Clinical Applications of Whole-Blood PCR with Real-Time Instrumentation

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Background: As the genetic basis of many human diseases is being discovered, there is increasing need for the detection of single-nucleotide polymorphisms/mutations in medical laboratories. We describe an innovative approach that combines PCR amplification directly on whole blood and real-time detection PCR technology (WB-RTD PCR).

Methods: We compared WB-RTD PCR with the method for extracted DNA-RTD PCR for the detection of mutations in the prothrombin (n = 94), factor V Leiden (n = 49), and hemochromatosis (n = 22) genes. Mutation detection on the Roche LightCycler was based on use of fluorescence resonance energy transfer (FRET) probes and melting curve analysis. We also compared the WB-RTD PCR on the LightCycler and the ABI PrismTM 7700 sequence detection system with minor groove–binding nonfluorescent quencher probes.

Results: We obtained complete concordance between both methods in assigning genotypes. We also demonstrated that the WB-RTD PCR method can be performed on real-time PCR instruments from Applied Biosystems and the LightCycler. Omission of the need for DNA extraction and gel electrophoresis allowed substantial labor and cost savings with this method.

Conclusion: This approach has applications for testing other medically relevant single-nucleotide polymorphisms.

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Real-time detection PCR (RTD-PCR)4 has improved the efficiency of testing for genetic variants, but sample preparation remains a bottleneck. We have previously described a method for DNA analysis based on PCR amplification directly on whole blood (WB), followed by gel electrophoresis and ethidium bromide staining (1). The procedure includes the addition of formamide to lower the melting temperature (T_m) of DNA and enable PCR to be performed at lower temperatures (2). The lower temperature lessens protein denaturation, which is presumed to trap DNA and prevent amplification (1). This approach removes the need for a DNA extraction step but requires use of gel electrophoresis. Here we combine amplification directly on WB with RTD-PCR on either of 2 different platforms.

Materials and Methods

TEST SAMPLES
We used both the WB-RTD PCR LightCycler® method and the routine LightCycler method with extracted DNA to test 49, 94, and 22 samples collected for factor V Leiden (FVL), prothrombin (PT), and hemochromatosis (HFE) mutation testing, respectively. Mutation detection on the LightCycler was based on use of fluorescence resonance energy transfer (FRET) probes and melting curve analysis. Subsequent analysis of the WB-RTD PCR LightCycler method for HFE testing was performed on 160 samples received for routine testing. To determine whether the WB-RTD PCR method could be applied to an alternative RTD PCR chemistry and platform, we performed a prospective study comparing the WB-RTD PCR LightCycler and the ABI Prism™ 7700 sequence detection system (7700 SDS), using minor groove–binding nonfluorescent quencher (MGB-NFQ) probes. Using both meth-

* Nonstandard abbreviations: RTD PCR, real-time detection PCR; WB, whole blood; T_m, melting temperature; FVL, factor 5 Leiden; PT, prothrombin; HFE, hemochromatosis gene; FRET, fluorescence resonance energy transfer; MGB, minor groove binding; NFQ, nonfluorescent quencher; and 6-FAM, 6-carboxyfluorescein.
ods, we tested 151, 310, and 46 samples for the presence of the FVL, PT, and HFE gene mutations, respectively. Subsequent analysis with the WB-RTD PCR method on the 7700 SDS was performed on 246 and 724 routine samples tested for FVL and PT polymorphisms, respectively. From 7 separate FVL analytic runs, the mean (SD) of the 6-carboxyfluorescein (6-FAM) and VIC® signals were determined.

DNA EXTRACTION

DNA was extracted by use of an in-house rapid-boil/ alkaline lysis method or the QIAamp® DNA Blood Mini Kit (Qiagen). For the former, WB samples were centrifuged for 10 min at 675g, and a 200-μL volume was aspirated from the buffy coat layer and added to 1 mL of a lysis buffer (2 mL/L Trition X-100, 50 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0) in a 1.5-mL polypropylene tube, mixed, and centrifuged at 2880g for 20 s. NaOH (400 μL of a 50 mmol/L solution) was added to the pellet, mixed on a rotating mixer for 5 min, and then heated to 95 °C for 30 min in a heating block. After the tubes were removed from the heating block, 100 μL of 1 mol/L Tris (pH 8.0) was added, and the tubes were capped and vortex-mixed. Samples were stored at 4 °C for up to 2 weeks before analysis. DNA quantity and purity were not determined.

FORMAMIDE LYSA TE SOLUTION AND PCR MIXTURE

Direct testing on WB treated with formamide was performed on either acid-citrate-dextrose– or EDTA-anticoagulated blood (1). Samples were mixed on a rotor (Ratex: R.S.M.6) for 5 min at room temperature. A 60% formamide lysate solution consisting of 1.5 μL of WB, 18.0 μL of deionized formamide (Sigma), and 10.5 μL of distilled water was made for each sample and heated at 95 °C for 5 min in a thermal cycler. This was then cooled on ice before addition to the appropriate PCR mixture.

For all LightCycler assays, 2 μL of the 60% formamide lysate solution was added to a LightCycler glass capillary containing 8 μL of reaction master mixture, giving in a formamide concentration of 12% in the final reaction mixture. Details of the reaction mixture are shown in Table 1, which shows the final concentrations of reagents in 10 μL of PCR reaction mixture. The thermostable DNA polymerase used was Tth plus DNA polymerase (Biotech International). The final PCR mixture also contained 0.2 mM of each deoxynucleotide triphosphate as well as 0.04 g/L gelatin, 3.32 mM ammonium sulfate, 0.9 mL/L Triton X-100, and 13.4 mM Tris-HCl, pH 8.8 (Biotech International). Patient samples were generally tested within 3–5 days of collection; however, WB samples stored at room temperature were successfully analyzed up to 6 weeks after collection. We do not know how long the formamide lysate solution can be stored before testing.

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<th>Table 1. Final concentrations of reagents in the PCR reaction mixture used for LightCycler WB-RTD PCR assays.</th>
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* BSA, bovine serum albumin (Sigma).
template for 1 cycle of 94 °C for 2 min, followed by amplification of target for 45 cycles of 94 °C for 1 s, 50 °C for 10 s, and 70 °C for 10 s, each with a temperature transition rate of 20 °C/s. Melting curve analysis was as follows: 1 cycle of 94 °C for 1 s and 40 °C for 1 min, each with a temperature transition rate of 20 °C/s, followed by 44 °C for 10 s and 85 °C for 10 s with a ramping rate of 0.2 °C/s. The cycling conditions for HFE were as follows: denaturation of the template for 1 cycle of 94 °C for 2 min, followed by amplification of the target for 35 cycles of 95 °C for 30 s, 50 °C for 10 s, and 72 °C for 10 s, each with a temperature transition rate of 20 °C/s. Melting curve analysis was as follows: 1 cycle of 94 °C for 1 s with a temperature transition rate of 20 °C/s, and then 50 °C for 30 s and 94 °C for 1 s with a ramping rate of 0.2 °C/s. Fewer cycles were used during the PCR amplification phase of the analysis to obtain efficient amplification of the template. The method was based on the procedures described by Neoh et al. (4) and Mangasser-Stephan et al. (5).

FVL AND PT GENE MUTATION DETECTION ON THE 7700 SDS USING WB-RTD PCR

Allelic discrimination for all methods on the 7700 SDS was based on use of MGB-NFQ probes. The FVL and PT primers and probe sequences used for the 7700 SDS reactions were recommended by the Primer Express assay design software and are different from the LightCycler primers and probes (Table 1 of the online Data Supplement). The reagents and conditions for allelic discrimination on the 7700 SDS were as follows: FVL and PT G20210A mutation PCR amplifications were performed in separate 25-μL reactions. Each reaction contained 5 μL of formamide lysate preparation, 5 mM MgCl₂, 0.2 mM dNTPs, 67 mM Tris-HCl, 16.6 mM (NH₄)₂SO₄, 4.5 mM Triton X-100, 0.2 g/mL gelatin, 0.5 μL of ROX reference dye (Life Technologies), 0.9 μM each of the sense and antisense primers, 0.6 μM each of the wild-type and mutant MGB probes, and 2.2 μL of Tth polymerase. Amplification was performed in a 96-well spectrofluorometric thermal cycler (7700 SDS) as follows: initial denaturation at 85 °C for 5 min followed by 45 cycles of denaturation at 82 °C for 15 s and annealing and extension at 50 °C for 2 min. Each PCR cycling temperature was decreased by 10 °C from that recommended by Applied Biosystems because of the presence of formamide in the reaction. The fluorescence for each sample was measured and analyzed immediately after thermal cycling by the allelic discrimination post-PCR plate reading software.

QUALITY CONTROL

Blood samples from individuals homozygous, heterozygous, and without the FVL and HFE mutations and individuals heterozygous and without the PT mutation were identified and stored in 50-μL volumes at −80 °C for use as controls. The genotypes of these samples were confirmed by our routine LightCycler method using extracted DNA. For each run, aliquots were thawed at room temperature and tested in parallel with the appropriate patient WB samples. Deionized water was used as the negative control. No WB was available for a homozygous PT mutant donor; therefore, DNA extracted from a known homozygous PT mutant control was used in the development of this technique.

Results

FVL GENOTYPING

The FVL genotypes were determined on the LightCycler by differences in melting curve temperatures of the wild-type and mutant alleles. Typical melting curve profiles for FVL alleles for WB-RTD PCR and DNA-RTD PCR are shown in panels A and B, respectively, of Fig. 1. For each assay, the means (SD) of the Tₘs and the number of samples tested during 4 separate analytical runs are shown in Table 2. There was clear separation (−8.0–8.9 °C) of the Tₘs for the wild-type and mutant alleles for both the DNA-RTD PCR and WB-RTD PCR methods. The Tₘ was highly reproducible for all analytical runs. No fluorescence was observed in the no-template control. There was complete concordance of the genotypes for the 2 methods on the 49 samples tested (see Table 2 of the online Data Supplement).

PT GENOTYPING

The PT genotypes were determined on the LightCycler by differences in melting curve temperatures of the wild-type and mutant alleles. Typical melting curve profiles obtained by WB-RTD PCR and the DNA-RTD PCR for the PT G20210A mutation are shown in panels C and D, respectively, of Fig. 1. For each assay, the mean (SD) Tₘ and the number of samples tested over 3 separate analytical runs are shown in Table 2. There was clear separation (−6.9–7.7 °C) between the Tₘs for the wild-type allele and the mutant allele for both methods. For all cases (90 samples) in which a result was obtained, there was complete concordance in the genotypes between the 2 methods (see Table 2 of the online Data Supplement). Four samples, however, failed to amplify in the DNA-RTD PCR method and also failed on repeat testing. There were no homozygous mutant genotypes identified by either method. No fluorescence was observed in the no-template control.

HFE GENOTYPING

HFE genotypes were determined on the LightCycler by differences in melting curve temperatures of the wild-type and mutant alleles. Typical melting curve profiles for the HFE C282Y and H63D mutations detected by WB-RTD PCR and DNA-RTD PCR are shown in panels E through H, respectively, of Fig. 1. For each assay, the means (SD) of the melting curve and the number of samples tested over 3 separate analytical runs are shown in Table 2. In all cases there was clear separation (~6.0–
Fig. 1. LightCycler melting curve profiles obtained by WB-RTD PCR and DNA-RTD PCR, respectively, for FVL (A and B), PT (C and D), HFE C282Y (E and F), and HFE H63D (G and H) gene mutations.

In all cases the melting curve profiles for identifying the wild-type, heterozygous, and homozygous mutant genotypes are shown, and distinct melting curves can be identified by the differences in Tm's of the amplicons. For FVL (A and B), the wild-type (W) allele dissociates at 57 and 65 °C in the WB and DNA-RTD PCR methods, respectively, whereas the mutant (M) allele dissociates at 48 and 57 °C in the WB and DNA-RTD PCR methods, respectively. Heterozygous (H) genotypes are shown by the presence of 2 melting curve peaks at 57 and 65 °C for the WB and DNA-RTD PCR methods, respectively. For the PT mutations (C and D), the wild-type allele dissociates at 52 and 60 °C in the WB and DNA-RTD PCR methods, respectively, whereas the mutant allele dissociates at 60 and 68 °C in the WB and DNA-RTD PCR methods, respectively. Heterozygous results are shown by 2 melting curves: 52 and 60 °C and 60 and 68 °C for the WB and DNA-RTD PCR methods, respectively. For HFE C282Y (E and F), the wild-type allele dissociates at 45 and 52.5 °C in the WB and DNA-RTD PCR methods, respectively, whereas the mutant allele dissociates at 52.5 and 57 °C in the WB and DNA-RTD PCR methods, respectively. Heterozygous genotypes are shown by the presence of 2 melting curve peaks at 45 and 52.5 °C and 57 °C in the WB and DNA-RTD PCR methods, respectively. For the HFE H63D mutation (G and H), the wild-type allele dissociates at 52.5 and 59 °C in the WB and DNA-RTD PCR methods, respectively, whereas the mutant allele dissociates at 60 and 67.5 °C in the WB and DNA-RTD PCR methods, respectively. Heterozygous results are shown by 2 melting curves: 52.5 and 59 °C and 59 and 67.5 °C for the WB and DNA methods, respectively. NTC, no-template control.
6.4 °C) between the $T_m$s for the wild-type allele and the mutant allele. There was complete concordance in the genotypes between both assays for all 22 samples tested (see Table 2 of the online Data Supplement). In a subsequent WB-RTD PCR study of 160 HFE C282Y samples tested over 11 analytical runs, we were able to assign a genotype to 99.4% of the samples (24 mutant, 87 wild-type, and 48 heterozygous genotypes).

**WB-RTD PCR can be used on the 7700 SDS**

We assessed the performance of the WB-RTD PCR technique for the detection of the FVL and PT genotypes, using an alternative real-time chemistry and thermocycler: MGB-NFQ probes on the 7700 SDS. We tested 151 and 310 WB samples over 11 analytical runs for FVL and PT mutations, respectively, on both the LightCycler and the 7700 SDS and obtained complete concordance for the FVL and PT genotypes. Similarly, we tested 46 HFE C282Y samples over 3 analytical runs and found complete concordance for both RTD PCR methods (see Table 3 of the online Data Supplement). Subsequently, we have shown that the WB-RTD PCR assay on the 7700 SDS is robust. In an analysis of 724 PT and 246 FVL routine samples tested over 3 months, we were unable to report a genotype in only fewer than 1.7% of samples (12 PT and 4 FVL samples), a result at least comparable to our previous experience with methods using extracted DNA.

A representative FVL analytical run using WB-RTD PCR on the 7700 SDS is shown in Fig. 2, in which there is clear differentiation of the 3 genotypes. To evaluate the reliability of genotype assignment, we calculated the mean (2 SD) intensity for the VIC signal (allele 1; x axis) and 6-FAM signal (allele 2; y axis) for each genotype from 7 analytical runs. For the homozygous mutant samples (n = 23), the means (2 SD) were 2.9 (0.4) and 3.0 (0.6) for alleles 1 and 2, respectively; for the heterozygous samples (n = 35), the means were 4.8 (0.6) and 2.5 (0.6) for alleles 1 and 2, respectively; and for the wild-type samples (n = 29), the means were 5.6 (0.4) and 1.2 (0.6) for alleles 1 and 2, respectively (Fig. 2). As shown in Fig. 2, there was minimal overlap of the 3 genotypes.

**WB-RTD PCR can be used on stored samples**

We assessed the suitability of these assays for use on samples stored at room temperature over time. A heterozygous PT sample was tested at multiple time points after collection. The results from days 3 and 39 showed very little difference in fluorescence readings (0.38 and 0.36 for the mutant allele and 0.44 and 0.41 for the wild-type allele, respectively) and for $T_m$s (60.2 and 59.8 °C for the mutant allele and 52.4 and 52.1 °C for the wild-type allele, respectively).

**Discussion**

We have developed a genetic testing protocol that combines RTD PCR and amplification on WB without the need for DNA extraction. We demonstrated that, after careful optimization of each assay, the WB-RTD method can determine the presence of commonly requested clin-
ical mutations in 3 genes on 2 of the more frequently used RTD PCR chemistries and platforms.

The WB-RTD PCR method has numerous advantages over conventional genetic testing. It is simple, highly reproducible, and inexpensive. Only a small amount of starting material is required, and as a result, amplification without DNA extraction has the potential to be used on alternative samples such as buccal cells, blood spots, and hair follicles. Furthermore, the method enables the testing of samples long after sample collection. We have shown that satisfactory results can be obtained on a PT sample stored at room temperature for 39 days after collection.

The use of WB-RTD PCR eliminates the need for DNA extraction and therefore allows a shorter turnaround time and can provide a result in 40 or 120 min for the LightCycler and the 7700 SDS instruments, respectively.

WB-RTD PCR tests for FVL, PT, and HFE gene mutations using real-time analysis were demonstrated to be a robust and simple protocol offering sensitivity and specificity. Analysis of clinical samples for the FVL and PT mutations for over 3 months has shown that, once optimized and validated, the approach is at least as robust as analysis of extracted DNA.

Discrimination of wild-type, heterozygous, and homozygous mutant genotypes by the use of FRET probes and melting curve analysis or TaqMan probes was highly reproducible. The FVL, PT, and HFE results for WB-RTD PCR were in complete agreement with the results obtained with routine DNA-RTD PCR. In fact, the WB-RTD PCR method resolved melting curves for 4 PT samples that could not be resolved on initial and repeat testing by DNA-RTD PCR. DNA quantification indicated low DNA yield, which presumably accounted for the assay failures.

Apart from the presence or absence of the mutation, several other conditions influence the shape of the melting curve for assays based on FRET probes, including hybridization-probe concentration and buffer composition. However, the reliability of our method was not markedly influenced by relatively moderate changes in any of these conditions. For example, our data showed that the Mg\(^{2+}\) concentration used on the LightCycler method influenced the fluorescence \(\langle dF/dT \rangle\) obtained, but variations in the range of 1–5 mM Mg\(^{2+}\) had little influence on the difference between the \(T_m\) for wild-type and mutant alleles (data not shown). However, it is important to note that the concentration of formamide does influence \(T_m\): the greater the final formamide concentration, the greater the reduction in \(T_m\) (2). With appropriate adjustments, we have shown the WB-RTD PCR method can be adapted to the 7700 SDS.

In conclusion, once optimized, the WB-RTD PCR method greatly reduces the time for testing, reduces the possibility of sample mix up, minimizes the number of steps for template amplification, and eliminates the need for dedicated post-PCR laboratory space. There are also substantial cost and labor savings because DNA extraction and gel electrophoresis are not required.

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References