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Research paper

# Analytical validation of an alternative method to quantify specific antibodies in 3 applications

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ABSTRACT

The detection and the quantification of specific antibodies represent essential tools for the diagnosis and for the biological monitoring of immune humoral response in many clinical situations in particular in autoimmune diseases or in the context of immunotherapy using monoclonal antibodies.

This article focuses on the development of a specific antibody measuring method (Patent n°PCT/IB2014/064437). The principle of this method is based on the combined use of a monoclonal antibody as standard and the protein G as immunoglobulins detecting agent.

We performed a complete analytical validation of this method for the quantification of antibodies in three different applications: autoantibodies, alloantibodies and therapeutic monoclonal antibody. The results showed good performances compatible with the use of these assays as diagnostic tools.

This method allows avoiding the use of products from human origin as reagent that causes ethical and infectious concerns but also storage and long term stock management problems. Moreover, this approach is particularly useful when no commercial reagent is available, especially in the case of rare diseases.

## 1. Introduction

The necessity of detection and quantification of specific antibodies is increasing since the last years in many situations of human and animal medicine: for the diagnosis of autoimmune diseases, the follow up of immune serology or of immunotherapy by monoclonal antibodies. This kind of assay is relevant for purposes of diagnosis but also in context of monitoring diseases or to assess the effectiveness of a treatment.

This research can be done by various methods including ELISA, the most commonly used. This method enables the detection of antibodies but also their quantification by introducing into the test a standard with known value, which serves as reference for converting an optical density in quantifiable units.

The antibodies contained in this reference sample must be revealed by the same method than the antibodies contained in the tested samples. Most frequently, the revelation system used immunoglobulins purified from immunized animals directed against human immunoglobulins. In consequence, the standard sample must contain also human immunoglobulins requiring the use of samples collected from positive patients. Therefore, this raises problems of legislative and ethical issues, of management of infectious risk, of conservation and of stock limitation with a risk of source depletion, especially for rare antibodies.

As a second consequence, the results of the antibodies quantification use frequently arbitrary units which vary from one assay to another, limiting the possibility of inter-reagent standardization.

To solve these various constraints, we developed a method applicable to all antibodies once a monoclonal antibody directed against the same antigen target is available. The principle of this method is based on the combination of a monoclonal antibody as a standard and the use of labelled purified protein G allowing the detection and the quantification in international units (g/L) of the antibody of interest.

Protein G is a bacterial protein from *Streptococcus*[1] having the particularity to bind Fc fragment of any mammalian IgG. Because of its capacity to capture the immunoglobulins, this protein and Protein A (SpA from *Staphylococcus aureus*) are important and very widely used tools, mainly to purify IgG by affinity chromatography (Koguma et al., 2013). However, Protein G binds with variable affinity to Fc fragments of immunoglobulins from dog, cat, mouse, rat, hamster, guinea pig, rabbit, monkey, donkey, sheep, goat and horse (Guss et al., 1986; Akerström et al., 1985; Sidorin and Solov'eva, 2011). Thus, it may allow

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revealing in the same assay a non-human IgG such as a monoclonal antibody used as standard and the human antibody of interest, which concentration may then be calculated from the monoclonal standard. In addition, this method is applicable for the antibody detection of many mammalian species.

We applied this method to detect and measure 3 types of antibodies: IgG anti-IgA, an alloantibody, IgG anti-Complement Factor H (CFH), an autoantibody and IgG anti-C5, eculizumab, a therapeutic monoclonal antibody.

IgG anti-IgA may be found in patients presenting with complete IgA deficiency, in whom an allo-immunization developed after administration of blood products containing IgA, mainly intravenous immunoglobulin (IVIg) prescribed as substitutive for chronic and recurrent infectious complications in immunodeficient patients. The detection and quantification of anti-IgA antibodies are highly recommended in patients presenting with clinical signs of intolerance reactions to the administration of blood derivatives (Mertes and Boudjedir, 2013).

Anti-CFH autoantibodies target Factor H, the main regulatory protein of the alternative complement pathway (Dragon-Durey et al., 2005). They have been reported mainly in the context of atypical Hemolytic Uremic Syndrome (aHUS) (Dragon-Durey et al., 2010; Józsi et al., 2008; Józsi et al., 2007; Strobel et al., 2010) and some cases in the context of C3 glomerulopathies (Meri et al., 1992; Sethi et al., 2011). In these diseases, the autoantibodies have been shown to neutralize factor H functions leading to complement alternative pathway activation.

The anti-C5 therapeutic antibody, Eculizumab, is a hybrid therapeutic monoclonal antibody composed by murine CDR regions on a structure of human IgG2 (light chains) and IgG4 (heavy chains) (Rother et al., 2007). This antibody targets the protein C5 of the complement system. Its binding on C5 impairs the C5 cleavage in C5b and C5a by the C5 convertases, preventing the generation of the anaphylatoxin C5a which is implicated in inflammation and of the membrane attack complex (MAC) C5b9 involved in the cellular destruction. This treatment is used in diseases mediated by complement activation, and currently its use has been approved in two diseases: Paroxystic Nocturnal Hemoglobinuria - PNH (since 2007) and in aHUS (since 2011). Several clinical trials are currently on going for other indications (organ graft acute rejection (NCT02113891, 2018; NCT01399593, 2018), autoimmune diseases such as myasthenia gravis (NCT02301624: ECU-MG-302, 2018; NCT01997229, 2018)). This treatment induces a complete complement activation blockage, measured by the CH50 (Complement hemolytic 50) test. However, this test has experimental limitations due to pre-analytic conditions which highly impact on the results. In consequence, false low levels of CH50 may be observed, impairing the good treatment adaptation. For this purpose, a more reliable dosage is necessary to monitor this particularly expensive treatment. We propose to measure the free circulating eculizumab, ie the drug not bound to the target protein. To date, this very expensive drug is only available for therapeutic purpose and may not be used as diagnostic tools. That's why we designed an ELISA approach using a murine monoclonal anti-C5 antibody as standard.

In this article, we present the results of the validation experiments necessary in the context of ISO 15189 accreditation, obtained for these three different assays. We demonstrate the efficacy and the robustness of the use of protein G for immunoglobulins detection to quantify antibodies.

## 2. Material and methods

The method was based on the principle of an indirect ELISA, in which the commonly used antibody conjugated with an enzyme (horseradish peroxidase (HRP) or alkaline phosphatase (AP)) was replaced by protein G coupled to HRP.

### 2.1. Buffers

#### 2.1.1. Coating buffer

We used commercially available PBS-Phosphate buffered Saline 1× (Biomerieux, Craponne, France), one vial to be dissolved in 1 L distilled water.

*Blocking buffer and sample dilution*: We used PBS 1×-BSA 1%. One g of BSA-Bovine serum albumin (PAA Laboratories GmbH, Paris, France) was added to 100 mL PBS 1×. In some assay, PBS 1×-Tween 0.01% can be used too. One mL of Tween 20 (Euromedex, Souffelweyersheim, France) was added to 1 L PBS 1×.

#### 2.1.2. Washing buffer

We used PBS 1×-Tween 0.01%.

*Substrate solution* for HRP revelation with Orthophenine di-Amine: it was prepared extemporaneously with 1 mL substrate buffer 10× (11,2 g NaH2PO4,2H2O + 650 mg C6H5Na3,2H2O, + distilled water for 50 mL final quantity), 9 mL distilled water, 1 tablet 10 mg OPD (Orthophenine di-Amine, Sigma, St. Quentin Fallavier, France), 80 µL of aqueous Hydrogene Peroxyde 30% (H2O2, VWR Prolabo, Fontenay-sous-Bois, France).

*Stop solution:* we used HCL 3 N.

### 2.2. Biological components

The references of the biological components are detailed in Table 1.

#### 2.2.1. Coating protein

the coating was composed by the target protein of the antibody we wanted to quantify.

#### 2.2.2. Standard

we used a monoclonal antibody specific to the same protein than the antibody to be quantified.

*Protein G-HRP*: we used recombinant protein G labelled with horse radish peroxidase (GenScript, Piscataway, NJ USA). This protein, able to bind to Fc region from mammalian immunoglobulins, replaced the conventionally used secondary antibody for both the calibration and the quantification assays.

#### 2.2.3. Positive control

we used a murine monoclonal antibody different from the one used

**Table 1**
Plasmatic and biological components specific of each assay.

| | Anti-IgA antibody | Anti-factor H antibody | Free eculizumab |
|---|---|---|---|
| Coating target protein | Human IgA kappa (Cappel, Cochranville PA, France) at 10 µg/ml | Human purified Factor H (Calbiochem, Fontenay sous Bois, France) at 4 µg/ml | Human purified C5 (Calbiochem, Fontenay sous Bois, France) at 5 µg/ml |
| Standard | Murine monoclonal anti-humain IgA (AD3, Abcam, Paris, France) | Murine monoclonal anti-human factor H (OX24, ThermoScientific, Waltham, MA USA) | Murine monoclonal anti-human C5 (A217, Quidel, San Diego, CA USA) |
| Positive control | Human sera | Human sera | Murine monoclonal anti-human C5 different ~~from the one used as standard~~ (HM2077, Hycult, Uden, The Netherlands). |
| Negative control | Healthy donors plasma | Healthy donors plasma | Healthy donors plasma |

**Table 2**
Optimal concentrations and dilutions used for each assay.

| | Anti-IgA antibody | Anti-factor H antibody | Free eculizumab |
|---|---|---|---|
| Coating target protein | 10 µg/mL | 4 µg/mL | 5 µg/mL |
| Standard (serial dilution) | 1/2000, 1/4000, 1/8000, 1/16000 | 1/250, 1/500, 1/1000, 1/2000, 1/4000, 1/8000, 1/16000 | 1/1000, 1/2000, 1/5000, 1/10000, 1/50000 |
| Screening samples'dilution | 1/100 | 1/50 | 1/2000 |
| Positive control dilution | 1/100 | 1/400 | 1/2000 |
| Negative control dilution | 1/100 | 1/50 | 1/2000 |

as standard or a human sera containing the antibody of interest.

#### 2.2.4. Negative control

we used plasma from healthy donors.

### 2.3. General procedure

The protein G's affinity for immunoglobulin depends on the isotype and the species origin. The optimal concentration of protein G had to be defined for each trio: target protein (antigen), monoclonal antibody (standard) and the antibody to be detected. The best concentration of protein G was the one giving the best ratio between the specific and the non-specific signals (best signal to background ratio).

*Coating of the target protein in the wells of a microplate* (ELISA 96 wells plate - Maxisorb NUNC, ThermoScientific, Waltham, MA USA). A solution of purified target protein (Table 1) was prepared at optimal concentration in PBS1X (Table 2), 50 µl were distributed in each well. Then, prepared plates were incubated overnight at +4 °C. After this step, plates could be storage at −20 °C during a month.

### 2.4. Saturation of the non-specific sites in the plate wells

After dumping the coating solution, 200 µL of saturation buffer were distributed in each wells and leaved at room temperature during 1 h.

After the saturation step, 3 washes were performed with 210 µL washing buffer per wells.

### 2.5. Incubation of the samples

All samples were applied in duplicate at 50 µL/well.

The blank was performed with 50 µl sample dilution buffer. Samples and controls were diluted in dilution buffer (Table 2). The dilution factor used for the samples was the same than in the respective routinely used assays. Highly positive samples were diluted more to obtain OD comprised in the ranges given by the standard curve. The Standard Curve was performed with serial dilutions of the monoclonal antibody standard.

After an incubation time (mainly 1 h at room temperature), 3 washes were performed with 210 µL washing buffer per wells to eliminate any non-bound immunoglobulins.

### 2.6. Incubation with the protein G HRP-labelled

Protein G labelled with HRP was diluted at 1/1500. Fifty µL of this solution were applied in each well and incubated during one hour at room temperature. Then 3 washes were performed with 210 µL washing buffer per well to eliminate any non-bound protein G.

### 2.7. Revelation

After incubation and washes, 50 µL of substrate solution per well were applied. Plate was incubated 5–7 min at room temperature in dark condition. This resulted in a peroxidation reaction coloring the solution and allowing quantification by spectrophotometry of the specific antibody bound in the wells. To stop the reaction, 25 µL of stop solution were added in each well. The plate was read immediately on a microplate reader at 490 nm.

### 2.8. Calculation of the concentration

The means of OD were calculated for each duplicate. The OD of the blank wells was subtracted to the mean of duplicates. The concentration was calculated according to the standard curve using a linear regression curve and a Lin/Lin axis scales and the standard values.

If the OD obtained with a patient's sample were higher than the higher OD of the standard curve, the sample has been tested in a new assay at higher dilution to obtain an OD comprised between the higher and the lower OD of the standard curve. For the final quantification, the dilution factor was applied to the result.

### 2.9. Validation and verification of methods

The validation of analytical methods is a major aspect of quality insurance in the laboratory. The assays have been validated according to NF EN ISO 15189. The agency responsible for laboratories' accreditation in France is named COFRAC (COmité FRançais d'ACcréditation) and has provided guidelines for method validation. We relied on these guidelines to assess the analytical performances of the proposed methods.

The validation of the method was based on a standardized set of experimental tests to determine the following parameters: specificity, linearity, limit of detection and lower limit of quantification, working range, precision (repeatability and reproducibility or intra and inter-run variability), accuracy (bias), stability, cut off (positive threshold) and when possible, methods comparison.

The specificity of an assay is defined by its capacity to ensure that the measured signal came only from the analyte of interest. To prove the specificity of our method, we used no-coated wells and blank with diluent buffer. In both cases, the signal must be closed to 0. A positive control and a negative control were also used in each series.

The linearity was evaluated by the dosage of serial dilutions of a highly concentrated sample (we used the standard antibody of each assay). An assay was considered as linear when there was a directly proportional relationship between the response and the concentration of the analyte. To prove the linearity, we studied linear regression analysis with Fisher's test as Goodness of Fit test. A high correlation coefficient ($R > 0.99$) was used as criterion of linearity.

The limit of detection is defined by the lowest concentration of the analyte which can be distinguished from the measure of the blank in the same conditions of analysis. To determine the limit of detection, we measured the blank (diluent buffer) 30 times in the same conditions. The limit of detection corresponded to 3 fold the standard deviation of the blank.

The limit of quantification is defined by the lowest concentration of the analyte which can be delivered with an acceptable level of confidence and given degree of certainty. It corresponded to 10 fold the standard deviation of the blank.

The working range was defined by the lower limit of quantification and the upper limit of linearity.

The precision was evaluated by repeatability test (or intra-run variation) and reproducibility test (or inter-run variation). Repeatability was calculated from 20 measurements in the same

condition of 3 samples with different antibodies titers (low, medium and high titers). The calculation of the coefficients of variation (CV) of these measurements used to assess the method repeatability. Reproducibility was calculated from at least 10 measurements spread over a period of at least 15 days, of 3 samples with different antibodies titers (the same samples were used in repeatability and reproducibility tests).

The accuracy of the method is defined by its ability to get results close to a theoretical value. Accuracy was estimated by analyzing samples at three different concentrations covering the working range.

The positive threshold (cut off) was determined by the mean of the values obtained with samples collected from 100 healthy donors plus 2 standard deviations.

The methods comparison can be done only once linearity, limit of quantification, repeatability, reproducibility and accuracy checked. The new method has been compared with the method routinely used in the laboratory of Immunology of the European Georges Pompidou Hospital.

The statistical tests used to compare the methods were linear correlation (Pearson correlation coefficient) and the Bland-Altman (Bland and Altman, 1995; Bland and Altman, 1986). The Bland-Altman test compares two methods that measure the same parameter to assess the consistency of the results produced by the new method compared to the reference method. This comparison is only possible if the values are expressed in the same unit. In the context of comparison method, and to detect systematic errors not identified by the coefficient of correlation of Pearson, we expressed the OD obtained for each sample, according to the standard used in the method reference. For this purpose, we tested this standard in the new technique. In the context of the comparison of our methods by Bland-Altman test, only the detection mode varied.

## 3. Results

### 3.1. Quantification of anti-IgA alloantibody

The performance parameters of the method applied on alloantibody anti-IgA assay are resumed in the Table 3. No interference was observed with hemolytic, lipemic or icteric samples. No cross-reactivity was seen with rheumatoid factor and human monoclonal Ig. The limit of quantification and the upper value of linearity limit determined the working range which was thus established to be from 50 to 500 ng/mL. Repeatability (intra-run variability) on 3 control levels (high = 2280 ng/mL, medium = 1099 ng/mL and low = 283 ng/mL) was evaluated. The CV were respectively 3%, 7% and 4%. Intermediate precision (inter run) was evaluated on these 3 control levels, with 15 measurements of each level over a period of 6 months, and the measured CV were respectively 10%, 13% and 14%. The accuracy was evaluated on the same control levels and the measured biases were respectively 1%, −2% and 0.2%. The method for the determination of IgA antibodies was compared with the reference technique, routinely used in the laboratory. The reference ELISA is based on the use of a patient's sample as standard and another patient's sample as positive control. Twenty eight positive sera have been tested with both methods. The correlation coefficient of the values obtained with the two methods of anti-IgA assay was 0.99 ($p$-value < 0.0001). The Bland-Altman analysis (Fig. 1A) showed that the differences between the two methods were much less pronounced in the low and medium titer of anti-IgA antibodies and tended to be more important in high rates. The difference between the concentrations obtained by the two methods at low levels (< 50 AU/mL) was close to zero. The differences tended to increase proportional to the average concentrations of anti-IgA antibodies. There was no systematic bias Table 3.

### 3.2. Quantification of anti-factor H autoantibody

The assay was performed with the monoclonal antibody OX24 as standard. This antibody is directed against the N-terminal part of Factor H. We have tested 2 other murine monoclonal antibodies C18/6 and L20/3 (Hycult, Uden, The Netherlands), both directed against the C-terminal portion. Results obtained with the 3 antibodies were similar. We selected OX24 antibody because it can't interfere with CFHR1 protein (Complement Factor H-related protein 1) which has 90% similarity with Factor H in the C-terminal domains.

Data from the performance assessment are summarized in the Table 4. We found no interference with hemolysis, icteric and lipemic samples as well as with presence of rheumatoid factor or monoclonal gammopathy.

We have tested 57 plasma samples collected from positive patients with anti-Factor H antibodies' titres ranging from 100 to 10,000 AU/mL as determined by the reference method[7].

The measurement range of the method was determined by the limit of quantification and the upper limit of the linearity and was from 20 to 480 ng/mL.

Repeatability and reproducibility have been tested on 3 samples with different antibodies titers determined from the reference method: high titer (1600 AU), medium titer (500 AU) and low titer (250 AU). Antibodies titers were measured 20 times for repeatability test and 10 times during a period of 5 months for reproducibility test. The study of repeatability showed excellent performance on high values (CV = 4%) and medium (CV = 10%) and reasonably good on the low values (CV = 21%). The coefficients of variation of the reproducibility tests

**Table 3**
Performance of the IgG anti-IgA assay.

| Performance settings | Results | | | | |
|---|---|---|---|---|---|
| Measurement interval | Linearity | Limit of linearity | 500 ng/mL | | |
| | | Correlation coefficient | $R$ = 0,98 ($p$-value < 0,0001) | | |
| | Quantification limit | | 50 ng/mL | | |
| | Working range | | 50–500 ng/mL | | |
| Precision | Repeatability | Coefficient of variation | Low titer N = 20 | Medium titer N = 20 | High titer N = 20 |
| | | | 4% | 7% | 3% |
| | Reproducibility | Coefficient of variation | Low titer $N$ = 15 | Medium titer N = 15 | High titer $N$ = 15 |
| | | | 14% | 13% | 10% |
| Accuracy | | | Low titer | Medium titer | High titer |
| | | Target value | 283 | 1099 | 2280 |
| | | Mean value | 278 | 1144 | 2255 |
| | | Bias | +0,2% | −2% | +1% |
| Reference range | | | Negative < 50 ng/mL | | |
| | | | Equivocal 50–100 ng/mL | | |
| | | | Positive > 100 ng/mL | | |
| Method comparison | | Bland-Altman plot | Bias = 5.9 AU/mL | | |
| | | Linear correlation | Y = 0,164× − 9,52 | | |
| | | | R = 0,99 (p-value < 0,0001) | | |

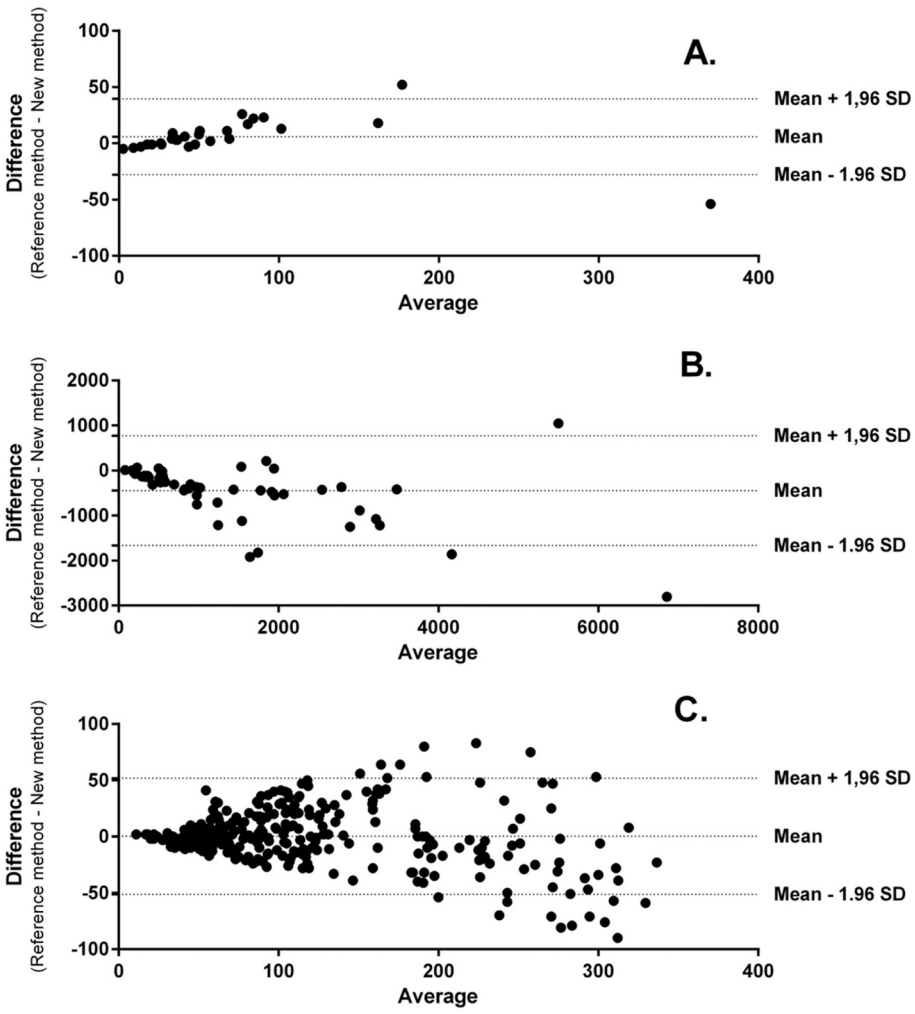


**Fig. 1.** Method comparison by Bland-Altman test plot.
The Bland-Altman graph is a scatter plot, in which the ordinate axis represents the difference between the two paired measurements and the abcissa axis shows the average of these measures. The mean represented on the plot is the average bias that is the average of difference. More the bias is close to 0, the more the methods overall tend to provide similar results. The limits of agreement (LoA 95% = mean ± 1.96 SD) delimit the ranges in which there is 95% of the differences values.
A. Anti-IgA (bias = 5.9 AU/mL, LoA 95% = [−28; 40]).
B. Anti-Factor H (bias = −401 AU/mL, LoA 95% = [−1311; 509]).
C. Eculizumab (bias = 0.28 µg/mL, LoA 95% = [−51; 52])

were respectively 17%, 16% and 26%. The accuracy, assessed through bias calculation, ranged from 0 to 9%.

The method comparison was performed by testing 57 plasma samples from positive patients in the same conditions, by the 2 techniques (the new one and the reference method). The reference method is an ELISA using a human plasma exchange product obtained from a positive patient, as standard (Watson et al., 2014).

The correlation coefficient R was 0.98 with a *P*-value < .0001. The Bland-Altman plot (Fig. 1B) showed that the new method tended to produce globally higher values than the reference method (−401 AU), due to very high levels of autoantibodies present in acute phase patients' samples, needing high dilutions.

**Table 4**
Performance of the anti-factor H IgG assay.

| Performance settings | Results | | | | |
|---|---|---|---|---|---|
| Measurement interval | Linearity | Limit of linearity | 480 ng/mL | | |
| | | Correlation coefficient | R = 0,99 (p-value < 0,0001) | | |
| | Quantification limit | | 20 ng/mL | | |
| | Working range | | 20–480 ng/mL | | |
| Precision | Repeatability | Coefficient of variation | Low titer N = 20 | Medium titer *N* = 20 | High titer N = 20 |
| | | | 21% | 10% | 4% |
| | Reproducibility | Coefficient of variation | Low titer *N* = 10 | Medium titer N = 10 | High titer *N* = 10 |
| | | | 26% | 16% | 17% |
| Accuracy | | | Low titer | Medium titer | High titer |
| | | Target value (ng/ml) | 55 | 131 | 354 |
| | | Mean value (ng/ml) | 52 | 131 | 385 |
| | | Bias (%) | +5% | 0% | −9% |
| Reference range | | | Negative < 20 ng/mL | | |
| | | | Equivocal 20–28 ng/mL | | |
| | | | Positive > 28 ng/mL | | |
| Method comparison | | Bland-Altman plot | Bias = −401 AU/mL | | |
| | | Linear correlation | Y = 2,98× + 68,9 | | |
| | | | R = 0,98 (p-value < 0,0001) | | |

**Table 5**
Performance of the anti-C5 therapeutic antibody (eculizumab) assay.

| Performance settings | Results | | | | | |
|---|---|---|---|---|---|---|
| Measurement interval | Linearity | Limit of linearity | 1,25 µg/mL | | | |
| | | Correlation coefficient | R = 0,98 (*p*-value < 0,0001) | | | |
| | Quantification limit | | 0,050 µg/mL | | | |
| | Working range | | 0,05–1,25 µg/mL | | | |
| Precision | Repeatability | Coefficient of variation | Low titer N = 20 | Medium titer *N* = 20 | High titer N = 20 | Very high titer N = 20 |
| | | | 7% | 3% | 3% | 3% |
| | Reproducibility | Coefficient of variation | Low titer N = 10 | Medium titer N = 13 | High titer *N* = 12 | Very high titer *N* = 13 |
| | | | 14% | 17% | 14% | 16% |
| Accuracy | | | Low titer | Medium titer | High titer | Very high titer |
| | | Target value (µg/ml) | 311 | 598 | 1086 | 4327 |
| | | Mean value (µg/ml) | 354 | 610 | 1056 | 4507 |
| | | Bias (%) | −14% | −2% | +3% | −4% |
| Reference range | | Cut off | 100 µg/mL | | | |
| Method comparison | | Bland-Altman plot | Bias = −0.28 µg/mL | | | |
| | | Linear correlation | Y = 1.02 x – 3.7 | | | |
| | | | R = 0,90 (p-value < 0,0001) | | | |

### 3.3. Quantification of an anti-C5 therapeutic antibody

This ELISA assay used a murine monoclonal anti-C5 antibody as standard. No interference was observed with hemolytic, lipemic or icteric samples. No cross-reactivity was seen in presence of rheumatoid factor and human monoclonal Ig.

The measuring range was between 100 and 2500 µg/mL (defined by the limit of quantification and the maximum of the reference curve), with a lower limit of detection of 30 µg/mL in the plasma. Considering the dilution factor (1/2000), the measuring range within the sample was between 0.050 µg/mL and 1.25 µg/mL (Table 5).

Repeatability was evaluated with 20 measurements in the same conditions of 4 samples at different levels (very high = 4327 µg/mL, high = 1086 µg/mL, medium = 599 µg/mL and low = 311 µg/mL). The CV were respectively 3%, 3%, 3% and 7%.

Reproducibility was evaluated with these four levels as well as on the positive control included in each series (titer = 660 µg / mL). The evaluation of reproducibility was based on 10 to 15 measurements over a period of 8 months. The coefficients of variation were respectively 14%, 17%, 14% and 16%, as well as 13% for the positive control.

The accuracy was evaluated on the same 4 samples and the measured biases were respectively −4%, 3%, −2% and − 14%.

Measurements of plasmatic free eculizumab were performed in 625 samples from 42 treated patients (6 to 40 serial samples per patient). For 7 out them, we tested also samples collected before treatment administration. No detectable eculizumab was found in these samples (< 30 µg/mL). In the others, the concentrations of free eculizumab ranged from 93 to 17,460 µg/mL.

The new method for the determination of eculizumab antibodies was compared with the technique used routinely in the laboratory (Peffault De Latour et al., 2015). This ELISA used the drug eculizumab as standard and another patient's sample as positive control. The comparison was performed on 303 sera tested with both methods. The Bland-Altman plot (Fig. 1C) shows that the two methods tends to give overall similar results. However, significant differences were observed in the highest values.

## 4. Discussion

The use of human biological samples as reagents causes problems related to the regulatory framework but also ethical issues. It requires managing the infectious risk, the storage, the stock thus also the risk of depletion of the source. This use also negatively impacts the effort of standardization of the different assays available for one analyte. The presented method solves these problems and enables to give results in quantifiable units. The principle is based on the use of a monoclonal antibody (from mammalian specie) as standard and of the protein G to reveal in the same conditions the standard and the patient's antibody of interest. The result is calculated according to the standard and uses the international units in g/L of the reference monoclonal antibody.

French medical biology laboratories are subject to accreditation according to NF EN ISO 15189. This standard provides guidelines on the laboratory quality approach and requires verification and/or validation of the analytical methods they use in order trace the initial quality and throughout their use. We used the guidelines provided by the French accreditation agency to perform the analytical validation of our method in three different assays.

The analytical method we developed must be efficient, specific, accurate, repeatable and robust for the detection and quantification of antibodies present in plasma because of alloimmune and autoimmune immune response or because therapeutic administration. To illustrate this, we have applied to the assays of anti-IgA antibodies, anti-factor H as well as the therapeutic antibody eculizumab. The comparison with the assays routinely used enabled us to affirm the reliability of our results. In the three analyses, the clinical interpretation was not modified by the results obtained with the new method as compared to the reference one.

Our results demonstrate that this method meets the need of standardized methods to quantify specific antibodies. With its original mode of revelation, it allows the use of monoclonal antibodies as calibration and control. This method enables the use of standardized calibration antibodies which may be produced in large quantities, adapted to an industrial scale.

However, this method is not intended to replace the well-established antibody quantification tests. In contrast, it is particularly relevant for the quantification of “rare” antibody for which no commercially standard is available, and for therapeutic antibodies because the drug's use as a laboratory reagent is not always possible.

The quantification of circulating antibodies for medical purposes shall be highly accurate and quantification accuracy requires relevant reference values for calibrating a quantification test. The availability of precise calibration reference values is particularly important for quantification of low level circulating antibodies.

Another advantage of this method of analysis is that it detects with the same reagents the antibodies of different species of mammals. This is particularly interesting for veterinary biology, while pets are diversifying more and more.

The three assays described in this article have been developed with the protein G which has better affinity for IgG3 subtype, more frequent in anti-FH autoantibodies, than protein A. There are also other immunoglobulin-binding bacterial proteins like protein A, the fusion protein A / G or protein L. These proteins have properties similar to those of the G protein but also their one specificities (Björck and Kronvall, 1984; Guss et al., 1986; Akerström et al., 1985; Sidorin and

Solov'eva, 2011; Eliasson et al., 1988; Björck and Protein, 1988; Biotechnology, 2013). The choice of the protein depends on the type of antibody that is desired to assay. It is important to note that neither protein A nor protein G does fixed chicken IgY immunoglobulins, which might enable the development of "sandwich" ELISA with a coating by chicken antibodies for the antigen capture.

These ELISA have been developed with a protein G coupled with the HRP enzyme. Other antigen-antibody complex revelation systems can be considered like the use of a coupling G-protein to biotin for amplifying the signal. It is also possible to use other detection systems with other types of labeling such as with a fluorescent marker.

The principle has been applied here to ELISA but we also adapted for other immunoassays, like Luminex® technology that allows multiplexing, ie simultaneous assay for detecting a panel of antibodies.

This method is patented (n°PCT/IB2014/064437).

## 5. Conclusion

Immunology is a medical specialty in perpetual evolution with the identification of new markers, new antibodies (autoantibodies, alloantibodies and therapeutic antibodies). This analytical method is potentially applicable to all antibody assays, since there is one equivalent monoclonal antibody available. This is a new antibody assay approach, both in the detection mode than in the calibration mode.

This approach allows the implementation of specific antibody assay easier and makes possible a more rapid transfer of technology to the diagnostic lab.

## Declarations of interest

none.

## References


Akerström, B., Brodin, T., Reis, K., Björck, L., 1985. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. J. Immunol. Baltimore MD 1950 135, 2589–2592.

Biotechnology, Pierce, 2013. Pierce Ig Binding Proteins (Protein A, G, A/G and L).

Björck, L., Kronvall, G., 1984. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol. Baltimore MD 1950 133, 969–974.

Björck, L., Protein, L., 1988. A novel bacterial cell wall protein with affinity for Ig L chains. J. Immunol. Baltimore MD 1950 140, 1194–1197.

Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet Lond. Engl. 1, 307–310.

Bland, J.M., Altman, D.G., 1995. Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet Lond. Engl. 346, 1085–1087.

Dragon-Durey, M.-A., et al., 2005. Anti-Factor H autoantibodies associated with atypical hemolytic uremic syndrome. J. Am. Soc. Nephrol. JASN 16, 555–563.

Dragon-Durey, M.-A., et al., 2010. Anti-factor H autoantibody-associated hemolytic uremic syndrome: review of literature of the autoimmune form of HUS. Semin. Thromb. Hemost. 36, 633–640.

Eliasson, M., et al., 1988. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. J. Biol. Chem. 263, 4323–4327.

Guss, B., et al., 1986. Structure of the IgG-binding regions of streptococcal protein G. EMBO J. 5, 1567–1575.

Józsi, M., et al., 2007. Anti factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. Blood 110, 1516–1518.

Józsi, M., et al., 2008. Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. Blood 111, 1512–1514.

Koguma, I., Yamashita, S., Sato, S., Okuyama, K., Katakura, Y., 2013. Novel purification method of human immunoglobulin by using a thermo-responsive protein A. J. Chromatogr. A 1305, 149–153.

Meri, S., Koistinen, V., Miettinen, A., Törnroth, T., Seppälä, I.J., 1992. Activation of the alternative pathway of complement by monoclonal lambda light chains in membranoproliferative glomerulonephritis. J. Exp. Med. 175, 939–950.

Mertes, P.-M., Boudjedir, K., 2013. Transfusion and allergy. Transfus. Clin. Biol. J. Société Fr. Transfus. Sang. 20, 239–242.

NCT01399593, 2018. Safety & Efficacy of Eculizumab to Prevent AMR in Living Donor Kidney Transplant Recipients Requiring Desensitization. Available at. https://clinicaltrials.gov/ct2/show/NCT01399593, Accessed date: 7 February 2018.

NCT01997229, 2018. Safety and Efficacy of Eculizumab in Refractory Generalized Myasthenia Gravis (REGAIN Study). Available at. https://clinicaltrials.gov/ct2/show/NCT01997229, Accessed date: 7 February 2018.

NCT02113891, 2018. Eculizumab Therapy for Subclinical Antibody-mediated Rejection in Kidney Transplantation. Available at. https://clinicaltrials.gov/ct2/show/NCT02113891, Accessed date: 7 February 2018.

NCT02301624: ECU-MG-302, 2018. An Extension Trial of ECU-MG-301 to Evaluate Safety and Efficacy of Eculizumab in Refractory Generalized Myasthenia Gravis. Available at. https://clinicaltrials.gov/ct2/show/NCT02301624, Accessed date: 7 February 2018.

Peffault De Latour, R., et al., 2015. Assessing complement blockade in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. Blood 125, 775–783.

Rother, R.P., Rollins, S.A., Mojcik, C.F., Brodsky, R.A., Bell, L., 2007. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. Nat. Biotechnol. 25, 1256–1264.

Sethi, S., et al., 2011. Proliferative glomerulonephritis secondary to dysfunction of the alternative pathway of complement. Clin. J. Am. Soc. Nephrol. CJASN 6, 1009–1017.

Sidorin, E.V., Solov'eva, T.F., 2011. IgG-binding proteins of bacteria. Biokhimiya/Biochemistry 76, 295–308.

Strobel, S., et al., 2010. Functional analyses indicate a pathogenic role of factor H autoantibodies in atypical haemolytic uraemic syndrome. Nephrol. Dial. Transplant. Off. Publ. Eur. Dial. Transpl. Assoc. 25, 136–144.

Watson, R., et al., 2014. Standardisation of the factor H autoantibody assay. Immunobiology 219, 9–16.