Design and Validation of a Non Cell-based Receptor Binding Assay for the Detection of Neutralizing Antibodies to a Biological Therapeutic

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Immunogenicity Testing

Test samples

Immunoassay

POSITIVE

Cell-based Assay

a.k.a NAb assay

NEGATIVE

Provide a functional biological system to assess if the Abs detected by the immunoassay have neutralizing capability.
Cell-based NAb Assay Designs

• Utilize intracellular signaling events triggered within a cell line that responds to:
  * the drug product (eg. cytokines)
  * the ligand inhibited by the drug product (eg. MAbs)

• May also utilize study of extracellular binding events at the cell surface (eg. MAbs)
Requirements for Cell-based NAb Assays

- Stable cell line responsive to drug
- Measurable Readout (cpm, OD, conc of secreted protein)
- Robust signal to noise ratio in serum matrix
- Availability of a positive control antibody to the drug
- Medium to High Throughput
- Adaptable to Automation
Justification For a Non-cell Based NAb Assay

- Drug product: blocked ligand activity
- Limited choice of cell lines expressing the receptor for ligand that was target for drug product
- Only cell line available yielded at best 2-fold signal when treated with 100-200 ng/mL ligand
- Cell line had finite life span
- Difficult cells to culture
- Low throughput (24-well plates)
- Irreproducible bioassay results
Meso-Scale Discovery (MSD) Technology

Meso-Scale Discovery (MSD) technology employs microtitre plates fitted with a series of electrodes associated with the bottom of each well.

Using an MSD Sector PR™ plate reader, an electrical current is placed across the plate-associated electrodes in the presence of a Tripropylamine (TPA) containing buffer. The result is a series of electrically induced oxidation-reduction reactions involving Ruthenium (from the captured complex) and TPA leading to a luminescent signal. The consequent electrochemiluminescent (ECL) signal is measured by photodiodes and is quantified as a relative unit (RU).

Courtesy of Mesoscale Discovery, Gaithersburg, MD
Assay Reagents

- Purified Soluble receptor (ruthenylated)
- Purified Ligand (biotinylated)
- Drug product (TP)
- Streptavidin-coated MSD Plates
- Cynomolgus monkey serum
- Affinity purified rabbit anti-TP antibody
**Assay Methodology**

1. **Incubate serum sample with TP – 15-30 Min**

2. **Add L-Biot – 15-30 Min**

3. **Add R-Ru – 15-30 Min**

4. **Add Mixture to blocked SA-Coated Multiarray Plate – 30-60 Min**

5. **Wash 2X and add TPA Containing read buffer Read on MSD reader.**

**Legend:**
- R-Ru
- Anti-TP Antibody
- L-Biot
- TP
- Streptavidin
Assay Development

- Optimized ratio between Ru-R and Biot-L for optimal signal
- Used 50% cyno serum for initial development expts
- Due to donor (n=10) variability serum was reduced to 15%
- Optimized order of mixing of reagents (capture vs. homogenous)
- Improved precision and inter-day repeatability in homogenous format with assay controls
Optimized Assay Conditions

- 15% cynomolgus monkey serum
- 250 ng/mL Biot-Ligand
- 900 ng/mL Ru-R
- 80 ng/mL TP
**Assay Controls**

- **N** = background (15% PCS with R-Ru)
- **M** = maximum binding (15% PCS with R-Ru and L-biot)
- **D** = drug control (15% PCS with TP, r-RU and L-biot)
- **P** = positive control (D with anti-TP antibody)
A cell line naturally expressing the receptor was used to demonstrate:

- L-Biot binding, inhibition of binding by TP
- Restoration of binding by the anti-TP NAb.

Binding of L-Biot to the receptor was detected using a SA-FITC conjugate.

Receptor expression was confirmed using a biotinylated goat polyclonal anti-receptor Ab and detected with SA-FITC.

Binding of L-Biot to the target receptor was inhibited by the TP and restored by the anti-TP NAb.
NAb Testing Strategy

IM-Positive Samples

NAb Receptor Binding Assay

Sample inhibits TP activity

Specificity Assay (in absence of TP)
Sample does not show non-Specific activity

NEGATIVE

Sample does not inhibit TP activity

(sample shows non-specific activity)

POSITIVE
Validation Experiments

- TP dose response curves
- Positive Control Ab curves
- NAb assay cutoff
- Specificity Assay Cutoff
- Interference by drug product
- Freeze-thaw stability
TP was incubated at increasing concentrations with 250 ng/mL L-Biot followed by incubation with 900 ng/mL R-Ru in 15% PCS.

TP showed a dose dependent inhibition of L-Biot/R-Ru binding.

A concentration of 80 ng/mL TP was chosen to be used for the Screening Assay.
Neutralization of TP and Restoration of Ligand Binding by Anti-TP Neutralizing Antibody in Pooled Cynomolgus Monkey Serum

- Rabbit Polyclonal anti-TP Antibody (NAb) was added to neat PCS.

- The Ab containing PCS was diluted to 15% and preincubated with 80 ng/mL TP followed by sequential incubations with 250 ng/mL L-Biot and 900 ng/mL R-Ru.

- Anti-TP antibody showed a dose dependent restoration of L-Biot/R-Ru binding to maximal binding signal (above curves represent 2 days testing).

- The lowest concentration of Anti-TP Nab to restore binding by twofold was 1 µg/mL.
## Precision and Analytical Recovery of the PAb

<table>
<thead>
<tr>
<th>Ab (ug/mL)</th>
<th>0.055</th>
<th>0.11</th>
<th>0.22</th>
<th>0.44</th>
<th>0.88</th>
<th>1.75</th>
<th>3.5</th>
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<tbody>
<tr>
<td>AR</td>
<td>356%</td>
<td>228%</td>
<td>117%</td>
<td>138%</td>
<td>98%</td>
<td>99%</td>
<td>98%</td>
<td>87%</td>
<td>68%</td>
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<tr>
<td>%CV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Inter-day</td>
<td>19%</td>
<td>29%</td>
<td>17%</td>
<td>8%</td>
<td>11%</td>
<td>2%</td>
<td>4%</td>
<td>25%</td>
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<tr>
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<td>35%</td>
<td>40%</td>
<td>44%</td>
<td>16%</td>
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<td>0.00</td>
<td>49%</td>
</tr>
<tr>
<td>Intra-assay</td>
<td>14%</td>
<td>20%</td>
<td>14%</td>
<td>13%</td>
<td>10%</td>
<td>2%</td>
<td>9%</td>
<td>26%</td>
<td>104%</td>
</tr>
</tbody>
</table>

- **Ab (ug/mL)**: Concentration of antibody in micrograms per milliliter.
- **AR**: Analytical Recovery percentage.
- **%CV**: Percentage Coefficient of Variation.
- **Inter-day**: Inter-day variability.
- **Inter-assay**: Inter-assay variability.
- **Intra-assay**: Intra-assay variability.
• Serum from 50 individual cynomolgus monkeys was tested at a final dilution of 15% with 80 ng/mL TP, 250 ng/mL L-Biot and 900 ng/mL R-Ru.

• Samples results were compared with control results in PCS to by dividing Sample Results/Control Results (Screening Ratio).

• Samples were tested on two separate days and the Ratio 1 values were combined to establish an assay cutoff using the upper bound of the 95% prediction limit.

• The assay cutoff (Screening Ratio) was determined to be 1.26.
Serum from 50 individual cynomolgus monkeys was tested at a final dilution of 15%, 250 ng/mL L-Biot and 900 ng/mL R-Ru in the absence of the TP to assess for nonspecific enhancement of ligand binding.

Samples results were compared with control results in PCS to by dividing Sample Results/Control Results (Ratio 3).

The Ratio 3 values were used to establish an assay cutoff using the upper bound of the 95% prediction limit.

The assay cutoff (Specificity Ratio) was determined to be 1.71.
AP Criteria for Positive/Negative

- Assay Acceptance Criteria
  - M/N > 10
  - M/D > 2.0
  - P/D > 1.26

Predose: Ratio 1 > 1.26
Ratio 3 < 1.71

Postdose: Ratio 2 > 1.26
Ratio 3 < 1.71

Post/Pre used because some individual animals yielded extremely low Ratio 1 values
Serum from 50 individual cynomolgus monkeys was tested treated and untreated with 2 µg/mL anti-TP NAb at a final dilution of 15% in the Screening or Specificity Assay.

A Ratio of Treated/Untreated samples (Post/Pre) was calculated and the Screening Ratio cutoff value was applied (1.26).

Samples results were compared with Post/Pre and Specificity Ratio cutoff values.

All samples treated with anti-TP NAb were above the Post/Pre Ratio cutoff.

All samples treated with anti-TP NAb were below the Specificity Ratio cutoff except for three. These represent false negative samples.

All untreated samples were below the Screening and Specificity Ratio cutoff (data not shown).
Excess Drug Interference

- Rabbit polyclonal anti-TP antibody was prepared in neat PCS at 20, 10, 4 and 2 μg/mL. At each concentration of antibody, the TP was titred from 68.6, 34.3, 17.15, 8.576, 4.288, 2.144, 1.072, 0.536 and 0 μg/mL in neat PCS.

- The ability of the assay to detect 2 and 4 μg/mL anti-TP antibody was inhibited by 80 ng/mL excess TP (536 ng/mL in neat serum). The assay is able to detect 10 μg/mL anti-TP antibody in the presence of up to 160 ng/mL (1.07 μg/mL in neat serum) excess TP and 20 μg/mL in the presence of up to 320 ng/mL (2.14 μg/mL in neat serum) excess TP.
Effects of Freezing and Thawing on Antibody Detection

- Positive control anti-TP antibody was added to PCS at the LOD of the assay (2 µg/mL).
- All samples containing antibody were positive up to 3 freeze thaw cycles while all untreated samples remained negative.
## Study Results

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose Group</th>
<th>Immunoassay Result</th>
<th>Neutralizing Antibody Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low</td>
<td>Negative</td>
<td>Not Analyzed</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
<td>Negative</td>
<td>Not Analyzed</td>
</tr>
<tr>
<td>3</td>
<td>Low</td>
<td>Negative</td>
<td>Not Analyzed</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>High</td>
<td><strong>Positive</strong></td>
<td><strong>Positive</strong></td>
</tr>
<tr>
<td>8</td>
<td>High</td>
<td>Negative</td>
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</tr>
<tr>
<td>9</td>
<td>High</td>
<td>Negative</td>
<td>Not Analyzed</td>
</tr>
</tbody>
</table>

*Serum Samples from Cynomolgus Monkeys dose with TP that were found to be Reactive in a Screening Immunoassay were tested in the Receptor-Binding Assay*

*Of the samples tested one sample was found to be positive for neutralizing antibodies which correlated with the Immunoassay result.*
Conclusions

A robust and sensitive receptor binding assay was develop for the detection of neutralizing antibodies to a TP in cynomolgus monkey serum.

The assay was able to detect 2 µg/mL rabbit polyclonal anti-TP antibody in monkey serum in the presence of 80 ng/mL TP.

The successful implementation of the assay detected antibodies in 1 animal from a preclinical pharmacokinetic study (11% incidence).
Concluding Comments

- Dependent upon quality of reagents
- Biological activity of reagents should be evaluated
- Sequence of assay steps extremely important
- Serum still an important factor
- Quick readout
- Assay development requires careful consideration
- Ratio of ligand/therapeutic important
- Not a slam dunk!
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