Nijmegen Modified Bethesda Assay

New Concepts

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Background

• Development of a neutralizing factor inhibitor is the most significant treatment complication in patients with hemophilia
  o Decreases effectiveness of treatment
  o Significantly increases treatment cost
  o Life threatening

• Laboratory plays an important role in providing a reliable and reproducible assay for the detection and quantitation of neutralizing factor inhibitors
  o Monitoring and management of hemophilia care
  o Evaluation of novel factor product safety
Factor Inhibitor Assays

Classical Bethesda Nijmegen Modified\(^1\) Assay

- **Dilutions**
  - Neat
  - 1:2
  - 1:5
  - 1:10
  - 1:20

- **Imidazole Buffer Factor deficient plasma**

- **Patient Sample**

- **1:1 Patient Mix**

- **Buffered Normal plasma**

- **Incubation at 37° C**

- **1:1 Control Mix**

- **Imidazole Buffer Factor deficient plasma**

- **Or 4 % albumin\(^2\)**

Measure Factor VIII or FIX Activity

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\(^1\)Verbruggen, B et al *Thromb Haemost* 1995; 73(2): 247

\(^2\)Kershaw GW et al *Thromb Res* 2013; 132:735
Performance of Nijmegen Modified Bethesda Assay (NBA)

- External quality assessment programs (ECAT, NEQAS, RCPA QAP) report high inter-laboratory variability with coefficients of variation (CVs) often greater than 30%.

- The percentage of false positive and negative results is unacceptably high.

- Surveys reveal that many laboratories use hybrid methods incorporating features of both the Bethesda and Nijmegen Modified assays.
In response to EQA surveys, there has been a concerted effort to standardize the assay and to identify factors contributing to the observed variability.

- Review critical reagents required for assay reproducibility
- Outline critical steps of the assay procedure
- Review options needed to address assay interference by residual circulating factor
Buffering the NPP with 0.1 M imidazole pH 7.4 prevents pH dependent loss of FVIII activity.

Variations in factor activities of the buffered NPP can influence the APTT measurement and hence the Nijmegen Bethesda assay results.

Activity of the factor under investigation contained in the buffered NPP should be as close as possible to 100% (ECAT 95-105%)

Use of factor deficient plasma rather than imidazole buffer helps to maintain constant protein concentration to prevent loss of FVIII activity.

Potential Issues with Factor Deficient Plasma

• For FVIII inhibitor assays, deficient plasma must contain normal levels of VWF
  • 30-50% lower NBA results have been observed in deficient plasma without VWF. ¹
• Immunodepleted plasma may be contaminated with capture antibody ¹
• Congenital deficient plasmas may contain inhibitors
• Chemically depleted plasma may result in activation of FV ¹

The presence of even 5% factor antigen in the deficient plasma can result in underestimation of the inhibitor titer even when measured in the presence heat inactivation.
Incubation at 37°C

- FVIII (and likely FV) inhibitors are time dependent, incubation for 2 hours at 37°C is required
- FIX inhibitors act rapidly, incubation for 10 minutes at 37°C is sufficient

<table>
<thead>
<tr>
<th>Incubation at 37°C</th>
<th>Inhibitor 1BU + 10 % Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FVIII Activity</td>
</tr>
<tr>
<td>Immediate</td>
<td>9.8 %</td>
</tr>
<tr>
<td>1 hour</td>
<td>5.2%</td>
</tr>
<tr>
<td>2 hour</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

Colorado Coagulation, unpublished observations 2014

1Kershaw, G et al, Semin in Thromb and Hemost, 2009: 35(8):760
Post 37°C incubation

- After incubation at 37°C, place sample in an ice bath (2-8°C) unless the factor assay is performed immediately\(^1\)

- There are several factors that influence inhibitor interactions: time \textit{and} temperature, (as well as others such as pH, inhibitor concentration etc.)

- Can inhibitor binding be affected when samples transition from 37°C to ambient temperature?

**Post 37°C Incubation**

FVIII and FIX inhibitor validation samples (sheep or goat anti-human inhibitor plasmas) tested during accuracy and precision assay runs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FVIII Inhibitor 25 NBU</th>
<th>FIX Inhibitor 5 NBU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Equilibration 2-8°C 10 min.</td>
<td>Equilibration 2-8°C 10 min.</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sample 1</td>
<td>29.3</td>
<td>25.7</td>
</tr>
<tr>
<td>Sample 2</td>
<td>24.7</td>
<td>24.5</td>
</tr>
<tr>
<td>Sample 3</td>
<td>32.3</td>
<td>27.5</td>
</tr>
<tr>
<td>Sample 4</td>
<td>31.8</td>
<td>25.8</td>
</tr>
<tr>
<td>Sample 5</td>
<td>42.4</td>
<td>27.5</td>
</tr>
<tr>
<td>Mean</td>
<td>32.1</td>
<td>26.2</td>
</tr>
<tr>
<td>SD</td>
<td>6.5</td>
<td>1.3</td>
</tr>
<tr>
<td>CV</td>
<td>20.2%</td>
<td>4.9%</td>
</tr>
</tbody>
</table>

Colorado Coagulation, unpublished observations 2014

Equilibration at 2-8°C for 10 minutes following 37°C incubation reduced intra-assay variability and drift in NBU titers.
Calculation of the Nijmegen Bethesda Titer

1:1 Patient Mix  1:1 Control Mix

Post incubation at 37° C
Measure Factor Activity

Residual activity = \frac{\text{Sample Factor Activity}}{\text{Control Factor Activity}}

NBU = 2 - \frac{\log[\% \text{Residual Activity}]}{0.301}

1 NBU = 50 \% residual Activity
If an inhibitor is suspected dilute patient plasma to obtain residual activity between 25 and 75%

Prepare sufficient dilutions of the patient sample to approach residual activity closest to 50%

Use the patient sample dilution that gives residual activity closest to 50% to calculate inhibitor titer

\[ ^1 \text{Verbruggen, B et al } Thromb Haemost } 2011, 9 :2003–2008 \]
### Determination of Precise Nijmegen Bethesda Titer (NBU)

Use of appropriate dilutions allows consistent reporting and may reduce variability in proficiency surveys.

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Factor Activity</th>
<th>Factor Activity</th>
<th>Residual Activity</th>
<th>Residual Activity</th>
<th>NBU Titer</th>
<th>Corrected NBU Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>0.102</td>
<td>0.550</td>
<td>15</td>
<td>1.18</td>
<td>2.72</td>
<td>5.4</td>
</tr>
<tr>
<td>1:5</td>
<td>0.221</td>
<td>0.550</td>
<td>33</td>
<td>1.52</td>
<td>1.61</td>
<td>8.0</td>
</tr>
<tr>
<td>1:10</td>
<td>0.441</td>
<td>0.550</td>
<td>66</td>
<td>1.82</td>
<td>0.61</td>
<td>6.1</td>
</tr>
<tr>
<td>1:20</td>
<td>0.500</td>
<td>0.550</td>
<td>74</td>
<td>1.87</td>
<td>0.43</td>
<td>8.6</td>
</tr>
<tr>
<td>1:40</td>
<td>0.617</td>
<td>0.550</td>
<td>92</td>
<td>1.96</td>
<td>0.13</td>
<td>5.0</td>
</tr>
</tbody>
</table>

| Example 2       |                 |                 |                   |                   |           |                    |
| 1:20            | 0.135           | 0.488           | 28                | 1.44              | 1.85      | 37.1               |
| 1:40            | 0.322           | 0.488           | 66                | 1.82              | 0.60      | 24.0               |
| 1:80            | 0.428           | 0.488           | 88                | 1.94              | 0.19      | 15.1               |

| Example 3       |                 |                 |                   |                   |           |                    |
| 1:20            | <0.050          | 0.477           | NA                | NA                | NA        | NA                 |
| 1:40            | 0.206           | 0.477           | 43                | 1.64              | 1.21      | 48.5               |
| 1:80            | 0.318           | 0.477           | 67                | 1.82              | 0.58      | 46.8               |

Colorado Coagulation, unpublished observations 2014
Interference from Residual Circulating Factor

• Use of prophylaxis therapy and longer acting factor products make it increasingly difficult to test a patient without residual circulating factor

• Presence of even a small amount of residual factor violates the principle of the assay “that the patient sample and control mixture are the same”

• Presence of residual factor can underestimate the inhibitor titer or result in a false negative titer
Approaches to Inhibitor Quantitation in the Presence of Residual Factor

The presence of > 5% factor activity must be taken into account when determining inhibitor titer\(^1\).

Options:

1. Add more factor to the control mixture to compensate for the amount factor in the patient sample - Can only be used if patient sample is tested undiluted

2. Mathematically correct for the presence of factor during the inhibitor titer calculation Complex!!!!

3. Heat inactivation – eliminates the residual factor while preserving the inhibitory antibodies

\(^1\)Kitchen, S et al 2010. *Diagnosis of Hemophilia and Other Bleeding Disorders, A Laboratory Manual, 2\textsuperscript{nd} Edition, WFH*
Heat Inactivation Procedure

Heat both at 56°C for 30 min.

Centrifuge at 2700 x g 5 min

Remove supernatant

Test in Nijmegen Bethesda Assay

Patient plasma
Control plasma

Nijmegen Modified Bethesda Assay (NBA)

Dilutions
- Neat
- 1:2
- 1:5
- 1:10
- 1:20

Factor deficient plasma

Buffered Normal plasma

Incubation at 37°C

1:1 Patient Mix

1:1 Control Mix

Equilibration 2-8°C

Measure Factor VIII or FIX Activity
Nijmegen Modified Bethesda Assay (NBA)

Dilutions
- Neat
- 1:2
- 1:5
- 1:10
- 1:20

Factor deficient plasma

Patient Sample

Buffered Normal plasma

Incubation at 37° C

1:1 Patient Mix

Factor deficient plasma

1:1 Control Mix

Ensure the factor activity is close to 100%

Measure Factor VIII or FIX Activity
Nijmegen Modified Bethesda Assay (NBA)

Dilutions
Neat
1:2
1:5
1:10
1:20

Factor deficient plasma

Patient Sample
Buffered Normal plasma
Incubation at 37°C

1:1 Patient Mix
1:1 Control Mix

Measure Factor VIII or FIX Activity

FVIII – ensure normal levels of VWF

Ensure inhibitor free
Nijmegen Modified Bethesda Assay (NBA)

- Dilutions:
  - Neat
  - 1:2
  - 1:5
  - 1:10
  - 1:20

- Factor deficient plasma

- Patient Sample

- Buffered Normal plasma

- Incubation at 37°C

- 1:1 Patient Mix

- 1:1 Control Mix

- Measure Factor VIII or FIX Activity

Ensure factor antigen is <5%
Nijmegen Modified Bethesda Assay (NBA)

Dilutions
- Neat
- 1:2
- 1:5
- 1:10
- 1:20

Prepare sufficient dilutions to approach residual activity close to 50%

Report the NBU titer based on the residual activity closest to 50%

Measure Factor VIII or FIX Activity
Nijmegen Modified Bethesda Assay (NBA)

Dilutions
- Neat
- 1:2
- 1:5
- 1:10
- 1:20

Factor deficient plasma

Incubate samples for the appropriate time to prevent underestimation of the NBU titer

Measure Factor VIII or FIX Activity

Incubation at 37° C
Nijmegen Modified Bethesda Assay (NBA)

- **Dilutions**
  - Neat
  - 1:2
  - 1:5
  - 1:10
  - 1:20

- **Patient Sample**
- **Buffered Normal Plasma**
- **Factor Deficient Plasma**

**Incubation at 37°C**

- **1:1 Patient Mix**
- **1:1 Control Mix**

**Equilibration 2-8°C**

Measure Factor VIII or FIX Activity

Consider equilibration at 2-8°C before factor activity testing to reduce assay variability.
**Nijmegen Modified Bethesda Assay (NBA)**

### Dilutions
- Neat
- 1:2
- 1:5
- 1:10
- 1:20

### Factor Deficient Plasma

### Patient Sample

### Buffered Normal Plasma

### Incubation at 37°C

### Equilibration 2-8°C

### 1:1 Patient Mix

### 1:1 Control Mix

### Presence of residual circulating factor should be considered when measuring inhibitor titer
- Adjust control sample
- Mathematically correct
- Heat inactivate sample

**Measure Factor VIII or FIX Activity**
Summary

- Limit assay variables
- Standardize assay procedures

- Improve accuracy and precision

- Reliable and Reproducible Nijmegen Modified Bethesda Assay
Thank you for your attention!

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