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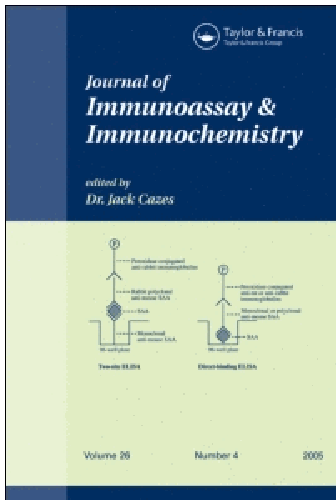

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Evaluation of a Protein Microarray Method for Immuno-Typing Erythrocytes in Whole Blood

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Evaluation of a Protein Microarray Method for Immuno-Typing Erythrocytes in Whole Blood

Nichola O'Looney,[2] Stewart T. G. Burgess,[1] Marisa Chong Kwan,[2] Alan J. Ross,[1] Janine Robb,[3] Thorsten Forster,[1] John S. Beattie,[1] Peter Ghazal,[1] Juraj Petrik,[2] and Colin J. Campbell[1]

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Abstract: All donor blood samples must be tested pre-transfusion to determine the blood type of donor erythrocytes, based on the ABO typing system. Current methods of testing are well characterised, but require a number of processing steps prior to analysis. In addition, standard testing protocols require additional assays such as hepatitis C and HIV testing be performed separately. We describe and evaluate a protein microarray platform for ABO blood typing that has the potential to be a simple reliable high throughput method, with the added capability for the integration of other important pre-transfusion tests. Sixty seven donor blood samples were incubated on microarrays printed with multiple spotted replicates of blood type antigen specific antibodies. We utilised a hold-out cross validation approach, combined with Receiver Operator Characteristic (ROC) curves to define thresholds within which a sample could be defined as being of a particular blood type. The threshold values from the ROC curve analysis demonstrated an excellent ability to accurately separate samples based on ABO blood type. The results obtained when the thresholds from the training sets were applied to test sets were also very encouraging, with misclassified samples being present in only 2 of the training sets and a mean

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classification error of 4.28%. When the mean thresholds were applied to the 67 donor samples, 95.5% were correctly blood typed (64 of 67 samples). We have demonstrated the ability of our protein microarray platform to successfully and accurately type human whole blood samples. We believe that this flexible platform provides a strong basis for an integrated approach for combined blood typing and pathogen testing in human whole blood.

Keywords: Microarray, Protein, Clinical, ELISA, Phenotype, Erythrocyte

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INTRODUCTION

The ABO blood group system is the most important in human blood transfusion medicine and was discovered over 100 years ago by Dr Karl Landsteiner.[1] This system is based on the presence or absence of either two antigens (A and B) on the erythrocytes, and the presence of naturally occurring antibodies against the absent antigen(s) in the serum (anti-A in type B individuals or anti-B in type A individuals). The incorrect assignment of blood donations can lead to 'transfusion reactions' that are caused by the agglutination or antibody-dependent complement-mediated lysis of erythrocytes in the recipient when blood is transfused to an individual of a different blood group from that of the donor.[2]

Our group recently published a new method of blood typing using a protein microarray.[3,4] The microarray consists of spots of antibody against blood group surface antigens; distinct patterns of reactivity were found for each blood type tested. Further, we developed a detection method for measuring binding between erythrocytes and immobilised antibodies, based on surface enhanced fluorescence that requires no fluorescent labelling and significantly reduces sample processing steps. Importantly, the microarray can be expanded to include other blood tests such as immunoassays for blood-borne pathogens such as HIV, HCV, and syphilis, and we anticipate that a single microarray could eventually carry out all the mandatory pre-transfusion tests required on a blood sample.

Current methods used for the typing of blood donations involve hemagglutination techniques, the most commonly used of these being column and microplate agglutination. Microplate agglutination involves the mixing of erythrocytes with antibodies prior to centrifugation; an example of this technology is the PK7200 automated microplate system from Olympus America (http://www.olympusamerica.com/dsg_section/dsg_bloodbank.asp). The aggregation of erythrocytes forms a film at the bottom of the well, indicating a positive reaction with the antibody. Ninety-six well microplates are used to allow the testing of multiple samples at once. With column agglutination technology, the antibody-antigen reaction takes place above a gel matrix (usually Sephadex® or glass-bead based); examples of this technology include the

Ortho BioVue System from Ortho Clinical Diagnostics (http://www.orthoclinical.com) and the DiaMed-ID Micro Typing System (http://www.diamed.ch). Following centrifugation, a positive (agglutinated) reaction product cannot pass through the column and forms a line at the top of the matrix, whilst a negative reaction is characterised by the presence of erythrocytes at the base of the column.

Although these methods are well characterised and are now widely used, they require that a number of centrifugation and washing steps be performed on the whole blood prior to analysis. Standard testing protocols also require that other assays such as hepatitis C and HIV immunoassays be performed separately. We believe that our microarray method has potential to be a simple reliable high throughput method, with the capability for integration of other important pre-transfusion tests.

In this paper, we evaluate the protein microarray method for ABO typing of whole blood, using samples obtained from sixty-seven blood donors.

To achieve this we utilised a robust statistical procedure to type sixty-seven donor samples as blood type A, B, or O.

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EXPERIMENTAL

Microarray Preparation

Arrays were printed with multiple spotted replicates of antibodies LA2 and LB2, specific for either A or B blood type antigens, respectively (See Table 1) along with PBS and IgG spots as negative controls. An antibody specific for A/B blood type antigens was also included on the array (4 replicate spots) but, since it did not react with any of the samples, it was excluded from the analysis. In total, 32 separate sub-arrays were printed per slide, with each sub-array containing 5 replicate spots of LA2 antibody (0.82 mg/mL), 7 replicate spots of LB2 (0.484 mg/mL), 2 replicate spots of IgG (0.31 mg/mL), and 7 replicate spots of PBS (pH 7.0). Therefore, each array contained the following numbers of replicate spots, LA2 (n = 160), LB2 (n = 224), IgG (n = 64), and PBS (n = 224).

Table 1. Summary of the characteristics of the blood typing antibodies used for the protein microarray

Antibody	Specificity	Ab class	Concentration (mg/mL)
LA2	Anti-A	IgM	0.82
LB2	Anti-B	IgM	0.484
IgG	Anti-IgG	IgG	0.31

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Blood Samples

Sixty seven whole blood samples (blood type A: n = 26, B: n = 8, O: n = 33) were obtained from the Scottish National Blood Transfusion Service (SNBTS). Samples were used with the donors' prior consent and ethical clearance was obtained from the SNBTS Sample Governance Committee. The ABO types of these samples were determined by SNBTS prior to microarray analysis using standard testing procedures.

Array Preparation

Microarrays were prepared by printing onto gold microarray slides (25 × 75 × 1 mm, Ssens BV) using a Genomic Solutions MG II microarray printer with 200 μm solid pins. Arrays were checked by microscopy after printing to ensure that probes were correctly printed and, when not in use, arrays were stored under N_2 at <5°C. Prior to use, arrays were submerged 10 times in phosphate buffered saline (PBS) pH 7.0 containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 before being placed in a fresh container of PBS pH 7.0 containing 1% BSA for 1 hr at room temperature, with constant agitation. Arrays were then submerged 10 times in a fresh container of PBS, pH 7.0, and centrifuged to dryness in an Eppendorf 5810R centrifuge at 1,000 rpm for 1 min.

Sample Incubation

Prior to incubation, the individual blood samples were diluted 1/40 in PBS pH 7.0. They were then contained on the array using hybridisation chambers (capacity = 450 μL, Whatman Schleicher & Schuell, UK) for 1 hr at room temperature with agitation. After incubation, the hybridisation chambers were removed and the slides were vigorously submerged 10 times in PBS, pH 7.0, with 0.05% Tween 20. Slides were then rinsed twice in PBS, pH 7.0, and centrifuged to dryness at 1,000 rpm for 1 min.

Data Processing and Analysis

Slides were scanned with a ScanArray 5000 confocal microarray scanner (Packard Biochip Technologies) using 488 nm excitation and measuring emission at 520 nm to detect bound red cells via their auto-fluorescence. For each array, five separate scans were taken, using a consistent photomultiplier tube (PMT) setting and incrementally increasing laser power settings. Microarray images were analysed with Quantarray software (Perkin Elmer, USA) using the fixed circle method; spot-specific background was corrected

by subtracting measured spot background from spot signal. From the five scans of each array, the optimal scan in terms of linear range was selected on the basis of comparative scatterplot analysis.[5] Representative signal for a probe x_i (probe x on array i) was robustly estimated as the median fluorescence across all replicates of a probe on an array (LA2, n = 160, LB2, n = 224). The relative expression (Index Score) between the anti-A (LA2) and anti-B (LB2) probes was then defined as the ratio $LA2_i/LB2_i$.

Cross Validation Approach and the use of Receiver Operator Characteristic (ROC) Curves to Determine Threshold Values for Blood Type Classification

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In order to determine a series of threshold values within which the different blood type responses could be defined, a hold-out validation approach using a random 90% of the data was utilised and repeated 10 further times.[6] This method involved partitioning the dataset into two subsets, one training set (90% of the data) and one test set (the remaining 10% of the data), such that the analysis was initially performed on the training set, while the test set was retained for subsequent use in confirming and validating the initial analysis. Therefore, the training set comprised of 90% of the total dataset that was selected at random for each of the blood groups (A Group, n = 23, O Group n = 30, B Group n = 7) and this data was then used to determine the threshold values. Receiver Operator Characteristic (ROC) Curves (sensitivity vs. {1-specificity}) were plotted to compare the Index Scores for each blood type (A, B, and O). ROC curves are regularly used in clinical diagnostic research as a plot of the true positive rate against the false positive rate for the different possible cut off points of a diagnostic test; therefore, they demonstrate the trade off between sensitivity and specificity.[7] During this study, ROC curves were used to obtain threshold values within which each blood type response could be defined. The threshold for type A blood was obtained by plotting a ROC curve of the Index Scores for the type A blood samples (positive) against the type B blood samples (negative), whilst the threshold for type B blood was obtained by plotting the Index Scores for the type B blood samples (positive) against those for the type A samples (negative). As two negative signals (anti-A and anti-B) are required for a type O blood sample, two separate thresholds were required, upper and lower. These thresholds were obtained by plotting the Index Scores for the type O blood samples against those for the type A blood (upper threshold) and type B blood (lower threshold). The optimum thresholds were calculated from the ROC curve data by selecting the index score value for each comparison that provided the maximum combined sensitivity and specificity.

In order to validate the threshold values, obtained above, these were applied to the remaining 10% of the total dataset, defined as the test set (A Group, n = 3, O Group n = 3, B Group n = 1). For each individual blood

sample within the test set an Index Score was calculated (LA2/LB2) and the samples were assigned a blood type based upon the thresholds obtained from the training set. The process described above was then repeated a further 10 times and the error was calculated for each set.

RESULTS

Scanned Image of Protein Microarray

Representative images of sub-arrays from three separate protein microarrays, one each of A, B, and O blood types, are shown in Figure 1. The reacted spots (red) in the A blood type array are those of the anti-A type antibody, whilst the reacted spots in the B blood type array are those of the anti-B type antibody. In addition, as expected, the A and B probes did not react with the O blood type samples.

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ROC Curves

ROC curves were constructed to obtain threshold values for each of the blood types; a representative ROC curve obtained from one of the randomly assigned testing sets for the A blood type is shown in Figure 2. Further ROC curves were prepared for the B blood type and also for the O blood type versus the A and for the O blood type versus the B, for each of the randomly assigned testing sets. For the ROC analysis, the A blood type sample index scores were used to represent the positive samples, whilst the index scores of the B blood type samples were used as the negatives. The straight diagonal line on Figure 2 denotes a level of no discrimination between positive and negative samples; in contrast, the ideal outcome

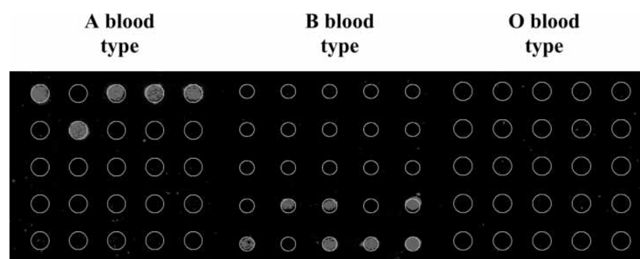

Figure 1. Representative images of A, B, & O blood typing sub-arrays. Red spots indicate that a reaction has occurred between the spotted antibody and the erythrocytes in the patient blood sample. The blue grid placed over the sub-arrays is used to highlight the spot positions.

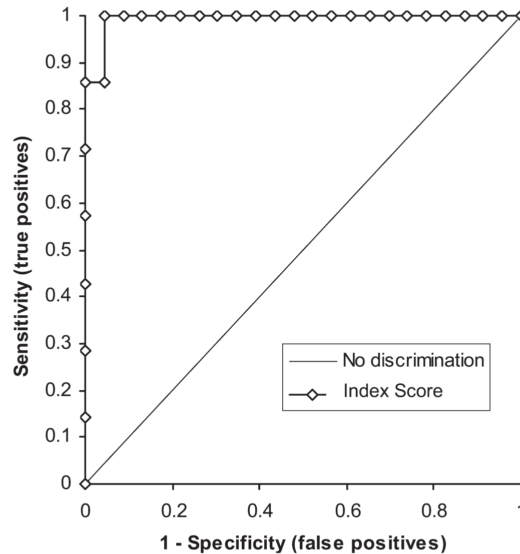

Figure 2. Receiver Operator Characteristic (ROC) curve for the A blood type samples versus the B blood type samples. The ROC curve is a graphical plot of sensitivity vs. (1-specificity); the example shown is of the Index Scores of the A blood type samples (True Positives) versus the B blood type samples (False Positives) for one of the randomly assigned testing sets (AUC = 0.994).

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would be a line which lies as close as possible to the top left corner of the graph, thus representing 100% sensitivity (all true positives found) and 100% specificity (no false positives found).

Area under the Curve (AUC)

The accuracy of the test in this study depends on how well it is able to separate the data set being tested into the different groupings, e.g., normal versus disease state or, in this case, type A blood versus type B blood. The accuracy of the test is measured by the area under the ROC curve, with an area of 1 representing a perfectly performing test with 100% sensitivity 100% specificity; in contrast, an area of 0.5 represents a worthless test with no discrimination between positive and negative values. Traditionally, the accuracy of a diagnostic test is classified using the following criteria: AUC = 0.9–1.0 (Excellent), 0.8–0.9 (Good), 0.7–0.8 (Fair), 0.6–0.7 (Poor), 0.5–0.6 (Fail).[7] For routine blood typing, higher standards are legislated for, e.g., at least 99.5% specificity (Commission Decision of 7 May 2002 on common technical specifications for in vitro-diagnostic medical devices).[8] The mean AUC values of the ROC curves prepared from the 10 training sets are displayed in Table 2.

Table 2. Mean area under the ROC curves (AUC) for each of the blood type comparisons obtained from 10 randomly assigned training sets

Blood type	Mean area under ROC curve (AUC)
A	0.995
B	0.995
O versus A	0.984
O versus B	0.9

As can be seen in Figure 2, the ROC curves obtained from the randomly assigned training sets demonstrated a good ability to separate the A and B blood types, as the mean area under the curve (0.995) was close to the maximum area of 1, indicating an excellent separation of the sample sets. This indicates that, for the chosen threshold cut-off value of 2.72 (obtained from the first of 10 training sets) for the A blood type values, a sensitivity of 100% and a specificity of 95.7% was obtained, with this threshold representing the highest combined values of sensitivity and specificity (See Table 3). Threshold values for the other blood types and from the remaining 9 training sets were obtained as described above and are presented in Table 3. Again, the ROC curves demonstrated an excellent ability to separate both the A and O, and the B and O blood types.

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Cross Validation of Threshold Values Obtained from 10 Randomly Assigned Training Sets Utilising the Blood Type Classification Algorithm

Using the threshold values obtained from the ROC curves (Table 3) from each of the 10 randomly assigned training sets, the test sets constituting the remaining 10% of the data for each blood group were assigned a blood type

Table 3. Mean threshold values of Index scores for each blood type were obtained following ROC curve analysis

Blood type	Mean threshold value of index score from ROC curve	Mean sensitivity (%) at selected threshold	Mean specificity (%) at selected threshold
A	$\geq$2.585	100	96.3
B	$\leq$0.612	100	88.6
O	0.929–2.58	88.6 (at 0.929) 95.7 (at 2.58)	100 (at 0.929) 97 (at 2.58)

without prior knowledge of the individual blood sample origins. A response of either 'Call' or 'No Call' was then assigned to each array for each of the possible blood types, based on the mean threshold values (Table 3). A correctly predicted sample was classified as one that received a correct 'Call' for only the correct blood type, whilst an incorrectly predicted sample was classified as one that received a 'No Call' for the correct blood type and a 'Call' for the incorrect blood type.

As can be seen in Table 4, the results obtained when the thresholds from the 10 training sets (90% of total dataset) are applied to their respective test sets (10% of total dataset) are very encouraging. Misclassified samples were only present in 2 of the training sets and, interestingly, 2 of these false positive results are obtained with the same sample (Sample 26). The mean classification error (defined as the sum of misclassified samples/total samples, multiplied by 100) is 4.28%, with a reported standard error of the mean (SEM) of 3.

In order to further test the mean threshold values, they were applied to the whole dataset of 67 samples and the blood type classification algorithm was used to determine the overall success rate. The results are described in Table 5.

In total, across all of the blood types, 95.5% of patient samples were assigned the correct blood type (64 out of 67 samples) and, for each of the blood types (A, B, and O), only one sample was incorrectly predicted. The O blood type had the highest overall percentage of correctly predicted samples with a success rate of 96.9% (32/33), whilst the success rates for the A and B blood types were 96.2% (25/26) and 87.5% (7/8), respectively.

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DISCUSSION

Previous results from our group have demonstrated the feasibility of using a protein based microarray platform to type blood samples. This study builds upon these results by demonstrating the first clinical application of this technique and by developing a novel algorithm based on receiver operator characteristic (ROC) curves in order to successfully assign blood samples with the correct blood type.

Table 4. Results following analysis of test sets using the thresholds obtained from the 10 training sets

Training set	A Threshold	B Threshold	O vs A Threshold	O vs B Threshold	False positives (FP)	False negatives (FN)	Classification error (%)
Mean	2.585	0.612	2.58	0.929	0.3	0	4.28
Median	2.72	0.64	2.56	0.929	0	0	0

Table 5. Number of blood samples correctly or incorrectly predicted using the mean threshold values of index scores for each blood type

Classification	A	B	O	Total samples
Total samples	26	8	33	67
Correctly predicted	25	7	32	64
Incorrectly predicted	1	1	1	3
% Samples correctly predicted	96.2	87.5	96.9	95.5

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Sixty seven whole blood samples were incubated on custom printed micro-array slides containing multiple sub-arrays, each of which contained multiple spots of antibodies specific for either type A or B blood type antigens and control spots. The median signal-background value for each probe, for each array was then used for comparative analysis. In order to assign the individual donor samples with a blood type, an index score was calculated for each of the samples by dividing the median signal-background value for the type A antigen by that of the type B antigen. The index scores for each blood type (A = 26, B = 8, O = 33) were then used to plot a series of ROC curves, from which a range of threshold values were obtained, based upon the maximum combined sensitivity and specificity for each blood type. In order to validate the success of the assay a cross-validation approach was utilised, whereby 90% of the total dataset was randomly assigned as a training set, from which threshold values defining the individual blood types were obtained. The remaining 10% of the total dataset was then used to validate the efficiency of these thresholds to correctly predict individual sample blood types. The threshold values obtained from the 10 training sets resulted in 2 samples being misclassified, one of which was on two separate occasions during the validation (Sample 26). This resulted in a mean classification error of 4.28% (SEM = 3) and a median classification error of 0%. The threshold values obtained were then further evaluated by applying the mean threshold values of the combined training sets to all 67 samples. This resulted in 95.5% of patient samples being assigned the correct blood type, with one of each blood type (A, B, and O) being incorrectly predicted. The lowest success rate was observed with the type B samples, where 7 of the 8 samples (87.5%) were correctly assigned; however, this result can be attributed to the low number of type B blood samples in this study.

ROC curves are commonly used in the field of medical diagnostics as means of determining whether or not a person has a particular disease based upon a classifier, i.e., high blood pressure as a means of diagnosing hyperten-sion. In this study, we have used the ROC curve principle, combined with a cross-validation approach, as a proof of concept to classify donor blood samples into one of three blood types (A, B, or O). The results shown here

have demonstrated that this technique is a viable means of successfully assigning samples with a blood type and, also, that we can utilise a series of threshold values with confidence. The areas under the ROC curves were compared; this value can be interpreted as the probability that, when we randomly pick one positive and one negative sample, the classifier will assign a higher score to the positive sample than to the negative. The area under each of the ROC curves in this study demonstrates that the model developed shows an excellent level of discrimination between positive and negative samples, indicating that we have been able to develop a robust classification algorithm. The algorithm is based on the threshold values for each of the blood types; as such, three cut-offs were utilised (mean threshold values): Type $A = \geq 2.585$, Type $B = \leq 0.612$, and Type $O = 0.929–2.58$. Using these thresholds, the samples were blinded and the index scores for each were then assigned a 'Call' or 'No Call' for each of the three outcomes. Samples were correctly predicted if they received a correct 'Call' for only the correct blood type, whilst an incorrectly predicted sample received a 'No Call' for the correct blood type and a 'Call' for the incorrect blood type. During this study, all of the samples were assigned a blood type and only three (4.5% of the total) were incorrectly predicted.

When performing blood typing, it is imperative that any diagnostic test correctly predicts the type of a random blood sample, since the consequence of an incorrect call may be a transfusion reaction which can be fatal.[2] Due to the nature of the algorithm used in this study, it is more likely that either an A or B blood type would be incorrectly classified as an O, or an O blood type would be incorrectly classified as either an A or a B. The reason for this is that the index score value used as the classifier for the algorithm is less susceptible to large shifts in its value and, as such, it is unlikely that an A blood type would be classified as a B or vice versa, as this would require a large change in the index score value. The consequences of an O type donor being incorrectly classified as an A or a B are minimal as type O negative blood is considered "the universal donor," since it is compatible with any blood type and, in this study, we have demonstrated one incidence of this (Patient 8, truth = type O but predicted as being type A). However, the consequences of incorrectly classifying either type A or B blood as type O could be more serious. In this study, there were two incidences where this occurred, Patient 23: truth = type A, but predicted as being type O and Patient 26: truth = type B, but predicted as being type O. The method used for assigning samples as type O blood essentially relies on a combination of negative responses, i.e., either no or a very low response for type A and B antigens, thus giving an index score close to 1. This creates the problem that the test is relying on a negative response as a diagnostic; therefore, a faulty array or a poor incubation could result in a sample being misclassified. One way of getting around this and ensuring that type O samples are not incorrectly predicted is to utilise a positive control for type O blood. The A and B antigens found on type A and B RBCs, respectively, are both derived from a

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common precursor, the H-antigen, which is a glycosphingolipid. On type O RBCs, the H-antigen remains unchanged whilst, on type A and B RBCs, the H-antigen is changed into A or B antigens, respectively, by enzymes coded by the blood group A or B genes.[9] Type A has an extra N-Acetylgalactosamine bound to the terminal galactose, while type B has an extra galactose bound to the terminal galactose. Antibodies against the H-antigen could be incorporated onto the arrays, to ensure that type O bloods were better classified. In this study, the overall success rate of correct blood typing was defined as 95.5% for the 67 donor blood samples used. We acknowledge that this falls short of the higher standards of specificity agreed by the Commission Decision of 7 May 2002 on common technical specifications for in vitro-diagnostic medical devices,[8] which is 99.5%; however, we believe that, with larger sample numbers, in particular in the B blood group (where only 8 samples were used) combined with further refinements to the protocol, we could obtain higher levels of specificity with this assay.

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CONCLUSIONS

During this study, we have demonstrated that it is possible to successfully assign blood group ABO types to human whole blood samples using a protein based microarray assay. In addition, by using Receiver Operator Characteristic (ROC) curves, combined with a cross-validation approach and a diagnostic algorithm, we have been able to set defined thresholds within which an individual blood sample can be successfully typed as type A, B, or O blood. In order to fully validate this blood phenotyping microarray, it will be necessary to perform validation on a much larger set of samples, in particular with the B blood type, where a limited number of samples were available for this study. Although this evaluation has focused on the ABO blood types, we have previously shown that we can successfully type Rh antigens D, C, and E using this technique. In the future, this assay could be further expanded to incorporate probes for other blood groups, e.g., Kell and Duffy.[10,11] In addition to blood typing, all donor samples are screened for specific blood borne pathogens prior to use. Since the cost of running a single microarray remains fairly stable, regardless of how many individual components are incorporated into it, the microarray platform used in this study lends itself to the use of multiple probes to detect multiple entities. As such, this assay platform could easily be used to type blood and perform pathogen detection at the same time on the same array.[12] This approach would add to the information obtained from the individual array but, more importantly, as the cost of the array itself would remain the same, it would also add value to the platform, as the cost per test would be much reduced. The technology developed in this assay could be further enhanced by developing a miniaturised system which could potentially offer a point of care test for combined blood typing and multiple pathogen testing.

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Nichola O'Looney and Stewart Burgess contributed equally to this article.

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