Preanalytical Factors That Influence the Abbott TDx Fetal Lung Maturity II Assay

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Background: The TDx Fetal Lung Maturity II (FLM II) assay uses amniotic fluid to assess lung maturity of the unborn infant. We investigated common preanalytical factors that influence FLM results, including centrifugation, sample storage, and contamination by whole blood.

Methods: We tested 18 specimens after centrifugation and after resuspension by vortex-mixing. We also analyzed 23 specimens stored at \(-20 ^\circ C\) for up to 448 days and then thawed (duplicate measurements), 20 specimens stored at 4 \(^\circ C\), and 24 specimens stored at room temperature. In addition, we evaluated the effects of whole blood diluted into 19 different specimens.

Results: Centrifugation significantly decreased FLM II results from baseline \((P < 0.0001)\), and resuspension returned results to baseline values \((P = 0.286)\). Storage at \(-20 ^\circ C\) produced highly variable results that demonstrated a nonsignificant negative trend associated with storage time. Specimens were stable for 24 h when stored at 4 \(^\circ C\) and 16 h at room temperature. Blood contamination produced significantly positive differences in results only in specimens with baseline values < 39 mg/g with a 5.8 mg/g increase in FLM II for every \(0.1 \times 10^{12}/L\) increase in the erythrocyte count \((slope = 58.4)\).

Conclusions: Resuspension of centrifuged specimens produces clinically valid FLM II results. Results from specimens stored at \(-20 ^\circ C\) can be highly variable and decrease over time. Results from specimens stored at 4 \(^\circ C\) and at room temperature are stable for 24 and 16 h, respectively. Blood contamination up to \(0.03 \times 10^{12}\) erythrocytes/L is acceptable for FLM II analysis.

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Respiratory distress syndrome currently ranks sixth among the leading causes of death in newborn infants (1). Also referred to as hyaline membrane disease, respiratory distress syndrome results from an insufficient amount of pulmonary surfactant that is produced by the neonatal lung and released into the amniotic fluid. Pulmonary surfactant is composed primarily of phospholipids and is contained within lamellar bodies that are synthesized and extruded by the type II cells of the alveolar epithelium.

The Abbott TDx Fetal Lung Maturity II (FLM II)\(^1\) assay uses fluorescent polarization to assess lung maturity of the unborn infant by measuring the ratio of surfactant to albumin in filtered, uncentrifuged amniotic fluid. The FLM II assay has been shown to correlate with fetal outcome (2–6). Although numerous studies have examined the clinical performance of the FLM II assay, few have analyzed the preanalytical variables that can potentially influence the results from this assay. We report here our evaluation of three commonly occurring preanalytical factors that impact FLM II results: centrifugation, sample storage (at \(-20 ^\circ C\), 4 \(^\circ C\), and room temperature), and contamination by whole blood.

Materials and Methods

Specimens

Amniotic fluid specimens received by the Barnes-Jewish Hospital laboratory for FLM II testing were processed and analyzed according to the manufacturer’s guidelines. Surplus amniotic fluid was then used for experimental analysis. All samples were filtered just before analysis as described in the manufacturer’s package insert. Institutional Review Board approval was obtained for these studies.

Centrifugation and Resuspension

We centrifuged 18 specimens at 1700g for 10 min and removed an aliquot for testing. We then resuspended the specimens by vortex-mixing for 15 s and removed a second aliquot for testing. Both sample aliquots were analyzed at the same time.

\(^1\) Nonstandard abbreviations: FLM, fetal lung maturity; RBC, red blood cell, and CI, confidence interval.
specimen stability at \(-20^\circ C\)
Twenty-three specimens that were stored unfiltered at \(-20^\circ C\) for 25–448 days and not subjected to freeze-thaw cycles were thawed at room temperature, vortex-mixed, filtered, and analyzed in duplicate within 3 h.

specimen stability at \(4^\circ C\) and room temperature
Twenty specimens stored unfiltered at \(4^\circ C\) and 24 specimens stored unfiltered at room temperature were vortex-mixed, filtered, and analyzed 2, 4, 8, 16, 24, and 48 h after being received in the laboratory.

effect of blood contamination
We added either donor (n = 17) or maternal (n = 2) whole blood to 19 amniotic fluid specimens without visible blood contamination. Whole blood was initially diluted into amniotic fluid specimens at a ratio of 1:10 (100 \(\mu L\) in 900 \(\mu L\)), and the amniotic fluid was then serially diluted twofold (500 \(\mu L\) in 500 \(\mu L\)) to a final ratio of 1:1280. Red blood cell (RBC) counts of each dilution were calculated using the RBC count from the donor blood divided by the corresponding dilution factor. All samples were filtered and analyzed within 1 h after the addition of whole blood.

FLM II assay
FLM was measured with the TDx FLM II assay (Abbott Laboratories). In our laboratory this assay has CVs of <4%, 3.6%, and <7.6% for control materials with FLM II values of 25, 50, and 100 mg/g, respectively. The CV of a pooled amniotic fluid control with a FLM II value of 25 mg/g was <5%. According to the manufacturer, FLM II values \(\geq 55\) mg/g are considered to be mature, whereas values between 40 and 54 mg/g are intermediate, and values \(<39\) mg/g are immature.

statistics
Results are reported as the mean (SE) unless otherwise indicated. Deming regression was used to estimate the linear relationships between original FLM II results and results obtained after centrifugation, resuspension, and storage at \(-20^\circ C\). A mixed-model ANOVA was used to analyze the effects of storage at \(4^\circ C\), storage at room temperature, and the effect of blood contamination (7).

results
centrifugation and resuspension
Centrifugation of amniotic fluid produced FLM II results that were significantly lower than the original values (Fig. 1A and Table 1). Deming regression analysis comparing centrifuged to baseline values produced a slope of 0.656 [95% confidence interval (95% CI), 0.495–0.817] and a y-intercept of \(-5.9\) mg/g (95% CI, \(-14.9\) to 3.2 mg/g) with an \(r^2\) of 0.824. The SD around the Deming regression line was 7.0 mg/g. Resuspension of amniotic fluid produced FLM II concentrations with a mean that was not significantly different from baseline (Fig. 1B and Table 1). Deming regression analysis produced a slope of 0.923 (95% CI, 0.786–1.061), a y-intercept of 5.7 mg/g (\(-2.1\) to \(13.4\) mg/g), and an \(r^2\) of 0.927. The SD around the Deming regression line was 6.2 mg/g. Sixty-one percent (11 of 18) of samples were within 10% of baseline values, and 89% (16 of 18) of samples were within 20% of baseline. No results were >30% different from baseline.

specimen stability at \(-20^\circ C\)
After thawing, the 23 amniotic fluid specimens that had been frozen at \(-20^\circ C\) for 25–448 days produced variable FLM II values. Only 48% (11 of 23) of samples were within

| Table 1. Effect of specimen centrifugation and resuspension on FLM II results. |
|-----------------------------|----------------|-----------------|
| Mean (SE), mg/g             | Range, mg/g    | \(P^a\)         |
| Baseline                    | 50.7 (5.9)     | 16.3–110.8      |
| Centrifuged                 | 27.2 (4.0)     | 6.4–68.9        | \(<0.0001\)   |
| Resuspended                 | 52.5 (5.5)     | 19.6–92.5       | 0.286         |

\(^a\) Compared with baseline (paired t-test).
10% of baseline values, and 65% (15 of 23) of samples were within 20% of baseline (Fig. 2A). The mean, however, was not significantly different from baseline (Table 2). Deming regression analysis produced a slope of 1.151 (95% CI, 0.959–1.342), a y-intercept of 9.2 mg/g (95% CI, 20.1 to 1.7 mg/g), and an $r^2$ of 0.882. The SD around the Deming regression line was 9.0 mg/g. When plotted relative to the number of days frozen at $20^\circ$C (Fig. 2B), FLM II results demonstrated a nonsignificant negative trend associated with length of storage time (slope, $-0.017$; 95% CI, $-0.034$ to 0.001). Specimens stored ≤55 days appeared to give FLM II results that were higher than the baseline values, but this difference was not statistically significant.

**Table 2. Effect of specimen storage at $-20^\circ$C on FLM II results.**

<table>
<thead>
<tr>
<th></th>
<th>Mean (SE), mg/g</th>
<th>Range, mg/g</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>52.6 (4.7)</td>
<td>10.0–90.4</td>
<td></td>
</tr>
<tr>
<td>Thawed</td>
<td>51.3 (5.3)</td>
<td>11.4–99.8</td>
<td>0.495</td>
</tr>
</tbody>
</table>

*Compared with baseline (paired $t$-test).*

**Specimen Stability at $4^\circ$C and Room Temperature**

The mean (SD) baseline FLM II result for the 20 specimens stored at 4°C was 55.9 (27.5) mg/g (range, 7.4–103.0 mg/g). FLM II results appeared to be stable for up to 24 h [mean (SD) change from baseline, $-1.3$ (5.5)%], and results were statistically significantly decreased from baseline results by 48 h ($P = 0.019$; Fig. 3). The mean (SD) baseline FLM II result for the 24 specimens stored at room temperature was 47.0 (30.7) mg/g (range, 12.5–136.0 mg/g). FLM II results appeared to be stable for up to 16 h [mean (SD) change from baseline, $-3.8$ (5.6)%], and results were statistically significantly decreased from baseline by 48 h ($P = 0.0008$; Fig. 4).

**Effect of Blood Contamination**

We added increasing amounts of whole blood to 19 different amniotic fluid specimens with FLM II values ranging from 5.1 to 105.3 mg/g. There were no differences between the addition of donor vs maternal blood. Six specimens were immature (mean, 18 mg/g), four were intermediate (mean, 48 mg/g), and nine were mature (mean, 73 mg/g). The mean RBC count for each dilution is given in Table 3. The instrument was unable to provide results and generated a “net I small” error from samples with RBC counts $>0.478 \times 10^{12}/L$ (1:10 dilution). The
influence of blood contamination on FLM II results varied according to the baseline result. Immature specimens demonstrated a significant positive difference with increasing amounts of blood \( (P < 0.0001) \), whereas intermediate and mature specimens were not significantly affected \( (P = 0.77 \) and 0.57, respectively; Fig. 5 and Table 3).

**Discussion**

As expected, centrifugation of amniotic fluid decreased FLM II values by \( \sim 50\% \) from baseline values. This finding is consistent with other studies that have reported a significant decrease in measurable phospholipid in centrifuged amniotic fluid \( (8–10) \). What has not been reported previously is the effect that resuspension has on the FLM II result. Our data suggest that vortex-mixing of centrifuged amniotic fluid for \( 15\) s adequately resuspends sedimented phospholipids in most samples \( (89\%) \).

The FLM II results from frozen and then thawed specimens demonstrated a high degree of variability compared with their original values. The SD of the data plotted in Fig. 2A is 17.8\%. This is 3.9 times greater than the expected SD based on the CV of the assay if the percentage difference between samples had been calculated in the absence of freezing and thawing (expected SD, 4.6\%). Possible reasons for this increase in variability include biochemical changes in amniotic fluid specimens stored frozen or changes in reagent lots during the time that samples were stored. These data are in contrast to a report by Lafler et al. \( (11) \), who reported that FLM II results demonstrated “remarkable reproducibility” after storage at \(-80^\circ\)C for up to 30 days.

In addition, there is the suggestion of a negative trend associated with prolonged storage time. Although this trend was not significant, it was close to significance with the upper limit of the CI just exceeding 0 (slope, \(-0.017; 95\%\) CI, \(-0.034 \) to 0.001). A larger sample of specimens would be needed to investigate this further.

FLM II results from amniotic fluid stored at \( 4^\circ\)C were stable for a longer period of time than results for samples stored at room temperature. At both storage temperatures, results were significantly different from baseline values by 48 h. Although the assay package insert specifies that samples can be stored at 2–8\°C for up to 72 h before testing \( (12) \), our data suggest that if analysis is to be delayed, storage at \( 4^\circ\)C or room temperature should not be extended beyond 24 or 16 h, respectively. These data are also in contrast to the study by Lafler et al. \( (11) \), who reported that results were minimally affected in specimens stored between 2 and \( 8^\circ\)C for 72 h. It is unclear why there is a difference between our results and those reported by Lafler et al. Both studies included 20 samples and appear to have been conducted in exactly the same manner. It is unclear whether samples in the study by Lafler et al. were filtered before storage. Samples in the present study were filtered after storage, just before analysis. It is possible that the presence of cells in the amniotic fluid leads to decreased stability of surfactants and may have produced the differences observed between the two studies.

Blood contamination produced a positive change in FLM II results but only when present in specimens with

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Immature ((\text{mean, 18 mg/g}))</th>
<th>Intermediate ((\text{mean, 48 mg/g}))</th>
<th>Mature ((\text{mean, 73 mg/g}))</th>
<th>RBCs, (10^{12}) cells/L</th>
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</thead>
<tbody>
<tr>
<td>1:1280</td>
<td>0.5</td>
<td>1.2</td>
<td>-1.6</td>
<td>0.004</td>
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<tr>
<td>1:640</td>
<td>1.0</td>
<td>0.9</td>
<td>-0.8</td>
<td>0.007</td>
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<tr>
<td>1:320</td>
<td>1.4</td>
<td>0.1</td>
<td>-2.5</td>
<td>0.015</td>
</tr>
<tr>
<td>1:160</td>
<td>2.7</td>
<td>-0.8</td>
<td>-4.1</td>
<td>0.030</td>
</tr>
<tr>
<td>1:80</td>
<td>4.7</td>
<td>-1.2</td>
<td>-6.2</td>
<td>0.060</td>
</tr>
<tr>
<td>1:40</td>
<td>8.1</td>
<td>-0.7</td>
<td>-6.5</td>
<td>0.120</td>
</tr>
<tr>
<td>1:20</td>
<td>14.2</td>
<td>0.2</td>
<td>-8.0</td>
<td>0.239</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>58.4</td>
<td>-3.0</td>
<td>-7.5</td>
<td></td>
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<tr>
<td>95% CI</td>
<td>36.4 to 80.5</td>
<td>-23.7 to 17.8</td>
<td>-35.3 to 20.2</td>
<td></td>
</tr>
<tr>
<td>(P) for trend</td>
<td>(&lt;0.0001)</td>
<td>0.77</td>
<td>0.57</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Difference = FLM II result after addition of whole blood – baseline FLM II result.
an immature (≤39 mg/g) baseline FLM II value and at very high RBC concentrations. Although increased, the effects of blood on FLM II results from immature specimens were gradual, and results were increased 5.8 mg/g for every 0.1 × 10¹²/L increase in the RBC count (Table 3). Even in the presence of very high concentrations of contaminating blood, none of the immature specimens were changed to a mature interpretation. The mean FLM II result for the six immature specimens was 18 mg/g. When we applied a 20% increase in FLM II results as a cutoff for unacceptable assay performance, specimens with a RBC count >0.03 × 10¹²/L would be considered unsuitable for analysis.

Although specimens with a baseline FLM II result that was either intermediate (40–54 mg/g) or mature (≥55 mg/g) were not significantly affected by the presence of blood at any concentration, values for mature specimens tended to decrease with increasing amounts of blood (Table 3). The mean FLM value for mature samples did not decrease >10% until the RBC concentration was >0.12 × 10¹²/L. These results are in conflict with those described by Carlan et al. (13). This group examined the influence of contamination by maternal blood (at the same dilution ratios used here) on FLM II results. They reported that blood significantly increased the value of nonmature (≤55 mg/g) results and significantly decreased the value of mature (>55 mg/g) results at all but the 1:1280 dilution (13). Our findings are similar to those reported by Russell (14), although the first-generation FLM I assay was used in that study, not the second-generation FLM II assay used here. Russell’s study examined the effects of whole blood diluted 1:100, 1:200, and 1:500 in four different pools of amniotic fluid. The author found that blood increased the FLM result most significantly in amniotic fluid pools with a phospholipid/albumin ratio <50 mg/g (immature and intermediate) but had little effect on mature pools (14). As he speculated, these findings may be explained by the high concentration of phospholipids in the RBC membrane. Russell, however, did not report the RBC counts in the amniotic fluid pools to which the blood was added, making interpretation difficult.

Our data suggest that amniotic fluid contaminated with whole blood need not be automatically considered unsuitable for FLM II analysis. Any specimen that returns an immature FLM II result regardless of the degree of blood contamination can be reliably interpreted. Applying the criterion of a >20% increase in FLM II results as being clinically unacceptable, specimens containing a RBC count >0.03 × 10¹²/L could produce a result that was sufficiently different from the expected result to consider the specimen unsuitable for FLM II analysis. Although the presence of blood exerted no significant influence on intermediate or mature specimens, we would recommend that specimens contaminated with blood that return results >39 mg/g be interpreted with caution.

Amniotic fluid samples are not easily collected and are therefore considered precious specimens by the laboratory. Care must be taken to avoid inappropriate handling that could produce invalid results. Our data suggest that although centrifugation will decrease FLM II values and should be avoided, resuspension by vortex-mixing can restore results to baseline values. In contrast to data reported by others, we found that storage of amniotic fluid at room temperature, 4°C, or −20°C can have a significant effect on FLM II results. Although short-term storage of specimens at room temperature (<16 h) or at 4°C (<24 h) had minimal effects, storage beyond these time points caused a considerable decrease in FLM II results. In addition, the highly variable results obtained from thawed amniotic fluid specimens suggests that the FLM II assay not be performed on previously frozen specimens. The minimal effect of blood contamination on FLM II results was perhaps the most surprising and clinically useful data. Our results suggest that rather than rendering an amniotic specimen unsuitable for FLM II analysis, the presence of RBCs can be quantified and used as a guide for cautious interpretation.

References