# Enhanced fully cellulose based forward and reverse blood typing assay

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## Abstract

This study presents an enhanced paper-based analytical device (PAD) for forward and reverse group blood typing. The proposed PAD uses a novel methodology, which provides highly reliable results on a fully cellulose based device. The PAD was printed on different cellulose substrates. These substrates were made of different cellulose fibers (sisal and eucalyptus), different grammages, refining steps and wet additive content. Best parameters were chosen to achieve high reliability on both forward and reverse blood typing. The substrates were patterned with five hydrophilic channels and two hydrophobic areas. For reverse blood typing, the hemo-agglutination reaction took place on the hydrophobic surface of the paper before being transferred to the paper web, where together with the forward blood typing tests were all washed with saline solution to read the results by elution. This device allows direct read-out of results; the stains show were agglutination happens. Different blood types were in full agreement between the reverse and forward method and in agreement with traditional methods. The time and simplicity of this methodology confirmed its utility.

Keywords: Sisal-based paper, Lab-on-a-Chip, Sisal paper, eucalyptus paper, paper-based microfluidics, blood typing test, Point-of-Care Testing (POCT).

## 1. Introduction

Human blood is characterized by the presence of certain antigens on the surface of red blood cells (RBCs). The most common antigens are A and B, and their presence generates the corresponding isoagglutinin in plasma (anti-A and anti-B isoagglutinins). Therefore, human blood is usually characterized in terms of ABO blood groups. Blood groups are commonly characterized as such, since in any non-programmed medical situation the transfusion of a different blood group could cause the patient's so-called "transfusion reaction," occurring immediately upon transfusion of incompatible blood and causing severe health problems or death [1]–[3].

The determination of blood type is typically done by localizing the antigens on the RBC's surface, called forward method, and by detecting the presence of certain isoagglutinin in plasma, called Reverse method [4].

Blood typing tests are based on haemagglutination, the RBCs' agglutination reaction in presence of the opposite antibody. Blood A type has isoagglutinin called Anti-B, and Blood B type has isoagglutinin called Anti-A, blood type O has no antigen and both isoagglutinins. Therefore when droplet of blood gets in contact with the antibody that it is not normally present in it, the blood will agglutinate [5]. The methods most commonly used to perform such characterization are tube, slide, and gel-based. Tube and slide methods are not highly sensitive, while gel-based methods can perform both methods (forward and reverse), providing more sensitive and reliable results but requiring a more time consuming protocol.

In 2006, Whitesides proposed for the first time the concept of microfluidics, which using inherited microfabrication processes offered the potential to miniaturize processes from the clinical labs [6]. Recent studies have proposed to take advantage of microfluidics, it reduces the volumes required to obtain results down to microliters and provides short turnaround times. However, some of the proposed designs require complex techniques, i.e. flow visualization methods [7], embedded fiber optics [8], quantum dots [9] or optical prisms [10]. In 2008, Whitesides group proposed a novel technique that uses paper as a substrate material to produce microfluidic patterns [11-13]. This technique can expand the advantages of microfluidics to low resource environments and therefore generalize the types of tests than can now be implemented [14]. Paper-based analytical devices (PADs) use a network of cellulose fibers to trap the RBCs once agglutinated and visually identify the blood group [15-17]. Most PADs for blood grouping use antibodies that have direct agglutinating capability (mostly IgM); therefore they provide only one method (forward) [16],[17]. Detection of RBC agglutination due to natural antibodies present in patient's serum is more challenging. In commercial kits (forward blood typing), the concentration of antibodies is optimal to create agglutination, however in patient's serum the concentration of natural antibodies can change from patient to patient and the concentration may not be the optimal to generate an strong agglutination in reverse blood typing. Recently, two studies proposed to use Whatman paper substrate to create a forward and reverse paper based blood typing test [18][19]. Both designs reported the results on a Barcode that differentiated

agglutinated results (short bar) versus non-agglutinated results (long bar). This method of reporting the results did not provide a clear threshold for determining a clear agglutination, and the weakness of the agglutination reaction in the reverse blood typing test decreased the test sensitivity. The overcome this issue, Li et al. proposed a method that combined a traditional slide test on a plastic surface with a paper-based readout of the sample [20].

In this study, a PAD for forward and reverse ABO blood group typing is developed in a single cellulosic substrate, taking advantage of hydrophobic patterning [21] to allow the hemoagglutination of plasma patient antibodies with RBCs on the surface of the cellulosic substrate before being transferred to the paper network for readout as circular stains. This method provides simple readout of forward and reverse blood group without the need of complex lab equipment. We investigated the effect of cellulosic fiber length, wet strength agent, channel patterning width and surface chemistry of cellulose to propose a novel fully cellulose based forward and reverse blood typing test. Lab papers with different basis weights and degree of refining were made with and without wet strength agent additive.

The influence of the paper manufacturing conditions (basis weight, wet strength agent) was examined assessing their impact on blood typing results. The paper and its pattern was optimized for a clear distinction between agglutination of RBCs by effect of antibody–antigen interactions and free to move RBCs. An alkylketene dimer (AKD) hidrophobic region was used to enhance the reverse blood typing.

## 2. Materials and methods

This section describes the methodologies used to develop a forward and reverse blood typing test on different cellulose papers.

### *2.1. Lab papers at different refining degree*

2.1.1 Sisal and eucalyptus lab paper at different refining degree

The lab paper sheets were produced from elemental chlorine free (ECF) bleached sisal and eucalyptus fibers. Sisal pulp (S-pulp) was obtained from CELESA mill in Tortosa (Spain) and Eucalyptus (E-pulp) from ENCE mill in Pontevedra (Spain). Initially, the pulp was disintegrated at 30,000 revolutions (S0 and E0 pulps), and then, in a PFI mill, one aliquot of pulp was refined at 1000 revolutions per minute (S1000 and E1000 pulps) following procedures from standards ISO 5263 and ISO 5264, respectively. Refining modify the fiber surface and increase the interfiber bonding capacity, therefore the paper network changed, achieving better sheet formation and enhanced mechanical properties. Following ISO 5267 and ISO 23714 standards, the samples were processed to determine the drainability by Schopper-Riegler method (ºSR degree) and the water retention value (WRV). Fiber length and percentage of fines were measured according to TAPPI standard method T271 using a Kajaani FS300 fiber analyzer. Rapid-Köthen method was used to prepare lab sheets, according to ISO 5269, at different basis weight from each unrefined and refined fibers samples. The EKA WS505 wet strength PAAE

(polyamidoamine-epichlorohydrin) resin from Akzo Novel Chemicals was added up to 6% in the paper pulp to see the effect on blood typing.

### *2.2. Paper properties characterization*

Following ISO187 standard, the papers were conditioned for at least 24h hours at 23ºC and 50% relative humidity and the main physical characteristics were measured (basis weight, thickness, apparent density and wet and dry tensile strength). All these properties were measured from ten replicates according to ISO 536, ISO 534, ISO 5636-3 and ISO 1924 standards, respectively. The wet strength development (W/D ratio) was calculated as the ratio between wet strength and dry strength. Klemm method was used to measure capillary rise following ISO 8787 doing 4 replicates for each sample of paper. Porometer 3G analyzer (Quantachrome Instruments) was used to measure the pore size distribution according to ASTM F316-03 standard. The Grubbs' Test was applied to detect outliers at a 0.05 significance level. Experimental errors were calculated as mean and standard deviation in accordance with the respective standards. **Table I** shows the nomenclature used.

### *2.3. Pattern printing on paper*

The method described by Casals-Terré et al. was used to deposit the patterns required using a PRUSA i3 Printer with a blunt G25 needle injector. The ink used was a hydrophobic mixture of alkylketene dimer (AKD) and colophony (Fennosize G7020F purchased from KERIMA) [21]. This product required a thermal curing to achieve the hydrophobicity (chemically bond their fatty acids to cellulose hydroxyl groups), therefore, the printed patterns were heated in an oven at 100ºC for 10 min just after printing.

### *2.4. Blood and antibodies*

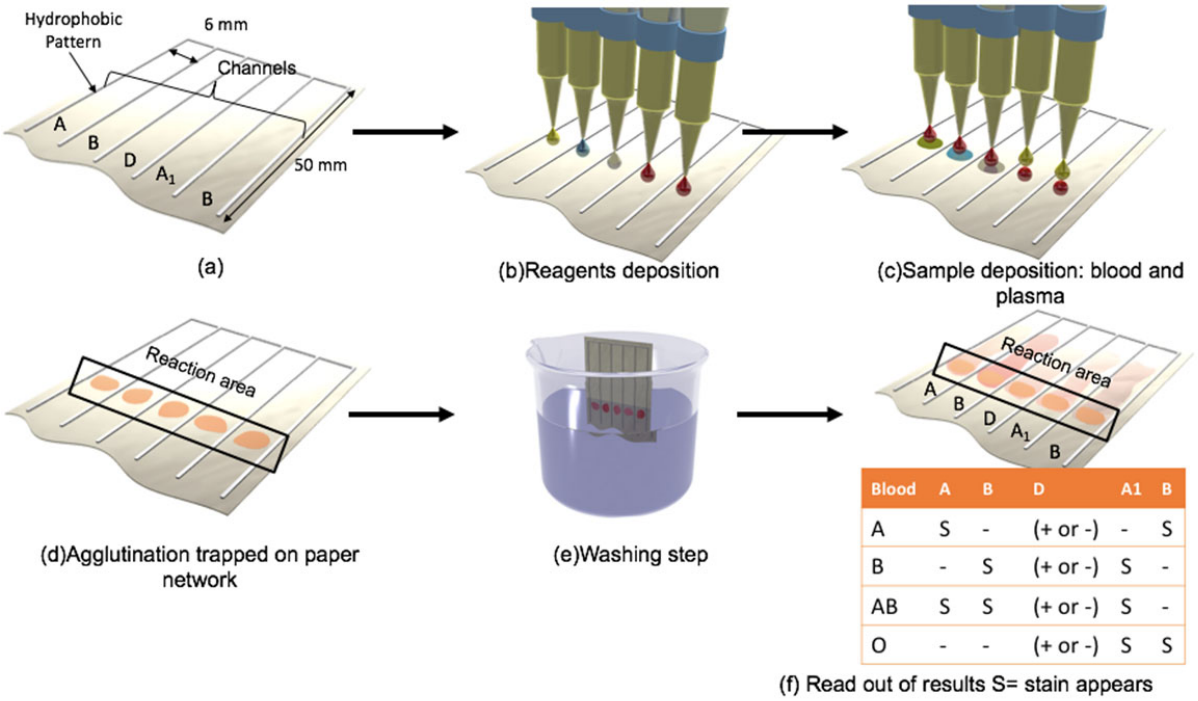
Blood was obtained from healthy donors and stored in ethylenediamine tetra-acetic acid (EDTA) tubes at 4º C less than 2 days before tested. Blood was initially characterized using conventional methods in Laboratori de Referencia de Catalunya.

Analytical grade 0.9% NaCl saline solution and phosphate buffered saline (PBS) were purchased from Sigma Aldrich. DiaClon Monoclonal IgM antibodies Anti-A, Anti-B, and Anti-D for blood grouping characterization were purchased from Bio-Rad. These antibodies are labeled A B and D respectively in Figure 1. From the blood typified, red blood cells with antigen A and red blood cells with antigen B were separated and resuspended in PBS at 30% in concentration. These RBCs are labeled A1 and B in Figure 1.

### *2.5. Methods for blood typing test*

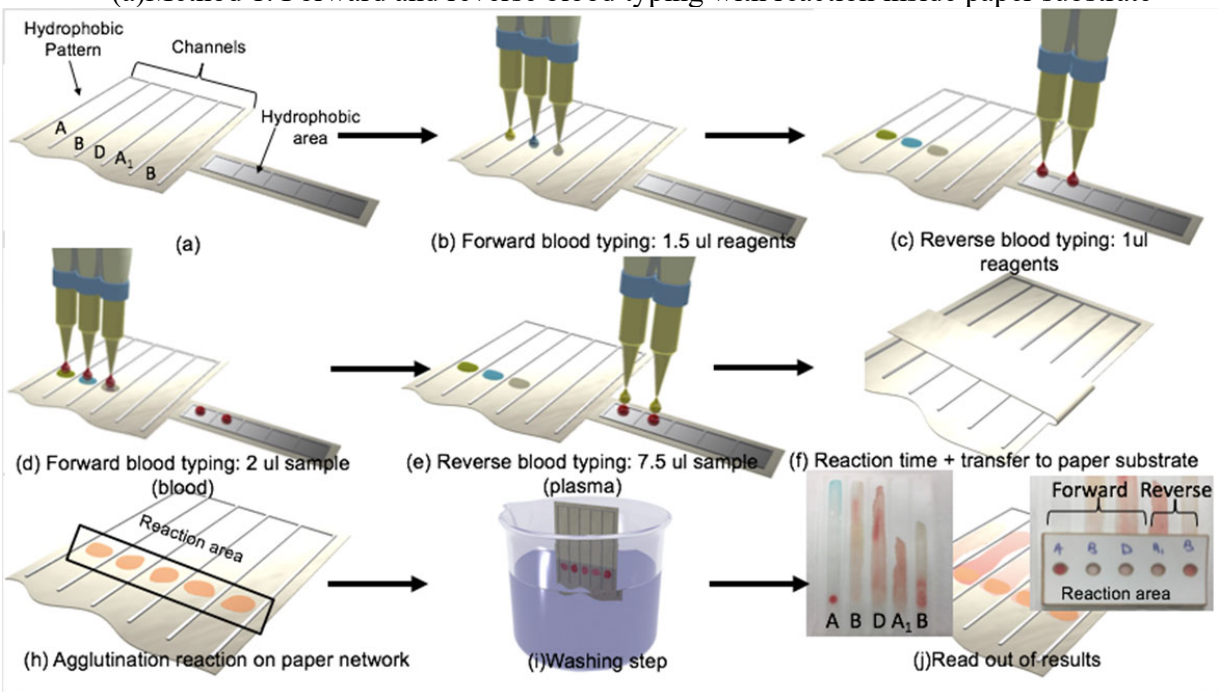
Forward and reverse blood typing tests were implemented on an array of rectangular channels. Three of the ends of the channels were allocated for the antibodies required for forward blood typing (anti-A, Anti-B and anti-D are deposited) and the other two were used for the reverse blood typing.

Two different methods were implemented to achieve results. First, one followed the method appointed by Songjaroen et al. [18] where all the reagents were directly deposited on the paper, and the sample was loaded afterwards.




| Blood | A | B | D | A1 | B |
|---|---|---|---|---|---|
| A | S | - | (+ or -) | - | S |
| B | - | S | (+ or -) | S | - |
| AB | S | S | (+ or -) | S | - |
| O | - | - | (+ or -) | S | S |

(a)Method 1. Forward and reverse blood typing with reaction inside paper substrate




(b)Method 2. Forward and reverse blood typing with reaction of reserve method on the surface of

a hydrophobic pattern.

**Figure 1.** Forward and reverse blood typing test on paper substrates.

**Figure 1** shows the testing procedure for blood typing using the two methods implemented. Initially 1.5 µL of DiaClon Monoclonal IgM antibodies Anti-A, Anti-B, and Anti-D from Bio-Rad and 0.5 µL of A1 and B red blood cells at 30% concentration were pipetted onto the reaction areas. Then, fresh patient blood was diluted (1:1) with PBS and a droplet of 2 µL was deposited in the middle of each channel for forward blood typing, and 2.5 µL droplet of patient plasma were deposited on the reverse channels. Finally the paper was immersed 1 cm into 0.9% NaCl saline solution for 90 sec.

In method 2, the forward blood typing followed the aforementioned procedure.

For the reverse blood typing method, two hydrophobic areas had been previously patterned next to the channels. Two droplets of 1µL of known RBCs at 30% concentration (diluted in PBS) were deposited and mixed with 7.5 µL of patient sample (see Supplementary Material 2 (video)). The mixture was allowed to react for 30s on the paper surface, previously coated with AKD (alkyl ketene dimer) for hydrophobization, see detail on **Figure 1** (b). The droplets from the hydrophobic pattern were then transferred to the paper network by folding the paper. Finally the paper was immersed 1 cm into 0.9% NaCl saline solution for 90 seconds. (See supplementary material 2: video)

Channels that had hemoagglutination reaction presented a blood stain in the reaction area, Figure 1. In both methods, a picture was taken with a conventional cell-phone camera where the stains had appeared and the intensity of the blood spot was quantified by ImageJ software. The gray scale mean optical density of spot was compared on each region. The grey intensity level value was measured to quantify the positive and negative result. A high grey intensity value means a clean area and therefore a negative result.

## 3. Results and discussion

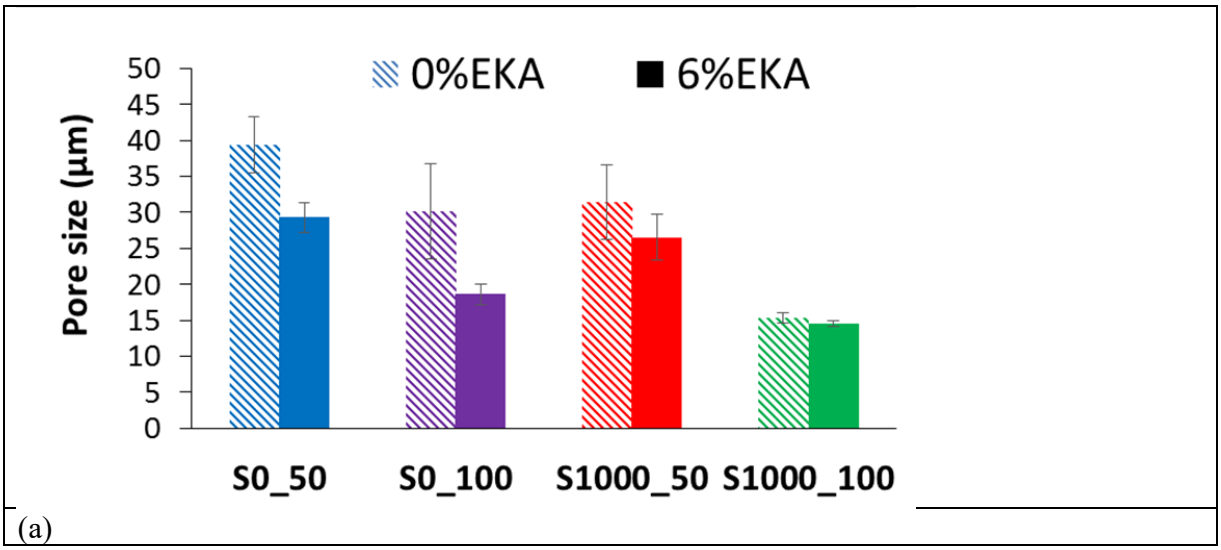
**Table I** shows selected characteristics and properties of hand sheets made from sisal and eucalyptus based paper obtained in different basis weights. Previous works form Casals-Terré et al. [22], had shown that papers with higher refining were not adequate for blood typing. However, the refining process is required in order to improve the bonding of fibers, to give more conformability and strength to the paper. For this reason, sisal samples were refined, but only up to 1,000 rev. Moreover, as low grammages are used, a wet strength resin was applied in some papers. The purpose of the wet strength agent is to increase the strength of the paper during the washing step of the blood typing test.

*3.1 Effect of refining and wet strength additive on fiber morphological properties*

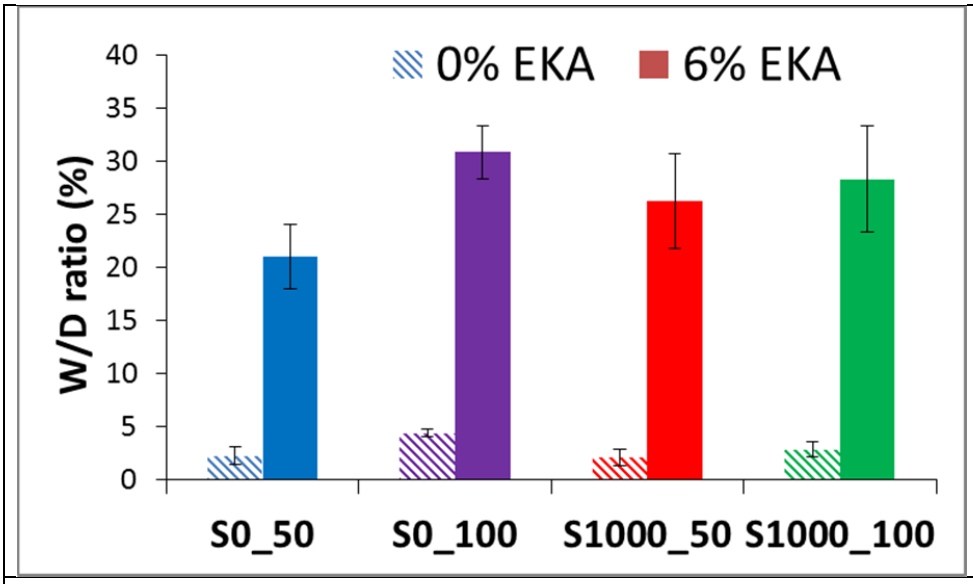
According to the results summarized in **Table I,** when the paper is refined, the apparent density of paper increases since the fibers are more flexible and conformable. Therefore a higher fiber bonding effect is produced. As a consequence, the pore size decreases, since the fiber network is better bonded (**Figure 2** (a)), which is in accordance with our previous research [22].

In general, paper loses almost all its strength when it is saturated with water, retaining only 2-10% of its original dry strength [23]. During the washing step of the blood typing test, the paper must sink in a liquid solution, which will rise by capillarity, sweeping along the non-agglutinated RBCs. Therefore, it is important that the paper can keep its behavior and strength until the end of this step. As it is shown in **Figure 2** (b), wet strength development was improved when the EKA resin was added to the paper, ensuring that paper will not break during the washing step.

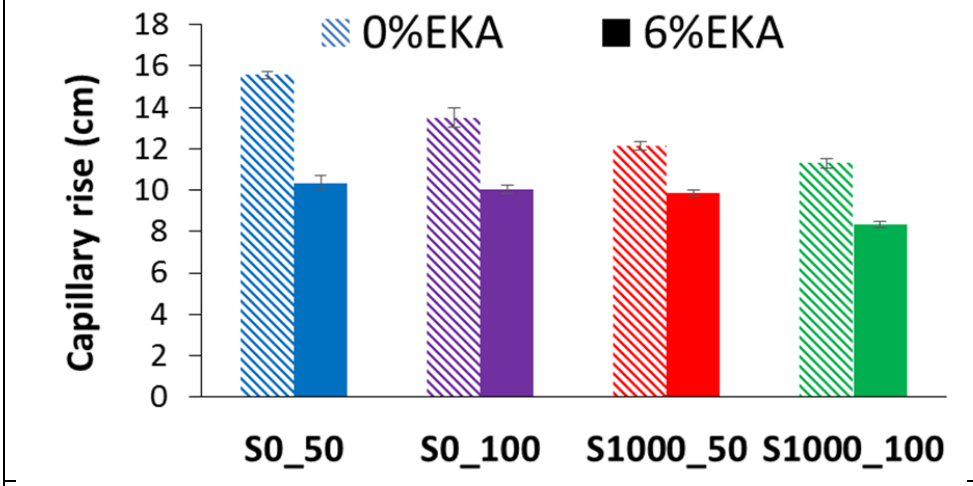
The capillary rise was measured using the Klemm method, which consists in a measurement of the height of the water rise after 10 min. During the test, the paper is suspended vertically and its lower end is immersed in water. Therefore, Klemm results provide information on the speed of the liquid through the paper by capillary ascension. **Figure 2** (c) shows that EKA resin reduced the capillary rise, which means that the speed of the water through the network of paper was lower. Corresponding to this trend, the pore size (**figure 2** (a)) was also decreased with the wet strength resin. Capillary rise and pore size also decreased with the basis weight due to the increase of fiber amount along the z-dimension (thickness) of paper.




(a)




(b)




(c)

**Figure 2.** (a) Pore size, (b) wet strength development and (c) capillary rise results of sisal papers at different basis weights, refining, and percentage of EKA (0% for striped pattern and 6% for plain pattern).

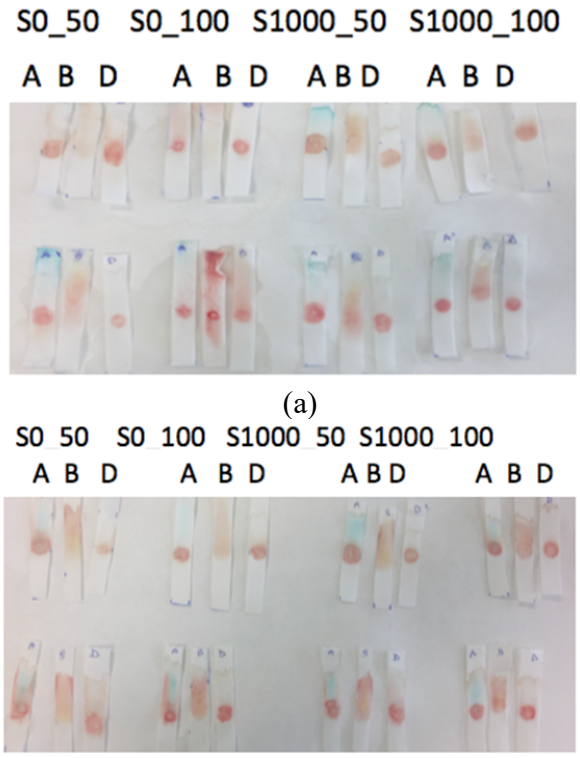
### *3.2 Effect of wet strength additive on blood typing*

#### 3.2.1 Forward blood typing

First, the forward blood typing test was implemented using all the sisal papers. **Figure 3** shows two repetitions of A+ test on different papers without EKA (a) and with EKA wet strength resin (b). The positive results are clearly readable in all papers, however the negative results do not clean completely in papers treated with the wet strength additive (EKA). The EKA papers showed a lower capillary rise and pore size (**Figure 2**), which agree with the results from

previous research [22] where papers with a pore size less than 15 um and with low capillary rise were the worst cleaned. However, another possible reason is that wet strength additives (cationic polymers) can interact with RBCs, which are negatively charged and decrease the freedom of RBCs to move through the paper network.

On the other hand, the non-treated EKA papers that are non-refined also present more cleanness than refined papers and therefore the difference between positive and negative is more clear. Therefore, in sisal papers, the capillary rise is more than 10 cm, and pore size higher than 30 µm can provide more contrast between positive and negative results.



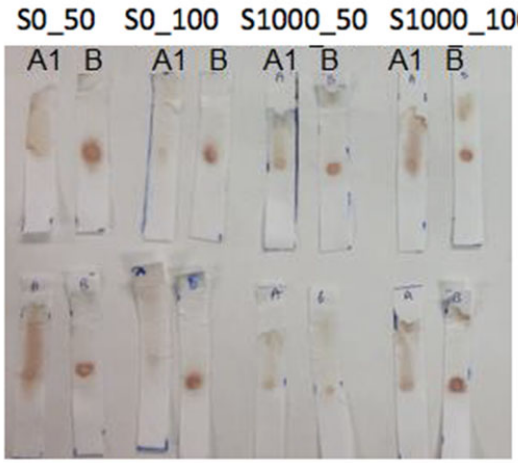

(a)

(b)

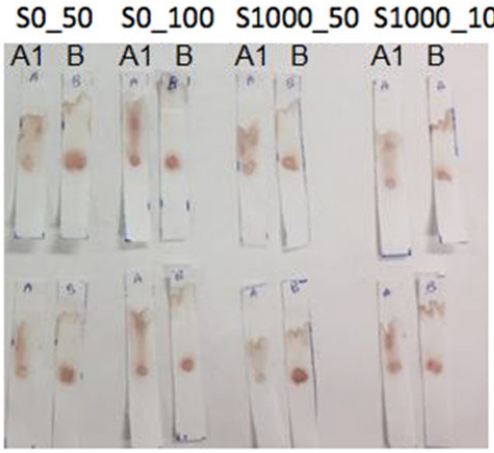
**Figure 3.** Forward blood typing test on A+ blood. a) 0% EKA papers. b) 6% EKA papers.

3.2.2 Reverse blood typing.

A similar approach was followed for reverse blood typing test. First, droplets of 0.5µL of 30% known RBCs with Antigen A (A1) and Antigen B (B) are deposited on the paper strip, and afterwards a 2.5µL droplet of patient plasma is deposited on top of the antigens. **Figure 4** shows two repetitions of the results of the hemoagglutination after the cleaning step (See supplementary material, **figure S1**, for other blood groups). The results are in agreement with the forward blood typing test: the papers with wet strength additive do not clean completely after the washing step, while the paper that do not have wet strength additive offer a quite clear result, especially when the paper is not refined. See **Figure 4** (a).




(a)




(b)

**Figure 4.** Reverse blood typing, A+ blood. a) Paper 0% EKA. b) Paper 6% EKA.

According to forward and reverse results, it could be suggested that the difficulty to clean EKA papers in negative samples was mostly due to the wet strength additive, while pore size and capillary rise are the predominant factors for a unclear negative result in the non-EKA papers.

### *3.3 Effect of the channel width*

Two channel widths were tested following method 1 with the same dose of reagents and patient samples. Böhn et al. worked with channels between 1 to 5 mm. According to their results, if the channel is wider than the average fiber length, more width means less velocity. In case of channel width similar to the average fiber length, they noticed a big influence that the amount of pores that were blocked by the hydrophobic pattern had in terms of modifying the velocity pattern, which became smaller [24]. Since we are working with channel widths of at least three times the average fiber length, the general pattern should be followed.

Figure 5 and 6 show the results of two channel's width (6 and 7 mm) after washing with the saline solution for 1.5 min, according to method 1. The stain of blood spreads more in channels

with less width, (see Figure 5) in agreement with Böhn et al. [18] results. The effect of the paper substrate is similar in both cases, since wet strength additive EKA decreases the pore size and the capillary rise. Therefore in papers with wet strength agent EKA the water front travelled less compared to papers without, see **Figure** 5 and 6.

The papers with smaller width required less wicking time and achieved a higher velocity diluting more RBCs. In this situation, the difference between positive and negative results was more clear. However in reverse blood typing, this effect decreases the positiveness of the results due to the low strength of the agglutination in this case. Therefore, the effect of the channel width influenced the clarity of the results, motivating a detailed analysis of the sample dilution.

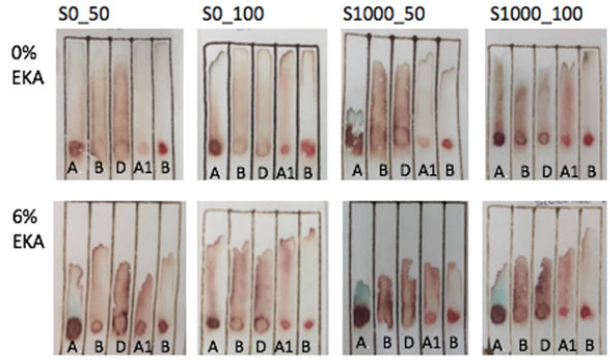



**Figure 5.** Blood typing results of A-blood type (Channel width 7 mm). In each test, forward typing area with anti-A (A), anti-B (B) and anti-D (D) antisera; reverse typing with A1 and B red cells

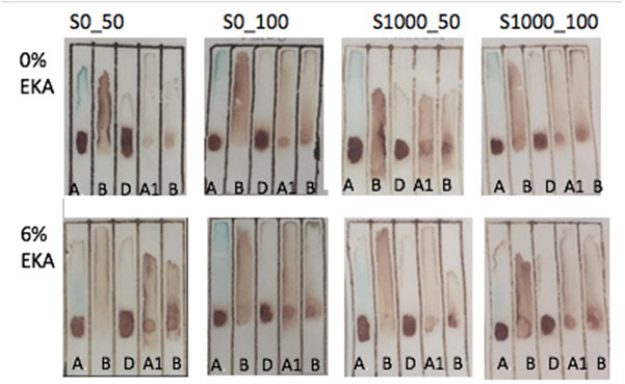



**Figure 6.** Blood typing results of A+ blood type (Channel width 6 mm). In each test, forward typing area with anti-A (A), anti-B (B) and anti-D (D) antisera; reverse typing with A1 and B red cells

### *3.4 Effect of sample dilution*

From the previous results, it can be concluded that the sample dilution can have an important impact on the readout of the results. Paper with high basis weight and refining increases the number of RBCs that are trapped. This seems to indicate that the RBCs even if they are not agglutinated, they are trapped in the fiber network and therefore it is more difficult to obtain clear negative results. Since it is important to have an important difference between negative and

positive results, we decided to work on papers without wet strength additives and without refining too. Several repetitions were made with papers S0_50_0 following the method 1, and as can be seen in **Figure** 7. Figure 7 shows the forward blood typing results on columns A, B and D and reverse blood typing results on A1 and B columns for an A- blood type. In this blood type column B and D should be negative results and should be clean. However S0_50_0 paper could lead to false positives in forward blood typing results since the number of RBCs trapped on these columns were still remarkable providing a certain color level, which could mislead the reader. For reverse blood typing, column A1 showed an acceptable negative results, however positive results were not strong enough (column B).

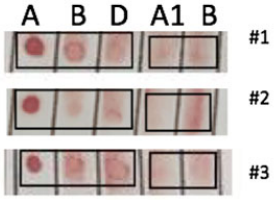



**Figure 7.** Forward and reverse blood typing tests on A- blood type using S0_50_0 paper following method 1. In each test, forward typing area with anti-A (A), anti-B (B) and anti-D (D) antisera; reverse typing with A1 and B red cells

In order to avoid false positive results, the concentration of reagents and samples were changed. **Figure 8** (a) shows that since the amount of RBCs is higher than in **Figure** 8(b) the negative results is not as clear as it is in (b). This is because in (b) the amount of antibodies has been decreased to 0.7µL and the sample has now been diluted to 30% blood and 70% of PBS.

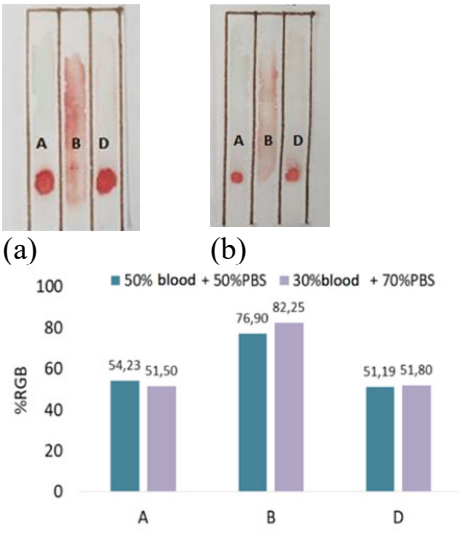


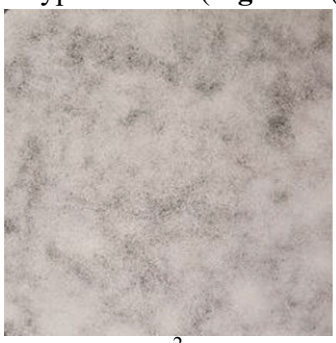

**Figure 8.** Test results of forward typing test on S0_50_0 paper using A+ blood by method 1. a) 50% blood+50% PBS. b) 30% blood+70% PBS. c) grey intensity analysis.

**Figure 8** shows the improvement of the difference between positive and negative results from the case (a) with a higher amount of sample compared to case (b) with less sample. **Figure 8** (c) shows the quantification of a set of different results, and we notice that in diluted samples the difference between positive and negative results increases from 22.67% to 30.55 %.

The new sample dilutions improved the difference between positive and negative results in forward blood typing, however the reliability of results on reverse blood typing were still poor.

### *3.5 Effect on fibre length and paper formation*

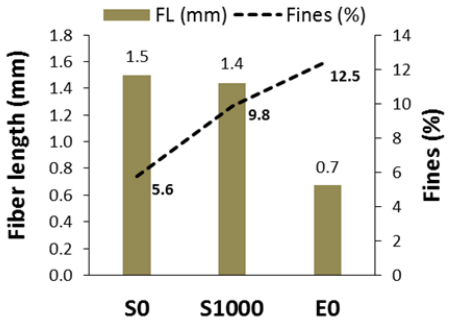
Since a higher basis weight had a detrimental effect on the negative results of the blood typing test, a lower grammage (30g/m$^2$) was tested. In fact, Li et al used basis weight from 20, 35, and 50 g/m$^2$ and showed the improved performance of low basis weight hardwood based papers [15]. Their work focused on forward blood typing, in which the haemoagglutination reaction is strong and easier to trap in the paper structure. When the sisal papers at 30 g/m$^2$ were tested for the forward and reverse blood typing test (**Figure S1b** supplementary material), the results were confusing with a very low repeatability. As it is shown in **Figure S1b,** in some cases the results of forward in S0_30 were wrong, depending on where the dots of the test were placed. And no right results were obtained for reverse method. The reason could be related to the paper formation of the sisal sheets, which was not uniform (**Figure 9** (a-b)). Sisal sheets show a structure more irregular, with zones more transparent (lower quantity of fibers), than the eucalyptus sheets (**Figure 9** (c)).



(a) Sisal 30 g/m$^2$

(b) Sisal 30 g/m$^2$ refined at 1000 rpm

c) Eucalyptus 30 g/m$^2$




(d)

**Figure 9.** Effect of different fibers on paper formation (a) Sisal 30 g/m$^2$ (b) Sisal 30 g/m$^2$ refined at 1000 rpm (c) Eucalyptus 30 g/m$^2$ (d) Measured fiber length and % of fines.

It is known that the shorter the fiber length is, the more uniform the paper formation becomes [25]. **Figure 9** (d) shows that when the fibers are refined, the length of the fiber decreases, improving the uniformity of the paper. Sisal has such a long length (1.38 mm in average compared to 0.48 of Eucalyptus), even though the refining process decreases this length, this is not enough to achieve the uniformity required for reverse blood typing test. The effect of paper nonuniformity influences on capillary rise and therefore the performance of the blood typing test. According to our previous results, the capillary rise is an important factor to select a paper [22]. The results obtained in the present research with Method 1 noted that capillary rise should be higher than 10 cm in order to have a correct discrimination between the positive and negative results. In the case of S0_30, the capillary rise was lower than 20 cm. Another parameter to be considered is the pore size, which it could not be measured in sisal papers at 30g/m2 due to their heterogeneous structure. To avoid this effect, and in order to check a lower grammage, we choosed eucalyptus fiber and eucalyptus paper was manufactured at low basis weight (30 g/m$^2$ and 50 g/m$^2$). **Table I** shows that pore size and capillary rise is similar to slightly refined sisal papers.

Forward and reverse blood typing were performed on eucalyptus paper. The test improved compared to Sisal results. However, the reverse result still showed poor reliability. Therefore, the multiple replicates performed by the same person provided different results, which was assumed to be related to a decreased interaction between antibodies from a patient sample with the reagent (red blood cells) and also due to the weakness of this reaction compared to forward reaction.

### *3.6 Novel method for forward and reverse blood typing test.*

To maximize the interaction between sample and reagents in reverse blood typing, two hydrophobic patterns were created. These patterns were used to allow the sample and the reagents to react before being transferred to the paper network (see **Figure** 1 (b)). Sisal S0_50 and eucalyptus E0_30 and E0_50 papers were tested with this new method. The implementation of this new methodology that allowed the interaction on the surface of the paper doubled the reliability of the test results. In all the tests performed, there were cells trapped in the positive region and at least 10% average grey intensity difference between stained areas (positive) and non stained (negative) (See **Figure 10**; See S3 to S5 supplementary material for test results with different blood types and different tests). Eucalyptus paper with basis weight 50 g/m$^2$ provided the same reliability as 30 g/ m$^2$ (100% trapping in positive results region), and the stains were more uniform. The test itself was easier to handle due to the fact that the paper is thicker since the basis weight is higher (see Figure 10 (c) and supplementary material S3 to S5 for different blood types).

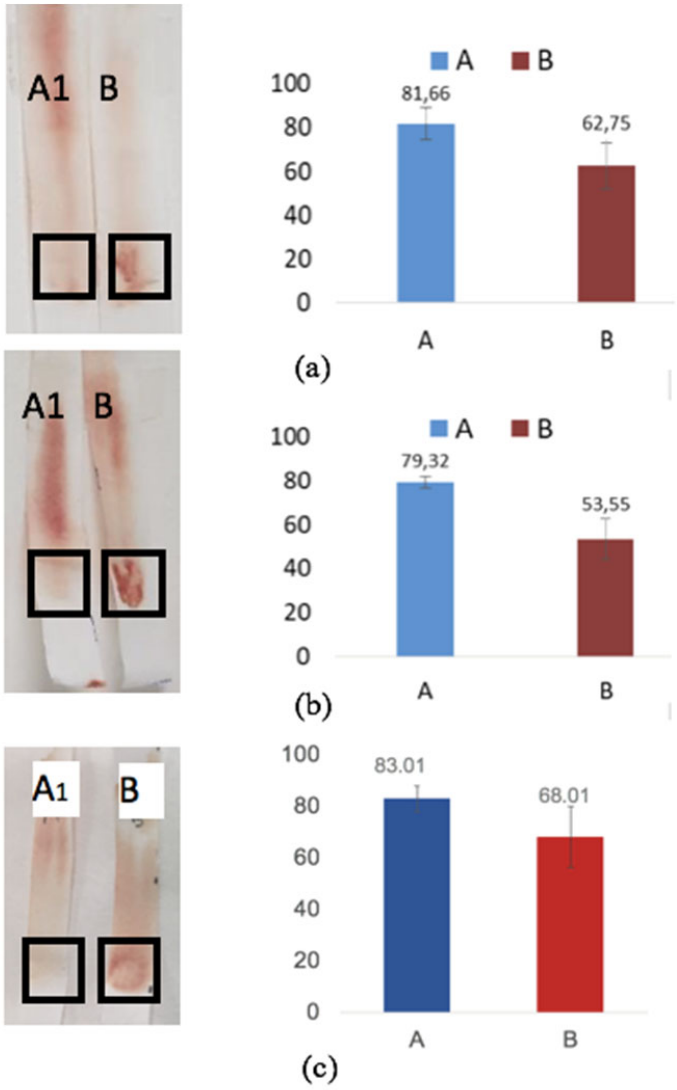
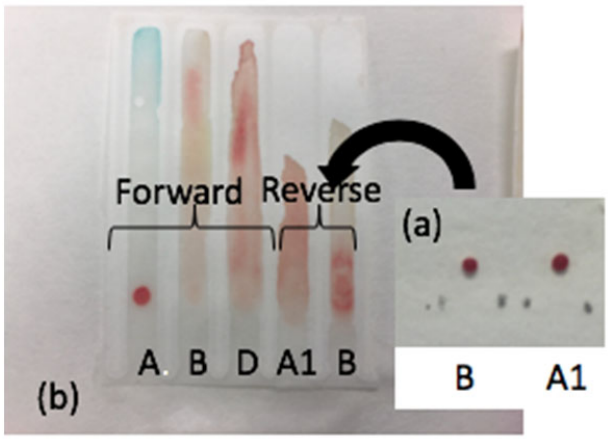
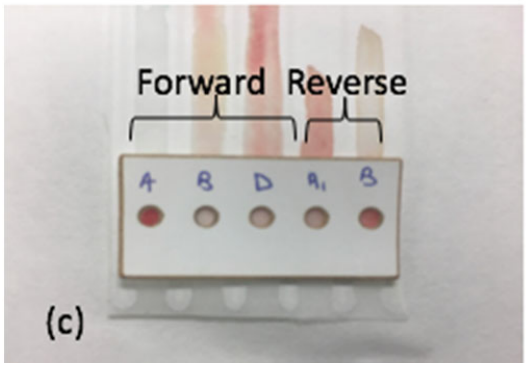
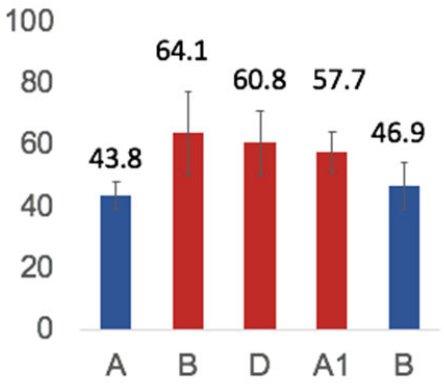



**Figure 10.** Method 2 reverse typing with A1 and B red cells for blood type A and average grey intensity. Test samples (a) Sisal paper S0_50. (b) Eucalyptus paper E0_30 (c) Eucalyptus paper E0_50.




(d)

**Figure 11.** Method 2 implemented on E0_50 for blood type A. (a) Reaction between sample and A1 and B cells on the hydrophobic area (b) Forward typing after cleaning anti-A (A), anti-B (B) and anti-D (D) antibodies with sample and reverse typing after cleaning A1 and B red blood cell with sample. (c) Read out of the results in the reaction area (d) Average grey intensity in each reaction zone.

According to previous results, a pattern of five columns 6 mm apart and two adjacent hydrophobic areas were printed on eucalyptus paper E0_50 (50 g/m$^2$) without refining nor adding wet strength agent. The test was performed with different samples and the results are

shown in Figure 11. 100% repeatability was obtained in the three repetitions done (See **Figure S5** of supplementary information), and a difference in gray intensity level was always higher than 10%, between positive and negative results.

## 5. Discussion

Cellulose is the most abundant natural polymer on earth and it can be easily obtained from a wide variety of natural species. There is an undeniable growing interest in using natural renewable resources such as non-wood plants at present. Therefore, to achieve a more sustainable methodology to perform blood typing assays, numerous investigations have focused on analyzing the anisotropic characteristics of paper to understand RBC motion along the fiber network, a key issue in forward blood typing. However, in reverse blood typing the antibodies are present in plasma in much lower concentrations compared to forward blood typing antibodies, which before being used, are concentrated. In natural cellulosic fiber surfaces, the major functional groups available for covalent conjugation (immobilization) of antibodies are hydroxyl groups. In reverse blood typing, the antibodies are suspended in plasma which is 90% water. Hydroxyl groups cross-react with water, making difficult the use of covalent conjugation reactions. Therefore, the direct absorption is the only realistic approach. However, in this paper we have locally modified the surface of the paper, adding AKD. AKD is mainly constituted by natural fatty acids and bonds chemically with hydroxyl groups in cellulose after heating, providing a hydrophobic behavior to the cellulosic surface.

Initial works that implemented both forward and reverse blood typing, used chromatography and Whatman filter papers [18][19]. However, none of these studies focused on the repeatability of the results. In our study, method 1 followed a similar approach. Both forward and reverse reactions took place in the paper network. In this situation, we achieve repeatable results for forward blood typing, but in reverse blood typing the repeatability was around 50%. Li et al. proposed a method to decrease the complexity of gel cards to achieve a forward and reverse blood typing test. They combined a traditional slide test on a plastic surface with a paper-based readout of the sample [20].

In this study, a single cellulosic substrate is used to create a PAD, which performs the ABO blood grouping using either antigens on red blood cell membrane (forward) and the antibodies on plasma (reverse). Two methods have been evaluated. Method 1 were all the reactions were done inside the paper and method 2 were the interaction between the plasma from the patient and the reagents (red blood cells) were in contact on a hydrophobic surface before being transfereed to the substrate. According to the results method 2 provides a simple and more reliable readout for forward and reverse blood group without the need of complex lab equipment. Previously reported approaches [18][19] rely on conventional Whatmann filter paper and the reaction was done all inside the cellulose with limited interaction between sample and reagents in reverse blood typing.

In this work, the cellulosic substrate (different cellulose sources (sisal and eucalyptus, which provide different cellulosic fiber length, wet strength agent, different basis weights and degree of refining were made). Moreover, the pattern as well reagent volume were optimized for a clear

distinction between agglutination of RBCs by effect of antibody–antigen interactions and free to move RBCs.

## Conclusions

This study demonstrates the use of a single cellulose substrate to perform paper-based analytical device (PAD) for forward and reverse group blood typing. The methods were evaluated to propose a PAD with a novel methodology, which provides highly reliable results on a fully cellulose based device. The effect of the different cellulose fibers, different grammages, refining steps, wet strength additive, and AKD patterning were evaluated in forward and reverse blood typing. Refining and adding wet strength additive decrease the pore size and the capillary rise, decreasing the clarity of negative results. Low basis weight and smaller channels improve the dilution of the blood during the washing step, increasing the clarity of negative results. Long fibers decrease the uniformity of paper, decreasing the reliability of test results. Probably, the cross-reaction of plasma with hydroxyl groups of cellulose decreased the immobilization of antibodies present in plasma during reverse blood typing, therefore an increased reliability is achieved in method 2, where the hemo-agglutination reaction took place on the surface of the paper before being transferred to the paper web. This device allows direct read-out of results—stains where were agglutination happens and provide 100% repeatability of the results in agreement with conventional tests, using a reduced amount of sample. Accurate blood typing is essential for safe blood transfusion, specially on systems used in the field settings. Eucalyptus papers with 50g/m$^2$ of basis weight clearly distinguish between negative and positive reactions and provide high reliability of the results.

## Acknowledgements.

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## Ethics.

The Universitat Politècnica de Catalunya ethics committee approved the tests performed during this study.

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**Table I. Nomenclature and Measured paper properties**

| Paper TYPE (S-Sisal, E- Eucaliptus) rev_BASIS WEIGHT_EKA % | Refining (rev) | Basis Weight (g/m2) | Thickness (µm) | Apparent Density (g/cm3) | Average Pore size (µm) | Capillary rise (cm) | W/D ratio (%) |
|---|---|---|---|---|---|---|---|
| S0_50_0 | 0 | 50.9± 1 | 143±11 | 0.359±0.022 | 39± 3 | 15.6±0.2 | 2.26±0.80 |
| S0_50_6 | 0 | 49.4±1.2 | 135±8 | 0.367±0.016 | 29± 2 | 10.4±0.40 | 21.0±3.07 |
| S0_100_0 | 0 | 104±2 | 301±50 | 0.356±0.046 | 30± 7 | 13.5±0.50 | 4.36±0.36 |
| S0_100_6 | 0 | 105± 1 | 254±7 | 0.416±0.009 | 19± 1 | 10.1±0.20 | 30.9±2.49 |
| S1000_50_0 | 1000 | 47.2± 0.9 | 109±5 | 0.436±0.023 | 31± 5 | 12.2±0.20 | 2.07±0.80 |
| S1000_50_6 | 1000 | 55.5± 1.1 | 132±4 | 0.422±0.013 | 27± 3 | 9.9±0.20 | 26.3±4.43 |
| S1000_100_0 | 1000 | 96.2± 2.2 | 203±5 | 0.474±0.012 | 15± 0.7 | 11.3±0.20 | 2.87±0.67 |
| S1000_100_6 | 1000 | 101± 5 | 222±9 | 0.458±0.015 | 14± 0.4 | 8.4±0.10 | 28.3±4.50 |
| E0_30_0 | 0 | 31.8± 0.4 | 77.2±1.8 | 0.412±0.010 | 19± 2 | 12.4 ± 0.2 | ND |
| E0_50_0 | 0 | 57.5± 0.9 | 121±1 | 0.474±0.005 | 14± 0.2 | 12.5 ± 0.1 | ND |

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| S1000_50_6 | 1000 | 55.5± 1.1 | 132±4 | 0.422±0.013 | 27± 3 | 9.9±0.20 | 26.3±4.43 |
| S1000_100_0 | 1000 | 96.2± 2.2 | 203±5 | 0.474±0.012 | 15± 0.7 | 11.3±0.20 | 2.87±0.67 |
| S1000_100_6 | 1000 | 101± 5 | 222±9 | 0.458±0.015 | 14± 0.4 | 8.4±0.10 | 28.3±4.50 |
| E0_30_0 | 0 | 31.8± 0.4 | 77.2±1.8 | 0.412±0.010 | 19± 2 | 12.4 ± 0.2 | ND |
| E0_50_0 | 0 | 57.5± 0.9 | 121±1 | 0.474±0.005 | 14± 0.2 | 12.5 ± 0.1 | ND |