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Efficient and Simple Paper-Based Assay for Plasma Separation Using Universal Anti-H Agglutinating Antibody

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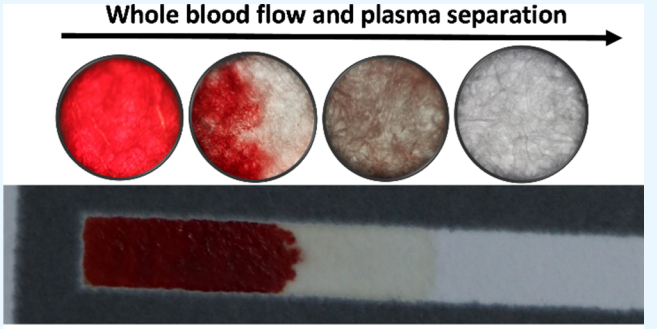
ABSTRACT: Background: Conventional laboratory tests require plasma separation using centrifugation by skilled personnel in well-equipped lab. Development of a simple, reliable, and cheap point-of-care (POC) test for plasma separation will overcome these limitations. Methods: Plasma separation was achieved in filter paper using the anti-H agglutinating antibody. Hydrophobic channels were created using a solid ink printer. The reproducibility, efficiency, recovery, and applicability of the assay were validated on a large number of blood samples. Results: A simple, fast, cheap, and direct paper-based assay for plasma separation from whole blood using universal anti-H agglutinating antibody was developed without equipment or pretreatment requirements. The purity of plasma separation using anti-H treated paper was confirmed by microscopy and biuret test for plasma albumin detection. Plasma separation was affected by paper structure, antibody concentration, donor gender, and hematocrit. The efficiency of the assay was 72% and the reproducibility was about 90% with minimal interassay and intra-assay variabilities. The assay successfully separated plasma from 116/119 samples, indicating high sensitivity (97.5%). Furthermore, the assay accurately recovers thyroid stimulating hormone from samples compared to standard methods with 107% recovery rate. Conclusions: Paper-based plasma separation using anti-H agglutinating antibodies would have numerous applications in paper-based POC tests and in resource limited areas.



Anti-H agglutinating antibody treated channeled filter paper

INTRODUCTION

Point-of-care (POC) testing is the emerging diagnostic procedure performed in clinical diagnostic labs, and by patient's bedsides. It is also called rapid testing or near-patient testing to describe its fast test results obtained and interpreted by medical and nonmedical professionals. Depending on the test target, it offers the diagnosis, screening, or monitoring of patient's diseases status.[1] For example, many POC devices are approved and marketed for monitoring diseases such as diabetes mellitus and hypertension.[2] Using this testing approach is cost-effective for both its cheap cost compared to clinical lab testing and saving millions of dollars spent on disease monitoring and treatment leading to reduced morbidity and mortality rates. Furthermore, POC tests' relatively easy procedure and result reading offer the ability to use it in hospitals, ambulances, specialized private clinics, public health-related campaigns, military centers, and at home by patients in rural areas with limited medical services.[3]

Although POC tests ease the detection of many pathological agents and the monitoring of many diseases, they still face many challenges that should be overcome to consider it a reliable and sensitive testing method. Achieving high sensitivity and precision of diagnosis depends on various factors related to presample processing and sample processing to ensure low to nontesting errors. Samples handled in POC testing range from blood, urine, serum, stool, or saliva. Hence, sample chemical composition variability requires proper treatment and separation approaches to target analytes in complex biofluids for qualitative and quantitative purposes.[4,5]

Recently, multiple studies have highlighted the promising use of bioactive paper for disease detection, diagnosis, monitoring food quality, detection of pathogens, and drug testing, especially in developing countries and for POC applications.[6,7] Paper is widely available, flexible, disposable, and very cheap; it wicks fluid through capillary absorption flow, is biologically compatible and recyclable, and is suitable for colorimetric assays. As a result, over the past years, there has been an increased interest in bioactive paper-based low-cost sensor development and fabrication. Paper-based sensors provide affordable platforms for the simple, accurate, and

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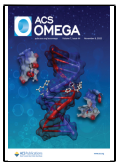

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rapid detection of biomarkers, cells, DNA, microorganisms, chemicals, and drugs.[6,7]

Blood plasma separation is one of the sample treatment steps that must be performed before target detection. Many separation techniques emerged using many approaches and resulted in varying accuracy levels. Complex approaches utilize separation devices such as microscale separation devices. These devices rely on mechanical separation methods (passive separation) such as sedimentation, cross-flow filtration, and cell deviation obstacles.[8] However, some separation devices that use these techniques suffer from a long separation time, resulting in higher coagulation and filter clogging risks.[9] Dynamic force-based devices (active separation) use more complex systems like magnetic or electric forces for separation achievement which results in a complex separation system, low input flow rate within short times, and could risk blood cell integrity.[10,11]

Membrane-based plasma separation is a technique used in both passive and active methods. Asymmetric pore-sized membranes facilitate the separation of large cellular-sized components without negatively affecting their integrity. Combining it with microfluidic channels fasten the separation process and limits any slow separation complications. Nevertheless, the separated plasma purity and volume remain the two challenging issues faced when using this approach.[12] A plasma separation method should have a biomarker high extraction yield to ensure the accurate detection of low concentration targets. Moreover, the method must not change the target analyte concentration or cause blood cell hemolysis.[13] Therefore, finding a simple yet fast and high yield separation method is a must for the sake of POC testing reliability.

The H antigen is the precursor of ABO blood group antigens and present in people of all common blood types.[14−16] The extremely rare "Bombay phenotype" does not express antigen H on red blood cells (RBCs) and can have circulating anti-H antibodies that could mediate the hemolytic transfusion reaction if they received H antigen positive blood.[14−16] The medical uses of anti-H monoclonal antibodies are usually limited to forward blood grouping of the suspected Bombay group.[14−17]

In this paper, plasma blood separation using simple treated microfluidic filter paper with anti-H agglutinating antibody was studied as a preanalyte targeting requirement for POC testing. The efficiency, purity, reproducibility, and applicability of this approach were investigated.

MATERIALS AND METHODS

Filter Paper and Hydrophobic Channel Creation. Hydrophobic wax barriers were made using two methods. The first one was based on manually waxing channels on filter papers using a wood burner. The filter paper that has channels drawn on it was put on top of a wax-soaked paper. Following that, a wood burner device was used to melt the wax onto the filter paper to draw the required channels.[18] The second method was waxing channels automatically using a Xerox 8580 solid ink printer, then melting the wax from the surface of the filter paper into its layers using a laminator device.[19] Different filter papers were chosen for the membrane filtration approach (Whatman Qualitative filter paper grades 5 (2.5 μm) and Whatman Quantitative ashless filter papers grade 42 and 44 (2.5 and 3 μm, respectively)).

RBC Agglutination and Plasma Separation. Anti-A, anti-B, and anti-H antibodies (Lorne laboratories, UK, Abcam, UK, respectively) were used for agglutinating RBCs using traditional slide and/or tube agglutination tests[14−17] or on the surface of filter papers. Anti-H antibody was diluted into 20 μg/mL using phosphate-buffered saline (PBS) with sodium azide added to it (1% w/v). About 7 μL of the antibody (anti-A or anti-H antibodies) was added to the channel's reaction zone followed by the addition of an equal volume of the blood sample. Plasma purity after separation using anti-H agglutinating antibody was analyzed using a 10× light microscope. The functionality of plasma separation was further tested using biuret reagent (Carolina Biological Supply Company, USA) for serum albumin detection with blue color for positive reaction. Assay optimization under different conditions (filter paper type and grade, anti-H concentration, antibody sample volumes, wax channel design and size, blood collection method, etc.) was investigated. Paper-based plasma separation using anti-H under optimized standard conditions were tested in duplicate (intra-assay variability) and in two different days (interassay variability). The coefficient of variability (CV%) was calculated by dividing the standard deviation on mean to determine reproducibility.

Blood Sample Collection and Analysis. Blood samples were collected using standard laboratory procedures using ethylenediaminetetraacetic acid (EDTA) tubes from random donors or directly using finger prick. Age and gender were recorded, and collective blood count (CBC) analysis was performed including hematocrit (HCT) %, hemoglobin (Hb) levels, white blood cell (WBC) count, RBC count, and platelet count. The efficiency of plasma separation was calculated by dividing the plasma separation band length mean of 45 samples over the total blood band length and compared to mean HCT levels of the same samples. To determine the sensitivity of the new separation method, 119 random EDTA blood samples were collected and separated. The sensitivity rate was calculated as the percentage of number of samples separated successfully divided by the total number.[15,20]

Thyroid Stimulating Hormone Enzyme-Linked Immunosorbent Assay. A thyroid stimulating hormone (TSH) enzyme-linked immunosorbent assay (ELISA) kit (DiaMetra, Italy) was incorporated in the study for TSH detection and comparing the result between the centrifuged plasma sample and filter paper-separated plasma sample (recovery samples) for the same patient. About 20 μL plasma was obtained using paper treated with anti-H antibody on wax designed channels followed by cutting plasma bands and adding them to 980 μL buffer (1:50 dilution ratio), then the plasma bands and buffer were vortexed for a minute. Following that, an ELISA assay was performed using original plasma and recovery samples in the same dilution ratio. The obtained sample concentrations were finally multiplied by the dilution factor. The TSH recovery rate was calculated as the percentage of TSH concentration measured by ELISA from recovered plasma separated by anti-H agglutination antibody on paper divided by TSH concentration measured by ELISA from plasma separated by standard centrifugation.[21]

Statistical Analysis. All the separated bands (in cm) were made in duplicate; then the mean of the results was taken and incorporated in the analysis. The used software for the statistical analysis was SPSS (version 21) using one sample T-test and one-way analysis of variance test.

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RESULTS

Plasma Separation Using RBC Agglutinating Antibodies on Filter Paper. Whole blood added directly to filter paper with no treatment (Figure 1A) or treated with buffer

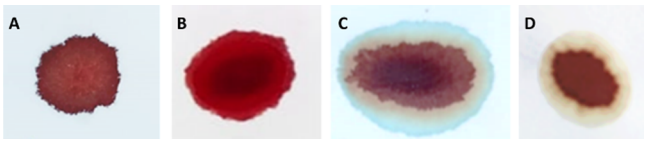

Figure 1. Plasma separation on filter paper grade 5. Whole blood was added to filter paper without any treatment (A), treated with PBS and 1% sodium azide (B), treated with anti-A antibody (C), or treated with anti-H antibody (D).

(Figure 1B) had no plasma separation, while whole blood (group A) added to filter paper treated with anti-A antibody (Figure 1C) or anti-H agglutinating antibody (Figure 1D) had a clear plasma separation zone (yellow color). Wax channels were printed on filter paper grade 5 and then used for plasma separation from EDTA samples. No plasma separation was seen with whole blood alone or treated with buffer only (Figure 2A,B), while plasma separation from whole blood was

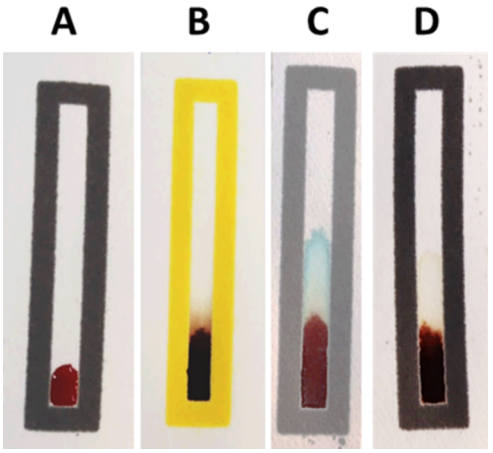

Figure 2. Plasma separation on wax channeled filter paper grade 5. Whole blood was added to filter paper without any treatment (A), treated with PBS and 1% sodium azide (B), treated with anti-A antibody (C), or treated with anti-H antibody (D).

successful upon using anti-A antibody to blood sample group A, and most importantly, using anti-H antibody to all blood types (Figure 2C,D).

Purity of Plasma Separation. To confirm successful separation of plasma, biuret reagent for serum albumin detection was added to plasma zone. As shown in Figure 3, the blue circles pointed out by the arrows display a positive biuret test for albumin detection proving the separation of albumin containing serum through the channel. The purity of plasma separated using anti-H antibody was further investigated through light microscopy (10×) on different locations on the tested channel. RBCs were seen in the agglutination zone (Figure 4A,B, section 4). Less RBCs were present in the area between the red and yellow zones (Figure 4A,B, section 3). Most importantly, no RBCs were present along the plasma zone (yellow zone) (Figure 4A,B, section 2).

Factors Impacting Plasma Separation. Plasma separation is dependent on two approaches. The membrane filtration

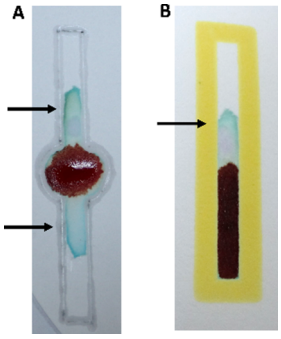

Figure 3. Whole blood added to channeled filter paper pretreated with anti-H antibody. (A) Manual wax printing, (B) automated printing with a solid ink printer. Biuret reagent was added for plasma albumin detection (arrows).

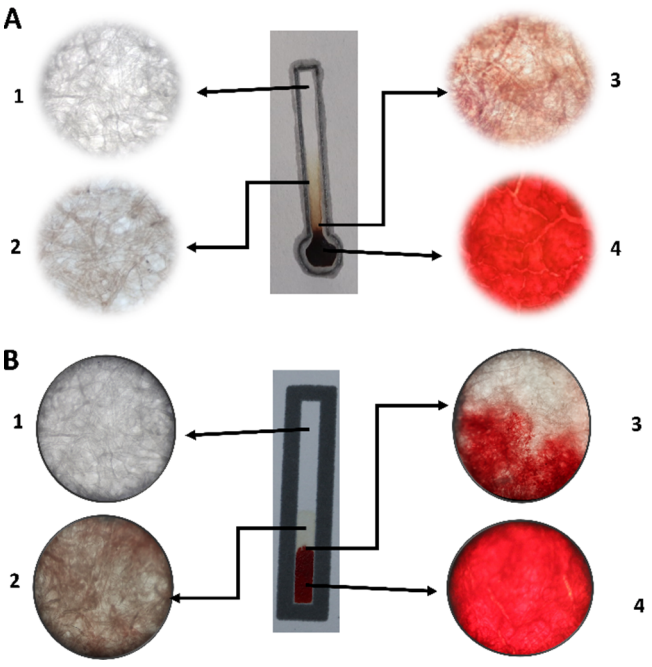

Figure 4. Purity of anti-H antibody plasma separation using the light microscope (10×). (A) manual wax printing, (B) automated printing with solid ink printer. Section 1 shows the channel location with no plasma reach. Plasma separated with high purity is shown in section 2. Early plasma migration with less purity is seen in section 3. RBC and anti-H antibody agglutination reactions are displayed in section 4.

approach uses filter papers (grades 44, 42, and 5) displaying pore sizes smaller than the RBC pore size (about 7 μm), and the cell agglutination approach uses anti-H antibody. No separation was seen using filter papers only but occurred on all filter papers using both antibody concentrations (1:25 and 1:50); thus, a lower antibody dilution was used in the rest of the reactions (1:50) (Figure 5A). Equal volumes of antibody and sample (7 μL antibody + 7 μL sample) resulted in the best separation results compared to the other ratios. Moreover, grades 42 and 5 had higher separation purity than grade 44 (Figure 5B). However, grade 5 had better membrane filtration ability within one paper layer fibers and with no sample loss from beneath the paper followed by grade 42, then grade 44 (Figure 5C). Therefore, filter paper grade 5 was used for the separation reactions using equal volumes of the blood sample and anti-H antibody in concentration 1:50.

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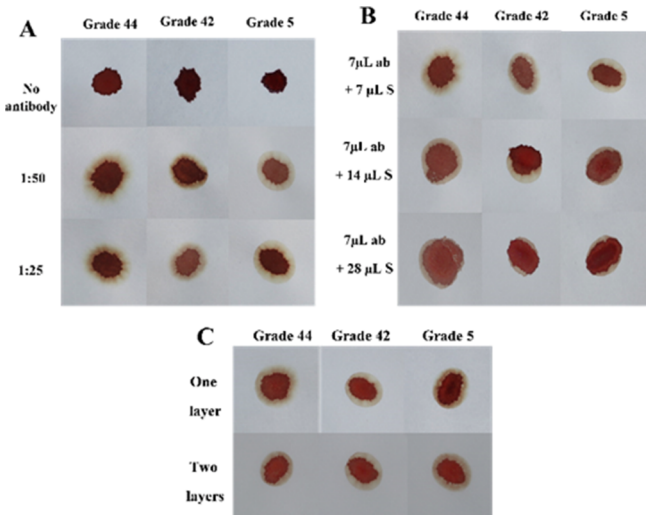

Figure 5. Plasma separation tested on different filter paper types, anti-H antibody concentrations (A), antibody sample volumes (B), and filter paper layers (C) for the determination of the proper separation conditions.

Sample Separation Success Rate. This plasma separation approach under optimized conditions was tested on 119 random EDTA blood samples using the same volumes and antibody concentration on all samples. The separation was successful on 116 samples (sensitivity rate 116/119 × 100 = 97.5%) but failed on three hemolyzed samples (Figure 6).

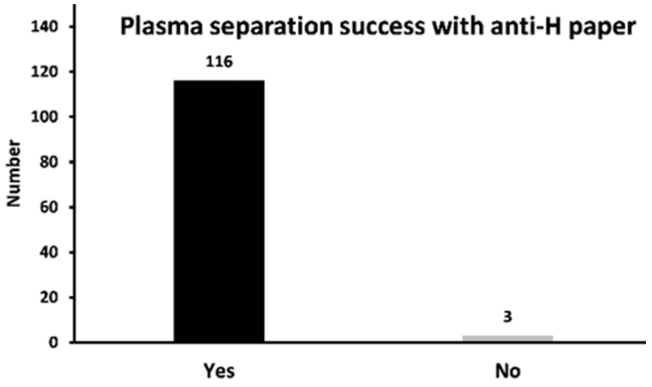

Figure 6. Plasma separation from whole blood success using anti-H antibody on 119 blood samples. All 116 samples were successfully separated but the remaining three samples had failed separation.

Plasma Separation from Fresh Samples. Direct fresh blood from finger prick was tested for separation using anti-H antibody treated filter paper after its success on EDTA blood samples. The five different tested samples showed successful separation (Figure 7).

Correlation between Separation Bands with CBC Test Results. In total, 45 different patient's samples were collected along with their CBC test results. They were used to correlate their separated plasma bands and RBC bands (in cm) across the wax channels with their age, gender, HCT, Hb levels, WBC count, RBC counts, and platelet count (Table 1). A positive association was found between both band's levels with gender and HCT% (P value < 0.05). This indicates that both separated bands' spread varies among samples and provide a relatively true reflection of their HCT% level on the channels.

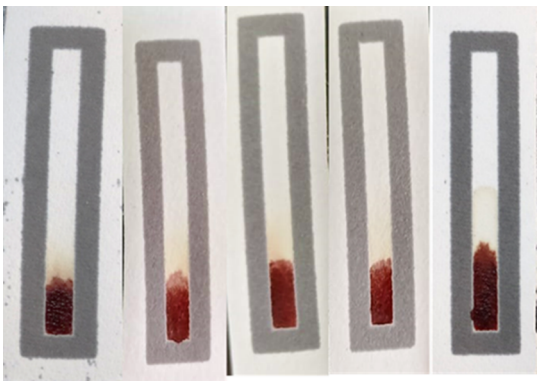

Figure 7. Plasma separation of fresh blood from finger prick using anti-H treated filter paper.

Also, a positive correlation was found between the Hb level and the RBC band (P value < 0.05). However, no correlation was seen between the separated bands and patient's age, WBC count, RBC count, and platelet count (P value > 0.05). The efficiency of plasma separation was 72% (mean of plasma bands 0.61 cm /mean of total bands length 1.41 cm, compared to expected plasma percentage of 60.1% according to HCT of 39.1%, n = 45 samples).

Biomarker Testing on Separated Plasma. This plasma separation approach's ability to be incorporated in future diagnostic tests was investigated to evaluate its capacity to preserve biomarkers quantitatively in the plasma band using the TSH ELISA assay. TSH results obtained from centrifugated samples using standard laboratory procedure were compared with results obtained from filter paper-separated samples from the same patient. The assay was performed three times with samples tested in duplicate or triplicate in each run. Consistent results were obtained, as the average of TSH results from filter paper-separated samples (recovery samples) was 11.8 ± 0.3 mlU/L (n = 3), while the average of TSH results from centrifuged samples was around 11.03 ± 0.05 mlU/L (n = 3) indicating 107% recovery rate (11.8/11.03 × 100).

Assay Variability Testing. Sample variability across different days and attempts was investigated using this separation approach. The obtained 45 samples were separated twice for two days. Results showed very close separation levels for all 45 samples during day 1 and day 2 as well as very close results for repeated attempts within the same day for both the RBC and plasma bands (Table 2).

DISCUSSION

Standard laboratory diagnostic assays require blood collection by venipuncture and separation of plasma or serum by centrifugation as blood cells can complicate analyses.[10] This process requires skilled technicians, well-equipped laboratories, electricity source, and large blood volume and is prone to artifacts.[20,21] To overcome these limitations, multiple POC assays were developed to separate plasma including for example a simple centrifugation utilizing hand-powered fidget-spinner.[22] Similarly, paper-based assays require plasma separation for similar reasons and because RBC color interferes with colorimetric assays.[21]

Paper-based plasma separation can be achieved using filtration, capillarity-driven force, microfluidic channels, membrane separation, and RBC aggregation, coagulation, and

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Table 1. Correlation between Patients' Separated Plasma Migration Level and RBC Agglutination Distribution across the Wax Channels with their Age, Gender, HCT %, Hb, WBC Count, RBC Counts, and Platelet Count[a]

	mean ± SD	category	number	RBCs band cm	plasma band cm
gender		male	17	0.77 ± 0.06	0.60 ± 0.13
		female	28	0.81 ± 0.09	0.62 ± 0.12
		***P* value**		**0.000***	**0.000***
age (years)	34.33 ± 19.1	1−10	4	0.79 ± 0.11	0.51 ± 0.09
		11−20	5	0.81 ± 0.11	0.63 ± 0.08
		21−30	17	0.78 ± 0.10	0.60 ± 0.13
		31−40	6	0.82 ± 0.04	0.67 ± 0.12
		41−50	3	0.83 ± 0.03	0.55 ± 0.08
		51−60	4	0.74 ± 0.07	0.65+0.16
		61−70	3	0.80 ± 0.05	0.68 ± 0.15
		70−80	3	0.85 ± 0.03	0.61 ± 0.12
		***P* value**		**0.67**	**0.56**
hematocrit %	39.98 ± 4.3	low < 35	7	0.85 ± 0.07	0.62 ± 0.14
		normal 35−47	37	0.79 ± 0.08	0.61 ± 0.12
		high > 47	1	0.63	0.40
		***P* value**		**0.000***	**0.000***
hemoglobin g/dL	13.92 ± 4.96	low < 12	8	0.86 ± 0.06	0.61 ± 0.12
		normal 12−16	35	0.79 ± 0.08	0.62 ± 0.12
		high > 16	2	0.66 ± 0.04	0.52 ± 0.17
		***P* value**		**0.007***	**0.59**
RBCs count × $10^6/\mu$L	4.77 ± 0.68	low < 42	11	0.84 ± 0.06	0.60 ± 0.12
		normal 4.2−6.1	33	0.78 ± 0.09	0.61 ± 0.13
		high > 6.1	1	0.77	0.65
		***P* value**		**0.12**	**0.95**
WBCs count × $10^3/\mu$L	7.01 ± 2.07	low < 4	2	0.90 ± 0.14	0.57 ± 0.03
		normal 4−11	41	0.78 ± 0.08	0.61 ± 0.12
		high > 11	2	0.86 ± 0.05	0.72 ± 0.03
		***P* value**		**0.12**	**0.43**
platelets count × $10^3/\mu$L	261.8 ± 80.28	low < 150	3	0.83 ± 0.07	0.76 ± 0.07
		normal 150−450	41	0.79 ± 0.08	0.60 ± 0.12
		high > 450	1	0.80	0.60
		***P* value**		**0.77**	**0.10**

[a]* Significant *P* value.

Table 2. Intra-assay Variability and Interassay Variability Results for 45 Patient's Samples

		day 1	day 2	average ± SD	CV%
interassay variability	RBCs band (cm)	0.78	0.80	0.79 ± 0.05	6.3
	plasma band (cm)	0.59	0.63	0.61 ± 0.07	11.5
		first attempt	second attempt	average ± SD	CV%
intra-assay variability	RBCs band (cm)	0.80	0.82	0.79 ± 0.05	6.3
	plasma band (cm)	0.63	0.62	0.62 ± 0.05	8.0

agglutination.[10,20,21,23−25] Different limitations were reported for different methods including complexity, cost, poor yield, large blood volumes, requirement of devices or diluents, long time, protein loss, and others.[21,23−26] In this study we report paper-based plasma separation assay using anti-H agglutinating antibody that would overcome most of these limitations.

The mechanism of separation in this assay depends mainly on mechanical passive filtration as large, agglutinated RBCs will be fixed/trapped in paper interfibers allowing the fluid phase (plasma) to separate and wick easily. Furthermore, using filter or chromatography paper with pore size less than 3 μm will also trap nonagglutinated RBCs of 6−8 μm size enhancing the efficiency of separation.[26−29] This mechanism was validated and characterized by many studies and were utilized in the development of many diagnostic applications.[20,28−30]

Paper-based RBC agglutination/aggregation was achieved using chitosan,[21] blood grouping antibodies,[27] or synthetic paper substrate.[26] Using anti-A, anti-B, and anti-AB blood grouping antibodies is clearly not applicable for the separation of blood group O samples accounting for over 50% of samples.[21,27] In this study, we use anti-H blood grouping antibody to induce universal RBC agglutination in all blood groups.[14−17] The ability of anti-H monoclonal antibodies to induce the agglutination of RBCs using different methods was characterized previously.[17] H antigen is present in virtually all RBCs including blood group A, B, AB, and O.[14−17,27] Using chitosan as the inducer of blood aggregation necessitates the addition of a diluent and EDTA, requires about 4 min, or requires a specific pattern which slightly complicates the final assay.[21] Anti-A, anti-B, and anti-AB blood grouping antibodies coupled with a synthetic paper substrate mediate efficient separation with minimal protein loss but require a larger blood volume and time for separation, increasing the required cost.[26] Other studies used the principle of RBC agglutination for blood group detection rather than plasma separation.[20,28−30]

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The paper-based separation with anti-H antibody assay reported in this study is simple, instant, and requires small volume.[21,23,27] The assay requires the direct addition of one drop of blood about 7 μL obtained by finger prick to filter paper soaked with anti-H antibody on patterned or unpatterned paper. Blood sample, anti-H antibody, and paper did not require any further treatment. Results are obtained immediately within seconds with high purity as indicated by the absence of RBCs in plasma bands under a microscope similar to other studies.[20−22,27]

While other assays have successfully separated plasma from RBCs on paper, however, most reports are proof-of-principle with minimal validation regarding reproducibility, functionality, efficiency, and applicability.[21,23,25−27] In this study, the reported reproducibility tested on 45 samples was high with minimum variabilities, the assay successfully separates 116/119 (97.5%) samples,[20,21,27] the recovery rate of plasma separated on paper was consistent with standard assays and similar or higher than other studies,[21,26] and the efficiency of plasma separation was higher (72%) compared to other studies which reported an efficiency of 30−60%.[24−26] For the three samples that did not exhibit separation, RBC hemolysis which limits agglutination and mechanical filtration was found to be the reason. Similarly, RBC hemolysis will affect other conventional or POC separation assays.[20−22,25]

The assay was affected by many variables related to paper type and structure, antibody, microfluidic channels, and blood sample as reported by other studies.[10,20,21,28] The assay was optimized under standard conditions to induce the best plasma separation. Factors related to blood sample including gender and HCT have a significant effect on plasma separation. This is expected and known to occur with conventional centrifugation or with other POC assay for plasma separation.[10,20,24] Importantly, almost all samples were separated successfully using our assay regardless of blood related variabilities.

The functionality and applicability of the anti-H treated paper separation assay was confirmed by the detection of plasma albumin by color change and by measuring TSH where levels obtained were comparable to levels obtained by standard centrifugation. Other studies using different methods of plasma separation on paper showed the functionality and applicability of these methods on different analytes like glucose,[21,27] proteins,[20,26] vitamin A and iron,[24] and human immunoglobulin G, interferon gamma, and HIV-1 RNA.[25]

The limitation of the anti-H paper-based assay is the requirement for antibody addition to the paper which could increase the cost and limit field applications. The assay requires a small amount and low concentration of anti-H antibodies to limit cost. Other studies have shown the interesting ability of filter paper to preserve antibodies at ambient temperature which can be enhanced and prolonged by the addition of simple materials like glycerol, tween,[25] and others.[26] Furthermore, a mild dilution factor might occur due to anti-H antibody solution depending on application time and temperature. This can be easily overcome by drying the antibody solution before the application of the blood droplet.[26] While H antigen is almost universal, individuals lacking H antigen reported as "Bombay" blood group are a rare occurrence at 1 of 10,000 individuals in India and 1 per million in Europe.[16]

CONCLUSIONS

A simple, instant, and direct paper-based assay for plasma separation from whole blood using the universal anti-H agglutinating antibody was reported and validated in this study. The assay was significantly affected by paper structure, antibody concentration, and donor gender and HCT. The efficiency of the assay was 72%, the recovery rate was in the range of 90−110%, the sensitivity was 97.5%, and the reproducibility was about 90%. The assay would have numerous applications in paper-based POC tests and in resource limited areas.

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Author Contributions

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