Methods 64 (2013) 241–249

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

DNA biosensor/biochip for multiplex blood group genotyping

S.A. Boccoz [a,b], L.J. Blum [a], C.A. Marquette [a,*]

[a] Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, Equipe Génie Enzymatique, Membranes Biomimétiques et Assemblages Supramoléculaires, Université Lyon 1 – CNRS 5246 ICBMS, Bâtiment CPE 43, bd du 11 novembre 1918, 69622 Villeurbanne Cedex, France
[b] AXO Science SAS, 66 Bd Niels Bohr, CEI 1, 69100 Villeurbanne, France

ARTICLE INFO

Article history:
Available online 27 September 2013

Keywords:
Blood group
DNA biochip
Microarray
Multiplex
Polymerase chain reaction

ABSTRACT

At present, 33 blood groups representing over 300 antigens are listed by the International Society of Blood Transfusion (ISBT). Most of them result from a single nucleotide polymorphism (SNP) in the corresponding DNA sequence, i.e. approx. 200 SNPs. In immunohematology laboratories, blood group determination is classically carried out by serological tests, but these have some limitations, mostly in term of multiplexing and throughput. Yet, there is a growing need of extended blood group typing to prevent alloimmunization in transfused patients and transfusion accidents. The knowledge of the molecular bases of blood groups allows the use of molecular biology methods within immunohematology laboratories. Numerous assays focused on blood group genotyping were developed and described during the last 10 years. Some of them were real biochips or biosensors while others were more characterized by the particular molecular biology techniques they used, but all were intending to produce multiplex analysis. PCR techniques are most of the time used followed by an analytical step involving a DNA biosensor, biochip or analysis system (capillary electrophoresis, mass spectrometry). According to the method used, the test can then be classified as low-, medium- or high-throughput. There are several companies which developed platforms dedicated to blood group genotyping able to analyze simultaneously various SNPs or variants associated with blood group systems.

This review summarizes the characteristics of each molecular biology method and medium-/high-throughput platforms dedicated to the blood group genotyping.

1. Introduction

Today, 33 blood group systems representing over 300 antigens are listed by the ISBT. Most of them have been cloned and sequenced [1,2] which means that the molecular bases of these blood group systems are known and mostly result from single nucleotide polymorphism (SNP).

Red Blood Cells (RBC) carrying a particular antigen can elicit an immune response if they are introduced in the blood circulation of someone who lacks this antigen. It is the antibody produced during the immune response which is problematic and is involved in donor/patient transfusion incompatibility, materno-fetal incompatibility and autoimmune hemolytic anemia. This immune response can be immediate or delayed and, in some cases, lead to death. That is the reason why antigen-negative blood is required for a safe transfusion. The method of reference for testing blood group antigens has been for decades the hemagglutination technique. However, this gold standard method has certain limitations (immunological reagent availability and specificity) when it comes to the determination of minor or rare blood group antigens in order to fulfill perfect matching between patient and donor [3].

The classical method for blood group typing is hemagglutination. This technique is simple and well established for all major blood groups, with specificity, sensitivity and security appropriate for clinical diagnostic environment. However, when it comes to extended blood group typing, this technique has some limitations [3] (see Table 1).

Reagents are specialized and must be obtained from immunized patients or donors (polyclonal and monoclonal antibodies) or from immunized mice (monoclonal antibodies). Reagents' cost is increasing, and many antibodies are not available, or are weakly reactive which is not suitable for the growing need of blood products typed for a large number of antigens. This limit led to a relatively low number of donors typed for larger number of antigens, which limits the establishment of an antigen-negative inventory. In addition, antigens may often be too weakly expressed on the surface of the RBCs to be detected by serology and discrepancies in serologic activity can occur between different manufacturer's reagents [4]. Finally, even if all the necessary antibodies required to determine all the blood group systems were available, there would remain the following problems:

* Corresponding author.
E-mail address: christophe.marquette@univ-lyon1.fr (C.A. Marquette).

1046-2023/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymeth.2013.09.011

Table 1
Limitations of serology.

Technical limitations	
	Manual data entry
	Reagents specialized and obtained from immunized patients or mice
	The cost of commercially licensed reagent increases
	Many antibodies are not commercialized or weakly reactive
	Not adapted to a high-throughput or high-content typing
Clinical limitations	
	Difficulties to phenotype a recently transfused patient or a multi-transfused patient, or when RBCs are coated with IgG
	Difficulties for the D-typing of the Rh blood group
	When a silencing gene is involved
	Indirect indication of the risk and severity of anemia or HDFN (Hemolytic Disease of the Fetus and Newborn)

- It is difficult to type recently transfused patients [5], multi-transfused patients like those with sickle cell disease [6], or those who RBCs are coated with IgG [4].
- D-typing is difficult due to the high number of antigens produced by the *RHD* gene [7].
- Cases where the phenotype does not exactly represent the genotype, due to silencing alleles, for example for the Duffy blood group [8].
- Hemagglutination only gives an indirect indication of the risk and severity of anemia or HDFN [9].

The knowledge of the molecular bases of most of the blood group systems allows the use of new tools like DNA based assays. One type of existing DNA-based assays uses DNA biosensors or microarrays (solid phase or suspended beads arrays). They are potentially high-throughput tools allowing testing a large number of donors on a large number of antigens [3]. Moreover, they help overriding the limitations of serology, replacing the immunochemical reagents by synthetic and reproducible probes. These methods usually associate a multiplex PCR step followed by detection with specific probes corresponding to the analyzed polymorphisms. Indeed, two thirds of all blood group antigens are defined by single nucleotide polymorphisms (approximately 200 SNPs) [10]. In this situation, a solution based on multiplex PCR which can analyze several parameters at the same time is required. The Fig. 1 gives an overview of the different techniques used to predict a blood type.

The purpose of this review is to summarize the different existing molecular biology methods which allow extended blood group genotyping. After seeing how DNA-based assays can be helpful, we will describe the principles of PCR-based assays applied to blood grouping and then summarize the different existing DNA biosensor/microarray-based platforms.

2. Classical PCR-based assays

This technique has revolutionized the field of molecular genetics, allowing the reproduction of a specific DNA sequence in an exponential way. After the isolation of DNA, the PCR reaction consists in three steps as represented on the Fig. 2.

(1) Denaturation: To dissociate the double stranded DNA and to obtain two simple strands. This step is usually performed at 95 °C during 30 s.
(2) Annealing: During this step, specific primers hybridize on their complementary sequences. The classic range of temperature is 55 °C–65 °C, depending on the melting temperatures of the primers.
(3) Extension: Between 68 °C and 72 °C, the DNA polymerase synthesizes strands complementary to oligonucleotides present in the reaction mix. The duration of this step depends on the length of the amplified fragment. The typical velocity of the DNA polymerase is 1 kb/minute.

These three steps are repeated approximately 30 times, which result in a number of DNA copies which increases in an exponential

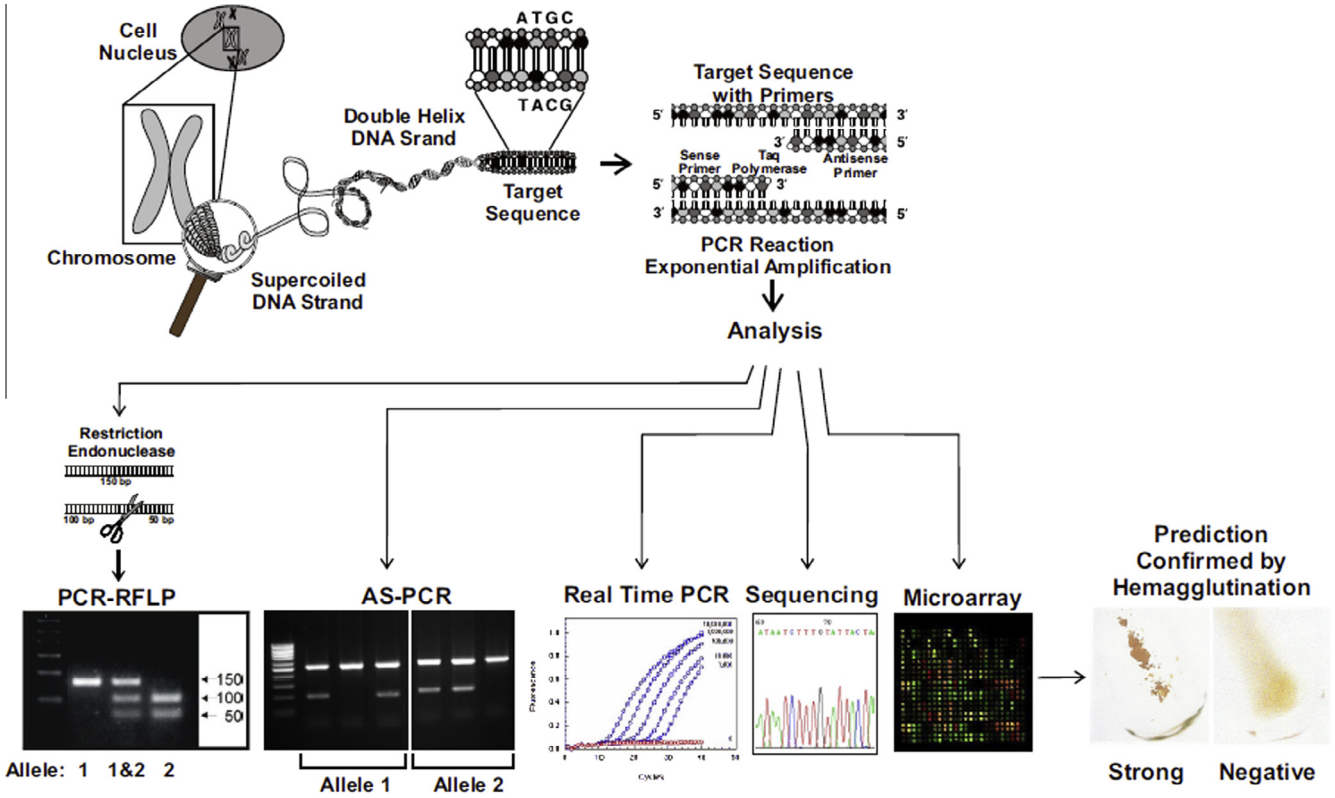

Fig. 1. Possibilities to go from cell nucleus to DNA and then assay, useful to predict a blood type [11].

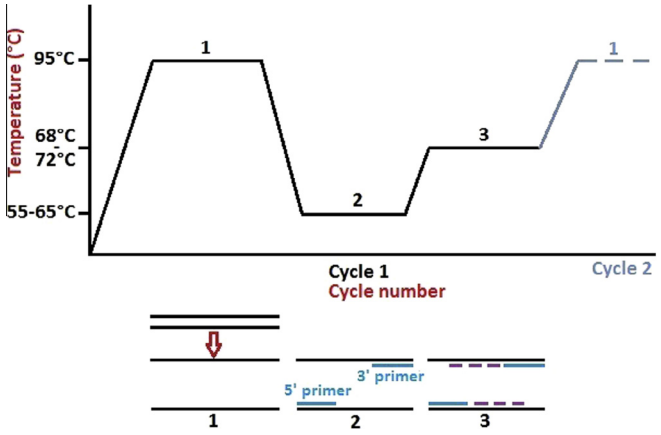

Fig. 2. Principle and major steps of the Polymerase Chain Reaction (PCR).

way. Before the beginning of the first cycle, one step of initial denaturation is usually performed to enable long fragments to dissociate, especially when genomic DNA is isolated. This step also allows the activation of the widely used hot-start DNA polymerase, which helps preventing non-specific amplifications. In a similar way, a final extension step of 5 min is performed to finish all the loading extensions.

After the amplification, the synthesized fragments have to be analyzed and here different methods can be used, from the low-throughput agarose electrophoresis, to the medium-throughput capillary electrophoresis and finally the high-throughput microarrays systems. It is interesting to note that going from low- to high-throughput is linked to the use of biosensor or biochip like systems.

For the sake of comparison, we are describing hereafter the full range of analysis systems (from non-biosensors electrophoresis to biochip microarrays).

2.1. Low-throughput

2.1.1. PCR-restriction fragment length polymorphism (PCR-RFLP)

This technique is relevant only if a SNP creates or removes a restriction site. Indeed, PCR is used to amplify region of interest including the SNP, followed by a step of digestion with a restriction enzyme. These enzymes are highly specific and cleave the DNA at a specific site, characterized by a unique sequence of nucleotides. Results are usually visualized using gel electrophoresis and fragments of different sizes are obtained, reflecting the presence or absence of the restriction site and thus the SNP of interest. Results are classically compared to known controls (homozygous samples for each allele, and a heterozygous sample) as internal calibrators.

Some studies have already been conducted using this technique, mostly for ABO genotyping [12–16], but also for other systems such as Lewis [17] and Knops [18].

This technique cannot be multiplexed in its current status but shall be in the future modified to fit a multiplex purpose.

2.1.2. Allele-specific-PCR (AS-PCR) or sequence-specific priming-PCR (SSP-PCR)

This method requires at least two reaction tubes per DNA sample. Each reaction tube contains one gene specific primer which is common to both alleles and one specific primer for one of the two possible alleles. If the complementary sequence of the allele specific primer is not present, annealing does not occur and there is no amplification. Results of the two reaction tubes are visualized by agarose gel electrophoresis. As the lack of PCR product reflects a missing allele, it is important to include an internal control to ensure that the PCR amplification step was successful.

Some articles have been published with this technique concerning the following blood group antigens: Duffy [19–21], RhD/CE [22,23], and ABO [24].

Some kits have been also developed and are commercialized: BAGene SSP Kits [25], by BAG Health Care (http://www.bag-health-care.com). These kits are CE-IVD marked and allow the determination of ABO blood groups, RH types, Kell Kidd and Duffy systems, MNS system, HPA and HNA specificities. They are composed of pre-aliquoted and dried reaction mixtures for SSP-PCR, optimized for a final volume of 10 μL. 10X PCR buffer and an internal control are supplied. However, Taq polymerase (not hot-start) and all the equipment for the detection of the PCR products by agarose gel electrophoresis must be added. This technique is compatible with multiplexing conditions as far as the determination of the two alleles is separated in two different vessels (see next paragraph).

2.2. Medium-throughput

2.2.1. Multiplex PCR

This technique allows the analysis of several SNPs or regions of interest in the same reaction, unlike AS or SSP-PCR, using multiple primer pairs. Number of reactions, time and quantity of reagents used are then drastically reduced.

The main problem in multiplex PCR is the degree of multiplexing. Indeed, increasing the number of primers promotes the emergence of non-specific hybridizations. Optimization of multiplex PCR can then be fastidious mostly because annealing temperatures of all primers must be similar.

In general, multiplex PCR products are visualized by agarose gel or capillary electrophoresis, and discriminated according to their sizes. In 2012, Jungbauer et al. published an article in which they developed a multiplex PCR to genotype 35 red blood cell antigens [26]. It consists of six different reactions, each with 5 to 7 primer pairs. At least one allele-specific primer is used in each primer pair. PCR products are analyzed by agarose gel electrophoresis and differentiated by their sizes. Sixteen people per 96-well plate can then be genotyped for 35 antigens. For all samples and markers tested, a 100% concordance was found between determined genotypes and phenotypes (from 1 to 469 samples were tested for each antigen). The group also compared the cost of classical serological typing (35 to 39€ per donor for 20 minor RBC antigens) with their own genotyping approach (15€ per donor for 35 RBC antigens). Thus, for 1 minor RBC antigen, serological typing would cost between 1.75 and 1.90€ (automated vs. manual), and genotyping around 0.43€.

This is one of many studies showing the relevance of genotyping compared to serology, from the standpoint of the cost, the throughput, and the fact that there are only approximately 20 minors RBC antigens for which serological reagents are available on a regular basis [26]. Kengkate et al. used this same technique to genotype HPA-1 to -7 and -15 in Thai population [27] and Wagner also used it to screen blood donors with rare phenotypes [28].

2.2.2. Real-time PCR

Real time PCR is an autonomous detection system based on PCR reaction. It can then almost be considered as biosensors on its own. The working principle is the same than classic PCR, except that the extension is detected and measured in real time. There are three common methods:

- The use of SYBR® Green. It is a non-specific intercalating dye which integrates to the forming double stranded DNA during extension and emits fluorescence. Multiplexing is here not possible but Novaretti et al. used real-time PCR with SYBR® Green to genotype blood donors for Diego blood group [29], as Sousa et al. for the genotyping of Duffy blood group [30].

- TaqMan probes. These probes have a fluorophore bound to one extremity and a quencher to the other one. In their initial configuration, the quencher captures the fluorescence of the fluorophore, turning off the signal. In the presence of DNA template, the TaqMan probe hybridizes and during the extension, the TaqMan probe is hydrolyzed by the 5′-3′ exonuclease activity of the polymerase. This results in the "break" of the proximity between fluorophore and quencher, turning on the fluorescence emitted by the fluorophore. Fluorescence detected is directly proportional to the amount of fluorophore and DNA template present in the reaction. Multiplexing is possible but limited due to the number of fluorescent dyes commercially available, and also due to the number of colors recognized by the instrument. In 2009, Atamaniuk et al. published an article in which they compared 3 molecular biology methods to predict fetal RhD in maternal plasma and concluded that TaqMan was one of the most accurate system [31].
- FRET detection. In this system, two probes are used: one with a transmitter fluorophore bound at the 3′ end, and the other with an acceptor fluorophore bound at the 5′ end. These two probes are chosen to hybridize to their target sequences in a configuration where the two fluorophores are separated only by one to five base pairs. When the two probes are separated, the transmitter fluorophore only emits background fluorescence whereas when they are hybridized and closer than 10 nucleotides away, the proximity of the two fluorophores allows the energy transfer from transmitter to acceptor. Fluorescence is measured during the annealing, and is proportional to the quantity of DNA synthesized. As probes stay intact, it is also possible to realize melting curves at the end of the reaction. Ansart-Pirenne et al. [32] used this technique for the genotyping of FY*X allele on 199 DNA samples.

Other probes exist for the measure of fluorescence during real-time PCR such as Molecular Beacons, and Scorpions, but their design is fastidious and their cost is high.

2.3. Non PCR-based assays

2.3.1. Sanger DNA sequencing

For this method, a complementary primer of region of interest is used. Extension is realized by Klenow fragment (DNA polymerase without 5′-3′ exonuclease activity). Dideoxynucleotides are added. They do not possess 3′OH group, and do not allow the addition of nucleotides and the extension is then stopped. Each dideoxynucleotide is labeled by a specific fluorophore which emits at a specific wavelength. The detection system measures the fluorescence emitted and a specific profile is obtained, allowing the determination of the desired sequence.

2.3.2. Pyrosequencing

In this technique, deoxynucleotides are not introduced in the reaction vessel all together, but one after the other. If the added nucleotide corresponds to the one expected by the DNA polymerase, it is incorporated in the strand being synthesized and a pyrophosphate is released. This pyrophosphate is transformed into ATP, by an ATP sulfurylase, and used by a luciferase bioluminescent enzyme to produce light. A CCD (Charge-Coupled Device) imaging sensor captures the emitted signal and reproduces it as a peak on a pyrogram. Peak height is function of signal intensity, itself function of the number of nucleotides incorporated at the same time. The sequence can be deduced from peak heights. In the case of single nucleotide polymorphism analysis, different nucleotides are incorporated at a same position and peak height helps identifying the presence of SNPs.

3. Integrated systems

3.1. Medium-throughput

3.1.1. Fluidic microarray system – xMAP

xMAP technology (http://www.luminexcorp.com) is based on the use of matrices in suspension. Polystyrene microspheres act as an immobilization support for oligonucleotide probes. Each bead (5.6 μm diameter) has its own optical encoding which allows its identification during result analysis. Two fluorophores are incorporated in different proportion in each bead, conferring them a unique spectral signature. Up to 500 different optical codes can then be created. Beads carrying the same code form a set, and each set carries a specific probe.

Oligonucleotide probes are synthesized with an amino C-12 modification at 5′ end and are covalently immobilized on the polystyrene microspheres through a carbodiimide activation method [33]. Once the beads coupled with probes, they are stable for at least 6 months.

The principle of the assay is summarized on Fig. 3. A multiplex PCR which generates biotinylated amplicons is performed in order to amplify regions of interest. Directly after the amplification, the mixture is transferred into a 96-well plate and denatured at 99 °C. Labeled PCR products are then hybridized with microspheres during 45 min. The plate is washed three times and incubated with R-phycoerythrine conjugated-streptavidin. The analysis is based on the flow cytometry principle. When a microsphere passes through the detection chamber, a laser excites red and infrared fluorochromes, allowing the bead classification among the different sets. Another laser excites orange fluorescence associated with the target binding.

Drago et al. used this technique for the identification of blood group SNPs [34]. They typed more than 2000 patients, whom 493 with multiplex assays, and they observed no discrepancies between Luminex results and serological results, except when there was a null/weak phenotype. According to the authors, there are some advantages with this method compared to other high-throughput techniques: fast reaction kinetics, rapid data acquisition, and excellent sensitivity. Due to its flexibility, probe sets can be reformulated, depending on the needs of the laboratory. No amplicon purification is required after PCR, and this is a technique which allows the use of a large range of DNA concentrations, avoiding fastidious steps of measures.

However, always according to the authors, even if it is possible to type from 1 to 96 samples, xMAP technology is cost-effective when a large number of samples is typed, then it may not be the method of choice for laboratories with low workload. There is also a lack of automation in the protocol, especially for sample loading and washing steps.

3.1.2. TaqMan open array – open array

This technique is based on TaqMan method, described earlier. Each probe contains a specific fluorescent dye to differentiate each allele (http://invitrogen.com) of one blood group SNP. The reporter dye VIC® is used at 5′end of each probe corresponding to allele 1. FAM™ is used at 5′ of each probe corresponding to allele 2. One Minor Groove Binding stabilizing agent is also present in the reaction mix, which allows the increase of melting temperature of probes while keeping their length constant. A non fluorescent quencher is included at 3′end of each probe. As it does not fluoresce, the real-time PCR system can measure the fluorescence of the reporter accurately. This technique allows the increasing of throughput using classical plates.

Hopp et al. used this platform to screen African American blood donors [35]. Out of the 4270 genotypes generated by the OpenAr-

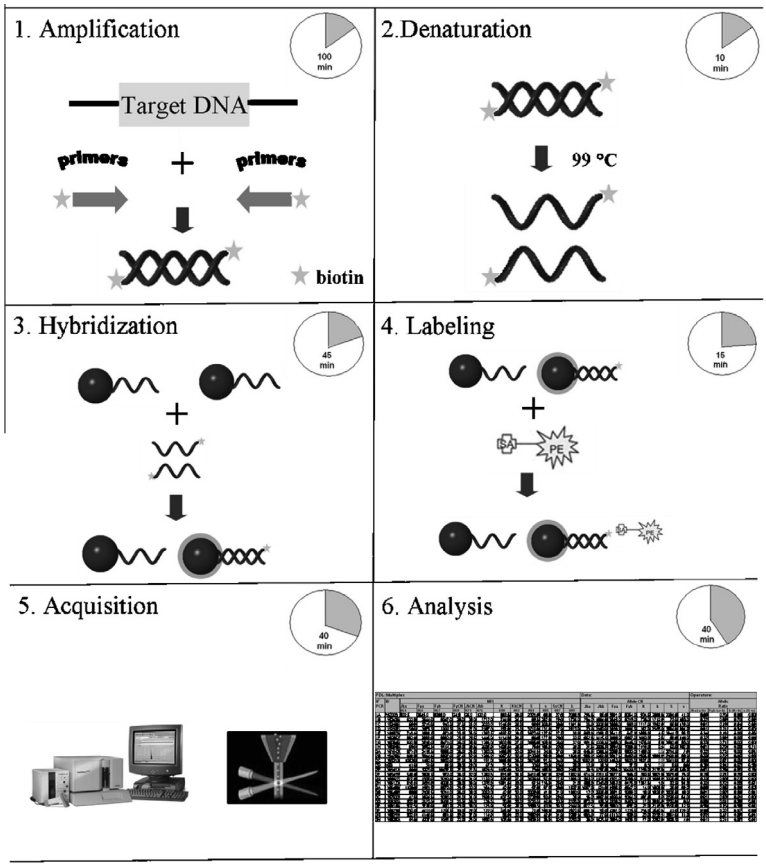

Fig. 3. Principle of xMAP assay [34].

ray® system, there were 5 discrepancies. Three have been resolved by either a new serological typing, a new OpenArray® genotyping, or using result obtained by Sanger sequencing. Each sample has been analyzed in triplicate, with over 99% correlation.

The great advantage of this method is the low reaction volume (33 nL) and speed (3 plates per approximately 5 h for a throughput of 9000 genotypes per run).

3.1.3. Mini sequencing or SNaPshot

This technique, developed by Applied Biosystems (www.appliedbiosystems.com) is based on an extension by only one nucleotide. Regions of interest are amplified by multiplex PCR so that each amplicon has a different length. Reaction mix is purified to remove non-incorporated nucleotides and primers. Amplicons are then mixed with a set of extension fragments. These fragments are complementary to the adjacent region of the SNP and will hybridize to the nucleotide preceding the SNP, at the 5′ side. A step of enzymatic extension of 1 nucleotide length is performed in the presence or four dideoxynucleotides labeled with four different fluorophores. Capillary electrophoresis allows the detection of fluorescence and length of the fragments, leading to the allelic determination.

One complete cycle allows to analyze up to 10 SNPs in a single reaction vessel per 24 h, and the estimated cost is 2$ per SNP [36]. One example of using this platform is reported in the publication of Di Cristofaro et al. [37]. They used SNaPshot reaction to type 18 blood group alleles. Their multiplex PCR amplified 9 regions of interest, with a length from 96 to 986 bp. Then, with the SNaPshot kit, they realized the one base extension and analyzed data by capillary electrophoresis with GeneMapper software. They analyzed only seven blood group systems: Kell, Duffy, MNS, Kidd, Dombrock, Cartwright and Colton but the assay also included the *FY*X* allele, silencing *FY* and silencing *MNS*.

3.1.4. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) - sequenom

This technique allows the differentiation of DNA fragments which differ from one nucleotide in length. It consists of two steps: (i) desorption/ionization of fragment induced by laser and (ii) separation/analysis of the different molecules, based on their physical properties.

Sequenom is a Californian company of biotechnology developing since 2008 bench analytical tools: MassARRAY systems. It is a compact equipment which allows the direct measurement of nucleic acid mass. This system is based on multi MALDI-TOF MS technology and finds applications for blood group genotyping (http://www.sequenom.com).

A cooperative study between Switzerland and Germany using the Sequenom platform has been published in January 2013 [38]. The aim of this study was the molecular detection of the following blood group systems: Rh, Kell, Kidd, Duffy, MNSs. This method has also been used for the characterization of a collection of low incidence antigens, platelet and granulocyte antigens for a total of 101 blood group antigens, encoded by 170 alleles.

The protocol starts by a multiplex PCR on genomic DNA, followed by an allele-specific single base extension of a primer. This primer anneals in a position directly adjacent to the SNP. The simple strands obtained (15–30 bases) are deposited on a silicon chip beforehand spotted with a matrix crystal containing patches. The entire mixture is then irradiated by a laser, inducing the desorption and ionization of the amplified fragments. Charged molecules accelerate through a flight tube towards a detector. Separation is

made by Time Of Flight (TOF). This time is proportional to the mass of the individual molecules. Low-mass molecules arrive faster than high-mass molecules. As a result, a mass spectrum is obtained.

This technique has some advantages: low quantities of DNA are required and the equipment is able to determine directly the mass of the molecules, without any labeling or dying step. The Sequenom platform supports multiplexing until 40 SNPs per reaction, with a rapid data acquisition and interpretation. Up to 150000 SNPs can be analyzed per day. Two formats are available: 384-well plate, suitable for up to 1000 samples per day, and 96-well plate, optimal up to 100 samples a day. Concerning the costs, list prices for Europe for full 96-well system and full 384-well system are 250000€ and 350000 € respectively, whereas each multiplex PCR costs 10€. There are 10 different multiplex PCR to cover all the antigens included in this study, and cost for the package of multiplexed PCR is 70€.

3.2. Medium- to high-throughput

3.2.1. BLOODChip®

The BLOODChip® system is dedicated to extended blood group genotyping and is commercialized by Progenika (www.progenika.com). It has been developed as a part of the European collaborative project BloodGen (2003–2006) which associated two blood banks (Barcelona and Prague) with two British academic research institutes (University of the West of England, Bristol Institute for Transfusion), the Sanquin (Netherlands), the universities of Ulm (Germany) and Lund (Sweden). The consortium also included two industrial partners: Progenika Biopharma SA (Spain), and Biotest (Germany) in charge of the integration on an existing SNP analysis platform. In version 2.0, which is CE marked, BLOODChip® allows the analysis of 128 SNPs associated with nine blood group systems and some platelet antigens.

Target sequences are amplified from genomic DNA through a multiplex PCR by hybridization of amplifiable probes, developed by Beiboer et al. [39]. Extracted DNA is mixed with a set of primers specific of the targeted polymorphisms. If the target sequence is present in the sample, the primers hybridize on genomic DNA. A washing step allows to isolate hybridized fragments and to amplify them. PCR products are then marked with two fluorophores, fragmented, and hybridized on the BLOODChip® array under stringent conditions. The chip is composed of a glass slide modified by an immobilized matrix of specific probes on which targets hybridize. Once hybridized, the chip is scanned with a fluorescence microscope and obtained signals are analyzed through a dedicated single software program to determine the different scores associated with the different alleles which allow the determination of the genotypes. Five hours after DNA extraction are required to achieve the results.

After clinical tests at a small scale on panels of rare donors in Spain, Germany, Czech Republic and Sweden, a validation on 3000 donors was performed and used to obtain the CE marking of the system. When compared to serology and other molecular typing methods, the results obtained by the authors highlighted an excellent performance of BLOODChip® v2.0 for an extended blood group genotyping.

3.2.2. HEA BeadChip™

Developed by BioArray Solutions Ltd. (held by Immucor Inc.), HEA BeadChip™ offers an extended genotyping solution for 11 blood groups and a panel of platelet antigens (http://www.immucor.com/bioarray). This product was approved by the *Food and Drug Agency* and obtained CE marking in July 2010. It has been evaluated on four hospital sites for the implementation of extended genotyping in routine [40,41].

It is a hybridization test on a matrix of beads (Fig. 4). Microbeads (3 μm of diameter) with carboxylic acid groups are functionalized with probe oligonucleotides modified with an amino arm (approximately 20 bases). Each bead carries about 50 copies of a unique probe, which 3′ end is specific to a target polymorphism. The different beads in suspension are collected (approximately 4000 beads) and coated on a silicon support where they form a monolayer. These silicon matrices are individualized and assembled on a glass slide. Two formats are available: 8×1 and 8×12, allowing testing several samples in parallel. Each bead has an optical encoding which allows its identification by spectral imagery and to deduce the sequence of the probe presents on its surface. From this stand point the technique is similar to the Luminex technology based ones.

After genomic DNA extraction, sequences with target polymorphisms are amplified using a multiplex PCR. The mixture of PCR products is then purified, denatured, hybridized on beads and fluorescently labeled using two strands specific enzymatic elongation. This step can be realized only with a perfect match between target and probe. The incorporation of a labeled nucleotide specifically labels the two strands. Genotypes are obtained through the comparison of fluorescence intensities between the two alleles of interest and a panel of reference values.

Main published validation studies of HEA BeadChip™ have been performed in collaboration with Marion Reid's team at the Immunohematology laboratory (New York Blood Center) [42,43]. In 2007, the team presented results of a study led on 2355 patients belonging to multiple ethnic groups, for the analysis of 24 blood group antigens and a panel of platelet antigens, with 99.5% of concordance compared to serological results.

HEA BeadChip™ test (version 1.2) has been also implemented by Kappler-Gratias et al. [40,41] for the evaluation of molecular methods in the characterization of erythrocytes used as reagent on the Laboratory and French National Reference Panel. The genotyping test has allowed analyzing 34 antigens involved in 11 blood group systems for a panel of 365 donors. Comparison of the predicted phenotype by genotyping results and serological phenotype for 25 of these antigens showed a concordance of 99.95%.

Brazilian teams have also reported the use of HEA BeadChip™ as a reference technique in a study evaluating the impact of extended molecular typing for the monitoring of multi-transfused patient panel [44] and for the monitoring of sickle cell patients [6].

3.2.3. Genome lab SNP stream

Genome Lab SNP Stream platform, commercialized by Beckman Coulter, use the SNPtream® UHT, developed by Orchid BioScience for ultra high-throughput genotyping in pharmacogenomics and in the drug screening [45]. It associates amplification by multiplex PCR with a fluorescent labeling step obtained by elongation, followed by hybridization on a chip. Canadian teams of Gregory Denomme (Toronto) and Maryse St-Louis (Quebec) developed the molecular biology tools required for the transfer of this technology to extended blood group genotyping [46,47].

The format of the chip is a conventional 384-well plate, with the same matrix of probes in each well, allowing then to test 384 samples in parallel. Oligonucleotides are covalently bound to a glass support activated by 3-mercaptopropyl-trimethoxysilan (MPTS). Then, the application of a neoprene mask allows individualizing 384 wells. Each matrix includes 16 probes: four are controls (positive and negative) and the other 12 correspond to the studied SNPs.

Each test allows the detection of two nucleotides among the four possible at the SNP position. For each studied SNP, a fragment SNP-IT is used and constructed as follows: 3′-end sequence of the fragment is complementary to one of the probes fixed in the bottom of the well and 5'-end sequence of the fragment is complementary to the target and immediately adjacent to the SNP.

The first step consists of a multiplex PCR to amplify specific sequences of targeted polymorphisms on the extracted DNA (Fig. 5).

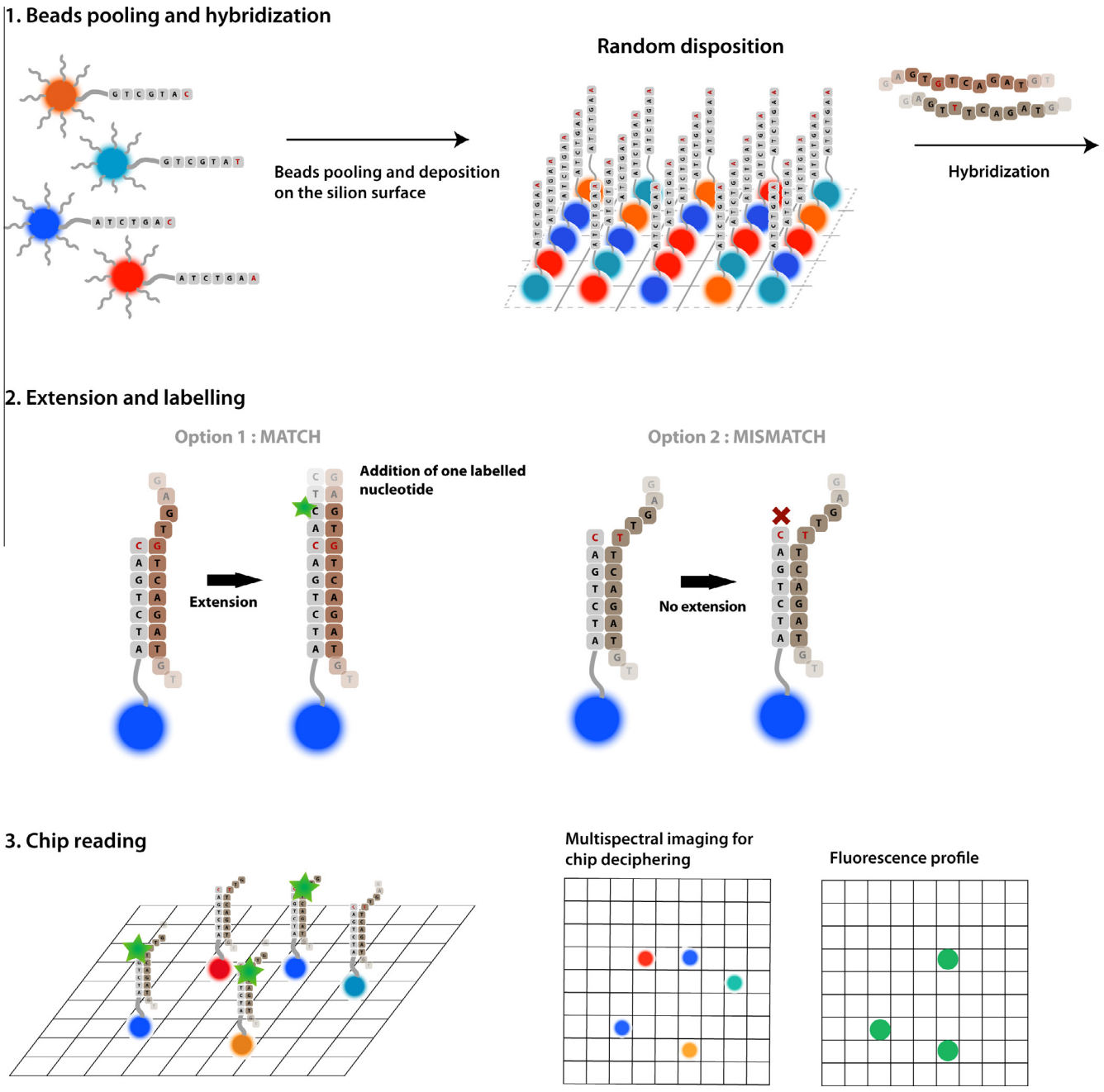

Fig. 4. Principle of HEA BeadChip™ assay.

Amplicons are mixed with SNP-IT fragments with which they specifically interact. Then a one-base enzymatic elongation is performed in presence of the two labeled nucleotides, one by BODIPY-fluorescein and the other by TAMRA. According to the base present on the target at the SNP site, the elongation will incorporate one or the other fluorophores. The reaction mixture is incubated on the chip and the 5′-end of the fragment hybridizes with its complementary probe. A plate reader enables to read the fluorescence intensity for each probe on each spot and to deduce the genotype.

In 2005, Denomme et al. [46] reported the analysis of 372 samples for 12 SNPs associated with RH, MNS, KEL, FY, JK, DI blood group systems and one platelet antigen, with a concordance above 98% for 11 of the 12 parameters.

Maryse St-Louis's team of Hema-Quebec was also interested in the development of this technology. In 2006, a study of Montpetit et al. focuses on the analysis of 22 minor blood group antigens (RHCE, KEL, FY, JK, MNS) and three platelet antigens [48]. In this study, blood samples were collected by FTA© cards. Very good results (concordance above 97%) were obtained despite varying DNA amounts in the extraction step.

Ensued at Hema-Quebec, a donor screening project involved 21 000 donors in less than 2 years [47]. A concordance of 99.6% was obtained and 55% of the discordances were resolved at the benefit of genotyping. This study also allowed evaluating the impact of implementation of blood group genotyping in routine laboratory [49].

3.2.4. HIFI Blood 96™

HIFI Blood 96™ is an emerging blood genotyping technique developed on the AXO Science HIFI assay platform which takes

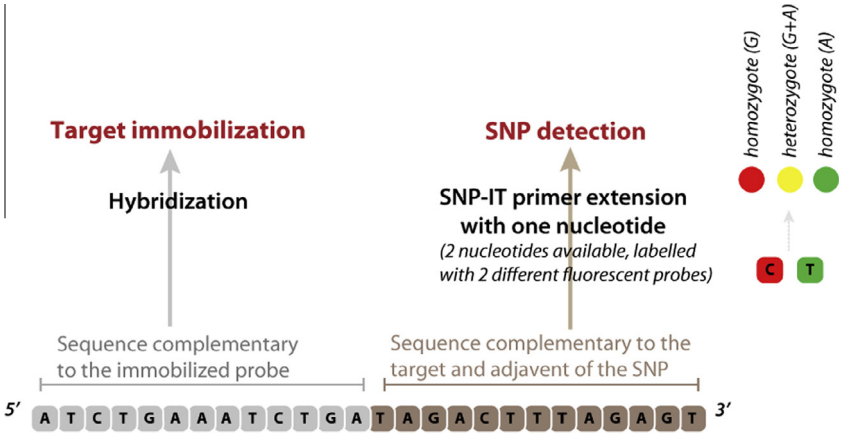

Fig. 5. Principle of Genome Lab SNP Stream assay.

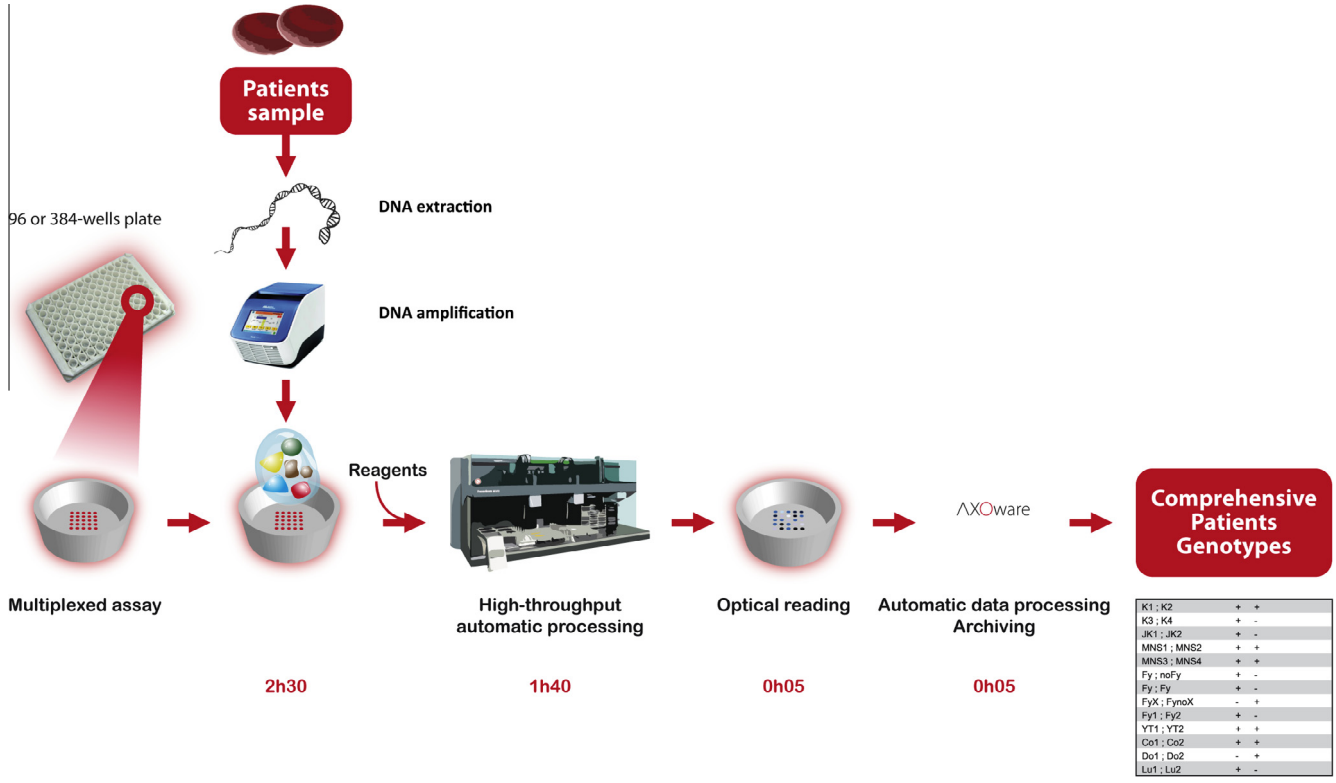

Fig. 6. Principle of the HIFI Blood 96™.

advantage of the HIFI technology for the assembly of microarrays with standard 96-, 384- and 1536-well plate (http://www.axos-cience.com). It associates fully automated steps from extraction to multiplex PCR, hybridization and reading. This is the first system on the market with real high-throughput capabilities.

The format of the test is based on classical 96- or 384-well plates with each well bottom modified with an array of probe oligonucleotides (Fig. 6). Each array is composed of 16 probes and three controls and allows the identification of eight genotypes concomitantly.

The whole blood samples are first processed through an automated DNA extraction and then amplified using biotinylated gene specific primers. Without any pre-treatment or purification step, the PCR products are hybridized on the HIFI platform and labeled to produce stained positive spots which are directly detected and quantified using optical imaging. Images of each well are then *in silico* processed to produce scores useful for the sorting of the sample according to their genotypes.

Two multiplex PCR were developed, allowing the identification of 22 blood group antigens (*KEL*01/02, KEL*03/04; JK*01/02; FY*01/02; MNS*01/02, MNS*03/04; YT*01/02; CO*01/02; DO*01/02*; *LU*01/02* ; JO(a+)/(a−); HY+/−), and also two variants which belong to the Duffy system (*FY*Fy and FY*X*). The molecular biology developments and validation of the platform were partially performed in collaboration with the *Etablissement Français du Sang [50]* and very good correlations (between 97% and 100%) with other genotyping and phenotyping assays were found. The *HIFI Blood 96*™, which is taking 4 h30 to generate 96 genotypes from whole blood, is currently under CE marking process.

4. Conclusion

As highlighted in this review, the integration of molecular biology techniques with microarray or biochip systems was the main

Table 2
Benefits and implementations of DNA-based assays for blood group typing.

Typing for a patient	
	To identify a fetus at risk for HDFN
	When serologic reagents are not available, or weakly reactive
	When antigen is weakly expressed
	In recently transfused patients
	In multi-transfused patients
	To distinguish alloantibodies from autoantibodies
	To determine zygosity, especially for *RHD*
	To identify Rh variants
	When RBCs are coated with Immunoglobulin
	To detect silencing genes or genes responsible of a weakly expressed antigen
Typing for a donor	
	When serologic reagents are not available, or weakly reactive
	When antigen is weakly expressed
	To screen for antigen-negative donors
	To detect silencing genes or genes responsible of a weakly expressed antigen
Other applications	
	High-throughput and high-content
	Can be fully automated (depending on the platform used), sometimes from DNA extraction until results acquisition
	To resolve reagent discrepancies
	Data entry in a donor/patient database

milestone leading to the achievement of really multiplex and high-throughput analytical tools based on DNA biosensing. Applied to the highly complex blood group genotyping field in clinical laboratory, these systems prove their capabilities and performances. Indeed, the knowledge of molecular bases of most of the 33 blood group systems [2] allows the implementation of molecular testing in classical molecular biology conditions. Furthermore, the use of multiplex PCR together with microarray or biochip systems, allowing the multiplex identification of PCR products, permitted the transfer of these laboratory techniques to clinical laboratory facilities.

It is assumed that high-throughput blood group genotyping using such biochip platforms will replace serological techniques in a near future. This change will only be accompanied by the emerging of a platform being cost-effective and easy to implement in clinical environment [51].

Finally, these new tools can override most of the technical and clinical limitations of serology (see Table 2) and one of the most important advances will here be the increasing of antigen-negative blood inventories, then overstepping a new milestone in safety and transfusion practices [9].

References

[1] L. Logdberg, M.E. Reid, R.E. Lamont, T. Zelinski, Transfus. Med. Rev. 19 (2005) 45–57.
[2] L. Logdberg, M.E. Reid, T. Zelinski, Transfus. Med. Rev. 25 (2011) 36–46.
[3] M.E. Reid, Program (2009) 171–177.
[4] C.M. Westhoff, Curr. Opin. Hematol. 13 (2006) 471–475.
[5] M.E. Reid, M. Rios, V.I. Powell, D. Charles-Pierre, V. Malavade, Transfusion 40 (2000) 48–53.
[6] L. Castilho, M. Rios, C. Bianco, J. Pellegrino Jr., F.L. Alberto, S.T. Saad, F.F. Costa, Transfusion 42 (2002) 232–238.
[7] C.M. Westhoff, Immunohematology 21 (2005) 155–163.
[8] L. Castilho, Transfusion 47 (2007). 28S–31S.
[9] M.E. Reid, Transfusion 47 (2007). 10S–16S.
[10] J.R. Storry, M.L. Olsson, Br. J. Haematol. 126 (2004) 759–771.
[11] M.E. Reid, Immunohematology 24 (2008) 166–169.
[12] Y. Fukumori, S. Ohnoki, H. Shibata, H. Yamaguchi, H. Nishimukai, Int. J. Legal Med. 107 (1995) 179–182.
[13] W. Haak, J. Burger, K.W. Alt, Anthropol. Anz. 62 (2004) 397–410.
[14] N.A. Mifsud, A.P. Haddad, J.A. Condon, R.L. Sparrow, Immunohematology 12 (1996) 143–148.
[15] H. Nishimukai, Y. Fukumori, T. Okiura, I. Yuasa, T. Shinomiya, S. Ohnoki, H. Shibata, U. Vogt, Int. J. Legal Med. 109 (1996) 90–93.
[16] Z. Tun, K. Honda, M. Nakatome, M.N. Islam, H. Bai, Y. Ogura, H. Kuroki, M. Yamazaki, M. Terada, C. Wakasugi, J. Forensic Sci. 41 (1996) 1027–1030.
[17] S. Ameno, K. Ameno, H. Kinoshita, N. Tanaka, I. Ijiri, Int. J. Legal Med. 110 (1997) 232–234.
[18] J.M. Moulds, B.J. Thomas, O. Doumbo, D.A. Diallo, K.E. Lyke, C.V. Plowe, J.A. Rowe, D.J. Birmingham, Transfusion 44 (2004) 164–169.
[19] F. Bauduer, M. Touinssi, A. Degioanni, S. Leroux, O. Dutour, L. Ducout, P. De Micco, J. Chiaroni, Am. J. Hum. Biol. 16 (2004) 78–81.
[20] C. Gassner, R.L. Kraus, T. Dovc, S. Kilga-Nogler, I. Utz, T.H. Mueller, F. Schunter, D. Schoenitzer, Immunohematology 16 (2000) 61–67.
[21] M.L. Olsson, C. Hansson, N.D. Avent, I.E. Akesson, C.A. Green, G.L. Daniels, Transfusion 38 (1998) 168–173.
[22] B. Faas, S. Simsek, P. Bleeker, M. Overbeeke, H. Cuijpers, A. von dem Borne, C. van der Schoot, Blood 85 (1995) 829–832.
[23] C. Gassner, A. Schmarda, S. Kilga-Nogler, B. Jenny-Feldkircher, E. Rainer, T.H. Muller, F.F. Wagner, W.A. Flegel, D. Schonitzer, Transfusion 37 (1997) 1020–1026.
[24] B. Hosseini-Maaf, A. Hellberg, M.A. Chester, M.L. Olsson, Transfusion 47 (2007) 2110–2125.
[25] M. Prager, Transfusion 47 (2007). 54S–59S.
[26] C. Jungbauer, C.M. Hobel, D.W.M. Schwartz, W.R. Mayr, Vox Sanguinis 102 (2012) 234–242.
[27] M. Kengkate, P. Butthep, P. Kupatawintu, S. Kanunthong, W. Chantratita, O. Nathalang, Transfus. Med. 22 (2012) 272–276.
[28] F.F. Wagner, R. Bittner, E.K. Petershofen, A. Doescher, T.H. Müller, Transfusion 48 (2008) 1169–1173.
[29] M.C. Novaretti, A.S. Ruiz, P.E. Dorlhiac-Llacer, D.A. Chamone, Immunohematology 26 (2010) 66–70.
[30] T.N. Sousa, B.A.M. Sanchez, I.P. Cerávolo, L.H. Carvalho, C.F.A. Brito, Vox Sanguinis 92 (2007) 373–380.
[31] J. Atamaniuk, K.M. Stuhlmeier, A. Karimi, M.M. Mueller, J. Clin. Lab. Anal. 23 (2009) 24–28.
[32] H. Ansart-Pirenne, S. Martin-Blanc, P.Y. Le Pennec, P. Rouger, J.P. Cartron, C. Tournamille, Vox Sanguinis 92 (2007) 142–147.
[33] R.J. Colinas, R. Bellisario, K.A. Pass, Clin. Chem. 46 (2000) 996–998.
[34] F. Drago, K. Karpasitou, F. Poli, Transfus. Med. Hemother. 36 (2009) 157–160.
[35] K. Hopp, K. Weber, D. Bellissimo, S.T. Johnson, B. Pietz, Transfusion 50 (2010) 40–46.
[36] P. Palacajornsuk, C. Halter, V. Isakova, M. Tarnawski, J. Farmar, M.E. Reid, A. Chaudhuri, Transfusion 49 (2009) 740–749.
[37] J. Di Cristofaro, M. Silvy, J. Chiaroni, P. Bailly, J. Mol. Diagn. 12 (2010) 453–460.
[38] C. Gassner, S. Meyer, B.M. Frey, C. Vollmert, Transfus. Med. Rev. 27 (2013) 2–9.
[39] S.H.W. Beiboer, T. Wieringa Jelsma, P.A. Maaskant Van Wijk, C.E. Van Der Schoot, R. Van Zwieten, D. Roos, J.T. Den Dunnen, M. De Haas, Transfusion 45 (2005) 667–679.
[40] S. Kappler-Gratias, T. Peyrard, M. Beolet, P. Rouger, P.Y. Le Pennec, B.N. Pham, Vox Sanguinis 99 (2010) 374.
[41] S. Kappler-Gratias, T. Peyrard, P. Rouger, P.Y. Le Pennec, B.N. Pham, Transfus. Clin. Biol. 17 (2010) 165–167.
[42] G. Hashmi, T. Shariff, M. Seul, P. Vissavajjhala, K. Hue-Roye, D. Charles-Pierre, C. Lomas-Francis, A. Chaudhuri, M.E. Reid, Transfusion 45 (2005) 680–688.
[43] G. Hashmi, T. Shariff, Y. Zhang, J. Cristobal, C. Chau, M. Seul, P. Vissavajjhala, C. Baldwin, K. Hue-Roye, D. Charles-Pierre, C. Lomas-Francis, M.E. Reid, Transfusion 47 (2007) 736–747.
[44] G.A.S. Guelsin, A.M. Sell, L. Castilho, V.L. Masaki, F.C. Melo, M.N. Hashimoto, T.T. Higa, L.S. Hirle, J.E.L. Visentainer, J. Clin. Lab. Anal. 24 (2010) 311–316.
[45] P.A. Bell, S. Chaturvedi, C.A. Gelfand, C.Y. Huang, M. Kochersperger, R. Kopla, F. Modica, M. Pohl, S. Varde, R.B. Zhao, X.J. Zhao, M.T. Boyce-Jacino, BioTechniques (2002) 70.
[46] G.A. Denomme, M. Van Oene, Transfusion 45 (2005) 660–666.
[47] M. St-Louis, J. Perreault, J. Lavoie, J. Émond, J. St-Laurent, A. Long, M. Richard, Transfus. Clin. Biol. 17 (2010) 242–248.
[48] A. Montpetit, M.S. Phillips, I. Mongrain, R. Lemieux, M. St-Louis, Transfusion 46 (2006) 841–848.
[49] J. Perreault, J. Lavoie, P. Painchaud, M. Cote, J. Constanzo-Yanez, R. Cote, G. Delage, F. Gendron, S. Dubuc, B. Caron, R. Lemieux, M. St-Louis, Vox Sanguinis 97 (2009) 61–68.
[50] S.A. Boccoz, P. Bailly, J.C. Bres, D. Rigal, L.J. Blum, C.A. Marquette, Vox Sanguinis 105 (2013).
[51] B. Veldhuisen, C.E. Van Der Schoot, M. De Haas, Vox Sanguinis 97 (2009) 198–206.