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RESEARCH PAPER

Digital camera and smartphone as detectors in paper-based chemiluminometric genotyping of single nucleotide polymorphisms

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Abstract Chemi(bio)luminometric assays have contributed greatly to various areas of nucleic acid analysis due to their simplicity and detectability. In this work, we present the development of chemiluminometric genotyping methods in which (a) detection is performed by using either a conventional digital camera (at ambient temperature) or a smartphone and (b) a lateral flow assay configuration is employed for even higher simplicity and suitability for point of care or field testing. The genotyping of the C677T single nucleotide polymorphism (SNP) of methylenetetrahydropholate reductase (MTHFR) gene is chosen as a model. The interrogated DNA sequence is amplified by polymerase chain reaction (PCR) followed by a primer extension reaction. The reaction products are captured through hybridization on the sensing areas (spots) of the strip. Streptavidin-horseradish peroxidase conjugate is used as a reporter along with a chemiluminogenic substrate. Detection of the emerging chemiluminescence from the sensing areas of the strip is achieved by digital camera or smartphone. For this purpose, we constructed a 3D-printed smartphone attachment that houses inexpensive lenses and converts the smartphone into a portable chemiluminescence imager. The device enables spatial discrimination of the two alleles of a SNP in a single shot by imaging of the strip, thus avoiding the need of dual labeling. The method was applied successfully to genotyping of real clinical samples.

Keywords Chemiluminescence · Single nucleotide polymorphisms · Genotyping · Smartphone · Digital camera

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Introduction

Chemi(bio)luminescent reactions have been employed widely as reporting systems for bioanalytical assays, including molecular imaging, because they provide higher detectability and wider dynamic range than spectrophotometric and fluorometric systems [1]. These advantages arise from the fact that, in chemiluminescence, the excited molecule is generated during the course of a chemical reaction, as opposed to techniques requiring light for the excitation. Consequently, the two main sources of background, namely, scattering of the excitation radiation and sample autofluorescence, are eliminated. Furthermore, contrary to fluorometric methods, photobleaching is not a concern in chemiluminometric ones.

The advantages described above have led to the development of various chemiluminometric assays for nucleic acid analysis addressing a plethora of challenges such as the detection of polymerase chain reaction (PCR) products [2, 3], quantitative PCR [4–6], determination of microRNA [7, 8], screening for unknown mutations [9], and genotyping of single nucleotide polymorphisms [10–12]. The chemiluminescent acridinium esters [8, 13], the electrochemiluminescent tris(bipyridyl) ruthenium(II) complex [14], and the photoprotein aequorin were used as reporters [5, 9, 12]. For signal amplification, enzymes, such as alkaline phosphatase, horseradish peroxidase, and galactosidase, were used as labels along with chemiluminogenic substrates [15, 16]. Further

signal amplification was introduced either by attaching multiple enzyme molecules, with the branched DNA system, or by using an enzyme-coding DNA sequence as label [17, 18].

Paper-based lateral flow devices for nucleic acid analysis involve immobilization of the target DNA sequences on the biosensor and detection, usually, by means of a hybridization assay. The continuous running of buffer due to capillary action ensures effective removal of the excess of reagents. Functionalized gold nanoparticles, quantum dots, carbon nanoparticles, and polysterene microspheres have been used as reporters [19–26].

Single nucleotide polymorphisms (SNPs) represent the most common type of genetic variation. SNPs may affect gene function by amino acid substitution, modification of gene expression, or alteration of gene splicing. As a consequence, SNPs constitute a new generation of biomarkers for disease susceptibility, prognosis, and response to medication. Genotyping of thousands SNPs per sample has been achieved by the microarray technology, which is a valuable tool for large association studies. However, in the clinical laboratory, one or a few SNPs are analyzed routinely for each disease. To this end, the development of simple, low-cost chemiluminometric genotyping methods is highly desirable, especially for areas of limited resources and minimal infrastructure.

Chemiluminometric assays for SNP genotyping comprise (i) exponential amplification of the target sequence, usually by PCR, (ii) allele-discrimination reaction, and (iii) detection of the reaction products, for each allele, by exploiting a chemiluminescent reporter [10–12]. Simultaneous detection of two or more alleles can be accomplished by employing multiple labels and/or exploiting differences in the kinetics of light emission from various reporters [15, 27, 28]. For instance, dual-allele genotyping can be achieved by combining a glow-type and a flash-type chemiluminescent reporter. It should be noted that, in all the above assays, chemiluminescence measurements were performed using luminometers with a photomultiplier tube as the detector.

The present work aims at the development of chemiluminometric genotyping methods that combine the following advantages: (a) detection is performed by using either a conventional digital camera or a smartphone, (b) a lateral flow assay configuration is employed for even higher simplicity and suitability for field testing, and (c) the device enables spatial discrimination of the two alleles of a SNP in a single shot by imaging of the lateral flow membrane, thus avoiding the need of dual labeling. Contrary to previous methods that used a cooled CCD camera for detection of the low chemiluminescence emission [29], in the present work, the imaging by a conventional digital camera takes place in ambient temperature. Furthermore, using a 3D printer, we constructed a smartphone attachment that houses inexpensive lenses and converts the smartphone into a portable chemiluminescence imager. Smartphones are ubiquitous and cost effective; they offer high computational power and provide wireless data transfer ability that facilitates rapid reporting of genotyping results. During the last 2 years, chemiluminometric immunoassays for bile acids, cholesterol, and cortisol, in a lateral flow format, were combined with smartphone-based detection [30, 31]. Herein, the research activity is expanded in the area of chemiluminometric nucleic acid analysis, particularly DNA genotyping. As a model, the genotyping of the C677T SNP of MTHFR gene was chosen. MTHFR is a key enzyme in folate metabolism. Individuals with the 677CC genotype are "normal." Homozygotes with the 677TT genotype and heterozygotes (677CT) have significantly decreased MTHFR activity that leads to hyperhomocysteinemia, which is associated with cardiovascular disease.

Materials and methods

Apparatus and reagents

PCR amplification and primer extension reactions were performed in the MJ Research PTC-0150 thermal cycler (Watertown, MA). A digital camera, Konica Minolta DYNAX 5D (Konica Minolta Holdings, Tokyo, Japan), was used for imaging of the test spots on the lateral flow membrane. The iPhone 4 (Apple, CA), with a 5-MP camera, was used for imaging of the test spots. An extra lens 47-460 (Edmund Optics, Barrington, NJ) was employed for better capture of the chemiluminescence. The Gel Analyzer software was purchased from Kodak (New York, NY). Immunopore FP nitrocellulose membrane was from Whatman (Florham Park, NJ). The wicking pad, glass-fiber conjugate pad, and absorbent pad were from Schleicher & Schuell (Dassel, Germany). Streptavidin-horseradish peroxide conjugate (SA-HRP) and the supersignal ELISA femto maximum sensitivity substrate were from Thermo Scientific (Waltham, MA). Carboxylated microspheres (5.68×10^6 particles μL^{-1}, 2 μm in diameter) were from Polysciences (Warrington, PA). Phusion DNA polymerase was from New England Biolabs (Ipswich, UK), and Platinum Tsp DNA polymerase was from Thermo Fisher Scientific. Ultrapure deoxyribonucleoside triphosphates (dNTPs) were purchased from Invitrogen (Carlsbad, CA). Biotin-16-dUTP was from Clontech (Mountain View, CA). QIAamp DNA blood mini kit was from Qiagen (Hilden, Germany). Bovine serum albumin (BSA), glycerol, Tween-20, and all other common reagents were from Sigma (St. Louis, MO). Oligonucleotides used as primers in the course of this study (Table 1) were synthesized from MWG-Biotech (Ebersberg, Germany) and Invitrogen (Carlsbad, CA).

Table 1 Oligonucleotide sequences used as primers and probes

Oligonucleotide	Name	Sequence (5′ → 3′)	Size (nt)
PCR primers	U-MTHFR	TCATCCCTCGCCTTGAACAG	20
	D-MTHFR	GGGAGCTTATGGGCTCTCCT	20
PEXT primers	dT-N	T_{24} GAAGGAGAAGGTGTCTGCGGGAGC	49
	dT-M	T_{24} GAAGGAGAAGGTGTCTGCGGGAGT	49
	Tag-N	CTTTTCATCTTTTCATCTTTCAATGAAGGAGAAGGTGTCTGCGGGAGC	48
	Tag-M	CAATATCATCATCTTTATCATTACGAAGGAGAAGGTGTCTGCGGGAGT	48
Capture probes	Antitag-N	ATTGAAAGATGAAAAGATGAAAAG	24
	Antitag-M	GTAATGATAAAGATGATGATATTG	24

Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4, and1.8 mM KH_2PO_4, pH 7.4. Saline sodium citrate (SSC) buffer contained 0.9 M NaCl and 90 mM $Na_3C_6H_5O_7$, pH 7.0. Tris-EDTA (TE) buffer contained 10 mM Tris and 1 mM EDTA, pH 8.0.

Preparation of oligonucleotide-functionalized microspheres

A 12.8-μL aliquot of carboxylated microspheres was used. The microspheres were washed with 125 μL of 0.1 M 2-(*N*-morpholino) ethanesulfonic acid buffer (MES, pH 4.5) and centrifuged for 2 min at 13,000 rpm. The pellet was resuspended in 40 μL MES buffer. A 1-μL aliquot of NH_2-oligo (400 μM) and a 1.25-μL aliquot of EDC (0.4 g mL^{-1}) were added to the mixture, followed by 30-min incubation at ambient temperature. The reaction was completed by another addition of 1.25 μL EDC (0.4 g mL^{-1}) and 30-min incubation. A 2-μL aliquot of 100 mL L^{-1} Tween-20 solution was then added and the mixture was centrifuged for 2 min at 13,000 rpm. The oligonucleotide-functionalized microspheres were washed twice with TE-Tween buffer (100 μL TE and 2 μL of 100 mL L^{-1} Tween-20) and centrifuged for 2 min at 13,000 rpm. Finally, the microspheres were resuspended in 100 μL TE buffer.

Fabrication of the lateral flow strip

The strip (4 mm × 70 mm) consisted of an immersion pad, a conjugate pad, a laminated membrane, and an absorbent pad assembled on a plastic adhesive backing that provides the required rigidity. The four parts were positioned in such a way that their ends overlapped in order to ensure continuous flow (by capillary action) of the developing solution from the wicking pad up to the absorbent pad. Aliquots (0.5 μL) of oligonucleotide-functionalized microparticles were deposited on the membrane to form the "test spots" of the sensor.

PCR of the MTHFR gene

Genomic DNA was isolated from whole blood by using the QIAamp DNA blood mini kit. PCR was performed to amplify the segment of MTHFR gene that contains the polymorphism. The PCR mixture (50 μL) contained 1 U Phusion DNA polymerase, 1× Phusion HF buffer, 1.5 mM $MgCl_2$, 200 μM of each of the dNTPs, 0.3 μM of upstream and downstream primer, and 2 μL of genomic DNA. Cycling parameters were initial denaturation at 98 °C for 45 s, 35 cycles at 98 °C (for 10 s), 58 °C (for 15 s), 72 °C (for 10 s), and a final extension step at 72 °C for 5 min. The size of the PCR product was confirmed by 2 % agarose gel electrophoresis and ethidium bromide staining and quantified by densitometric analysis.

Paper-based chemiluminometric genotyping assay using a digital camera or a smartphone as detectors

Single-allele genotyping assay

Each PCR product was subjected to two separate allele-specific primer extension reactions, containing either normal primer (N) or the mutant primer (M). The PEXT mixture of 20 μL contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgSO_4$, 10 μM each of dATP, dCTP, dGTP, 5 μM dTTP, 5 μM biotin-dUTP, 5 pmol of PEXT primer, 1 U Platinum Tsp DNA polymerase, and ~100 fmol of PCR product. The reactions were performed in the thermal cycler under the following conditions: an initial denaturating step of 95 °C for 3 min followed by 5 cycles of 95 °C for 15 s, 68 °C for 15 s, and 72 °C for 10 s. The PEXT products, before applying onto the strip, were denatured at 95 °C for 5 min and placed immediately on ice.

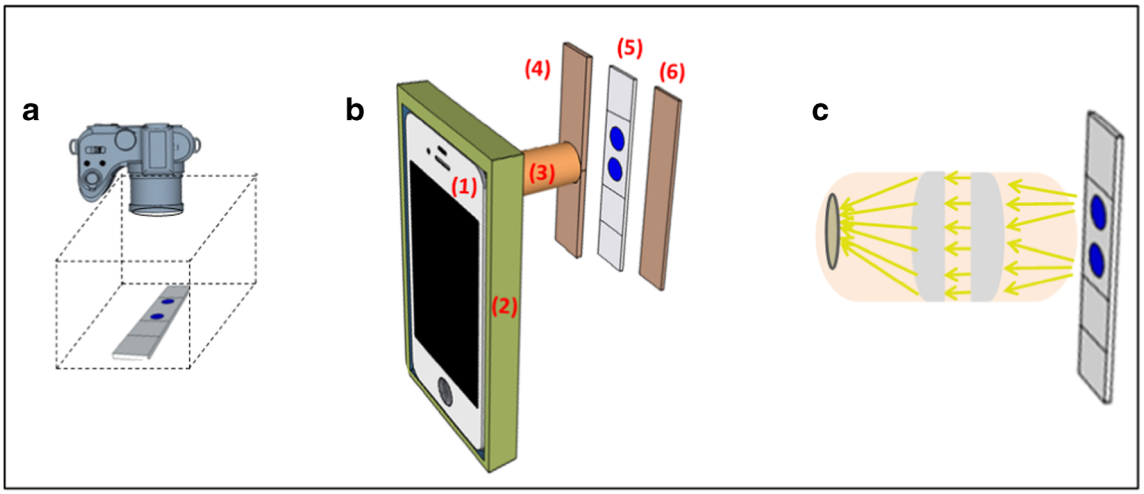

Fig. 1 (**a**) Scheme for chemiluminescence imaging of the genotyping strip by a digital camera. (**b**) Layout of the portable device for the chemiluminescence imaging of the genotyping strip using a smartphone. The device consists of a simple smartphone case, a focus lens hood, and a case with cover for the lateral flow strip. (*1*) Smartphone, (*2*) smartphone case, (*3*) lens holder, (*4*) case for the genotyping strip, (*5*) strip, and (*6*) cover of the strip case. (**c**) Close up of the lens holder. Two plano-convex lenses are used to direct and focus the light emerging from the sensing areas (spots) of the strip onto the camera of the smartphone

A 5-μL aliquot of the denatured extension product and 5 μL of streptavidin-HRP (diluted 1000 times in PBS containing 6 % BSA and 0.2 % Tween-20) were applied onto the membrane of the strip. The strip was then immersed into 250 μL of the developing solution (3 % glycerol, 1 % BSA, 0.2 % Tween in SSC buffer) for 25 min. The strip was placed into a dark box (25-cm distance between the strip and the camera) in order to avoid ambient light (Fig. 1). The light emission was triggered by the addition of 10 μL of the chemiluminogenic substrate onto the membrane. The emitted light was captured immediately by the digital camera with an exposure time of 10 s.

The presence of the normal and/or the mutant allele was detected by the formation of a blue spot at the designated positions of the membrane.

Dual-allele genotyping assay

The PCR-amplified DNA was subjected to a dual-allele primer extension reaction. Both the normal (N) primer and the mutant (M) primer were added into the reaction mixture (5 pmol of each). Each primer contained a characteristic oligonucleotide tag at the 5′ end. The extension reaction was carried out in a total volume of 20 μL as above (2.5.1.1). Fifteen extension reaction cycles were performed, and the cycling parameters were as described above ("Single-analyte (single-allele) lateral flow genotyping assay" section).

For image acquisition with a smartphone, an iPhone 4 with a 5-MP camera was used. An extra lens 47-460 of Edmund Optics was employed for better capture of the signal. A mini darkbox smartphone accessory was designed with the Google Sketch Up software and printed using the 3D printer Reify 3D Solus and Makergear M2. The smartphone accessory consisted of the following parts: (i) a simple smartphone case, (ii) a focus lens hood, and (iii) a case and cover for the lateral flow device. The dimensions of the focus lens hood were 8 mm height and 15 mm external diameter, and the dimensions of the case for the lateral flow device are 4 × 15 × 90 mm. The layout of the accessory is shown in Fig. 1.

A 5-μL aliquot of the denatured extension product and 5 μL of streptavidin-HRP solution were applied to the membrane of the lateral flow strip. The strip was then immersed into 250 μL of the developing solution for 25 min. The strip was placed in the smartphone accessory, and 10 μL of the chemiluminogenic substrate was added onto the membrane. The emitted light was captured immediately by the smartphone with an exposure time of 8 s. The presence of the normal and/or mutant allele appears as bright blue spot(s) in the image.

Results and discussion

Single-allele chemiluminometric assay

The principle of the assay is illustrated in Fig. 2. The sensing area (test spot) of the lateral flow strip consists of immobilized oligo(dA)-conjugated microspheres. For the genotyping, each PCR product is subjected to two separate primer extension reactions, each containing an allele-specific primer. These primers consisted of (a) a $(dT)_{24}$ sequence at their 5′ end, (b) a sequence complementary to the PCR product, and (c) the

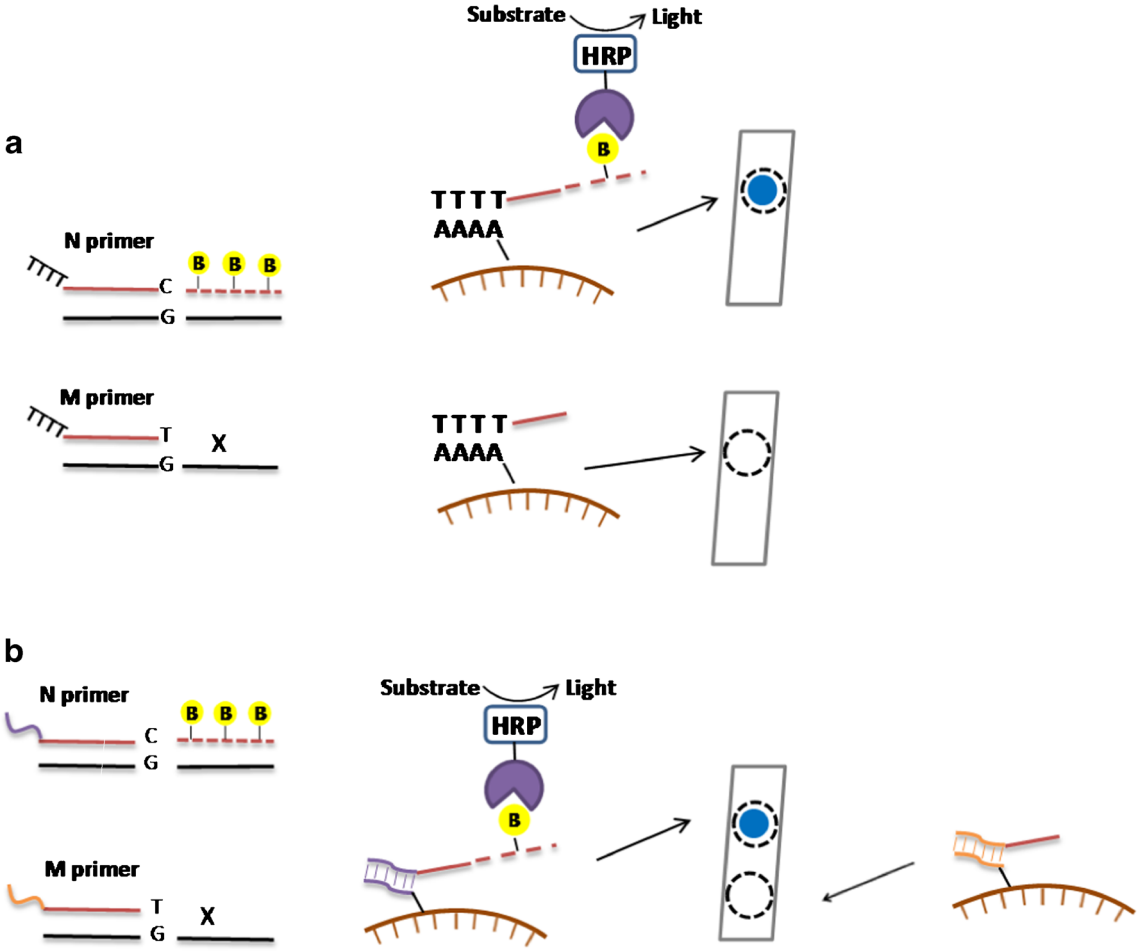

Fig. 2 Principle of chemiluminometric strip tests for genotyping of single nucleotide polymorphisms (SNPs). (**a**) Single-allele assay that requires two strips for the genotyping of one SNP. (**b**) Dual-allele assay in which both alleles (normal and mutant) are detected on the same strip. *B* biotin, *SA* streptavidin, *HRP* horseradish peroxidase

base of the mismatch at the 3′ end that differs for each primer. The primer is extended by the specific DNA polymerase, only if it is perfectly complementary to the target. During extension, biotin-dUTP is incorporated in the newly synthesized strand. The products of the two extension reactions are denatured and applied to two strips, along with streptavidin-HRP. The strips are immersed into the developing solution. As the developing solution migrates, by capillary action, the extension products are bound to the oligo(dA)-microspheres at the test spots via (dA)/(dT) hybridization. It should be noted that the amount of the oligonucleotide-functionalized microspheres deposited at the test spots is adequate for hybridization with the extension primer. The streptavidin-HRP conjugate is then captured by the hybrids via streptavidin–biotin interaction. A blue chemiluminescent spot is generated after the addition of the substrate. The genotype is obtained by taking the images of the membranes of the two strips. Thus, a sample with normal genotype (N/N genotype) gives a chemiluminescent spot only from the strip that detects the extension product of the N primer. On the other hand, a homozygote for the mutation (M/M genotype) gives chemiluminescence signal only from the strip that detects the extension product of the M primer. A heterozygote (N/M genotype) gives positive result with both strips. A separate negative control is not necessary because a sample with a normal genotype constitutes the negative control for the mutant homozygote sample and vice versa.

The proposed genotyping assay was optimized with respect to SA-HRP concentration, substrate incubation time, and the camera shutter speed for image acquisition. The effect of SA-HRP concentration on the signal intensity of the spots was studied by preparing serial dilutions of the conjugate in PBS buffer containing 6 % BSA and 0.2 % Tween-20. Biotinylated oligonucleotide-conjugated microspheres were used as a target. Detection was performed with a digital camera. The results are presented in Fig. 3a. We observed that the signal

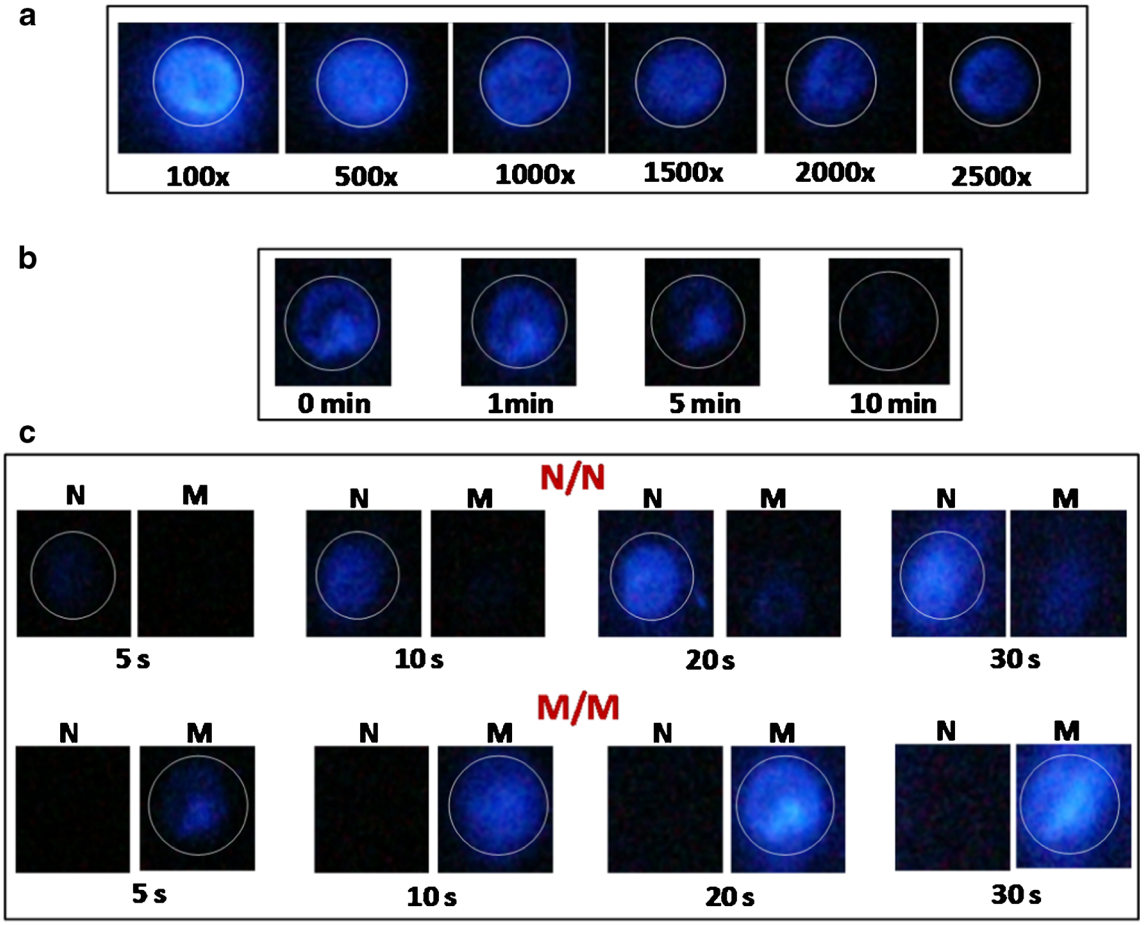

Fig. 3 Optimization studies for the chemiluminometric strip test with respect to (**a**) SA-HRP concentration, (**b**) substrate incubation time, and (**c**) the camera shutter speed for image acquisition. Microspheres conjugated with a biotinylated oligonucleotide were used as a target for the optimization in (**a**) and (**b**). For the optimization of the camera shutter speed (panel (**c**)), a normal sample (N/N genotype) and a mutant homozygote (M/M genotype) were used. Two strips were required for each sample, one strip for the detection of the extension product of the normal primer and the other strip for the detection of the extension product of the mutant primer. *N* normal, *M* mutant

decreases with increasing dilution factors and that a 1000-fold dilution of SA-HRP provided a high signal with minimum background level. The signal dependence on the incubation time of the chemiluminogenic substrate was studied at 0, 1, 5, and 10 min. The most intense signal with a low background (Fig. 3b) was obtained at immediate capture of the image (0 min). For the optimization of the shutter speed (exposure time) of the digital camera, a normal sample (N/N genotype) and a mutant homozygote (M/M genotype) were used. The effect of shutter speed was studied at 5, 10, 20, and 30 s. The optimum exposure time was at 10 s, although the signal was higher at 20 and 30 s, as it combines high intensity of the spots with low background signal (Fig. 3c). The data from the densitometric analysis of the spots, for the optimization studies, are presented in Fig. S1 of the Electronic Supplementary Material (ESM).

The repeatability of the single-allele genotyping assay was assessed by analyzing, in triplicate, the extension products of a normal (N/N), a heterozygote (N/M), and a homozygote for the mutation (M/M) sample. The results are shown in Fig. 4. The CVs were estimated by densitometric analysis of the images and were found to be 4.8 and 10.2 % for the normal and the mutant sample, respectively ($n = 3$). The heterozygote sample gave CVs of 8.2 and 10.6 % for the normal and mutant allele, respectively ($n = 3$). The densitometric data are also presented in Fig. 4.

Dual-allele chemiluminometric genotyping assay

PCR-amplified DNA was subjected to a dual-allele extension reaction, using both the normal (N) primer and the mutant (M) primer. Each primer contained a unique oligonucleotide tag at the 5′ end and differed from the other primer only in one base at the 3′ end. Due to the specificity of DNA polymerase, the primer is extended only upon perfect complementarity with

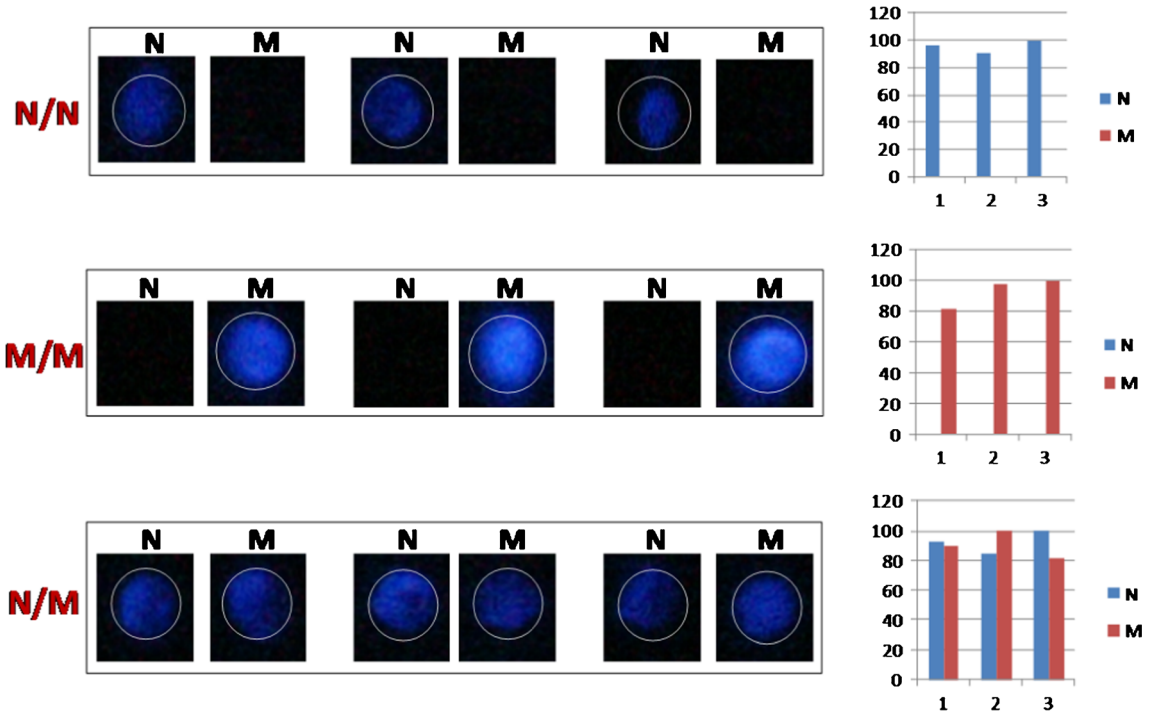

Fig. 4 Repeatability of the single-allele genotyping assay. The extension products of a normal (N/N), a heterozygote (N/M), and a homozygote for the mutation (M/M) sample were analyzed, each by two strips. The densitometric data are shown next to the spots

the target sequence. Biotin-dUTP was incorporated during the extension step.

The lateral flow membrane contains two independently functionalized sensing areas (spots). As the sample migrates along the strip, the extension primer that corresponds to the normal allele is captured at the bottom spot through hybridization of its tag-N sequence with the immobilized antitag-functionalized microspheres. The extension primer for the mutant allele hybridizes to the top spot through hybridization of its tag-M sequence with the antitag-M conjugated microspheres. The streptavidin-modified HRP was captured to the hybrids via streptavidin–biotin interaction, forming a chemiluminescent spot after the addition of the substrate. The genotype was obtained by taking the results of a single strip detecting the extension product(s) of both the normal primer (N) and the mutant primer (M). Thus, a normal genotype (N/N genotype) gives chemiluminescent signal only for the extension of the N primer, forming a blue spot only at the bottom of the membrane. A homozygote (M/M genotype) gives chemiluminescent signal only for the extension of the M primer generating blue emission at the top of the membrane. A heterozygote (N/M genotype) gives positive results for both test spots.

The accuracy of the method was assessed by using genomic DNA from eight samples that were previously genotyped and include all genotypes (N/N, M/M, and N/M). The results are presented in Fig. 6. In all cases, the proposed method gave the correct genotype. Data from the densitometric analysis of the spots are presented in Fig. S2 (ESM).

Data pertaining to the repeatability of the dual chemiluminometric genotyping assay were obtained by analyzing in triplicate samples representative of all three genotypes. The results are shown in Fig. 5. The CVs of the spot intensities were found to be 14 and 9 % for the normal and mutant alleles, respectively.

The genotyping assay was performed using smartphone camera as a chemiluminescence imager. iPhone 4 was utilized for this study. An accessory device was constructed with a 3D printer and adjusted in front of the camera. The accessory is accompanied by a removable case where the biosensor is placed. The whole layout of the accessory is shown in Fig. 1. The LongExpo pro (version 3.2.2) application was used for image capturing. The exposure time of the smartphone camera was set at 8 s. Typical results for the eight clinical samples are presented in Fig. 6. Data from the densitometric analysis of the spots are shown in Fig. S2 (ESM). We observe that the genotyping results achieved by using the smartphone are in full concordance with those obtained by the digital camera.

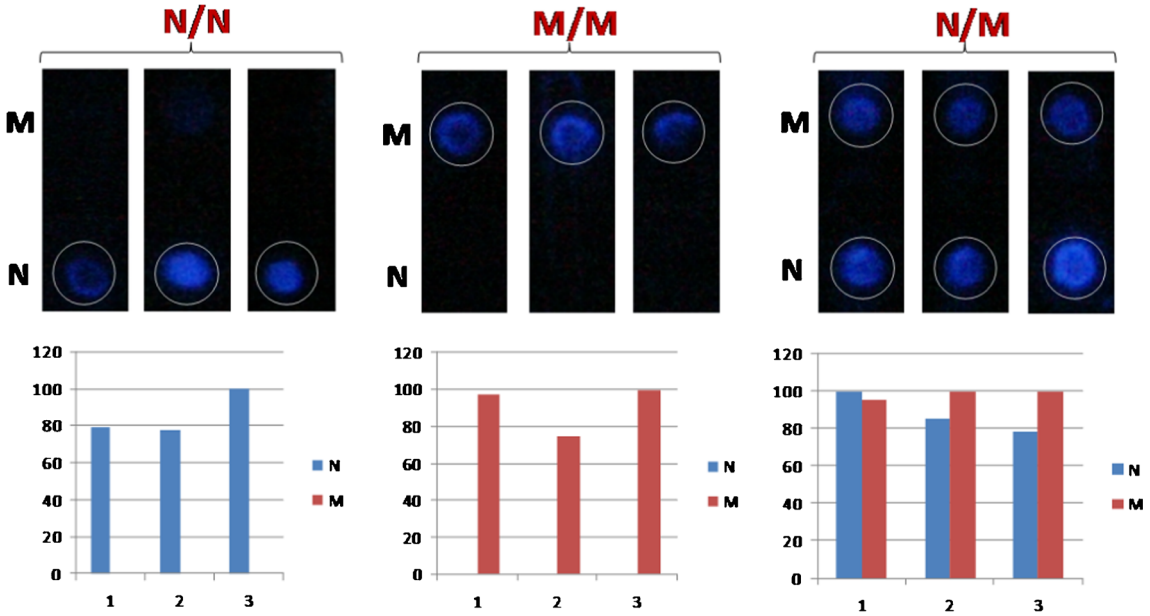

Fig. 5 Repeatability of the dual-allele genotyping assay. The extension products of a normal (N/N), a heterozygote (N/M), and a homozygote for the mutation (M/M) sample were analyzed, each by one strip only. The densitometric data are also presented at the bottom

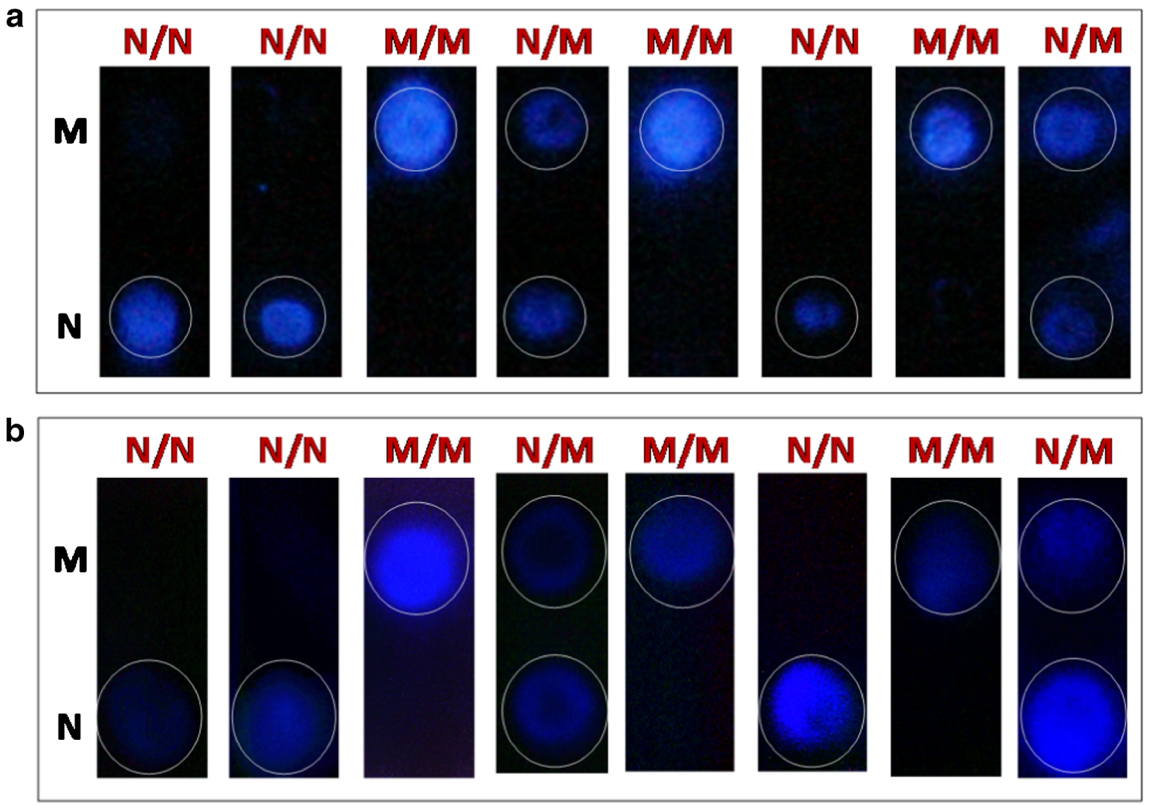

Fig. 6 Performance of (**a**) the digital camera-based and (**b**) the smartphone-based chemiluminometric paper test for genotyping of the MTHFR C677T SNP in eight clinical samples. The genotypes (N/N, M/M, or N/M) are denoted above the strips. The sensing areas for the normal (N) and mutant (M) alleles are shown on the left of the strips

Conclusions

We have developed chemiluminometric strip tests for SNP genotyping by using a digital camera or a smartphone as low-cost imagers. Following PCR, the method requires a primer extension reaction (20 min) and detection of the extension products (25 min). The PCR products are pipetted directly to the extension reaction mixture without prior purification. Similarly, the extension products are pipetted directly on the strip without prior treatment. The amount of amplified DNA required for the extension reaction is about 100 fmol. Since 1/4 of the extension reaction volume was applied to the sensor, the signal corresponds to an amount of 25 fmol of amplified DNA. The method was applied successfully to genotyping of real clinical samples. The lateral flow strip format simplifies the assay because it eliminates several pipetting, washing, and incubation steps. The use of a digital camera and especially a smartphone circumvents the need for costly equipment and enhances the portability of the device and its suitability for point of care or field testing as well as facilitates rapid wireless communication of test results. Because of these distinct advantages, and the pervasiveness of the smartphones, we believe that the proposed devices will transform the way chemiluminometric nucleic acid tests are performed.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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