Quantitative Measurement of Anti-D Antibody in Human Serum Using a Flow Cytometry PK Method

Dr C. de COUPADE
Head of Cell Biology Lab
LFB Biotechnologie (France)
Sensitization to Rh D antigens may lead to the production of maternal IgG anti-D antibodies which can pass through the placenta.

The mother often receives an injection of anti-D antibodies at 28 weeks gestation and at birth to avoid the development of antibodies.
Background (II): Prevention of HDN

- Polyclonal anti-D antibodies purified from human plasma to prevent hemolytic disease of the fetus and newborn
  - Rhophylac® (CSL Behring GmbH)
  - RhoGAM® (Kedrion)
  - WinRHO® (Cangene/Baxter)

- Anticipate potential shortage due to donors aging + avoid volunteers immunization
  => get an alternative to current treatment
  => secure anti-D supply
   replacement therapy

⇒ Risk on Sourcing : BIOHAZARD + DONORS decline
Anti-D: Needs an Alternative to Plasma Derived Products

- Fully human recombinant anti-D antibody developed by LFB (IgG1)

- Variable human region binds specifically to D antigens on red blood cells
- Human constant k-regions
- Human IgG1 Fc region

**Advantages of non plasma derived anti-D:**

- No infectious hazards
- No repeated immunization of volunteers
- No limitation in supplies

- Phase I ✓
- Phase IIa ✓
- Phase IIb In Progress
Assessment of pharmacokinetic profile of monoclonal anti-D antibody

1. Develop the BioAssay
2. Validate the BioAssay
3. Run the BioAssay

R&D
Validated method for clinical trials
Step 1  Develop the BioAssay

- Development of a ligand-binding assay (ELISA)
  - Soluble D antigen not available
  - Conformational epitope

- Alternatives
  - Murine anti-idiotype antibody for ELISA format
  - Cell-based assay => binding to target cells
A flow cytometry method was developed at LFB to quantify anti-D antibodies in human serum.
Principle

- Binding of anti-D to Red Blood Cells (group OR1R1)
  - O blood group to avoid isoagglutinin interference (anti-A/anti-B hemagglutinins from human serum)
  - R1R1 phenotype selected due to high RhD antigens/cell
- Detection of immune complexes using a fluorescent marker

![Diagram of Cytometry method applied to anti-D determination (I)]
Material

- Flow cytometer (one laser-based cytometer) with high-throughput sample loader

Reagents

- O RhD-positive red blood cells (cryoconserved, stored at +4°C in Alsever solution for 15 days after thawing)
- Reference standard for monoclonal anti-D: LFB internal control used for calibration curve and QCs samples
- Fluorescent-labeled secondary antibody Fab fragment anti-human IgG(H+L)
Red Blood Cell Preparation: Optimization step

=> Treatment of red cells by papain allows better detection of the Rh system on the cell surface by reducing the negative charges and polypeptid chains.

- Increased reactivity of RhD antigen to anti-D antibody leading to an increased response (fluorescence intensity)
- Decreased background due to the serum matrix
Cytometry method: Operating procedure

- **Calibration Standards/ QCs samples**
  - 8 concentration levels (0.5 to 7.5 ng/mL) in 1% PBS-BSA
  - 3 QCs levels (High, Mid and Low)

- **Quantification and adjustment of RBC concentration**
  - Flow Count beads used for direct quantification of RBC/mL

- **Incubation** of RBC with samples (diluted 1 in 2 in 1% PBS-BSA)
  - in a microplate at 37°C for 2h shaking
  - washings in 1% PBS-BSA

- **Addition of the fluorescent antibody**
  - at room temperature for 30 mn
  - washings in 1% PBS-BSA

- **Reading** of the microplate directly on a Flow cytometer
RBC gated on C (50,000 events) to allow good measurement of the fluorescence (MFI - gate A)

**Data processing**

- Signals processed using flow cytometer and Microsoft Excel softwares to give a Mean Fluorescence Intensity

**Acceptance criteria**

- Slope must be > 10
- $R^2 > 0.99$
50,000 events/well => reading time around 1h

Minimize decrease of fluorescence over time => Triplicate over the plate

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Triplicate
Flow cytometry

Step 2 Validate the BioAssay

Assay Validation of a Flow cytometry PK method for the measurement of anti-D antibodies in human serum

=> The Flow cytometry-based assay was validated according to EMA (Feb 2012) and FDA (May 2011) Guidelines on Bioanalytical Method Validation
Validation Parameters

- Calibration model suitability
- QC Precision, Accuracy and Total error
- Dilution Linearity in buffer
- Stability studies

Instrument set-up: Beckman Coulter FC500 MPL flow cytometer equipped with dual laser system; 488 nm argon laser and 635 nm diode laser
Calibration model suitability

- CV ≤ 20% for all concentration levels
- RE ± 20% (± 25% LLOQ and ULOQ)
- Standard curve must contain a minimum of 6 standards within the quantification range

<table>
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<th>Calibration curve data</th>
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<tr>
<td>Concentration (ng/ml)</td>
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<tr>
<td>Rep 1</td>
</tr>
<tr>
<td>7.50</td>
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<tr>
<td>6.00</td>
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<tr>
<td>4.00</td>
</tr>
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<tr>
<td>0.50</td>
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- R² > 0.99
- Slope > 10
QC Precision, Accuracy and Total error

- CV of triplicate ≤ 20%
- RE ± 20% (± 25% LLOQ and ULOQ)

<table>
<thead>
<tr>
<th>QC sample (ng/mL)</th>
<th>Mean Fluorescence Intensity</th>
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<tr>
<td>(ULOQ QC 1) 15.0</td>
<td>250</td>
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<tr>
<td>(High QC 1) 12.0</td>
<td>200</td>
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<tr>
<td>(Mid QC 1) 5.0</td>
<td>150</td>
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<tr>
<td>(Low QC 1) 2.5</td>
<td>100</td>
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<tr>
<td>(LLOQ QC 1) 1.0</td>
<td>50</td>
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\[
y = 26.16x + 24.34 \\
R^2 = 0.9920
\]
## Linearity, Precision and Accuracy

- **5 QC levels (LLOQ up to ULOQ)**
- **6 independent runs**

<table>
<thead>
<tr>
<th>Assay Run number</th>
<th>LLOQ (1.00 ng/mL)</th>
<th>Low QC (2.50 ng/mL)</th>
<th>Middle QC (5.00 ng/mL)</th>
<th>High QC (12.00 ng/mL)</th>
<th>ULOQ (15.00 ng/mL)</th>
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<td>2.90</td>
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<td>2.60</td>
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<td>8</td>
<td>0.90</td>
<td>2.38</td>
<td>4.72</td>
<td>11.55</td>
<td>13.96</td>
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| Mean             | 1.04              | 2.66                | 5.27                   | 12.46                 | 15.27             |
| SD               | 0.10              | 0.23                | 0.47                   | 0.82                  | 0.99              |
| CV (%)           | 9.4               | 8.6                 | 8.9                    | 6.5                   | 6.5               |
| RE (%)           | 3.7               | 6.4                 | 5.3                    | 3.8                   | 1.8               |
| TE (%)           | 13.1              | 15.0                | 14.3                   | 10.3                  | 8.2               |
| N                | 6                 | 6                   | 6                      | 6                     | 6                 |

**Acceptance Criteria**
- ≤ 20%
- ± 20% and ± 25% LLOQ & ULOQ
- ≤ 30% and ≤ 40% LLOQ & ULOQ

SD - Standard Deviation
CV - Coefficient of Variation
RE - Relative Error
TE - Total Error
Stability Investigations

- **Stability QC levels**
  - Low QC (2.5 ng/mL)
  - High QC (12 ng/mL)

- **Acceptance criteria**
  - CV ≤ 20%
  - RE ± 20% nominal value or T0 value if appropriate

- Room temperature for 24h and 48h
- At +5°C for 24h and 48h
- After 3 F/T cycles at -70°C
- Frozen (-70°C and -20°C) from T1M to T24M
Concluding remarks

Flow cytometry-based assay

- High sensitivity
- Related to Ig binding to target cells
- « Easily » transferable
- Validation: Regulatory compliant method
- High throughput clinical sample testing
- Useful tool for pharmacokinetic studies

Good alternative to ELISA

Used during all clinical phases
Special thanks to...

- Bruno PICOT
  Cell Biology Lab Senior Technician
  Department of Non-Clinical Study
  LFB-BIOTECHNOLOGIES