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ABSTRACT

Conventional blood group phenotyping by hemagglutination assays, carried out pretransfusion, is unsuitable in certain clinical situations. Molecular typing offers an alternative method, allowing the deduction of blood group phenotype from genotype. However, current methods require a long turnaround time and are not performed on-site, limiting their application in emergency situations. Here, we report the development of a novel, rapid multiplex molecular method to identify seven alleles in three clinically relevant blood group systems (Kidd, Duffy and MNS). Our test, using a dry-reagent allele-specific lateral flow biosensor, does not require DNA extraction and allows easy visual determination of blood group genotype. Multiplex linear-after-the-exponential (LATE)-PCR and lateral flow parameters were optimized with a total processing time of one hour from receiving the blood sample. Our assay had a 100 % concordance rate between the deduced and the standard serological phenotype in a sample from 108 blood donors, showing the accuracy of the test. Owing to its simple handling, the assay can be operated by non-skilled health-care professionals. The proposed assay offers the potential for the development of other relevant single nucleotide polymorphism (SNP) panels for immunohematology and new applications, such as for infectious diseases, in the near future.

INTRODUCTION

Blood transfusion is a lifesaving procedure that replaces blood cells lost through severe bleeding, during surgery when blood loss occurs, or to increase the blood count in anemic patients. Alloimmunization is one of the main side effects of blood transfusion that could severely complicate further red blood cell transfusions, especially for patients with diseases requiring multiple transfusions (e.g., those with sickle cell disease or thalassemia)[1]. Therefore, blood group compatibility between the recipient and the transfused blood is mandatory. This process involves determining the patient's blood group and identifying the presence of alloantibodies against relevant red blood cell antigens. Hemagglutination is the standard method for the determination of red blood cell phenotype. This serological technique is simple and sensitive, but is not adapted in some limited situations, such as recently transfused patients or the presence of interfering autoantibodies[2-4]. Molecular typing for red blood cell antigens offers an alternative for blood group phenotype deduction, and the benefits of genotyping strategies in pretransfusion practice have been reported[5-7]. Several blood group genotyping platforms are commonly used in clinical environments[8-10]; however, current platforms using high-throughput devices are more suited for mass-scale screening[11-13], and these analyses have to be performed by highly skilled personnel in accredited molecular testing laboratories[14]. Moreover, these methods cannot rapidly provide red blood cell genotypes and thus cannot be utilized for emergency transfusions.

Tests performed near or on-site, where the patient encounters the health care system, can be advantageous when a rapid decision is needed or when suitable facilities and logistic chains are unavailable. In the field of classical on-site diagnostics, lateral flow biosensors are the best-established commercial products and several studies for blood group phenotyping have been reported[15-19]. The technical advantage of these sensors is that they are self-operating devices that perform rapid assay from a single sample, and results can be obtained by visual

inspection[20-22]. Recently, emerging molecular diagnostics have met requirements for speed, low cost, and ease of use for POC applications. Another advantage of POC molecular diagnostics is their ability to multiplex analyses, which allows simultaneous detection of multiple nucleic acid sequences on a single device[23]. However, most current amplification procedures require nucleic acid isolation before DNA amplification.

Here, we present a novel method for rapid visual detection of blood group genotype with an approximately one hour turnaround time. Our genotyping assay was designed for use in immunohematology laboratories and it is a complementary method to conventional hemagglutination assays. It is based on a multiplex LATE-PCR amplification performed directly from whole blood without DNA extraction. The single-stranded amplicons migrate on a dry-reagent lateral flow dipstick, where they interact with allele-specific capture probes. Gold nanoparticles are used as a reporter and permit the detection of blood group SNPs by the generation of red dots that are visible to the naked eye. The test was developed for deducing clinically relevant blood group antigens of Duffy (FY), MNS (glycophorin B; GYPB) and Kidd (JK) blood group systems. Four SNPs corresponding to seven alleles (*FY*01, FY*02, FY02N.01, GYPB*03, GYPB*04, JK*01* and *JK*02)* were investigated. The validation study carried out on a cohort of 108 samples from phenotyped donors is presented.

Materials and methods

Chemicals and materials. Methanol, sucrose, sodium dodecyl sulfate (SDS), Tween® 20, and formamide were purchased from Sigma-Aldrich. Anti-biotin antibodies conjugated gold nanoparticles (40 nm, 9 x 10^{10} particles/mL) were purchased from BBI Solutions (Cardiff, United Kingdom). Sodium chloride-sodium citrate (SSC) buffer 20x concentrate (pH 7.0) and biotinylated bovine serum albumin (BSA) were purchased from Thermo Fisher Scientific (Waltham, MA). KAPA2G Fast HotStart DNA Polymerase and HotStart PCR kit were

obtained from Kapa Biosystems (Wilmington, MA). Oligonucleotides primers and probes were synthesized by Eurogentec (Seraing, Belgium). Nitrocellulose membranes, immersion pads, glass-fiber conjugate pads and absorbent pads were purchased from Merck Millipore (Darmstadt, Germany). PCRs were performed with a TProfessional instrument (Biometra, Germany). Membrane and conjugate pads were dried in an incubator with natural convection from Binder (Tuttlingen, Germany). The strips were incubated in a hybridization incubator from Techne (Cole-Parmer, Staffordshire, UK) and scanned on a desktop scanner, Epson perfection V600 photo (Epson, Japan).

Blood donor samples. The validation of the assay was conducted on a cohort of 108 blood donation samples. After informed consent, whole blood samples were collected from random donors in EDTA tubes. Samples were collected in the South of France by the Etablissement français du sang (EFS) Occitanie and were mainly from donors of Caucasian descent. The study protocol was approved by the local ethics committee (DC-2016-2689) and by the research ethics committee (CPP). Extended blood group phenotypes for FY1, FY2, JK1, JK2, MNS3 and MNS4 antigens were determined by routine serological hemagglutination techniques in the Blood Donation Qualification Laboratory (Montpellier, France) with licensed antisera (Diagast, Loos, France) associated to AHG anti-IgG PK and LISS PK solution (Diagast, Loos, France) with the PK system from Beckman Coulter (Brea, CA).

Preparation of lateral flow strips. The lateral flow biosensor was composed of four components: an immersion pad, a glass-fiber conjugate pad, a nitrocellulose membrane and an absorbent pad (Figure 1A). All parts were assembled on an adhesive backing card (5 mm x 93 mm) as follows. The nitrocellulose membrane (6.5 cm x 0.5 cm) was placed first. The absorbent pad was positioned above the membrane, overlapping by 5 mm. The conjugate pad

was placed below the membrane, overlapping by 2 mm. The immersion pad was placed below the conjugate pad, overlapping by 1 mm.

The eight capture oligonucleotide probes were manually spotted on the membrane in spotting buffer (6X SSC, 2 % methanol, 2 % sucrose) with a final volume of 0.1 μL at optimized concentration (Table 1). These probes were designed using OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA)[24], with the allele-specific SNPs placed in central position to enhance allele discrimination. A poly(dT)15 spacer was introduced in the 5′ position between the amino-linker and probe's sequence. A solution of 25 μg/mL biotinylated-BSA in spotting buffer (0.1 μL) was applied in triplicate at the top of the strip to define a control zone for the assay. After spotting, the membranes were dried in an incubator at 80°C for 40 minutes. Anti-biotin conjugated gold nanoparticles (1.5 x 10^8 particles) were loaded on the conjugate pad and were dried in an incubator at 60°C for 15 minutes. The strips were stored dry in a desiccator cabinet at room temperature.

Direct multiplex LATE-PCR amplification of whole blood. OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA)[24] was used to design the eight PCR primers, including four 5′-biotinylated forward primers (Table 2). In accordance with LATE-PCR primer design rules, primers were designed to flank the SNP site of each blood group antigen, which generate single-stranded products with a higher efficiency[25,26]. The PCR fragment size ranged from 86 to 133 bp.

Multiplex LATE-PCR amplification was carried out on 2.5 μL of whole blood in a final volume of 20 μL containing 1X KAPA2G Buffer, 3 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.5 U of KAPA2G Fast HotStart DNA Polymerase (5 U/μL), and primers at selected concentrations (Table 2). PCR amplification was optimized by testing biotin-labeled excess primer (1 μmol/L) at different primer ratios with limiting primer (1:5, 1:10 and 1:20) as well

as the number of PCR cycles (35, 40 and 45 cycles). Multiplex LATE-PCR started with enzyme activation at 95°C for 30 seconds, followed by a number of cycles composed as follow: 5 seconds at 95°C, 10 seconds at 55°C, and 10 seconds at 72°C. Negative controls containing water instead of whole blood were included in each series of PCR.

Visual detection of blood group genotype. Without any purification or denaturation, PCR products (1.5 μL) were applied to the bottom of the membrane, above the conjugate pad (Figure 1B). The immersion pad was dipped in 200 μL of pre-warmed running buffer in a 50 mL conical tube (Greiner Bio-one, Germany) and incubated at the optimized temperature for 20 minutes. During optimization migration/hybridization temperatures of 53, 55 and 57°C were tested. The running buffer contained 1X SSC, 0.5 % SDS, 10 % Tween® 20 and formamide. The effect of formamide concentration (0, 2.5, 5 and 10 %) was also studied during the optimization. Visual detection of red dots on control zone allows validation of the assay. The genotype of the sample can be determined from the positions of red dots on the detection zone (Figure 1A). An interpretation card was designed primarily as an aid in deducing phenotype from genotype data and as an archiving tool.

RESULTS AND DISCUSSION

Assay for visual blood group genotyping

Currently, molecular-based platforms for blood group genotyping are time-consuming, labor intensive, and they are ill-adapted to clinical situations in which urgent transfusion is needed. Considering the potential of dry-reagent dipstick assays in making detection of nucleic acid sequences rapid and simple, we developed a visual lateral flow-based assay of whole blood for multiplex detection of blood group SNPs (Figure 1B).

Amplification of four genomic DNA targets is performed by multiplex LATE-PCR from whole blood to produce 5′-biotinylated single-stranded products. PCR amplicons are applied to the ready-to-use lateral flow strip. The migration/hybridization is achieved in a dry oven. Under the effect of capillary migration, the dried anti-biotin conjugated gold nanoparticles are rehydrated with the running buffer and released from the conjugate pad. As the solution moves along the membrane, the PCR products migrate and hybridize to their complementary capture oligonucleotide probes in the detection zone. Hybridized biotinylated amplicons are detected by anti-biotin antibodies conjugated to gold nanoparticles. Aggregation of gold nanoparticles enables visual detection as red spots. Excess nanoparticles are captured at the control zone of the dipstick by immobilized biotinylated-BSA, forming three red spots that indicate the proper reagent flow in each device. After the assay has been completed, the lateral flow strip is placed on the interpretation card to deduce blood group phenotype based on the visual detection of dots. The validity of an assay is determined by the two following criteria: (i) generation of three red dots on the control zone and (ii), there is at least one positive allele per SNP in the FY and JK probe groups. Both *GYPB*03* and *GYPB*04* probes can have a negative result, consistent with the rare MNS:-3,-4 phenotype found in some Black populations. This infrequent phenotype (4 in 300 blood donors in Mali[27]) is due to a deletion of the *GYPB* gene[28].

Optimization of multiplex LATE-PCR amplification directly from whole blood

The KAPA2G Fast HotStart DNA Polymerase is based on a second-generation polymerase with an ability to synthesize DNA faster than other DNA polymerases, allowing reduction of the duration of the steps during each cycle of PCR. This enzyme remains functional in the presence of PCR inhibitors, such as hemoglobin or immunoglobulin present in blood samples[29], supporting its use for PCR amplification directly on blood collected on EDTA.

We used LATE-PCR since it produces single-stranded DNA, allowing hybridization without purification and denaturation steps[25,26]. To generate enough single-stranded DNA, we optimized for the 4-plex amplification both the primer ratio, between the limiting and the excess primers (1μmol/L), and the number of PCR cycles. Ten blood donor samples, with known blood group phenotype, were used to develop the amplification protocol. Our optimization criterion was the naked-eye detectability of the different genotypes for each blood group SNPs. As shown in Figure 2A, a 1:5 primer ratio (0.2 μmol/L of limiting primer) resulted in undetectable signals for both *GYPB*03* and *GYPB*04* alleles (samples 1-3) and a weak signal for the *JK*02* allele in a heterozygous sample (sample 2). At a 1:10 primer ratio (0.1 μmol/L of limiting primer), *GYPB*04* is detected only in homozygous samples (samples 1 and 2). As before, *JK*02* is barely visible to the naked eye in a heterozygous sample (sample 2). Stronger signals were obtained for all probes with a 1:20 ratio primer (0.05 μmol/L of limiting primer) allowing clear determination of sample genotype.

Thereafter, optimization of the number of LATE-PCR cycles was performed (Figure 2B). After 35 cycles, label intensities were low for all probes, with almost undetectable signals for any allele in sample 5. Again, detection of *GYPB*03* and *GYPB*04* probes appear to be particularly vulnerable to PCR conditions. The color intensity increased on all probes with the number of cycles without observing nonspecific signals, and 45 PCR cycles were shown to be suitable for unambiguous detection of blood group genotypes (*GYPB*04* probe in sample 6).

Thus, optimized LATE-PCR protocol consists of 45 PCR cycles in the presence of 0.05 μmol/L of limiting primer and 1 μmol/L of excess primer (1:20).

Optimization of migration conditions on the dry-reagent lateral flow dipstick

Using the optimized parameters determined in the previous section, we evaluated effects of the migration/hybridization temperature and the running buffer composition on the

determination of blood group genotype (Figure 3). The temperature of migration is one of the most critical parameters for the performance of nucleic-acid-based assays[30]. Based on the calculated melting temperature (T_m) of capture probes, lateral flow migration was performed at 53, 55 and 57°C (Figure 3A). At 53°C, weak nonspecific signals were observed for *FY*01* and *JK*02* in sample 3. At 57°C, the incubation conditions were too stringent, which resulted in low signals in all samples with dots that were almost undetectable for *JK*01* (sample 1), *JK*02* (sample 2) and *FY*wt* (samples 1-3). At 55°C, specificity was achieved, and hybridization of non-complementary amplicons with immobilized probes was eliminated.

The effect of formamide concentration (0, 2.5, 5 and 10 %) in the running buffer was also investigated. In accordance with its function as denaturing agent for DNA duplex preventing unspecific hybridization, the absence of formamide leads to nonspecific signals on all capture probes in all samples (Figure 3B). With 2.5% of formamide, nonspecific red spots were still visible for *FY*01* (sample 6), *FY*02* (sample 5), *GYPB*03* (sample 4 and 6), *GYP*04* (sample 5) and *JK*02* (sample 5). An increase of formamide up to 5% leads to the elimination of nonspecific signals while maintaining high color intensity. In the case of 10% formamide, the solution was too stringent, resulting in the absence of red spots in the detection zone, while signals in control zone remained intense.

Therefore, optimal conditions for migration/hybridization were a temperature set at 55°C in the presence of 5% formamide.

Prospective evaluation of the lateral flow-based assay on blood donor samples

The analytical performance of our lateral flow-based assay for visual blood group genotyping was assessed using a validation cohort made of 108 blood samples from random donors with known blood group phenotypes. All samples showed red spots in the control zone and therefore all were considered as valid. The genotype for the three blood group systems was

determined using the interpretation card. Phenotypes deduced from genotypes were compared with phenotypes obtained by serology. There was a 100 % concordance between the lateral flow-based and standard hemagglutination assays for the 864 alleles investigated (Table 3). The allele distribution in the validation cohort allowed the specificity of hybridization to be demonstrated, with a minimum of 15 negative samples for each allele (except the *FY*wt* allele, which had only one negative sample). Blood group frequencies observed in our cohort were coherent with previous prospective analysis of blood donors[31].

The interpretation card was developed for two main reasons. First, it allows proper interpretation of genotypes by non-skilled healthcare professionals, especially for the Duffy blood group, which includes detection of two SNPs for three alleles (*FY*01, FY*02 and FY*02N.01)*. Secondly, the card ensures the traceability of analyses. Indeed, at the end of the analysis, the strip is fixed and, after air drying, it can be archived, scanned or photographed in accordance with regulations (Figure 4).

Altogether, these results show the accuracy and the performance of our rapid and easy-to-use visual genotyping assay using dry reagent dipstick for rapid deducing blood group phenotype.

CONCLUSION

We developed a novel approach for rapid detection of SNPs using a ready-to-use lateral flow DNA microarray strip. Our full assay is based upon nucleic acid specificity within <1-hour turnaround time, without the necessity for gel electrophoresis, and with 100 % accuracy in concordance with standardized procedures. Compared to other commercially available blood group genotyping tests, such as BLOODchip ID[32] (Grifols, Spain), BeadChip[33] (BioArray Solutions - Immucor, Warren, NJ) or BAGene SSP Diagnostics[34] (BAG Healthcare, Germany), our assay provides significant improvements in terms of processing time. Hence, the simplicity of the procedure allows our assays to be performed with minimal training

requirements. The test was designed for use in immunohematology laboratories, since it allows the determination of deduced extended phenotype for six clinically relevant blood group antigens (*i.e.* JK1, JK2, FY1, FY2, MNS3 and MNS4). The assay was designed for carrying out unitary testing in specific clinical situations, such as pre-transfusion testing for patients when serological methods have failed or in emergency situations. The short turnaround time (approximately one hour) is highly compatible with such situation. Current developments are in progress to increase the performance of the assay, such as the automated dispensing of probes with the aim to make deduced phenotype determination more reader-friendly.

To avoid false positive as far as possible, we designed the test by taking into account both the ethnicities and the frequencies of variant alleles in the French population. We include detection of *FY*02N.01* allele which is frequent in people of African ancestry[27]. False positive remain possible for the MNS3 antigen due to the presence of silent alleles (*GYPB*03N.01* and/or *GYPB*03N.03*). The detection of these alleles was not included in our panel because of their very low frequencies (0.002 and 0.020 in Mali)[27]. However, taking advantage of the simplicity to update both LATE-PCR and dipsticks by adding new SNPs of interest while keeping the same protocol, further developments are already in progress for designing probes targeting the SNPs corresponding to *GYPB*03N.01* and/or *GYPB*03N.03* and upgrading our test.

Such a test may also be considered for elucidating ambiguous or inconsistent phenotype in blood group systems where weak and partial phenotypes were described. As the molecular identification of weak D type 1, 2, or 3 carriers which rationalizes the use of RhD-negative stock units. On the basis of the proof-of-principle reported here, our novel SNP genotyping assay system would be easily developed potentially applicated in POC testing for the detection of infectious diseases or to diagnosis genetic diseases.

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TABLES

Table 1. Capture probes spotted on the membrane. The SNPs in the allele-specific probes are indicated by bold and underlined nucleotide.

Blood group System	Allele	Encoded antigen	Probe sequences	Conc. (μM)	T_m (°C)
Duffy (FY)	*FY*01*	FY1	5′- C_6 NH_2-$(dT)_{15}$AGGTTGGCA**C**CATAGTCTC-3′	100	54.2
	*FY*02*	FY2	5′- C_6 NH_2-$(dT)_{15}$AGGTTGGCA**T**CATAGTCTC–3′	100	51.5
	*FY*wt*	-	5′- C_6 NH_2-$(dT)_{15}$GCTTCCAAG**A**TAAGAGCCA-3′	150	52
	*FY*02N.01*	none	5′- C_6NH_2-$(dT)_{15}$GCTTCCAAG**G**TAAGAGCCA-3′	100	54.7
MNS	*GYPB*03*	MNS3	5′- C_6NH_2-$(dT)_{15}$AGGAGAAA**T**GGGACAACTTG-3′	100	52.4
	*GYPB*04*	MNS4	5′- C_6 NH_2-$(dT)_{15}$ATAGGAGAAA**C**GGGACAACTT -3′	200	55.4
Kidd (JK)	*JK*01*	JK1	5′- C_6 NH_2-$(dT)_{15}$AGTAGATGT**C**CTCAAATGG–3′	200	48.7
	*JK*02*	JK2	5′- C_6 NH_2-$(dT)_{15}$ AAGTAGATGT**T**CTCAAATGGG –3′	65	51.8

Table 2. Primers used for LATE-PCR amplification.

Blood group System	Allele	Primer sequences	Conc. (μM)	T_m (°C)
Duffy (FY)	*FY*01/FY*02*	5′- Biotin-ATGATTCCTTCCCAGATGGAGAC-3′	1	58
		5′-TGCAGAGTCATCCAGCAGGTTACAGGAGT-3′	0.05	62.4
	*FY*wt/FY*02N.01*	5′-Biotin-CCCTCATTAGTCCTTGGCTCTT-3′	1	58.2
		5′-CTCACCCTGTGCAGACAGTTCCCC-3′	0.05	61.6
MNS	*GYPB*03/GYPB*04*	5′- Biotin-ACCTGGTACAGTGAAACGATG-3′	1	56.5
		5′- GCCGCATGACCCTTCTTTGCATG -3′	0.05	59.5
Kidd (JK)	*JK*01/ JK*02*	5′-Biotin-CAGTCTTTCAGCCCCATTTGAG-3′	1	57.9
		5′-GGTGAGCGCCATGAACATTCCTCCC-3′	0.05	62

Table 3. Results of blood group genotyping assay on blinded panel of 108 blood samples.

Blood group system	Serology		Multiplex lateral flow		Number of Discordances
	Phenotype	**n**	**Genotype**	**n**	
Duffy (FY)[#]	FY : 1, -2	20	*FY*01/*01* *FY*01/*02N.01*	17 3	0
	FY : -1, 2	35	*FY*02/*02* *FY*01/*02N.01*	34 1	0
	FY : 1, 2	52	*FY*01/*02*	52	0
	FY : -1, -2	1	*FY*02N.01/*02N.01*	1	0
MNS	MNS : 3, -4	16	*GYPB*03/*03*	16	0
	MNS : -3, 4	42	*GYPB*04/*04*	42	0
	MNS : 3, 4	50	*GYPB*03/*04*	50	0
Kidd (JK)	JK : 1, -2	38	*JK*01/*01*	38	0
	JK : -1, 2	15	*JK*02/*02*	15	0
	JK : 1, 2	55	*JK*01/*02*	55	0

[#]The *FY*02N.01* allele is a silent allele of *FY*02*. Homozygosity for the *FY*02N.01* allele give rise to the FY:-1,-2 or FYnull phenotype, common in Black populations but extremely rare in Caucasian populations[35].

Figure captions

Figure 1.

Lateral flow test for rapid visual detection of blood group genotypes and deduced phenotype. (A) Schematic view of the lateral flow strip. (B) Lateral flow procedure to perform visual detection of blood group SNPs.

Figure 2.

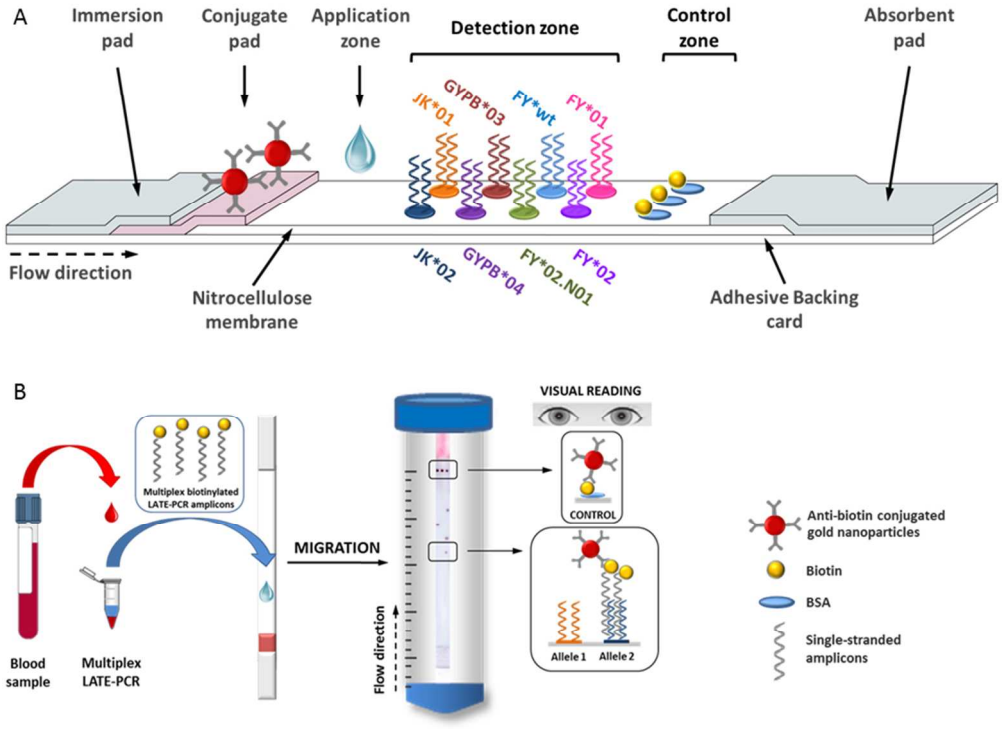
Optimization of multiplex LATE-PCR: (A) effect of the primer ratio and (B) the number of PCR amplification cycles on color intensity of allele-specific probes for each blood group SNP. Visual detection of blood group genotypes was performed using the interpretation card (Figure 4). NC: negative control. * indicates the optimum conditions for each parameter.

Figure 3.

Optimization of migration conditions on the lateral-flow dipstick: (A) effect of migration/hybridization temperature and (B) effect of formamide concentration in the running buffer. Visual detection of blood group genotypes were performed using the interpretation card (Figure 4). NC : negative control. * indicates the selected conditions for each parameter.

Figure 4.

Examples of interpretation card use for deduced phenotype determination and traceability. The upper part of the card is dedicated to patient ID. The strip is stuck in the TEST zone. After validation of the assay (check the Control zone), deduced phenotype is filled in the right box. The “FY reading guide” associates the different SNP profiles for FY with the corresponding deduced phenotypes. After drying, the card can be scanned or photographed and archived for traceability.



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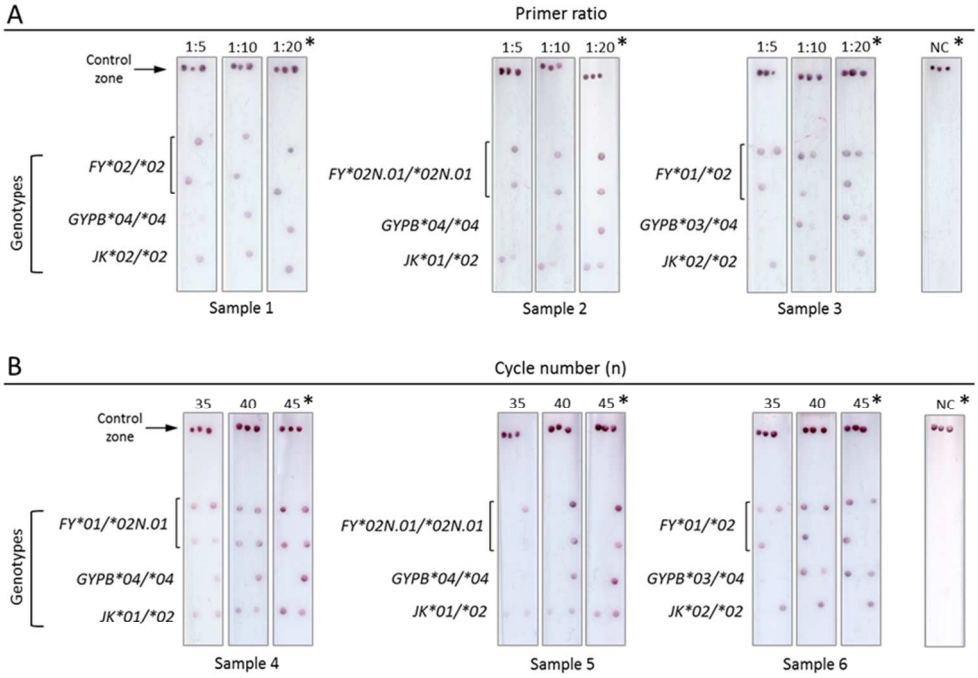
254x190mm (96 x 96 DPI)



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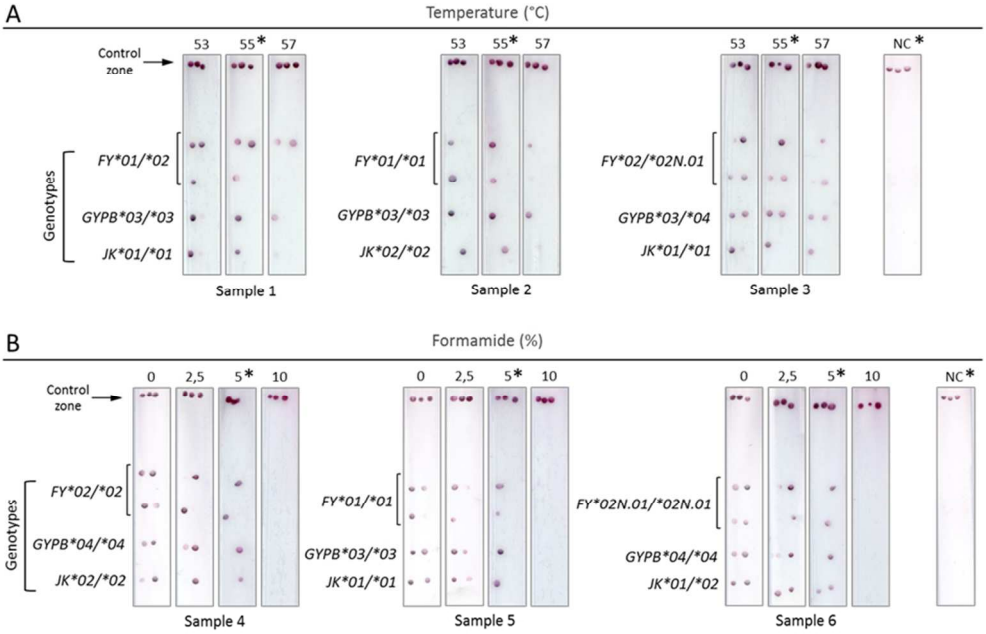

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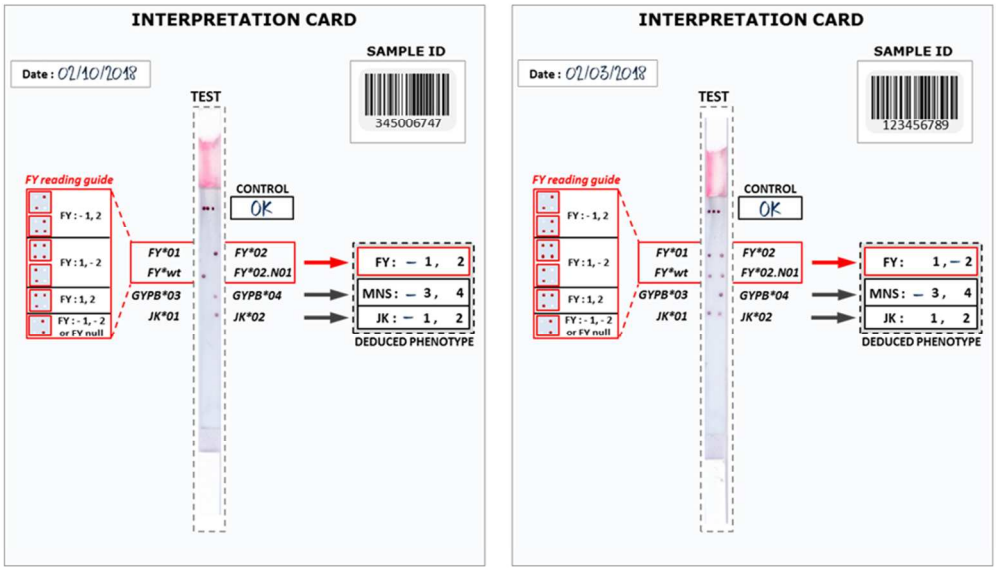

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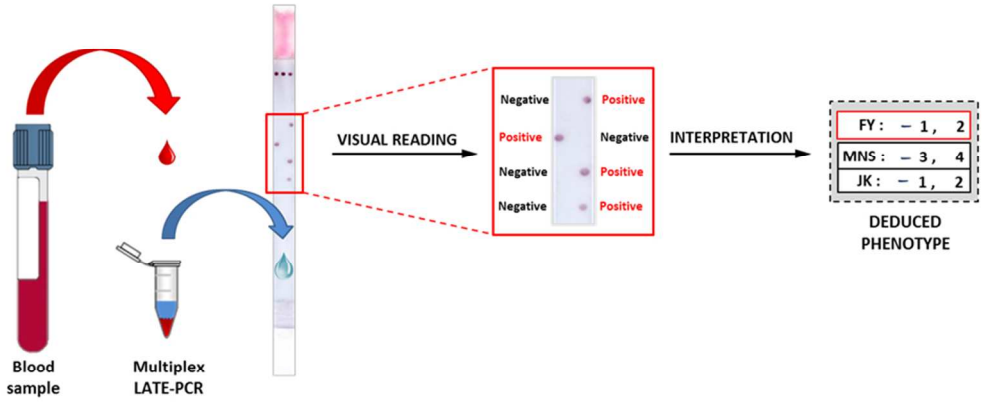

for TOC only

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