Single Nucleotide Polymorphism (SNP) Genotyping in Unprocessed Whole Blood and Serum by Real-Time PCR: Application to SNPs Affecting Homocysteine and Folate Metabolism, *Arve Ulvik* and Per Magne Ueland* (LOCUS for Homocysteine and Related Vitamins, University of Bergen, Armauer Hansens hus, N-5021 Bergen, Norway; *author for correspondence: fax 47-5597-4605, e-mail arve.ulvik@farm.uib.no)

Several single nucleotide polymorphisms (SNPs) have been identified that affect folate and homocysteine metabolism, which in turn are implicated in the pathogenesis of cardiovascular disease *(1)*, neural tube defects *(2)*, and

colorectal cancer *(3)*. These SNPs include methylenetetrahydrofolate (*MTHFR*) C677T *(1)*, *MTHFR* A1298C *(4)*, methionine synthase (*MTR*) A2756G *(5, 6)*, and methionine synthase reductase (*MTRR*) A66G *(7)*.

To determine the relationships of these and other SNPs with the risk of chronic diseases, along with possible interactions with environmental factors, large population-based epidemiologic studies are necessary. This requires high-throughput methodologies for SNP determination.

SNP genotyping can generally be divided into two steps: sample preparation, e.g., purification of DNA from blood, and allele detection. To date, great advances have been made in allele detection because novel technologies for DNA analysis have been developed *(8)*. The DNA purification step is required because the enzymes used for manipulation of DNA (e.g., polymerases) are susceptible to inhibition by substrate impurities. Purification of DNA is often labor-intensive, time-consuming, and costly and enhances the risk of back- or cross-contamination of samples. The development of automated DNA purification methods has been aimed at alleviating some of these problems *(9)*, but may increase the need for expensive equipment. Other researchers have investigated the feasibility of PCR amplification without prior DNA purification *(10–12)*.

Allele-detection methods based on real-time PCR are attractive because signal amplification and allele detection are accomplished in a single, closed tube *(13)*. For this type of assay, DNA purification seems mandatory because blood constituents such as hemoglobin may interfere with the acquisition of the fluorescence signals.

In this report, we demonstrate the successful detection of SNPs in untreated whole blood and serum by use of real-time PCR [using the 5′nuclease (TaqMan®) assay *(14)*] as applied to the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphisms.

Samples of ~1 μL of whole blood or 7 μL of serum (archival samples, collected in standard, non-gel barrier tubes) were deposited at the bottom of 96-well microtiter plates (polypropylene plates supplied by Abgene or Applied Biosystems) used for real-time PCR. This was carried out manually or by a robotic pipetting station. The samples were allowed to dry at room temperature, and could be stored at 4 °C for several months before analysis.

Table 1 shows the sequences for the primers and probes (Eurogentec) used for genotyping *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G. 4-(4-Dimethylaminophenylano)benzoic acid (DABCYL) was used as quencher for the probes, 6-carboxyfluorescein was used as the reporter for the wild-type allele, and 6-carboxy-4,7,2′,7′-tetrachlorofluorescein was used as the reporter for the variant allele. PCR reactions were initiated by overlaying the in-well dried samples with 40 μL of PCR mastermix, including DNA polymerase from *Thermus brockianus* (DynAZyme II; Finnzymes), and immediately mounting the plate in the real-time PCR instrument (model SDS 7700; Applied Biosystems). The reaction buffer was 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 1 mL/L Triton X-100, as supplied by Finnzymes, with the addition of 3.5 mM $MgCl_2$, 125 μM each dNTP, 300 nM each primer, probes, and 0.25 U of DNA polymerase. Optimal concentrations of the probes varied for each assay (Table 1). After a preincubation of the reaction mixture at 95 °C for 3 min, thermocycling was 94 °C for 10 s and 58 °C 40 s for 45 cycles. Data handling and generation of the output graphics were carried out by the Sequence Detector program (Ver. 1.6.3; Applied Biosystems) for Macintosh.

The multicomponent view for the detection of the three genotypes of the *MTRR* A66G polymorphism with the use of dried whole blood as template is shown in Fig. 1. The color-coded traces, here designated by labels (A and G),

Table 1. Primers and probes for genotyping of four SNPs.

Gene and genotype	Primer/Probe	Reporter[a]	Sequence[b]	Probe concentration, nM
MTHFR	Primer fw		TGACCTGAAGCACTTGAAGGAGAA	
	Primer rev		GGAAGAATGTGTCAGCCTCAAAGA	
C677T	Probe C	FAM	ATGAAATCGGCTCCCG	125
	Probe T	TET	ATGAAATCGACTCCCG	85
MTHFR	Primer fw		GGAGGAGCTGCTGAAGATGTG	
	Primer rev		TCTCCCGAGAGGTAAAGAACAAA	
A1298C	Probe A	FAM	AAAGACACTTTCTTCACTG	100
	Probe C	TET	AGACACTTGCTTCACTG	120
MTR	Primer fw		AGGAAATCATGGAAGAATATGA	
	Primer rev		TACCACTTACCTTGAGAGAC	
A2756G	Probe A	FAM	ATTAGACAGGACCATTATGA	100
	Probe G	TET	TTAGACAGGGCCATTATG	60
MTRR	Primer fw		CAAAGGCCATCGCAGAAG	
	Primer rev		AAGATCTGCAGAAAATCCATGT	
A66G	Probe A	FAM	AATATGTGAGCAAGCTG	65
	Probe G	TET	AATGTGTGAGCAAGCT	120

[a] FAM, 6-carboxyfluorescein; TET, 6-carboxy-4,7,2′,7′-tetrachlorofluorescein.
[b] The variant base in underlined. Sequence is 5′ to 3′.

show the progression of the PCR reaction according to genotype. A signal from the probe specific for the A or G allele only indicates a homozygous AA or GG genotype, respectively. Simultaneous signals from both probes demonstrate a heterozygous AG genotype. Similar results were obtained for the three other polymorphisms, *MTHFR* C677T, *MTHFR* A1298C, and *MTR* A2756G, detected by the probe pairs given in Table 1 (data not shown).

We compared the allele-specific fluorescence signals from dried blood deposits with the signals from the same amount of whole blood mixed and dissolved in the PCR solution (n = 16). We observed no fluorescence signal for most of the dissolved blood samples (n = 11). For the remaining samples (n = 5), the output signal was characterized by an increased cycle threshold value and low intensity. Mixing of 7 μL of serum into the PCR solution also led to complete inhibition of the reaction. These observations suggest that DNA in the dried blood and serum spots is available for PCR amplification and that inhibitory substances, or material quenching the fluorescence signal, are not efficiently released into the PCR mixture.

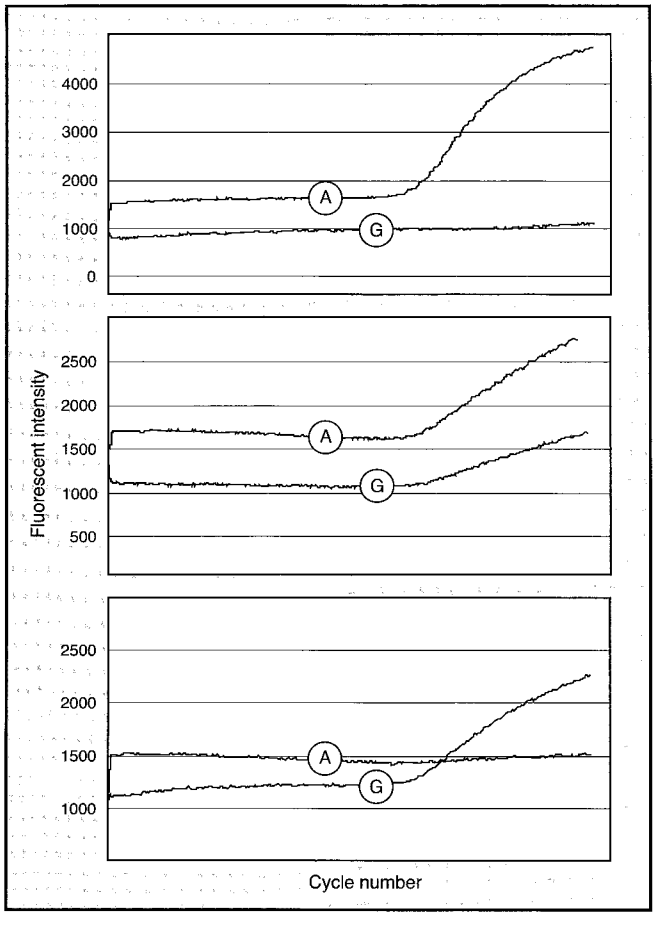

Fig. 1. Allele calling of the *MTRR* A66G polymorphism by real-time PCR.

The separate traces are denoted *A* and *G*, according to the actual base substitution defining the alleles. The *top*, *middle*, and *bottom* panels show the homozygous AA, heterozygous AG, and homozygous GG genotype, respectively.

Inhibition of the PCR reaction by components in whole blood has been shown to be dependent on the thermostable DNA polymerase. DNA polymerases from *T. aquaticus* (e.g., AmpliTaq Gold, which is the enzyme most frequently used in the 5′nuclease assay) are highly susceptible to inhibition *(15)*, whereas the polymerase derived from *T. brockianus* used in the present work is more resistant to impurities *(16)*. However, we have previously genotyped samples of dried blood cell spots using a polymerase from *T. aquaticus (17)*, strengthening our view that the drying of blood material entails efficient entrapment of inhibitory material.

The drying conditions were not critical to the success of the assay. Plates were dried for 1 h to overnight at ambient conditions or in the refrigerator (low humidity) for up to several months.

The assay for *MTHFR* C677T was in complete agreement (n = 100) with results obtained with an alternative technique based on allele-specific amplification and capillary electrophoresis *(18)*. In addition to whole blood samples, we applied the same procedure (drying of 1 μL into microtiterplates) to large sample collections of packed blood cells obtained after centrifugation of EDTA blood and to cell fractions obtained after centrifugation of serum. More than 6000 packed blood cell samples from the Hordaland Homocysteine Study *(19)*, 1000 serum cell fractions, and 2500 serum samples from the JANUS serum bank *(20)* have been genotyped. The success rates were >99% for whole blood, packed blood cells, and cell fraction, and ~95% for the serum samples. For all sample types, the typical fluorescent signal response (difference in signal intensity at cycle 45 compared with baseline) was ~50% of that obtained with purified DNA (QIAamp; Qiagen). The throughput of the assay with the SDS 7700 for PCR and detection is ~400 samples per an 8-h workday.

In conclusion, we have demonstrated the utility of real-time PCR for the determination of SNPs in whole blood and serum. The samples are spotted and dried at the bottom of the PCR tubes and not mixed into the PCR solution. Under these conditions, inhibition of the PCR reaction and/or quenching of the fluorescent signal do not interfere with detection of the alleles, and successful genotyping of >99% of samples is obtained in 1 μL of whole blood. Thus, this homogeneous assay for SNP determination sidesteps the need for sample purification, leading to considerable savings in time, cost, and effort.

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