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A uniform method for the simultaneous blood group phenotyping of Fy^a, Fy^b, Jk^a, Jk^b, S, $\bar{s}$, P1, k applying lateral-flow technique

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Background and Objectives A lateral flow assay for simultaneous blood group typing of ABO, RhD, C, E, c, e, Cw and K with stable end-point and without centrifugation is in routine use since several years (MDmulticard®).
The typing of extended phenotype parameters belonging to the Duffy, Kidd, MNSs blood group systems and others, however, has not yet been demonstrated for this technique.
Reliable detection of Fy^x, a weak Fy^b phenotype with a pronounced quantitative reduction of the number of Fy^b antigens on the erythrocyte surface, remains a weakness of current serological blood grouping techniques.

Material and Methods The performance characteristics of the following reagents were evaluated in donor and patient samples in lateral flow technology (MDmulticard®): Anti-Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -$\bar{s}$, -P1 and -k. The sensitivity to detect Fy^x was in addition evaluated with Fy^x positive samples, which had been preselected by MALDI-TOF MS-based genotyping.

Results All results obtained with the MDmulticard® were in full accordance with those of the CE-certified reference products for all the eight reagent formulations used: Anti-Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -$\bar{s}$, -P1 and –k. Also, all Fy^x phenotypes of the selected population of 93 positive samples, originally identified by MALDI-TOF MS-based genotyping, were reliably detected by the lateral flow assay.

Conclusion Extended phenotype blood group parameters, including the serologically challenging Fy^x phenotype, can be determined simultaneously, rapidly and accurately using the lateral flow (MDmulticard®) technology, even in cases when IgG class antibodies are the only source of diagnostic antibodies.

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Introduction

The majority of current blood group serotyping techniques are based on the haemagglutination reaction between erythrocytes with unknown antigens and diagnostic antibodies. Typically, the reaction mixture is centrifuged either in a matrix-free glass tube (tube method), or a microtiter plate (solid-phase technique [1]) or passed through a gel matrix contained within a microcolumn (gel technique [2, 3]), before interpretation of agglutination is done. All these techniques share two disadvantages. Firstly, only one parameter may be determined per sample and tube, and secondly, centrifugation is needed.

Alternatively, migration of red blood cells (RBC) through a porous matrix has been described as rapid and

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reliable blood group serotyping technique [4]. These techniques may use different matrixes and test principles, such as nitrocellulose- and nylon-based dipstick [5], paper-based lateral flow and flow through assays [6, 7], and nitrocellulose-based lateral-flow technique (LFT) [8, 9]. Most typically, test RBCs bind to defined areas, impregnated with antigen-specific IgM antibodies. Here, several parameters can be detected per single sample application and without the need for centrifugation. Thereby, number of pipetting steps, hands-on-time and overall testing time is greatly diminished.

One limitation for the nitrocellulose- and paper-based LFTs has been the possibility to use only directly agglutinating mAb, which are usually IgM class antibodies [6, 10]. This may not affect the detection of ABO, RhD, RhCE and K antigens, as several different IgM mAbs are commercially available for each of these parameters. However, for other blood group systems, for example Fy, Jk and MNSs (extended phenotype antigens), some of the only commercially available reagents are mAb of the IgG class, and/or polyclonal antibodies (pAb). Using a paper-based assay, the use of IgG antibodies has recently been shown. Again, this approach would need a time-consuming step, that is an indirect agglutination, relying on the preincubation of the RBC with the antigen-specific mAb [7].

Beside the diagnostic antibodies, other factors are contributing to the challenge of having one common, quick, extended serotyping protocol. Those are the nature of the antigens themselves, their epitope presentation, and the extremely varying number of antigens per cell, even in normal phenotypes, and their even lower in weakly presenting phenotypes.

Enhancing haemagglutination in weakly presenting phenotypes may be achieved by preincubating RBCs with antibodies at different temperatures, or by treating RBCs with enzymes (e.g. papain, bromelain), or using polymeric potentiators (such as polyethylene glycol) and addition of 'bridging' antibodies (anti-human IgG) to agglutinate IgG sensitized RBC. However, although some of these strategies are ideal for the detection of certain antigens, they may be deleterious for others.

Occasional blood group phenotyping errors in the detection of a number of weak phenotypes became more evident with the advent of molecular techniques. This has been reported, for example for RhDs [11], the Kell blood group system [12, 13] and the Duffy blood group system [14].

In this study, performance characteristics in blood group typing for the parameters Fy^a, Fy^b, Jk^a, Jk^b, S, $\bar{s}$, P1 and k using nitrocellulose-based LFT are described. For the first time, we show the harmonization of the methods for the detection of the extended phenotype antigens. Moreover, this technique requires only few pipetting steps and is independent from electric power supply and centrifugation, and mAbs of the IgG class are utilized without the need for preincubation with RBCs. Additionally, we were able to show on a large number of genetically predefined Fy^x positive individuals that the nitrocellulose-based LFT reliably recognizes Fy^x in routine serotyping.

Material and methods

Blood samples

In the performance evaluation for Fy^a, Fy^b, Jk^a, Jk^b, S, $\bar{s}$, P1 and k using nitrocellulose-based LFT, for each parameter, 160 freshly drawn blood samples with historic phenotypes recorded have been tested. Twelve additional samples with negativity for k (Cellano) were included. Sample material was provided in tubes with standard concentrations of ACD, EDTA, CPDA or Citrate. Alternatively, sample material was clotted or directly derived from blood bag segments. The population of 160 fresh samples came from 148 donors, 10 clinical patient and two newborns.

The totality of the patient and neonatal blood samples, and 89 of donor blood samples were provided by the German Red Cross, DRK-Blutspendedienst Baden-Württemberg-Hessen gGmbH. A total of 90 donor blood samples were provided by Service Regional Fribourgeois de transfusion sanguine CRS, Hôpital cantonal, Fribourg and 59 by the internal blood donor station of Medion Grifols Diagnostics AG. All material was derived from excess routine diagnostic material, and all individual data were blinded. All blood group testing was performed in accordance with the Swiss national legislation.

For the Fy^x typing performance evaluation, 137 EDTA-anticoagulated blood donor samples were provided by the Blood Transfusion Service Zurich (Swiss Red Cross, Schlieren, Switzerland) from excess routine diagnostic material and with all individual and genetic prevalue data blinded. All sampling was performed in accordance with the Swiss national legislation. Duffy phenotypes and *ACKR1* (formerly known as *DARC*) genotypes were obtained as described previously in Meyer *et al.* (2014) [15] and were both available for 95 donors. For the remaining 42 donors, only standard Fy serotypes were known.

Nitrocellulose-based lateral-flow technique

The test device (MDmulticard®) is composed of a nitrocellulose membrane contained in a plastic, credit card-sized cassette housing. On the membrane, there are two equidistant detection areas left and right of a central application zone. Each detection zone can contain up to

five parallel lines of antibody reagents plus a process control spot (val) and an auto control spot (ctl) [4]. In this study, the following antibodies are formulated and dispensed on the membrane: Anti-Jk^a clone P3HT7 and Anti-Jk^b clone P3·143 (Diagast, Loos, France), Anti-Fy^a clone P3TIM, Anti-Fy^b clone SpA264LBg1, Anti-S clone MS-94, Anti- $\bar{s}$ clone P3BER, Anti-P1 clone P3MON2 and Anti-k clone P3A1180L67 (Merck Millipore, Darmstadt, Germany).

Briefly, in the nitrocellulose-based LFT, blood is prediluted by mixing 200 µl of a diluent (Diluent F, Medion Grifols Diagnostics, Duedingen, Switzerland) with either 50 µl of whole blood, 25 µl of erythrocyte sediment or 200 µl of clotted blood. Then, 100 µl of this premix is added to the application zone. After approximately 30 s, the test is rinsed by repeating the addition of the diluent to the application zone. End results may be interpreted after 5-10 min processing at room temperature. Positive results clearly impose as distinct red bands, whereas negative results lack the respective bands (Figs. 1a and b).

Performance evaluation of Anti-Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -$\bar{s}$, -P1 and –k in nitrocellulose-based LFT

In the nitrocellulose-based LFT, results obtained with the whole blood, erythrocyte sediment and clotted blood method were compared with the blood group typing carried out with the following CE-certified reagents: Anti-Fy^a,-Fy^b,-Jk^a,-Jk^b for DG Gel, Anti-S and Anti-k (Cellano) Mono-Type (Medion Grifols Diagnostics) and ID-Anti-s and DiaClon Anti-P1 (Bio-Rad, Cressier, Switzerland).

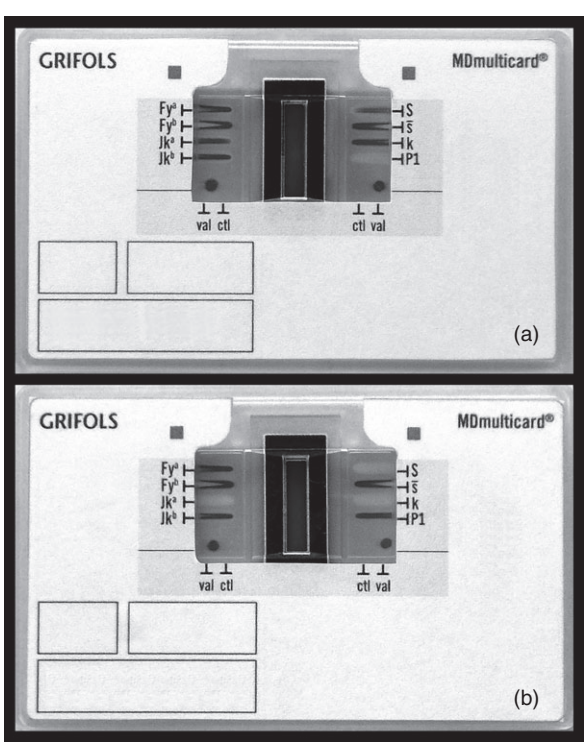

Fig. 1 Examples of the extended phenotyping using nitrocellulose-based lateral-flow technique. (a) Donor Fy(a+b+) Jk(a+b+) S+$\bar{s}$+ k+ P1-; (b) Donor Fy(a+b+) Jk(a-b+) S- $\bar{s}$+ k- P1+.

Fy^x typing performance evaluation in nitrocellulose-based LFT

All 137 samples for Fy^x typing performance evaluation were investigated at Medion Grifols Diagnostics by one single technician without prior knowledge of their pheno- and genotype prevalues. For the nitrocellulose-based LFT, testing with the whole blood and erythrocyte sediment method has been carried out with all samples. Afterwards, the phenotyping results were compared to pheno- and genotype prevalues, earlier obtained at the Blood Transfusion Service Zurich.

Reference typing with serological methods for the Fy^x study

An additional and extended serological analysis was carried out manually on these 137 samples with the following reagents and material: Test Serum ID-Anti-Fya/Fyb, ID-Card Fya/Fyb, ID-Diluent 2 and ID-Centrifuge (DiaMed-ID Microtyping System, Bio-Rad, Cressier, Switzerland), Anti-Fy^a for DG Gel, Anti-Fy^b for DG Gel, Anti-Fy^a, Anti-Fy^b, Anti-Fy^a Mono-Type® and Anti-Human Globulin Mono-Type (Medion Grifols Diagnostics, Duedingen, Switzerland), BIOSCOT® Anti-Fy^b (Merck Millipore Ltd, Livingston, UK), DG Gel® Neutral and DG Gel® Coombs cards, DG Gel® Sol, DG Therm, DG Spin (Diagnostic Grifols, Barcelona, Spain). All reagents were used according to the manufacturers' instructions.

Results

Performance evaluation of Anti-Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -$\bar{s}$, -P1 and –k in nitrocellulose-based LFT

For each parameter analysed, the phenotypic distribution of the blood samples tested was similar to the natural occurrence in Caucasians with the exception of the k (Cellano) antigen. Considering that this is a high-prevalence antigen, the number of Cellano negative samples was artificially increased in this study to have a more significant evaluation of the antibody specificity.

For all the eight reagent formulations used, that is Anti-Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -$\bar{s}$, -P1 and –k, all results obtained using the nitrocellulose-based LFT were in full concordance with those of the CE-certified reference products (Table 1). Therefore, each of these antibodies displays a sensitivity

Table 1 Specific performance characteristics of Anti-Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -$\bar{s}$, -P1 and -k in nitrocellulose-based LFT

	Anti-Jk^a	Anti-Jk^b	Anti-Fy^a	Anti-Fy^b	Anti-S	Anti-$\bar{s}$	Anti-k	Anti-P1
Total sample tested (*n*)	160	160	160	160	160	160	172	160
Positive samples (*n*)	118	121	100	120	85	139	157	117
Positive samples (%)	73·8	75·6	62·5	75	53·1	86·9	91·3	73·1
Sensitivity (%)	100	100	100	100	100	100	100	100
Negative samples (*n*)	42	39	60	40	75	21	15	43
Negative samples (%)	26·3	24·4	37·5	25	46·9	13·1	8·7	26·9
Specificity (%)	100	100	100	100	100	100	100	100

and specificity of 100% for the typing of the respective blood group. In addition, the results obtained were consistent no matter which anticoagulant was used for the blood specimen collection or if whole blood, erythrocyte sediment or clotted blood was used for the preparation of the diluted suspension for the application to the card. Finally, erythrocyte sediment obtained from blood bag tubing segments was used as test sample material and found to deliver correct results in all cases, delivering a formal proof for suitability of such material for performing pretransfusion compatibility checks in the laboratory or directly at the patient bedside. Some typical results obtained with the parameters Fy^a, Fy^b, Jk^a, Jk^b, S, $\bar{s}$, P1 and k using the nitrocellulose-based LFT are shown in Fig. 1.

Fy^x typing performance evaluation

In the course of preliminary studies, an improved Fy^x detection using the nitrocellulose-based LFT was noticed on two *FY*A/ FY*02W* heterozygous donor samples (data not shown). Thus, a performance evaluation focusing on Fy^x typing was carried out, including a total of 42 blood samples, randomly chosen for their Duffy phenotypes, and 95 samples with known genetic *FY*02W* positivity. The latter 95 samples consisted of 39 heterozygous *FY*A/ FY*02W*, 54 heterozygous *FY*B/FY*02W* and two homozygous *FY*02W* genotypes, encoding for the blood group Duffy phenotypes Fy(a+bweak+), Fy(a-b+) and Fy (a-bweak+), respectively. The final 137 sample panel had a strong statistical overrepresentation of samples positive for Fy^x (69·3%), normally expected to range at about 2% in an average Caucasian population. The 39 *FY*A/ FY*02W* heterozygous individuals were of specific interest for evaluation purpose as they included 21 previously serologically 'unrecognized' cases, that is samples falsely typed as Fy^b negatives.

Using whole blood method, only one of the 95 samples with known genotypic positivity for Fy^x was typed Fy^b negative. However, using erythrocyte sediment method, all 95 samples were correctly recognized as Fy^b positives (Table 2).

With respect to the 42 samples with randomly chosen Duffy phenotypes, but unknown *ACKR1* genotypes, all results but one obtained with the nitrocellulose-based LFT were concordant with the serological prevalues from the Blood Transfusion Service Zurich (Table 2). The discrepant sample has been typed Fy(a+b-) with routine serology in the Blood Transfusion Service Zurich previously, but showed a Fy(a+Fybweak+) phenotype by nitrocellulose-based LFT. Retesting using polyclonal Anti-Fy^b reagents in Gel Technique and Tube Method showed inconclusive results. Blood group genotyping on new sample material of this donor was thus requested and finally confirmed Fy^x positivity and a *FY*A/FY*02W* genotype.

An in-depth comparison of different serological Duffy typing techniques/reagents included gel technique, tube method and nitrocellulose-based LFT on all 137 samples (examples shown in Fig. 2). For the Fy^b antigen determination of known Fy^x positive samples, the percentage of concordance between each particular serological method and the molecular typing is shown in Table 3.

The mAb Anti-Fy^b evaluated using the nitrocellulose-based LFT is identical to the clone as used for the manufacturing of one of the liquid reagents tested in this study. However, using the Gel technique and the above-mentioned mAb, 46·3% of the 95 Fy^x positive samples tested in this study, and 100% of the 39 genetically predefined *FY*A | FY*02W* heterozygous individuals were non-reactive for 'Fy^b'.

Discussion

Nitrocellulose-based LFT had been presented earlier for ABO, Rh and K antigen typing with unique features, such as quick multi-antigen determination per sample application and without the need for centrifugation [8, 10]. In this study, comparable performance characteristics could be shown also for the detection of Fy^a, Fy^b, Jk^a, Jk^b, S, $\bar{s}$, P1 and k. Indeed, performance evaluation showed 100% sensitivity and specificity, while simultaneously avoiding the need for different phases, incubation times and

Table 2 Fy^x typing performance evaluation using nitrocellulose-based LFT

MALDI TOF MS			Serological Prevalues			Nitrocellulose-based LFT					
						Erythrocyte Sediment Method			Whole Blood Method		
allel-1 | allel-2	Phenotype deduced from genotype	*n*=	Fy (a+b-)	Fy (a+b+)	Fy (a-b+)	Fy (a+b-)	Fy (a+b+)	Fy (a-b+)	Fy (a+b-)	Fy (a+b+)	Fy (a-b+)
*FY*A* | *FY*02W*	Fy(a+b+)	39	21	18	0	0	39	0	1	38	0
*FY*B* | *FY*02W*	Fy(a-b+)	54	0	0	54	0	0	54	0	0	54
*FY*02W* | *FY*02W*	Fy(a-b+)	2	0	0	2	0	0	2	0	0	2
ND	n.a.	42	15	15	12	14	16[a]	12	14	16[a]	12
Total		137	36	33	68	14	55	68	15	54	68

LFT, Lateral-flow technique; ND, not determined; n.a., not applicable;

[a]1 sample which has been typed Fy(a+b-) in the serological prevalues, was found Fy(a+b+) with the nitrocellulose-based LFT and later confirmed *FY*A* | *FY*02W* by MALDI-TOF genotyping.

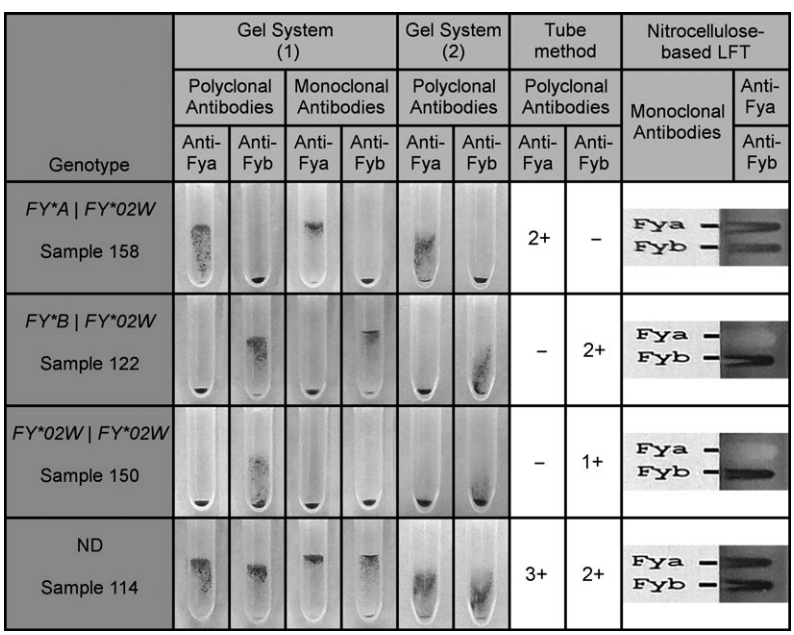

Fig. 2 Comparison of results obtained with Fy^x positive samples using different techniques. (1) DG Gel® System, Diagnostic Grifols; (2) DiaMed-ID Microtyping System, Bio-Rad; LFT, lateral-flow technique; ND, not determined.

temperatures as usually required with current serological techniques. Moreover, and although for some antigens only IgG mAbs were available, the uniformed process parameters allowed for the direct typing of the extended blood group phenotype, that is without the need for preincubation of RBCs with antigen-specific antibodies. In comparison with currently available technologies, testing time for the Fy antigens was pronouncedly reduced from about 25 to 8 min.

The importance of extended phenotype matching in blood typing has been emphasized as preventive measure for chronically transfused recipients with increased risk for alloimmunization, such as Sickle Cell Disease and Thalassaemia patients [16]. The most relevant antigens to be typed for these patients have historically been C, E and K. However, there is agreement that a most efficient prevention of alloimmunization may be achieved by comprehensive typing for C, c, E, e, K, Fy^a, Fy^b, Jk^a, Jk^b, S, $\overline{s}$ [17–20]. Although several authors pointed to the fact that molecular typing might be superior in comparison with serological typing [21], our study showed that nitrocellulose-based LFT could be useful in prescreening and might be seen complementary to genotyping, especially when considering the speed of time-to-result and the independence of the presented method of an excessive laboratory infrastructure.

Table 3 Concordance in Fy^x detection between Molecular typing and different serological methods and reagents

MALDI-TOF			mAb[a] Anti-Fy^b in nitrocellulose-based LFT[b]		pAb[c] Anti-Fy^b in gel system[d]		pAb[c] Anti-Fy^b in gel system[f]		pAb[g] Anti-Fy^b in tube method		mAb[h] Anti-Fy^b in gel system[f]	
			Fyb+		Fyb+		Fyb+		Fyb+		Fyb+	
allel-1 | allel-2	Phenotype deduced from genotype	*n*=	*n*	%[i]	*n*	%[i]	*n*	%[i]	*n*	%[i]	*n*	%[i]
*FY*A | FY*02W*	Fy(a+b+)	39	39	100	5	12·8	17	43·6	12	30·8	0	0
*FY*B | FY*02W*	Fy(a-b+)	54	54	100	54	100	54	100	54	100	50	92·6
*FY*02W | FY*02W*	Fy(a-b+)	2	2	100	2	100	2	100	2	100	1	50
TOT		95	95	100	61	64·2	73	76·8	68	71·6	51	53·7

[a]mAb Anti-Fyb, clone SpA264LBg1 (Merck Millipore Ltd).
[b]Lateral-flow technique; using Erythrocyte sediment method.
[c]ID-Anti-Fyb (Bio-Rad).
[d]DiaMed-ID Microtyping System (Bio-Rad).
[e]Anti-Fyb for DG Gel (Medion Grifols Diagnostics).
[f]DG Gel® System (Diagnostic Grifols).
[g]Anti-Fyb (Medion Grifols Diagnostics).
[h]BIOSCOT® Anti-Fyb (Merck Millipore Ltd).
[i]Sensitivity compared to the phenotype deduced from the genotype.

Additionally, the method provided improves sensitivity in the detection of the weak phenotype Fy^x and equals molecular typing. Not only we were able to identify all genetically known Fy^x positive samples but also another sample falsely typed as Fy^b negative by standard serological methods which had not been uncovered until our finding. As shown within this study, molecular blood group typing is an excellent tool to provide specifically preselected testing panels, fully representative of a wide variety of specific antigens, though consisting of a relatively small sample number, only. Developing more sensitive serological reagents is thereby facilitated due to genotypically defined phenotypes.

The study underlines a pronounced difference in sensitivity between different serological techniques, even in using identical clones (in this case, Anti-Fy^b antibody, clone SpA264LBg1). These differences may be dependent on the formulation of the reagents and/or the conditions and properties of a given test format. With respect to the nitrocellulose-based LFT, several technical properties may contribute to its improved performance.

Firstly, the nitrocellulose membrane matrix may be interpreted as an open lateral immuno-chromatographic column. Thereby, non-reacting erythrocytes are removed from the detection system, when leaving the chromatographic matrix opposite to the application area. As a consequence, compared to other techniques, a larger number of cells can be loaded into the system without compromising end-point readability. The signal-to-noise is thus optimized, leading to more distinct end-points of the reactions, especially in cases of blood group antigens with weak expression. Looking at other techniques, small agglutinates of RBCs and non-agglutinated RBCs share the same space, making it difficult to recognize weak agglutination reactions.

Secondly, the nitrocellulose-based LFT is independent of centrifugation. Thus, it avoids elevated shear forces present in centrifugation-dependent techniques [22]. This is most important to maintain agglutinates formed by RBCs with weakly expressed antigens, and/or antibodies with low affinity and/or avidity to specific antigens.

Lastly, diagnostic antibodies are concentrated, dried and immobilized at distinct positions in the matrix of the nitrocellulose-based LFT. This allows adherence of single RBCs to their specific immobilization sites inside the antibody-coated matrix area with or without formal agglutination events. Therefore, in comparison with haemagglutination-based techniques, LFT allows increased number of red cells to interact with the respective diagnostic antibody, adding to improved diagnostic sensitivity. Also, antibodies are dried in a relatively small area, which may facilitate the capture of incoming RBC by increasing the rate of contact [23]. Drying and immobilization of antibodies may further lead to modifications in their physico–chemical properties, stability and activity compared to antibodies presented in liquid form [24].

In summary, the use of well-selected clones with the proposed diagnostic nitrocellulose-based LFT may provide

an improved method for rapid and reliable blood group phenotyping for potentially all blood group antigens, requiring less resources and time as compared to traditional serological typing.

The presented study extends previously reported typing capacity of nitrocellulose-based LFT from ABO, Rh and K to Fy^a, Fy^b, Jk^a, Jk^b, S, $\overline{s}$, P1, k, including reliable Duffy x (Fy^x) detection. Furthermore, it extends the range of mAb to be used with the LFT, as not only IgM, but also IgG mAbs can be used in one simple and uniform protocol.

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Conflict of interests

A Caesar and P Schwind are employees of Medion Grifols Diagnostics AG. S Meyer, N Trost, K Neuenschwander, C Geisen, BM Frey and C Gassner have no conflict to declare.

Authorship

A Caesar, C Gassner and P Schwind designed and supervised the study. A Caesar, S Meyer, N Trost and K Neuenschwander performed the experiments. C Geisen contributed essential material. A Caesar, N Trost and K Neuenschwander analysed the experimental data. A Caesar, BM Frey, C Gassner and P Schwind wrote and/or commented the manuscript. All authors reviewed and approved the manuscript.

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