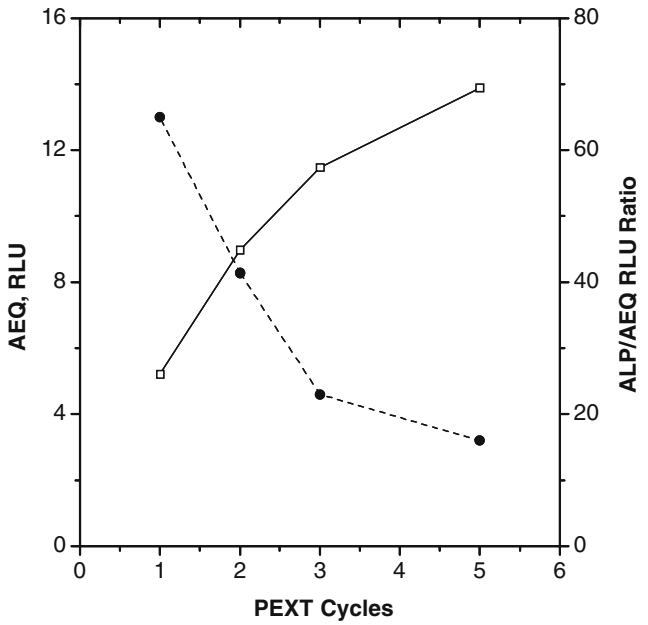

Fig. 3 Effect of the number of cycles in PEXT reaction. The signal obtained from aequorin reaction (AEQ) as well as the ALP/AEQ signal ratio are plotted versus the number of cycles. The sample was from a homozygote (M/M) for CYP2D6*3

signal for a M/M sample increases over two times when the Mg^{2+} concentration increases from 1 to 2.5 mM and then remains practically unchanged. The background of both ALP and AEQ reactions is independent of Mg^{2+} concentration. The background is defined as the luminescence obtained from the M-PEXT reaction of an N/N sample and the luminescence obtained from an N-PEXT reaction of an M/M sample. The above data demonstrate that an increase

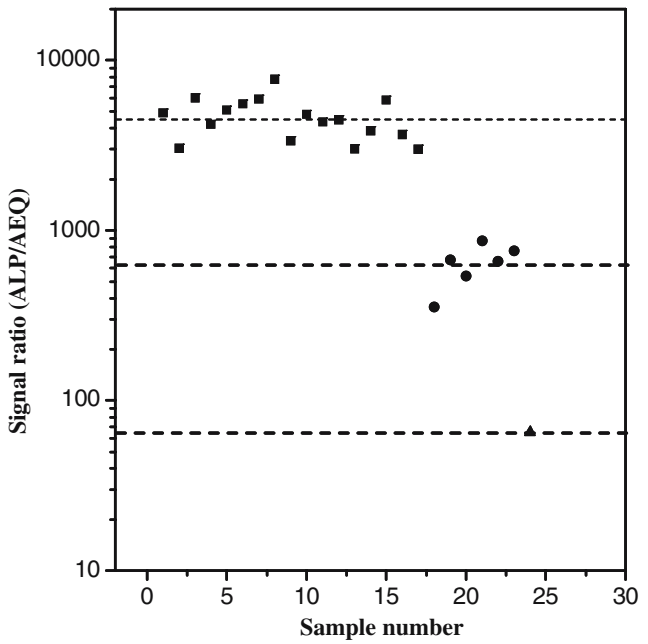

Fig. 4 Application of the proposed PEXT dual-analyte bio/chemiluminometric assay to the genotyping of the CYP2D6*3 mutation

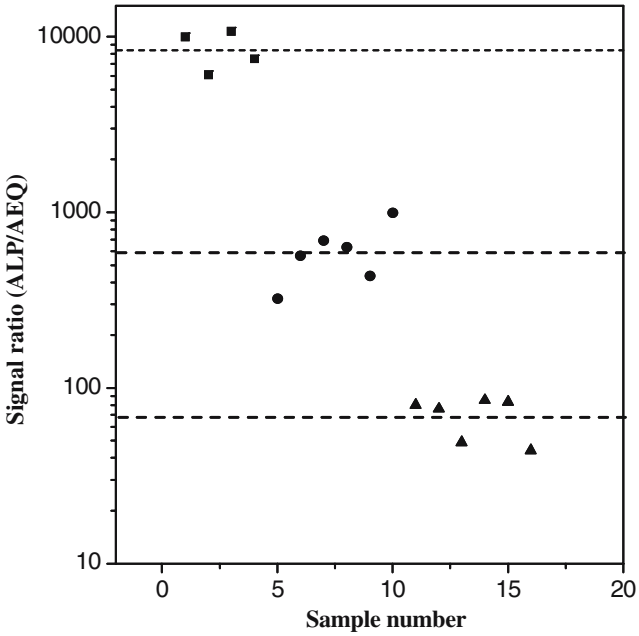

Fig. 5 Application of the proposed PEXT dual-analyte bio/chemiluminometric assay to the genotyping of the MBL2 -550 polymorphism

in Mg^{2+} concentration results in a higher yield of PEXT reaction in the case of a perfectly matched primer but does not affect the nonspecific extension reactions. The highest discrimination between the two alleles was observed at a Mg^{2+} concentration of 2 mM.

We tested the effect of primer annealing temperature in the range 55–70 °C and found that the discrimination of the two alleles was satisfactory in the entire range, despite of the fact that both the ALP and AEQ signals decreased as

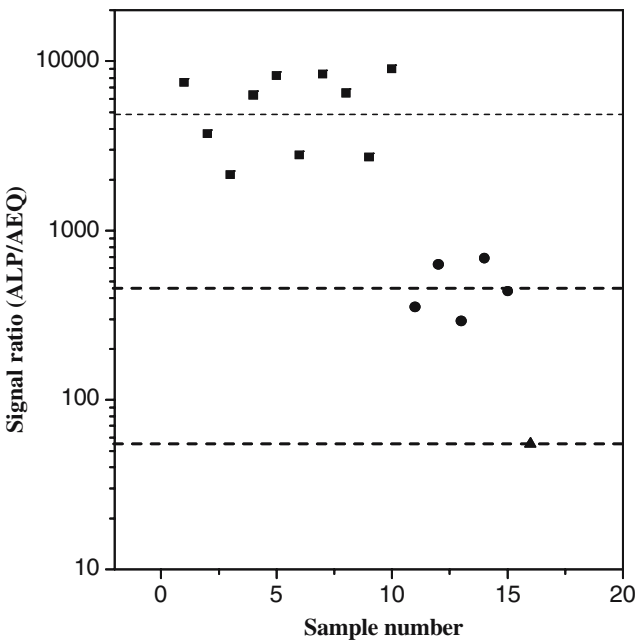

Fig. 6 Application of the proposed PEXT dual-analyte bio/chemiluminometric assay to the genotyping of the MBL2 -221 polymorphism

the temperature increased (due to decreasing efficiency of primer/template hybridization). An annealing temperature of 65 °C was chosen for further studies. The DNA polymerase concentration required for the extension of primers annealed to target DNA was optimized in the range 0.125–1 U per reaction using a sample from a normal individual. The ALP/AEQ signal ratio is slightly affected by increasing enzyme concentration. The optimum enzyme concentration for allele discrimination was found to be 0.25 U per reaction.

PEXT reaction consists of repeated cycles of target denaturation annealing of the primer and final extension of the primer. The effect of the number of cycles on the ALP/AEQ signal ratio was studied in the range from one to five cycles using an M/M sample. The results are presented in Fig. 3. We observe that the luminescence signal increases with the number of cycles and (as expected) the ALP/AEQ ratio for the M/M sample decreases. A PEXT of three cycles was selected for further studies because is provides both good discrimination of alleles and short assay time. The effect of the primer/target (template) molar ratio to the yield of PEXT reaction was also studied. We found the signal was not affected by the presence of a 2.5- to 40-fold molar excess of primer. Thus, a fivefold molar excess of primer was selected.

All optimized parameters, even though these were established for the detection of CYP2D6*3 mutation, were proven to be robust when applied to −550 and −221 mutations of the MBL2 gene with the only exception that Mg^{+2} concentration was optimal at 2.75 mM.

Application to clinical specimens

The proposed method was evaluated by detecting the CYP2D6*3 point mutation in 24 blood samples and two SNPs (−550 and −221) in the promoter region of the human mannose binding lectin gene, MBL2, in 16 blood samples. Extraction of genomic DNA, PCR and PEXT reactions were performed as described in the Experimental section and a pool of the two PEXT reaction products (N-PEXT and M-PEXT) was analyzed in the same microtiter well. The genotype for each sample is given by the ratio of the luminescence signals obtained from ALP and AEQ (ALP/AEQ). The results for CYP2D6*3, MBL2 -550, and MBL2 -221 polymorphisms are presented as scatter plots (i.e., plots of the ALP/AEQ signal ratio vs. the sample number) in Figs. 4, 5, and 6. The ALP/AEQ ratio is high (>3,000) for a normal sample, low in the case of a mutant homozygote (<100), and medium for a heterozygote (<1,000). Consequently, the proposed method provides very good discrimination between the two alleles.

The overall reproducibility of the method including PEXT reaction and the dual hybridization assay was assessed by analyzing samples of the three genotypes (N/N, N/M, M/M). The entire procedure was performed six times for each sample (same day). The reproducibility of the detection of the mutant allele in each sample, given by the coefficient of variation (%CV) of the signals obtained for AEQ, was found to be 18, 14, and 25% for N/N, N/M, and M/M genotypes, respectively. The reproducibility of the detection of the normal allele, given by the %CVs of the signals obtained from the ALP, was 10, 17, and 18% for N/N, N/M, and M/M genotypes, respectively.

Conclusions

The proposed method enables rapid genotyping of SNPs by selective labeling of PEXT reaction product with either biotin or digoxigenin and detection of the products by a dual-analyte (bio)chemiluminometric hybridization assay. The detection of both alleles is performed in the same reaction vessel and is based on the difference in light-emission kinetics between flash- and glow-type (bio) chemiluminescent reactions. Contrary to most PEXT-based genotyping methods, our method does not require time-consuming amplicon purification steps prior to PEXT reaction. The assay is performed in microtiter wells thus enabling automation and high-throughput genotyping.

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References

1. Collins E, Green ED, Guttmacher AE, Guyer MS (2003) Nature 422:835
2. Kruglyak L, Nickerson DA (2001) Nat Genet 27:234
3. Hirschhorn JN, Daly MJ (2005) Nat Rev Genet 6:95
4. Syvanen AC (2001) Nat Rev Genet 2:930
5. Kwok PY (2001) Annu Rev Genomics Hum Genet 2:235
6. Shi MM (2001) Clin Chem 47:164
7. Verhaegen M, Christopoulos TK (1998) Anal Chem 70:4120
8. Tannous BA, Verhaegen M, Christopoulos TK, Kourakli A (2003) Anal Biochem 320:266
9. Turner MW (2003) Mol Immunol 40:423
10. Petersen SV, Thiel S, Jensenious JC (2001) Mol Immunol 38:133
11. Ohlenschlaeger T, Garred P, Madsen HO, Jacobsen S (2004) N Engl J Med 351:260
12. Yarden J, Radojkovic D, De Boeck K, Macek M Jr, Zemkova D, Vavrova V, Vlietinck R, Cassiman JJ, Cuppens H (2004) J Med Genet 41:629
13. Daly AK (2003) Fundam Clin Pharmacol 17:27
14. Chen S, Chou WH, Blouin RA, Mao Z, Humphries LL, Meek QC, Neill JR, Martin WL, Hays LR, Wedlund PJ (1996) Clin Pharmacol Ther 60:522

15. Christopoulos TK, Ioannou PC, Verhaegen M (2006) Photoproteins in nucleic acids analysis. In: Daunert S, Deo SK (eds) Photoproteins in bioanalysis. Wiley, Weinheim
16. Roda A, Guardigli M, Michelini E, Mirasoli M, Pasini P (2006) Luminescent proteins in binding assays. In: Daunert S, Deo SK (eds) Photoproteins in bioanalysis. Wiley, Weinheim
17. Kricka LJ (1995) Nonisotopic probing, blotting and sequencing, 2nd edn. Academic Press, San Diego
18. Nakashima Y, Okano K, Kojima K, Shirakura H, Ishida S, Watanabe M, Maeda K, Tsunoda H, Imai Y, Nagai K (2004) Clin Chem 50:1417
19. Zhou G, Kamahori M, Okano K, Chuan G, Harada K, Kambara H (2001) Nucleic Acids Res 29:E93
20. Zerefos PG, Ioannou PC, Traeger-Synodinos J, Dimissianos G, Kanavakis E, Christopoulos TK (2006) Hum Mutat 27:279
21. Glynou K, Kastanis P, Boukouvala S, Tsaoussis V, Ioannou PC, Christopoulos TK, Traeger-Synodinos J, Kanavakis E (2007) Clin Chem 53:384
22. Zerefos PG, Ioannou PC, Christopoulos TK (2006) Anal Chim Acta 558:267
23. Emmanouilidou E, Tannous B, Ioannou PC, Christopoulos TK (2005) Anal Chim Acta 531:193