Application of the ADVIA cerebrospinal fluid assay to count residual red blood cells in blood components

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Background and Objectives

There is no automated, accurate assay for the enumeration of residual red blood cells (rRBCs) in non-RBC components for transfusion, despite the potential risk of allo-immunization when mismatched components are transfused.

Materials and Methods

The automated ADVIA 120 cerebrospinal fluid (CSF) assay, which is approved to count RBCs and WBCs in CSF samples, was optimized and tested to measure rRBC in platelet concentrate (PC) and plasma components.

Results

Sample dilution, incubation time and reagent volume were optimized for use with non-RBC blood products. The assay was linear ($R^2 = 0.99$), even at low rRBCs counts. Intra- and inter-assay variation gave coefficients of variance (CV) between 2-2 and 9-4% and 2-6 and 14-9%, respectively, depending on rRBC levels. Good correlation ($r = 0.995$) was found between the automated assay and manual counting, which is considered the gold standard. Using the automated assay, the range of rRBCs (count/unit) in buffy-coat platelet concentrate (PCs) was $27–5505 \times 10^6$ and in apheresis PCs was $1–361 \times 10^6$.

Conclusion

The ADVIA CSF assay is a sensitive, precise and accurate means to assess rRBC counts in non-RBC components.

Key words: automated haematology analysers, plasma, platelet, residual red blood cells, transfusion.
in plasma and PCs [1, 11, 12], and manual counting using counting chambers and microscopy remains the gold standard. Canadian Blood Services (CBS) developed a visual guide that includes images of PCs containing the maximum recommended volume of rRBCs to aid hospital blood banks in estimating whether the levels of rRBC contamination exceed the recommendation [13]. However, this method is subjective, and it is difficult to accurately detect acceptable vs. unacceptable contamination levels visually, especially as amounts of rRBCs below the recommended threshold will result in a markedly red unit.

An automated, accurate and reproducible assay for the determination of rRBCs in non-RBC components would permit routine measurement of rRBCs in visibly contaminated products, information that could then be provided with the blood component. This could reduce waste (i.e. discard of products based on their ‘redness’) and unnecessary administration of prophylactic RhIG. We assessed a method for the ADVIA automated haematology analyser, originally developed to measure RBCs and WBCs in cerebrospinal fluid (CSF), as a means of accurately and conveniently determining rRBC counts in PC and plasma components. Initial studies focused on optimizing this assay for use with PC and plasma components, which contain a higher amount of protein than CSF and can be lipemic, either of which could potentially interfere with the assay. The ADVIA assay, which has a detection range of 0–1500 cells/μl for RBCs [14, 15], was assessed by comparison to the gold standard chamber counting method and applied to determine the level of rRBC contamination in CBS PC products.

Materials and methods

Blood components

Blood components were produced using standard CBS protocols. Leucoreduced PCs were prepared by the buffy-coat (BC) method, as previously described [16], or by apheresis. Briefly, for BC PCs, whole blood units were rapidly cooled and held overnight at room temperature. The following day, units were centrifuged at 3496 × g (hard spin), and the layers were separated into platelet-poor plasma, RBC and BC using a semi-automated extracting device (Compomat G4; Fresenius-Kabi, Bad Homburg, Germany). A pooled BC PC was generated from the BCs of four ABO-matched donors and a plasma unit from one of the four donors. The pooled BC components were then centrifuged at 1258 × g for 5 min (soft spin) to separate the platelet concentrate from the residual red blood cells, and the platelet concentrate was leucoreduced. Apheresis PCs were prepared using either an automated blood collection system (MCS+; Haemonetics, Braintree, MA, USA) or an apheresis system (Trima Accel; TerumoBCT, Lakewood, CO, USA). PCs were sent from the production site or the hospital blood bank to a central laboratory in Vancouver, BC. Once received, PCs were stored at 20–24°C in a platelet agitator (Thermo Forma; Thermo Scientific, Asheville, NC, USA or Helmer, Noblesville, IN, USA) under standard CBS storage conditions. PCs were tested at or beyond expiry (storage day 5); the majority were tested on day 6, although testing ranged between days 5 and 9. Plasma components, processed from whole blood by the BC method, were also sent to the testing laboratory and were analysed before freezing. Components were sampled for analysis using aseptic techniques, and samples were held for no more than 5 h at room temperature prior to testing.

ADVIA 120 cerebrospinal fluid assay

Sample preparation and measurement

Samples were prepared and analysed following the manufacturer’s instructions with modifications [15]. Plasma or PC samples were diluted at least 1 in 10 in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 5 mM phosphate buffer). Samples with an RBC count higher than the upper range of the assay (1500 cells/μl) were diluted 1 in 20 or 1 in 50 as required. Samples were then diluted 1:1 with 100% or 25% (v/v with PBS) ADVIA CSF reagent (Siemens Healthcare Diagnostics, Mississauga, ON, Canada), mixed well by vortexing, incubated for 10 min at room temperature and then analysed within 20 min. Samples were aspirated into an ADVIA 120 in CSF program mode, which detects and enumerates RBCs (and WBCs) by light scattering. Results are reported as cell count/μl. TESTpoint controls (Siemens Healthcare Diagnostics) were prepared and run following the manufacturer’s instructions.

Assay evaluation

Assay reagent volume. The ADVIA CSF assay recommends mixing samples 1:1 (300 μl sample:300 μl reagent) with the assay reagent, which spheres and fixes the cells. The linearity and accuracy of using 25% (i.e.: 300 μl sample:225 μl PBS:75 μl reagent) vs. 100% reagent was tested using non-RBC components with low and high numbers of rRBCs.

Linearity testing in non-RBC components. According to the manufacturer’s instructions [15], high protein concentrations, such as those found in plasma and PC samples, can affect ADVIA CSF assay measurements. To assess this, serial dilutions (from undiluted to 1 in 64) of PC and plasma components in PBS were prepared, and linearity testing was performed.

Intra- and inter-assay variation. To test intra-assay variation, large volume samples from 3 non-RBC components
containing high, medium and low levels of rRBCs were aspirated into the ADVIA 120 analyser 11 times each and analysed using the CSF assay. For inter-assay variation, 11 samples from the same component were separately prepared and analysed using the ADVIA 120 in CSF assay mode. Assessment of inter-assay variation was conducted on PCs containing a range of rRBCs and a plasma component. Three samples from 1 PC and the plasma component were also analysed using manual Nageotte chamber counting for comparison.

Manual counting using the Nageotte chamber

Plasma or PC samples already diluted for the ADVIA CSF assay were further diluted with PBS (1 in 1000). One hundred and fifty microlitres of diluted sample was loaded into a Nageotte chamber (Hauser Scientific, Horsham, PA, USA) and left to settle in a Petri dish for 15 min. Residual RBC counts were obtained using light microscopy (Nikon; Mississauga) with 200× magnification.

Determination of the range of rRBC counts in platelet concentrates

Sample size calculations

For sample size calculations, the population size was based on the number of PCs in the CBS inventory on any given day (i.e. those that have been end-labelled but not yet issued to hospitals; approximately 274 PCs). For this population, at a 95% confidence level and a 5% margin of error, a sample size of 160 was needed.

Sample preparation and analysis

Outdated APCs and BC PCs from the blood bank at Vancouver General Hospital or from CBS production sites across Canada were sent to the laboratory for analysis. PCs were sampled using aseptic technique, and rRBC counts were conducted using the method optimized earlier in this study. Briefly, BC PCs were either diluted 1 in 10 or 1 in 20 with PBS, while APCs were diluted 1 in 10. All samples were mixed with the ADVIA CSF assay reagent (300 μl sample:225 μl PBS:75 μl reagent), incubated for 10 min at room temperature and analysed by the ADVIA 120 in CSF program mode as described above.

Results

Evaluation of the ADVIA cerebrospinal fluid assay for rRBC counting in platelet and plasma components

Reagent amount

Parallel analysis of PC and plasma samples containing higher and lower levels of rRBCs (based on colour), prepared using the manufacturer’s recommended volume of assay reagent [15] and by a modified method using 25% of the recommended reagent volume to avoid potential interference from polymerized protein, revealed similar results. Seven non-RBC blood components, with rRBC counts/μl ranging from approximately 140–60 000, were tested using both reagent volumes. The correlation coefficient between the two methods (r) was 0.999 and the gradient of the line was 0.993, indicating very good agreement between the two measurements across the entire range of rRBC levels tested. On the basis of these results, subsequent samples were analysed using 25% reagent volume.

Linearity of rRBC counts in PC and plasma components

Serial dilution of three non-RBC components was conducted to investigate linearity and determine suitable dilution factors for analysis. Undiluted samples were sometimes viscous and led to clogging of the ADVIA intake, as was the case with one PC sample and one plasma sample (Fig. 1). At 1/2 dilution, the PC sample was still too viscous to be analysed. Overall, at dilutions below 1/2, the samples displayed good linearity, with R² = 0.990, 0.998 and 0.986 for plasma samples 1 and 2 and PC, respectively. This indicates that protein concentration and lipemia do not affect linearity or impede the assay at these dilutions. Linearity appeared to be maintained even at RBC counts <50 cells/μl. A 1/10 dilution was chosen as being favourable for most samples to prevent sample viscosity, to bring samples within range of the assay (<1500 cells/μl) and to avoid any potential...

Fig. 1 Linearity of rRBC counts in PC and plasma components using the ADVIA 120 cerebrospinal fluid assay. Serial dilutions (undiluted, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64) in phosphate-buffered saline were conducted for PC [●] and 2 plasma components [plasma 1 [○] and plasma 2 [▲]] containing a range of rRBCs. For the PC sample and plasma sample 1, undiluted samples clogged the ADVIA intake, and results were not obtained. All samples displayed good linearity, with R² = 0.990, 0.998 and 0.986 for plasma samples 1, 2 and PC, respectively.

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Vox Sanguinis (2012) 103, 186–193
interference resulting from high protein or lipid concentrations in the samples.

**Intra- and inter-assay variation determined using non-RBC components**

Intra-assay variation, which assesses the precision of the instrument, resulted in coefficients of variance (CVs) of 2.6% and 2.2% for 2 PCs examined, and 9.4% for a plasma component, which contained rRBCs a level of magnitude lower than in the PCs (Fig. 2). For inter-assay variation, which examines repeatability, the CVs for PCs with a variety of different levels of rRBCs ranged from 2.6% to 14.9%, while for a plasma component, the CV was 14.4% (Table 1). The plasma component and the PC

![Fig. 2](image)

**Table 1** Inter-assay variation. Eleven consecutive samples were taken from PC or plasma components with a range of rRBC counts (based on colour), prepared separately and analysed using the ADVIA 120 cerebrospinal fluid assay. Samples from one of the platelet concentrate and the plasma component were also counted using the Nageotte chamber. Coefficients of variance were calculated to determine inter-assay variation.

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Mean (rRBC/μl)</th>
<th>Standard deviation (rRBC/μl)</th>
<th>CV (%)</th>
<th>95% Confidence Interval (rRBC/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC sample 1</td>
<td>11</td>
<td>3491</td>
<td>89</td>
<td>2.6</td>
<td>3431–3551</td>
</tr>
<tr>
<td>Nageotte Chamber</td>
<td>3</td>
<td>3613</td>
<td>61</td>
<td>1.7</td>
<td>3462–3765</td>
</tr>
<tr>
<td>PC sample 2</td>
<td>11</td>
<td>8085</td>
<td>566</td>
<td>7.0</td>
<td>7705–8466</td>
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<tr>
<td>ADVIA 120</td>
<td>11</td>
<td>8954</td>
<td>401</td>
<td>4.5</td>
<td>8684–9223</td>
</tr>
<tr>
<td>PC sample 3</td>
<td>11</td>
<td>183.7</td>
<td>27.2</td>
<td>14.9</td>
<td>164.4–201.0</td>
</tr>
<tr>
<td>ADVIA 120</td>
<td>11</td>
<td>125.5</td>
<td>18.1</td>
<td>14.4</td>
<td>113.3–137.6</td>
</tr>
<tr>
<td>Plasma sample 1</td>
<td>11</td>
<td>138.3</td>
<td>2.5</td>
<td>1.8</td>
<td>132.1–144.6</td>
</tr>
<tr>
<td>Nageotte Chamber</td>
<td>3</td>
<td>138.3</td>
<td>2.5</td>
<td>1.8</td>
<td>132.1–144.6</td>
</tr>
</tbody>
</table>

CV, coefficients of variance.
giving higher CVs contained low levels of rRBCs; therefore, these CVs were deemed acceptable. Two of the samples were also counted using the Nageotte chamber, with similar results, although at low rRBC counts, a smaller CV was observed for the Nageotte chamber than for the ADVIA 120 assay.

Comparison of ADVIA cerebrospinal fluid assay to Nageotte chamber counting

Passing and Bablok [17] regression analysis was used to compare the results of 34 samples, analysed in parallel by the ADVIA CSF assay and the gold standard Nageotte chamber counting. Good correlation was found between the methods ($r = 0.995$, Fig. 3). The majority of the points fell within the 95% confidence interval. The data were also analysed using a Bland-Altman [18] plot, which revealed an average bias between the methods of 1099 counts/μl, suggesting that the ADVIA under-estimated RBCs counts (Fig. 3b). However, this analysis was conducted over a wide range of rRBC levels, and the bias was more pronounced at higher rRBC counts. There appeared to be greater agreement between the methods at rRBC counts/μl <20 000.

Number of rRBCs in CBS platelet products

Buffy-coat BC PCs and APCs were assayed for rRBCs using the ADVIA 120 CSF assay. The range of rRBCs in BC PCs (based on a sample size of 166) was 80–15 040 counts/μl with a median of 605 counts/μl (95% confidence interval for median: 448–749 counts/μl). The range of rRBCs in APCs (based on a sample size of 58) was between 5 and 1380 counts/μl with a median of 70 counts/μl (95% confidence interval for median: 46–100 counts/μl). The rRBC count/μl was converted into rRBC counts/unit based on the volume of each unit. Frequency histograms summarizing these results are shown in Fig. 4. For BC PCs (Fig. 4a), the range of counts/unit was between 27 and 5505 $\cdot 10^6$ RBCs with a median of 202 $\cdot 10^6$ RBCs (95% confidence interval for median: 157–251 $\cdot 10^6$ RBCs). For APCs (Fig. 4b), the range of counts/unit was between 1 and 361 $\cdot 10^6$ RBCs with a median of 16 $\cdot 10^6$ RBCs (95% confidence interval for median: 12–23 $\cdot 10^6$ RBCs).

![Fig. 3 Comparison of ADVIA 120 cerebrospinal fluid (CSF) assay to Nageotte chamber counting. Thirty-four platelet concentrate (PCs) were analysed in parallel using ADVIA 120 CSF assay and Nageotte chamber counting, the gold standard method. Results were analysed using the Passing-Bablok method, which displays the correlation between the two methods with 95% confidence intervals for the slope and intercept (dashed lines) and the line of identity (grey). Good correlation was observed between the two assays, with $r = 0.995$. Results were also displayed using a Bland-Altman plot (b), which displays the bias between the methods (the mean of all the differences between results obtained using the Nageotte chamber and the ADVIA 120 CSF assay). The bias (solid line) was 1099 counts/μl, and the dashed lines are $+2$ and $-2$ standard deviations around this mean.](image-url)
Discussion

The ADVIA CSF assay enumerates cells using light scatter and absorbance, has a stated range of 0–1500 RBC/μl, and is approved by the food & drug administration as having equivalent accuracy, precision, linearity, sensitivity and specificity as manual methods for counting RBCs and WBCs in CSF samples [15]. However, the assay was designed and optimized to detect and count low numbers of RBCs and WBCs in CSF. Plasma contains protein concentrations two orders of magnitude greater than CSF, and this had to be accounted for while adapting the assay for use with plasma components and platelet products suspended in plasma. The CSF assay reagent spheres cells and fixes them using aldehyde, which cross-links proteins and cells. In samples with high protein concentrations, this could lead to inaccurate counts due to cell aggregation or trapping of cells within protein precipitates and clogging of the ADVIA intake. In our experience, undiluted samples sometimes led to clogging of the ADVIA intake, and a 1 in 10 dilution was determined to be appropriate for most samples to avoid this complication and also to generate samples that fell within the range of the assay (<1500 RBC/μl). Due to the high protein concentrations, the use of 25% of the recommended reagent volume was tested, and this did not negatively affect the performance of the assay. Samples were analysed between 10 and 30 min after the addition of the assay reagent, as the manufacturer recommends that samples with protein concentrations of 500–1000 mg/dl (which is approximately 10 times lower than the protein concentration in plasma) are analysed within an hour of the addition of the reagent to minimize potential interference related to protein cross-linking. Finally, lipemia can also affect the assay and although several lipid, opaque samples were encountered during this study, 1 in 10 dilution of these appeared to avoid any interference.

The ADVIA CSF assay was found to be as sensitive in plasma as previously reported in CSF [14]. The inter- and intra-assay variation was good, with CVs below 15% even at rRBC counts around 100/μl. These compare well with the precision reported previously for this assay in CSF, in which CVs for between-run repeatability were 20.7% [at a mean of 9.8 RBC/μl] and 9.3% [at a mean of 97.5 RBC/μl] [14]. This previous study also found that the RBC count in CSF was accurate to 5 cells/μl over 0–50 cells and to 10% over 1–1500 cells, and that it was linear between 15 and 537 RBC/μl [14]. In our experience, there was excellent correlation between the ADVIA CSF assay counts and the gold standard manual chamber counting. However, there was some bias, and while most results fell within the 10% accuracy previously reported for the assay in CSF [14], there was an indication that the ADVIA CSF assay gave lower results than the manual method, especially at RBC counts >20000/μl. In the Mahieu et al. [14] study that assessed the assay for measuring RBCs in CSF, the automated assay also appeared to tend towards under-estimating the number of RBCs compared to manual counting. When testing PC samples from the CBS inventory, the majority had rRBC counts below 20000/μl, but the tendency of the assay to under-estimate counts highlights the need for this to be assessed in each laboratory implementing this method, and for careful validation to be conducted, particularly if the laboratory plans to assess samples containing higher rRBC levels. The automated assay enumerates cells using low-angle light scatter to determine cell size and high-angle light scatter to determine cell granularity (refractive index) and can distinguish between RBCs and ghost cells based on differences in refractive index and size. RBCs and ghost cells should also be distinguishable using manual counting. For both methods, errors could be introduced if samples are not well mixed before analysis and therefore not a homogenous suspension.

The range of rRBC in BC PCs and APCs was examined using the ADVIA assay, with the sample size for BC PCs being representative of the Canadian platelet inventory.
The major guideline regarding rRBC levels in platelet products comes from the AABB, who recommend there be <2 ml RBCs in apheresis platelet units [9]. The highest rRBC count in the BC PCs surveyed was 5505 ± 10^5/unit, which is equivalent to approximately 0.5 ml RBCs when converted to an RBC volume (using 90 fl as the volume of the average RBC as per reference [6]), well below the AABB recommendation for a single dose. For APCs, the level of rRBCs was very low, representing a volume range of <0.1–32.5 µl rRBC/unit. These rRBC ranges are similar to those previously reported, for example Santana and Dumont reported [1] <27 × 10^6 rRBCs per 300 ml APC dose using a flow cytometry method. Ranges of 0–3–0.5 ml RBCs/adult therapeutic dose in pooled BC PCs and 0.2–7 µl RBCs/adult therapeutic dose in APCs are commonly cited [2, 3, 19, 20], and our findings correspond well with these. Approximately 74% of the BC PC and all the APCs units surveyed were outdated from hospitals, and depending on demand, units that appear less contaminated with rRBCs are generally chosen for transfusion before ones that look overly red; therefore, the range of rRBCs presented here could represent a slight over-estimate of rRBCs levels in CBS products.

The ADVIA CSF assay is fast, precise and sensitive and demonstrates reasonable accuracy for enumeration of rRBC in non-RBC blood components. Despite decreasing levels of rRBCs in PCs produced with modern production practices, particularly apheresis methods, rRBCs in non-RBC products remain clinically relevant, making automated, accurate enumeration of these as important as ever. One benefit of this method is that the provision of an rRBC count with non-RBC products could reduce unnecessary waste of components that appear overly red but are within the recommended range. In addition, its use on a high-throughput analyser may facilitate testing in jurisdictions where counting residual RBCs is a regulatory quality assurance measure. Although it will remain to be seen whether providing an rRBC count will be sufficient to change the practice of discarding grossly red units, this study was born out of a need expressed by transfusion scientists and clinicians for more definite information regarding rRBC counts in components, as the colour of a component can often be misleading. Together with education and clearer guidelines, the provision of rRBC counts could impact practice, and most importantly, this information could aid in decision-making regarding transfusion of mismatched components and administration of prophylactic RhoIG. More immediately, implementation of this method will allow for routine rRBC measurement that could generate data to inform the development of future guidelines.

Acknowledgements

Brankica Culibrk helped design the experiments, collected and analysed data for the assay validation portion of the study and wrote the original draft of the paper. Elizabeth Stone conducted experiments and analysed data for the enumeration of rRBCs in CBS products. Elena Levin helped design and collect data for both the assay validation and enumeration of rRBCs in CBS products and helped draft the manuscript. Sandra Weiss was involved in the study design of the assay validation portion of the study. Katherine Serrano helped design the study and conducted statistics. Dana Devine designed the study and guided the work. All authors were involved in revising and approving the manuscript. The authors would like to thank everyone who provided components for this study: the staff at the Transfusion Medicine Laboratory at Vancouver General Hospital, particularly Dr. Kate Chipperfield, Luca Rizzetto from the University of British Columbia Hospital, and Olivia Wong and Judy Hyrtzak from CBS BC and Yukon. The authors would also like to acknowledge the assistance of Dr. Geraldine Walsh, CBS Scientific Writer with manuscript preparation and editing, and Theresa McCaskill of Siemens for assistance with assay implementation.

Competing interests

The authors have no conflicts of interest.

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Vox Sanguinis (2012) 103, 186–193
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