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

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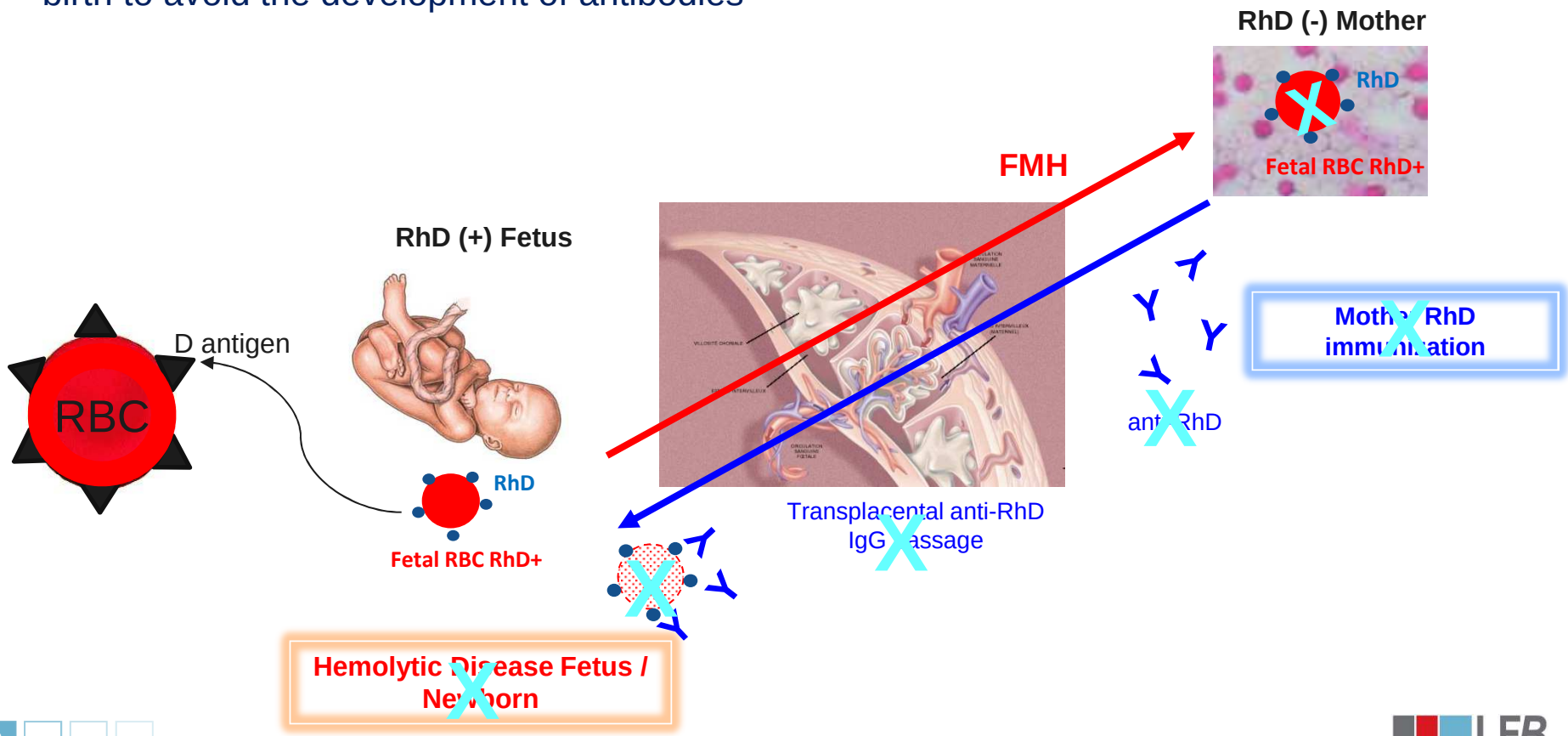
L'ENGAGEMENT ÉTHIQUE

Background (I): Hemolytic disease of the fetus and newborn (HDN)

- 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)

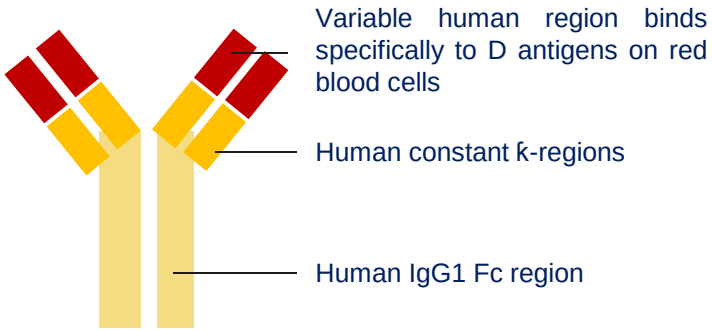
- Anticipate potential shortage due to donors aging + avoid volunteers immunization
  => get an alternative to current treatment
  => secure anti-D supply

⇨**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)



• Advantages of non plasma derived anti-D:

• No infectious hazards

• No repeated immunization of volunteers

• No limitation in supplies

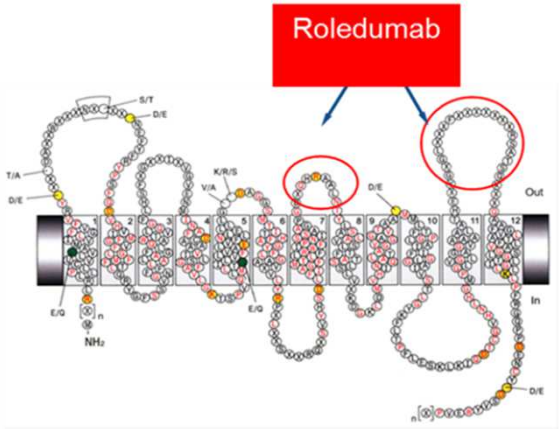
- Phase I ✓
- Phase IIa ✓
- Phase IIb **In Progress**

Clinical Trials and BioAnalysis Needs

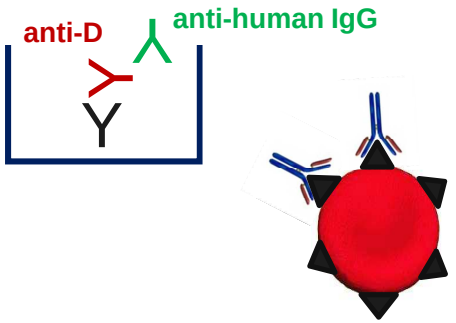
- Assessment of pharmacokinetic profile of monoclonal anti-D antibody

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



Flow cytometry

Develop the BioAssay

A flow cytometry method was developped at LFB to

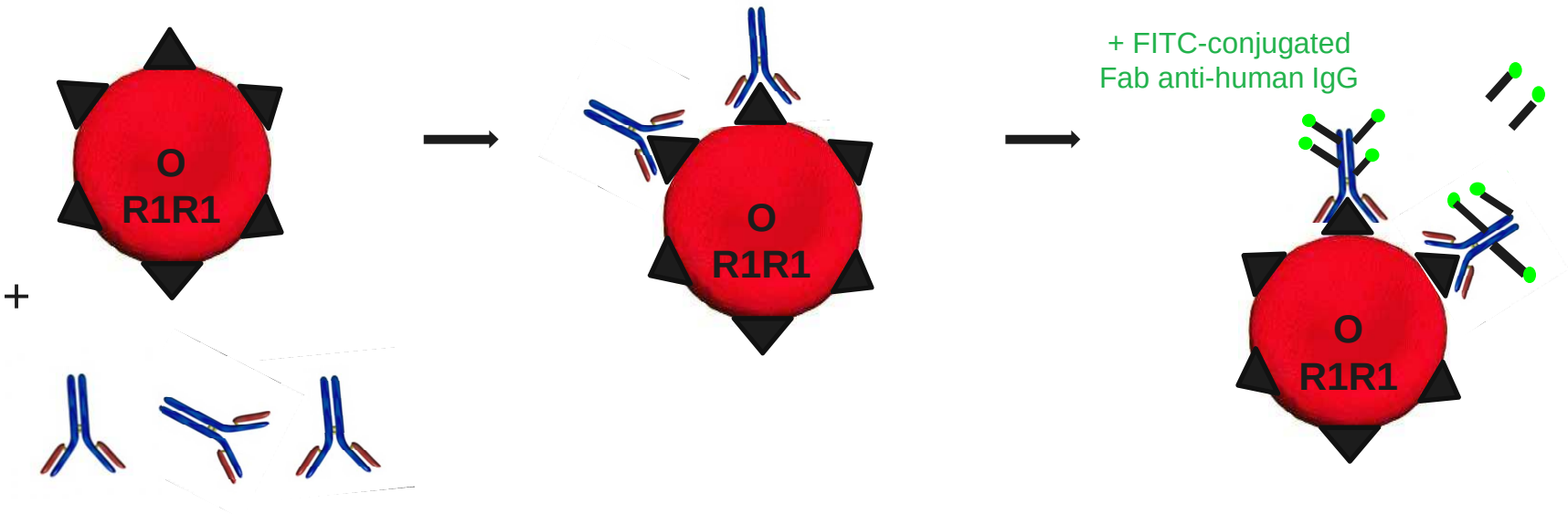
quantify anti-D antibodies in human serum

Cytometry method applied to anti-D determination (I)

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

Group	No of antigen sites per cell
DCe/dce (R_1r)	D sites: 9900–14 600
Dce/dce (R_or)	D sites: 12 000–20 000
DcE/dce (R_2r)	D sites: 14 000–16 600
DCe/dCe (R_1R_1)	D sites: 14 500–19 300
DCe/DcE R_1R_2	D sites: 23 000–31 000
DcE/DcE (R_2R_2)	D sites: 15 800–33 300



Cytometry method applied to anti-D determination (II)

Material

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

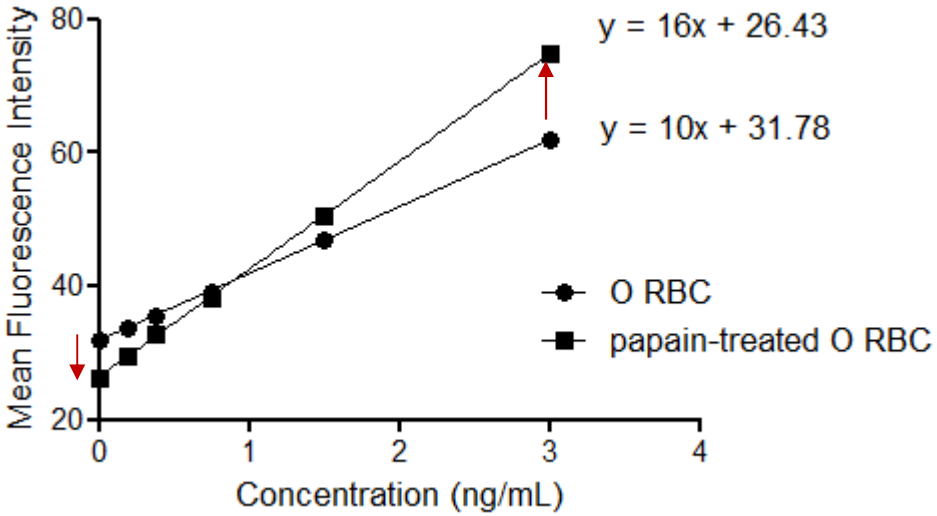
Reagents

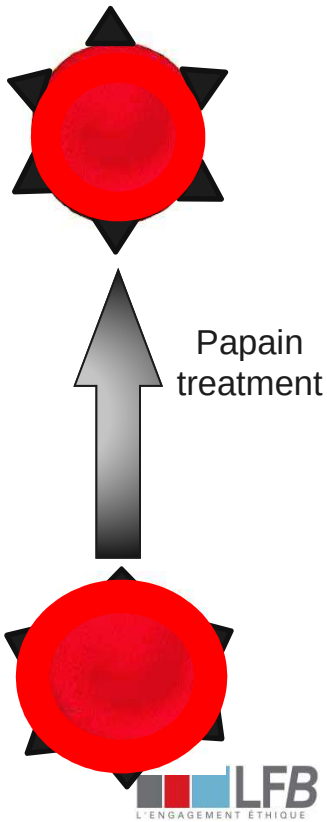
- O RhD-positive red blood cells (cryoconserved, stored at +4℃ in Alsever solution for 15 days after thawing)
- Reference standard for monoclonal anti-D: LFB internal control used for calibration curve and QCs samples
- Fluorescent-labeled secondary antibody Fab fragment anti-human IgG(H+L)

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



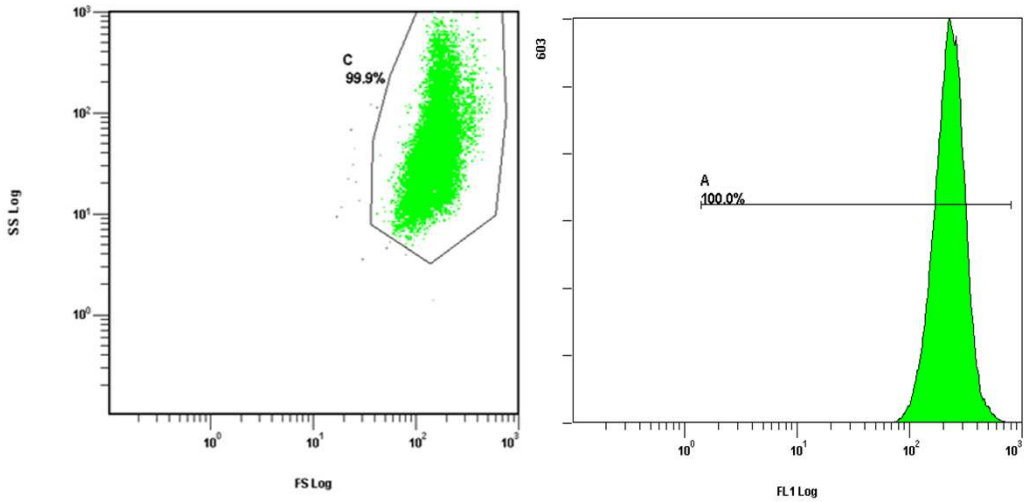


Cytometry method : Operating procedure

- 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℃ 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

Cytometry method : Settings

RBC gated on C (50 000 events) to allow good measurement of the flourescence (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

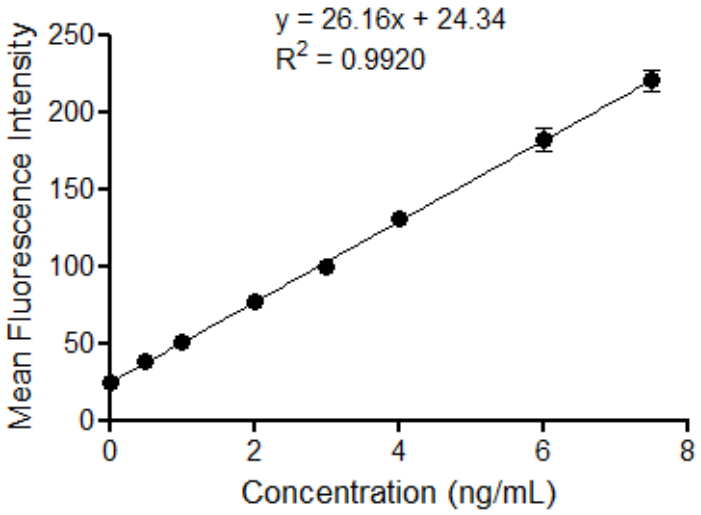
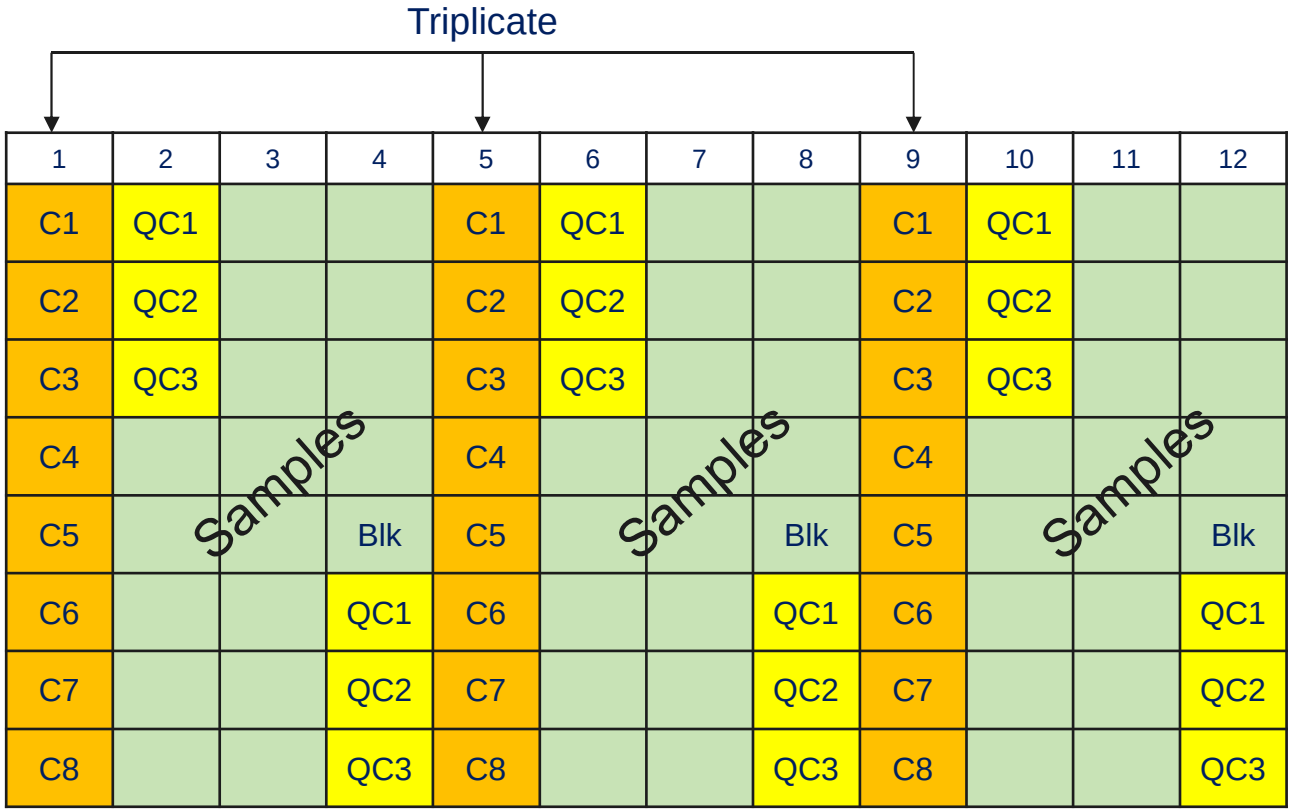


Plate Layout

- 50 000 events/well => reading time around 1h
- Minimize decrease of fluorescence over time => Triplicate over the plate



Flow cytometry

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

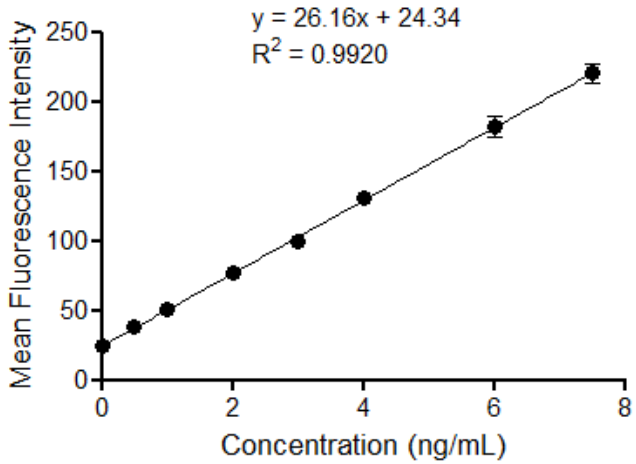
- Instrument set-up: Beckman Coulteur FC500 MPL flow cytometer equipped with dual laser system; 488 nm argon laser and 635 nm diode laser

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

Calibration curve data											
Concentration (ng/ml)	Rep 1	X-mean Rep 2	Rep 3	Mean	SD	CV (%)	Pass/ Fail	Back Calculation	RE %	Pass/ Fail	Replicates excluded
7.50	234.0	216.0	211.0	220.3	12.10	5.5	Pass	7.49	0.1%	Pass	0
6.00	195.0	181.0	170.0	182.0	12.53	6.9	Pass	6.03	0.5%	Pass	0
4.00	139.0	127.0	126.0	130.7	7.23	5.5	Pass	4.07	1.6%	Pass	0
3.00	102.0	101.0	95.6	99.5	3.44	3.5	Pass	2.87	4.2%	Pass	0
2.00	79.4	75.8	74.3	76.5	2.62	3.4	Pass	1.99	0.3%	Pass	0
1.00	53.6	50.5	47.3	50.5	3.15	6.2	Pass	1.00	0.1%	Pass	0
0.500	39.8	35.5	38.1	37.8	2.17	5.7	Pass	0.51	2.9%	Pass	0
0.0	26.5	26.0	22.9	25.1	1.95						

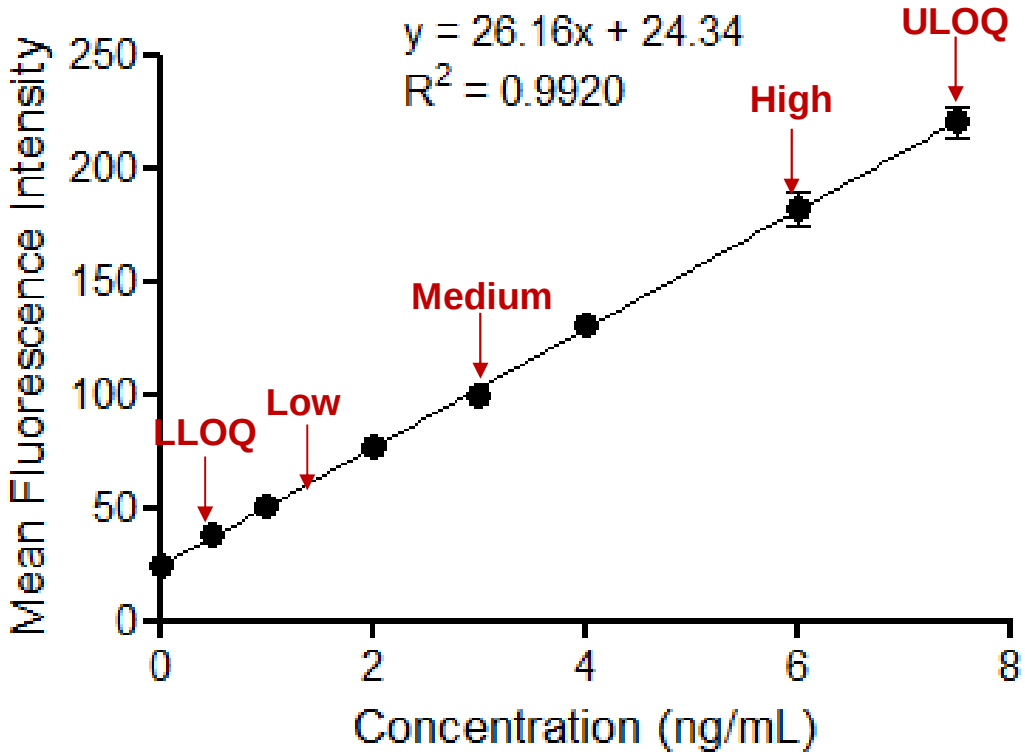
- $R^2 > 0.99$
- Slope > 10



QC Precision, Accuracy and Total error

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

QC sample (ng/mL)
(ULOQ QC 1) 15.0
(High QC 1) 12.0
(Mid QC 1) 5.0
(Low QC 1) 2.5
(LLOQ QC 1) 1.0



Linearity, Precision and Accuracy

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

Assay Run number	LLOQ (1.00 ng/mL)	Low QC (2.50 ng/mL)	Middle QC (5.00 ng/mL)	High QC (12.00 ng/mL)	ULOQ (15.00 ng/mL)
2	0.94	2.95	5.76	13.00	15.92
3	1.12	2.68	5.25	12.46	15.31
5	1.10	2.90	5.85	13.69	16.74
6	1.12	2.46	4.80	11.63	14.52
7	1.05	2.60	5.22	12.40	15.16
8	0.90	2.38	4.72	11.55	13.96
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

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

Acceptance Criteria

≤ 20%

± 20% and ± 25% LLOQ & ULOQ

≤ 30% and ≤ 40% LLOQ & ULOQ

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℃ for 24h and 48h
- After 3 F/T cycles at -70℃
- Frozen (-70℃ and -20℃) from T1M to T24M

Concluding remarks

Flow cytometry-based assay

- High sensitivity
- Related to Ig binding to target cells

} Good alternative to ELISA

- « Easily » transferable
- Validation: Regulatory compliant method
- High throughput clinical sample testing
- Useful tool for pharmacokinetic studies

} Used during all clinical phases

Special thanks to…

- Bruno PICOT

Cell Biology Lab Senior Technician

Department of Non-Clinical Study

LFB-BIOTECHNOLOGIES