Research Article

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Bioanalysis

Design and validation of an immuno-PCR assay for IFN-α2b quantification in human plasma

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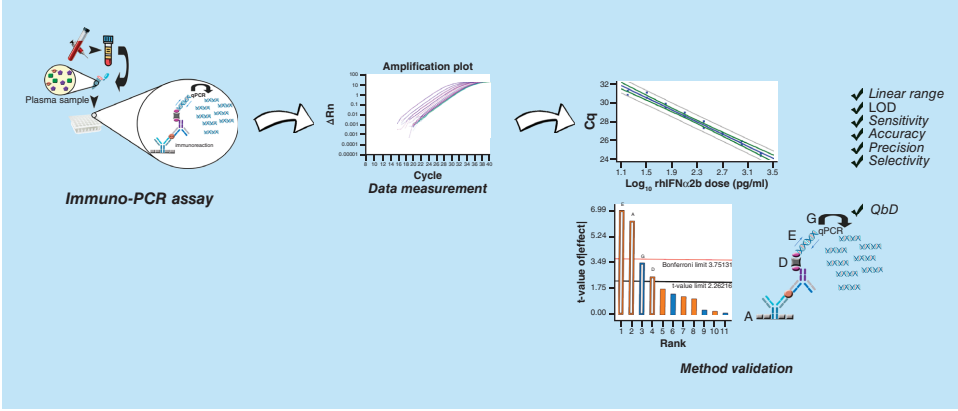
Aim: Nowadays, IFN-α is considered a promising therapeutic target for systemic lupus erythematosus. An immuno-PCR (iPCR) was developed to quantify low amounts of IFN-α in human plasma followed by a deep analysis of the methodologic robustness throughout quality by design approach. **Results:** An accurate, sensitive, selective and versatile iPCR was validated. The critical iPCR procedural steps were identified, applying a Plackett–Burman design. Also, this assay demonstrated an outstanding LOD of 0.3 pg/ml. A significant aspect relies on its high versatility to detect and quantify other cytokines in human plasma as the appropriate biotinylated antibody is employed. **Conclusion:** This reliable iPCR assay can be clinically used as an alternative method for quantitating and detecting low IFN-α2b concentrations in human plasma samples.

Graphical abstract:



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Keywords: accuracy • ELISA • IFN-α • immuno-PCR • linear range • precision • quality by design • robustness • selectivity • sensitivity

Type I interferons (IFN-I) are a family of cytokines characterized by their antiviral, antiproliferative and immunomodulatory activities [1]. IFN-I comprise mainly IFN-α and IFN-β, which are expressed from 13 functional IFN-α genes and 1 IFN-β gene, codifying 12 and one protein, respectively. All IFN-I have similarities in structure, like the length of the protein (161–167 amino acids), and their highly conserved protein sequence (75–99% amino acid sequence identity between IFN-α subtypes, and 35% between IFN-α and IFN-β) [2,3]. In spite of this evidence, IFN-α subtypes probably have a range of similar, but nonidentical, biological activities [4].

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Since 1979, IFN-α was associated with systemic lupus erythematosus (SLE). The plasma of SLE patients was found to contain immune complexes composed of DNA or RNA capable of inducing the production of IFN-α. Even a potential causative role for IFNs in SLE arose when the use of IFN-α2 for blood cancer or hepatitis-virus type C treatment shown to correlate with induction of lupus-like symptoms [5]. Nowadays, this cytokine is considered one of the most promising therapeutic targets for lupus [6].

In the most available purified leucocyte IFN and lymphoblastoid IFN-α preparation the IFN-α2 subtype is the major component [7]. There are three IFN-α2 subvariants (a, b and c) as different allelic forms of the IFN-α2 gene locus. The IFN-α2 subvariants a, b and c differ by only one or two amino acids at positions 23 and 34 of the mature protein [8]. However, the IFN-α2b is the predominant allelic variant [9].

Currently, the determination of plasmatic IFN-α occurrence is performed by analytical methods or functional measurements (bioassays), as antiviral and antiproliferative activity [10], or reporter cells methods [11]. Nevertheless, the latter have been limited by their low reproducibility and poor correlation [12]. Considering the analytical methods, the IFN-α quantification in human serum or plasma is often undetectable by ELISA since the cytokine is in the order of picogram/milliliter [13]. Zarrin *et al.* [14] reported a simple and rapid HPLC method with LOD and LOQ of 0.125 and 0.25 MIU/ml, equivalent to 0.2 and 0.4 μg/ml, respectively [15]. However, chromatographic methods have some drawbacks as the need of special columns and equipment, the use of organic solvents, the high expertise for using and interpreting the results and the lack of long-term reproducibility. Also, tandem mass spectrometric techniques were used. Thus, Izrael-Tomasevic *et al.* [16] compared a sample preparation workflow based on affinity enrichment to the one based on generic multidimensional chromatography coupled to mass spectrometric techniques. Despite the high specificity of the MS/MS (nearly over 95%), this method required high complex sample pretreatment. For instance, it is known that plasma albumin is a carrier protein for other plasma proteins and the depletion step has the potential to remove proteins of interest as well, leading to misinterpretation. In addition, these techniques require high-cost equipment and skilled personnel [16]. Therefore, all the above-mentioned methods tend to be complex, costly and time consuming, presenting high variability or dynamic range limitations.

In 1992, Sano *et al.* [17] designed a new technique called immuno-PCR (iPCR), replacing the enzyme (used as the detection system in ELISA) by a biotinylated DNA probe connected with an antibody. This DNA probe acts as the DNA template by a quantitative PCR (qPCR) [18]. Combining the high sensitivity of the PCR and the possibility of using specific molecules such as antibodies, iPCR assay allows quantifying low analyte levels in complex samples. In addition, compared with ELISA, iPCR enhances 100–10,000-fold the LOD [19], allowing to work with higher dilution of the sample and avoiding the matrix effect.

Hence, a novel, simple, precise and accurate iPCR for IFN-α2b quantitation in human plasma samples was developed followed by a deep analysis of its methodologic robustness using quality by design approach.

Materials & methods

Materials

Two *Escherichia coli*-derived human IFN-α2b (rhIFN-α2b) preparations, used as internal references, were provided by Zelltek S.A. (Santa Fe, Argentina). Mouse monoclonal and rabbit polyclonal antibodies (mAb and pAb) anti-rhIFN-α2b were previously obtained in our lab using rhIFN-α2b as immunogen [20,21].

Plasma samples collection

Seventeen individual volunteers (eight females, 23–75 years old; nine males, 27–74 years old) provided donor blood samples for the current study. Among the volunteers, nine are healthy donors and eight are patients diagnosed according to the comprehensive diagnostic criteria for IgG4-related disease [22]. Informed consent was obtained from all the volunteers according to the ethical guidelines for human subject research. After whole blood centrifugation in a Ficoll-Hypaque density gradient (Sigma, MO, USA), plasma sample was separated and three centrifugations were made using physiologic solution to remove cells and debris. These samples were stored at -80°C.

Biotinylated DNA probe preparation

A PCR was made from pBluescript SK (+) plasmid to prepare the biotinylated DNA probe (244 pb). The primers sequences were: 5′-biotin_GTAAAACGACGGCCAGT-3′ and 5′-GCGGATAACAATTTCACACAGG-3′. The PCR reaction was performed according to the following conditions: 30 cycles of amplification at 95°C for 30 s,

60°C for 30 s and 72°C for 30 s. The PCR product was purified by a Nanosep Centrifugal Devises (Pall Corporation, NY, USA) and quantified by a NanoDrop™ Lite (Thermo Fisher Scientific, MA, USA).

To evaluate the amplification efficiency on the DNA probe using the primers: 5′-GACTCACTATAGGGCGAATTGG-3′ and 5′-CACTAAAGGGAACAAAAGCTGG-3′, a qPCR was made. The DNA fragment was amplified using 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Estonia) in a StepOne™ Real-Time PCR System (Applied Biosystems, CA, USA). The qPCR reaction was performed according to the following conditions: predenaturation at 95°C for 15 min, followed by 40 cycles of amplification at 95°C for 15 s, 60°C for 20 s and 68°C for 20 s. For the standard curve, the threshold cycle (Cq) values were plotted against the DNA probe concentration (in the log form), fitting a linear trend line. Thereafter, the slope of the trend line was used to deduce the PCR efficiency (E) [23]. An optimal efficiency (100%), reflecting the doubling of the PCR product each cycle, is characterized by a slope of -3.32 (Equation 1). A melting curve was performed to evaluate the sensitivity and specificity of the assay.

$$E = \left(10^{\frac{-1}{Slope}} - 1\right) \times 100 \quad \text{(Eq. 1)}$$

Quantification of circulating IFN-α2b

Sandwich ELISA

The quantification of circulating IFN-a2b in plasma samples was carried out by a sandwich ELISA. Ninety-six well plates were coated with 100 ng per well of the anti-rhIFN-α2b mAb diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6). Coating was carried out for 1 h at 37°C and overnight at 4°C. After blocking 1 h at 37°C with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 140 mM, KCl 2.7 mM, pH 7.4), plates were incubated with twofold serial dilutions of rhIFN-α2b from 2000 to 15.625 pg/ml for the standard curve or with the plasma sample dilutions, for 1 h at 37°C. Then plates were incubated with 2 µg/ml of a biotinylated anti-rhIFN-α2b pAb prepared as described by Ceaglio *et al.* [20] and Mao [24]. After 1 h of incubation at 37°C, plates were incubated with Amdex™ Streptavidin Horseradish Peroxidase Conjugate (GE Healthcare, MA, USA) for 1 h at 37°C. Finally, plates were incubated for 10 min with the substrate solution containing 3 mg/ml o-phenylenediamine, 0.12% (v/v) H_2O_2 in 50 mM phosphate citrate buffer. The assay signal (absorbance) was registered at 492 nm using a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). Between every step, plates were washed six-times with the wash buffer containing PBS 0.05% (v/v) Tween 20. Dilutions of tested samples and antibodies were prepared in the wash buffer, supplemented with 0.1% (w/v) BSA. The assay was performed in triplicate.

Immuno-PCR assay

The quantification of circulating IFN-a2b in plasma samples was carried out by an iPCR assay based on Niemeyer *et al.* [25]. Coating was achieved overnight, at 4°C, in polypropylene 48-well plates, as described by Abud *et al.* [26,27], by incubating the plates with 100 ng per well of the anti-rhIFN-α2b mAb diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6). After blocking 1 h at room temperature (RT) with 1% (w/v) BSA in PBS, plates were incubated with twofold serial dilutions of rhIFN-α2b from 2000 to 15.625 pg/ml (standard curve) or the plasma sample dilutions, during 1 h at RT. Then, plates were incubated with 2 µg/ml of a biotinylated anti-rhIFN-α2b pAb prepared as described before. After 1 h at RT, plates were incubated with neutravidin 1 µg/ml (Thermo Fisher Scientific) during 30 min. Then plates were incubated with a solution of 0.5 pM biotinylated DNA probe during 30 min. After washes, a qPCR was carried out using 5× HOT FIREPol EvaGreen qPCR Mix Plus (ROX) in a StepOne Real-Time PCR System. The qPCR step was performed using the primers and reaction conditions described in section 2.3. Each sample was performed in duplicate. PBS was used as a negative control. A melting curve was carried out to evaluate the sensitivity and specificity of the test.

Validation method

Validation procedure was performed according to the Bioanalytical Method Validation Guidance for Industry established by ICH [28]. Hence, the analyzed figures of merit were linear range, LOD, sensitivity (SEN), selectivity, accuracy, precision and robustness. For validation purpose, two rhIFN-α2b internal references were employed (Zelltek S.A.). To obtain a normal distribution of the data, a logarithmic transformation was applied to the rhIFN-

α2b dose (pg/ml). Thus, a relationship between the dose and the response (Cq) can be fitted by a simple linear regression model over the range of doses used.

Linear range, LOD & sensitivity

The linear relationship in the dose–response curves should be demonstrated using an appropriated statistical method. The correlation coefficient is frequently used to guarantee the linearity, regularly by visual inspection, obtaining a value close to one. However, some organisms as the International Union of Pure and Applied Chemistry (IUPAC) discourage the use of correlation coefficient (R) as a good indicator of linearity. Thus, a test called lack-of-fit of variance through an F-test (Equation 2) was applied; where the statistical hypothesis are H_0 (null hypothesis, the data are linear) and H_a (alternative hypothesis, the data are nonlinear). The H_0 would be rejected if the calculated F-value exceeds the F critical value (p-2; m-p; 1-α), using a significance of 0.05.

$$F_{calc} = \frac{MSlof}{MSE} \quad \text{(Eq. 2)}$$

$$M_{slof} \frac{SSR - SSE}{p - 2} \quad \text{(Eq. 3)}$$

$$MSE = \frac{SSE}{m - p} = \frac{\sum_{p=1}^{p} \sum_{n=1}^{np} (y_{p,n} - \overline{Y}_p)^2}{m - p} \quad \text{(Eq. 4)}$$

$$MSR = \frac{SSR}{m - 2} = \frac{\sum_{p=1}^{p} \sum_{n=1}^{np} (y_{p,n} - \widehat{Y}_p)^2}{m - 2} \quad \text{(Eq. 5)}$$

Where M_{Slof} is the lack of fit; MSE (mean square error) the pure error; MSR (regression mean square) the residual error (S_{fit}); $Y_{p,n}$ and $\widehat{Y}$ are experimental and predicted response values, respectively; $\overline{Y}$ is the mean response for p concentration levels; m, n and p are the total number of calibration samples, the replicates and the concentration levels of the calibration curve, respectively.

Considering the ICH guidelines, LOD (pg/ml units) represents the lowest amount of the analyte of interest that could be detected. The detection limit was estimated based on the standard deviation of the blank. Taking into account the type I error or α-errors and type II or β-errors, the SD was calculated as follows:

$$LOD = 3.3 \times S_0 \quad \text{(Eq. 6)}$$

$$S_0 = \frac{S_{fit}}{A} \times \sqrt{\frac{1}{n} + \frac{1}{m} + \frac{(X_{inc} - \overline{X})^2}{\sum_{i=1}^{m} (X_i - \overline{X})^2}} \quad \text{(Eq. 7)}$$

Where A is the slope; S_{fit} is the residual SD, X_i is each concentration level; $\overline{X}$ is the mean calibration concentration or dose, and X_{inc} was considered the minimum concentration level in the linear range.

Considering the IUPAC guidelines, SEN (Cq × ml/pg units) represents the change in the response (signal, Cq) due to a change in the stimulation (rhIFN-α2b dose, pg/ml). To compare assays with different analytical signal (e.g., absorbance measurement respect to fluorescence intensity) it is better to use the analytical SEN (γ) because it is independent of the kind of signal. The equations below were applied:

$$SEN = A \quad \text{(Eq. 8)}$$

$$\gamma = \frac{SEN}{S_y} \quad \text{(Eq. 9)}$$

Where Sy (Cq units) is the pure error, which is a measure of instrumental noise.

The units of SEN are Cq × milliliter/picogram, whereas the units of γ are milliliter/picogram, as the Sy unit is Cq. Thus, the comparison between different methods presenting different signals (such as the ELISA assay in absorbance and the iPCR assay in Cq), could be carried out.

According to Equation 7, it is possible to infer that as greater the SEN (A, slope), lower the LOD. Thus, a more sensitive method (greater SEN) is related to a method that demonstrates lesser LOD values.

Selectivity

Considering the IUPAC guidelines, selectivity is defined as the degree in which a method can be used to quantify an analyte in mixtures without interference of other compounds of similar behavior. Molecules that are structurally similar to or closely related to the analyte must be analyzed to confirm that a positive response is not obtained. Thus, potential cross-linking with other proteins presents in plasma samples, like IFN-β, was analyzed. Hence, in this assay, an amount equivalent to 2000–250 pg/ml of IFN-β was evaluated.

Nonparallelism analysis was performed to compare the slope of the dose–response curves of the pure standard and the analyte embedded in an adequate dilution of the sample matrix (human plasma). A statistical F-test was applied to assess whether there are significant difference between the slopes with 0.05 confidence level (H_0 = slopes of the lines are all equal; H_a = slopes of the lines are not all equals).

Accuracy

The accuracy refers to the proximity between the value considered as a true amount and the value determined by the analytical method. This parameter was evaluated through a recovery assay, applying a *t*-test that guarantees if the average recovery is significantly different from 100% or not, with a 95% confidence level and seven degrees of freedom (nominal value: 350 pg/ml IFN-α2b). The $H_0 = \overline{R} = 100\%$ and $H_a = \overline{R} \neq 100\%$. The analytical method will be considered accurate when $t_{cal} < t_{critical}$ ($\alpha/2 = 0.025$; n-1 = 7):

$$T_{cal} = |100 - \frac{\overline{R_{\exp}/\sqrt{N}}}{DSR}| \quad \text{(Eq. 10)}$$

In addition, the recovery test was carried out from matrices (plasma sample) spiked with rhIFN-α2b over the linear range. The recovery was calculated, as:

$$R(\%) = \frac{C_A * V(final\ \ volume) - C_B * V(final\ \ volume)}{m_C} \quad \text{(Eq. 11)}$$

Where C_A is the concentration of the recombinant form of IFN (rhIFN-α2b) and the naturally present plasma hIFN-α2b, both determined in the spiked sample, C_B is the hIFN-α2b concentration determined in the plasma sample (without spiking with rhIFN-α2b) and m_C is the amount of rhIFN-α2b added to the plasma sample (internal reference 1).

Three IFN-α2b levels were evaluated (1000, 500 and 250 pg/ml), considering that the total added amount of analyte remained within the linear range. Moreover, a regression analysis of the estimated (found) against the added (spiked) was evaluated. An elliptical joint confidence region (EJCR) test was applied, by which if the ideal point (1.0) is contained inside the ellipse, the method is accurate. Bilinear least square was applied as the regression method, by which individual nonconstant errors were found in both the axes (found and added).

Precision

Precision refers to the degree of concordance between measures obtained for the same sample. Precision was considered at two levels: repeatability and intermediate precision, and it was analyzed by inter and intra CV%. Plasma samples from three patients were evaluated in duplicate in three independent assays.

Robustness by design of experiments approach

Considering the ICH guidelines, the robustness refers to the capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Design of experiments (DoE) was applied to determine the method's robustness and the critical parameters. The most critical parameters of the iPCR assay reported above, were varied simultaneously, modifying the most critical factors levels between expected outer limits that could change the performance of this assay (e.g., blocking time, Neutravidin incubation or number of washes post-DNA probe incubation). One of the main DoE's advantage is the possibility to study simultaneously many factors in a reduced number of experiments [29]. By the contrary, in the traditional univariate procedures (one variable at a time, OVAT), numerous experiments have to be done, varying one factor at a time. The Plackett–Burman (P–B) design is one of the most widely used statistical models because it allows the evaluation of many factors (n-1) with a minimum of experiments (n) [30,31]. The interaction between factors can be ignored, calculating only the main effect. Hence, in this work, a P–B design was applied with a triplicate in the central point to study the SD of the effects in N = 15 (number of experiments). The effect of eight real factors and three dummies (virtual variables) on IFN-α2b quantitation was evaluated. The experiments were performed in a randomized order.

The analysis of variance test (ANOVA) was performed to determine the factors that affect the response (dependent variable). Stat-Ease Design-Expert trial Version 7.0.3 was applied to analyze the design's results. If there is statistical significance ($p < 0.05$), this factor is considered a critical variable for the assay.

Results & discussion

iPCR design

The goal of this assay was to establish a standard method to quantify IFN-α2b in human plasma samples from patients with IFN-α-related diseases. Indeed, the iPCR was developed to replace the sandwich ELISA by a more sensitive method, aiming to detect lower levels of this cytokine. As it was mentioned above, the quantification of IFN-α in human serum or plasma is often undetectable by ELISA methods, or the LOQ is too elevated. Before performing the iPCR assay, the amplification efficiency was determined resulting of 83%. After this result, a sequential iPCR was achieved using the capture anti-rhIFN-α2b antibody and the biotinylated detection anti-rhIFN-α2b antibody, both also used to carry out the ELISA sandwich. Neutravidin was used as a bridge between the biotinylated detection antibody and the biotinylated DNA probe. Finally, a qPCR was developed under the previously evaluated conditions. A good amplification performance was demonstrated, obtaining a Cq value of 33 for the negative control (data not shown in the graph) and a specific melt curve (Figure 1A & B).

iPCR validation

As it was previously mentioned, the validation procedure was carried out to assess the reliability of the iPCR assay. Linearity was evaluated on one lot of rhIFN-α2b, applying a lack-of-fit test (ANOVA). The Cq values were inversely proportional to the rhIFN-α2b dose (Figure 1C). The standardized residues plot showed a normal and homoscedastic distribution around zero (Figure 1D). The nonlinearity test was valid between 15.6 and 2000 pg/ml (the linear part of the Sigmoid curves, Figure 1C), obtaining a $p = 0.3638$. This method displays low LOD values. As is was indicated in materials and method section the LOD was calculate considering the blank deviation, being able to detect even 0.30 pg/ml, which is partly linked with the high method SEN, that is, the slope (SEN: 3.1 ± 0.1 Cq × ml/pg). The analytical SEN (γ) was 10 ml/pg. These results are according to other previously reported iPCR methods [32,33]. The wide ΔCq, from 24 (2000 pg/ml) to 31 (15.6 pg/ml) (Figure 1A), could be related with a better prediction of IFN-α2b concentration in real samples, giving rise to a more accurate analytical method.

IFN-α and IFN-β are structurally related cytokines belonging to IFN-I family. Thus, to study potential cross-linking with IFN-β present in plasma samples, an iPCR was performed using IFN-β concentrations from 2000 to 250 pg/ml obtaining no lineal correlation between the IFN-β added and the Cq values (data not shown).

The iPCR method was designed to quantify IFN-α2b in patients' plasma samples. Therefore, the parallelism of dose–response curves of both reference and samples was a requisite. Thus, a nonparallel test was applied, meaning that the calculated probability value was not <0.05. The statistic test confirmed that the curves of the reference and the pure analyte embedded in plasma matrix (with a proper dilution of 1:10 or 1:20), were parallel, thereby thwarting any potential matrix effect (Figure 1E & F).

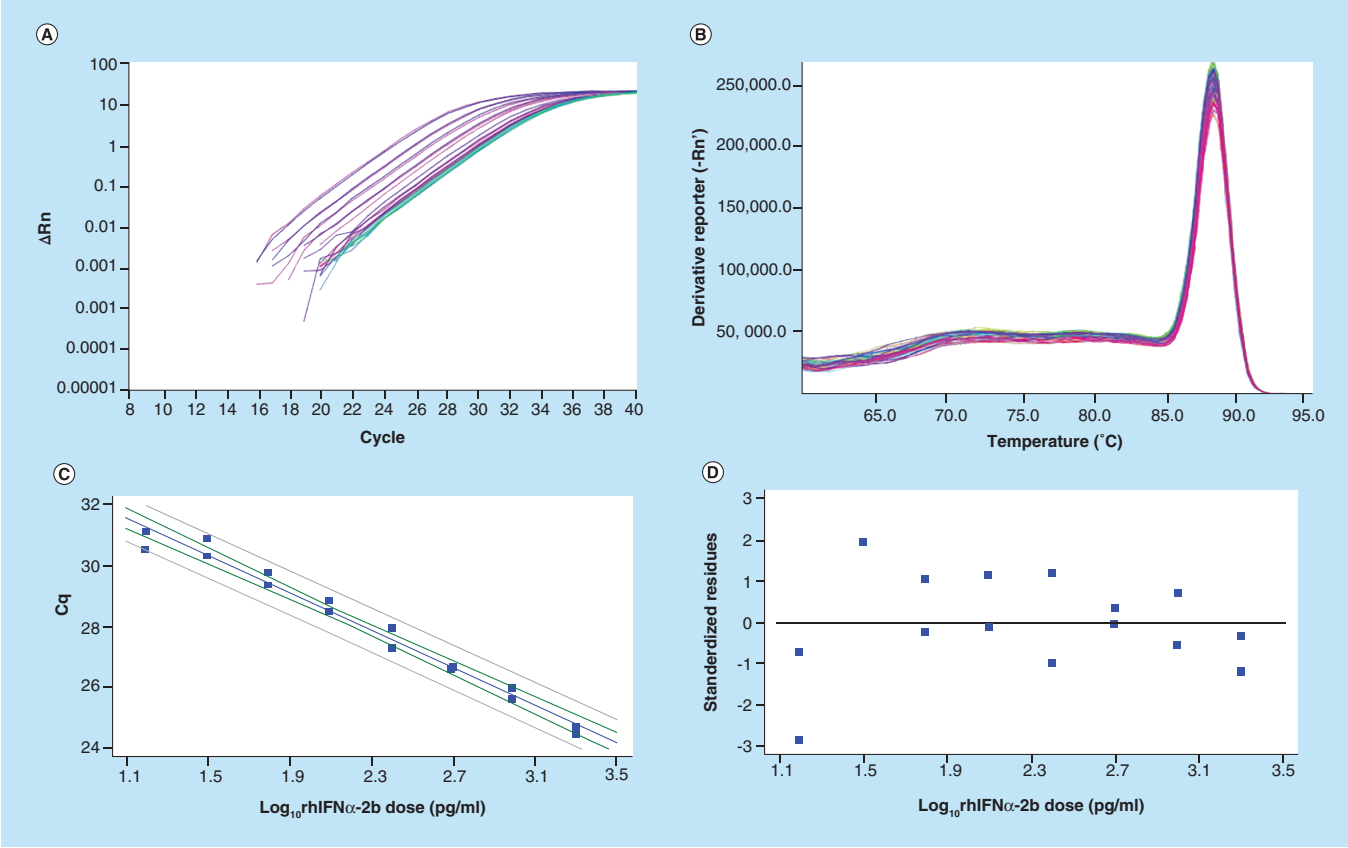

Figure 1. Analytical parameters of the validated iPCR. (A) Amplification plot. Signal (ΔRn) versus cycles of an immuno-PCR from 2000 to 15 pg/ml (from the left to the right). **(B)** Melt curve. Signal (derivative reporter -ΔRn′) versus temperature (°C). **(C)** Dose–response plot. A logarithmic transformation was applied to the rhIFN-α2b dose (pg/ml). A lack-of-fit of variance (ANOVA) test was applied, being valid between 2000 and 15.6 pg/ml. **(D)** Residuals plot. Standardized residues measure the difference (in standard deviation [SD]) between the observed and predicted values of the dose–response plot, at each point level (p level, 2000–15.6 pg/ml IFN-α2). No residues higher that three SD were found. **(E)** Nonparallelism analysis. Curves of the internal reference (1000–125 pg/ml) and the pure analyte embedded in serum matrix (1000–250 pg/ml) were parallel (p: 0.9294). **(F)** Residuals plot versus predicted Cq values of both reference and spiked serum matrix curves showed no residues higher than three SD. **(G)** Regression analysis of estimated (found) against added (spiked). Each dot represents a mean of two individual results for both found and spiked amount (reference). Three levels of concentrations were studied (1000, 500 and 250 pg/ml). **(H)** Elliptical joint confidence region plot applying a bilinear least square regression method. The calculated intercept and slope were compared with ideal value (1,0).

Since in univariate calibration, interference always affects the accuracy, it must be guaranteed during method validation procedures. Accuracy could be reported as the percent recovery of known added amount of analyte in the sample [28]. When discussing recovery results, it is usually stated that they are satisfactory only by visual inspection or their closeness to 100% recovery values. However, statistical tests are highly recommended [34]. Therefore, a *t*-test was applied as indicated in Equation 10, evaluating an amount equivalent to 350 pg/ml rhIFN-α2b (internal reference 2) as reference value. No statistical difference was found (t_{calc} = 1.6; $t_{critical}$ [0.025; 7] = 2.37), concluding that the method is accurate. As this test assumes constant variance, a complementary test was applied. Recovery values, as indicated in Equation 11, were calculated in three concentration levels (1000, 500 and 250 pg/ml), obtaining recoveries between 104 and 140%. Thereafter, the elliptical joint confidence region assay using bilinear least square regression method allowed us to compare the predicted value with that of the added IFN amount corresponding to the nominal reference value spiked in a human plasma sample. As the ideal point (1,0) was included in the ellipse (Figure 1G & H), we could assume, once more, that the method is accurate in the evaluated range.

Precision was evaluated using three patients' plasma samples by duplicated in three independent assays. Intra-assay CV (%) values from 2 to 35 (mean value: 15) and interassay CV (%) values from 11 to 25 (mean value: 16) were obtained. These results showed acceptable repeatability and intermediate precision (Table 1).

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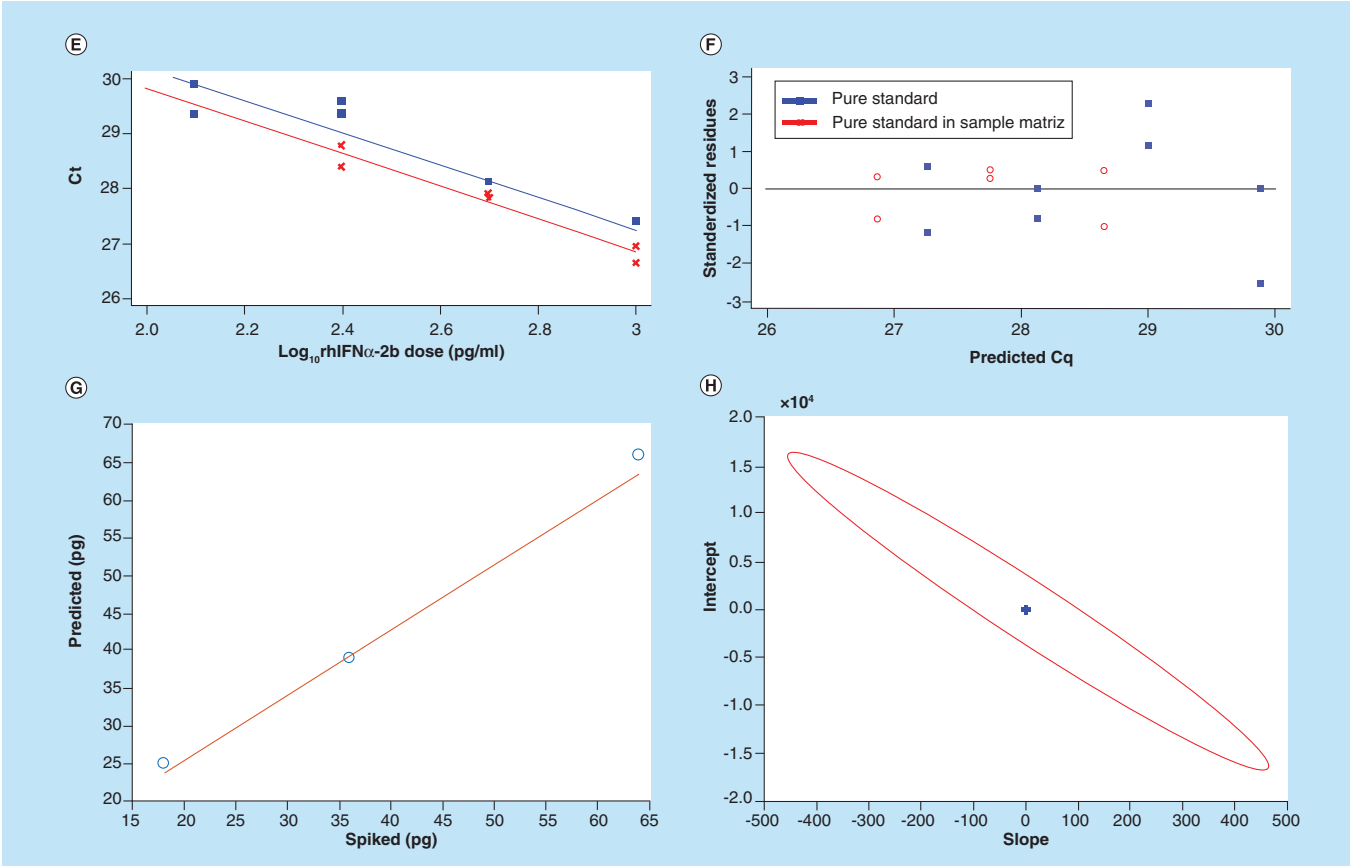

Figure 1. Analytical parameters of the validated iPCR (cont.). (A) Amplification plot. Signal (ΔRn) versus cycles of an immuno-PCR from 2000 to 15 pg/ml (from the left to the right). **(B)** Melt curve. Signal (derivative reporter -ΔRn′) versus temperature (°C). **(C)** Dose–response plot. A logarithmic transformation was applied to the rhIFN-α2b dose (pg/ml). A lack-of-fit of variance (ANOVA) test was applied, being valid between 2000 and 15.6 pg/ml. **(D)** Residuals plot. Standardized residues measure the difference (in standard deviation [SD]) between the observed and predicted values of the dose–response plot, at each point level (p level, 2000–15.6 pg/ml IFN-α2). No residues higher that three SD were found. **(E)** Nonparallelism analysis. Curves of the internal reference (1000–125 pg/ml) and the pure analyte embedded in serum matrix (1000–250 pg/ml) were parallel (p: 0.9294). **(F)** Residuals plot versus predicted Cq values of both reference and spiked serum matrix curves showed no residues higher than three SD. **(G)** Regression analysis of estimated (found) against added (spiked). Each dot represents a mean of two individual results for both found and spiked amount (reference). Three levels of concentrations were studied (1000, 500 and 250 pg/ml). **(H)** Elliptical joint confidence region plot applying a bilinear least square regression method. The calculated intercept and slope were compared with ideal value (1,0).

Table 1. Immuno-PCR precision.

Plasma (dilution; concentration)	Day 1 (%CV) (n = 2)	Day 2 (%CV) (n = 2)	Day 3 (%CV) (n = 2)	Interassay (%CV) (n = 6)
Plasma I (D:1/20; 1400 ± 400 pg/ml)	12	2	10	25
Plasma II (D:1/10; 1800 ± 200 pg/ml)	16	16	N/D	11
Plasma III (D:1/10;1500 ± 200 pg/ml)	11	35	N/D	12

D: Dilution; N/D: Non data.

Robustness by DoE approach

Multivariate analytical technique is a powerful tool commonly used in analytical methods optimization step. The screening step is one of the first procedures in the design of experiment approach allowing the selection of significant factors, followed by an optimization design. Indeed, the DoE procedure can be used as a tool assessing the robustness

Table 2. Plackett–Burman design and interferon concentrations obtained for each run.

Standard run N°	Factors								IFNα-2b (pg/ml)
	A (min)	B (min)	C (min)	D (min)	E (min)	F (n°)	G (n°)	H (n°)	
1	60.0	60.0	45.0	30.0	30.0	5.0	3.0	8.0	154.3
2	45.0	60.0	60.0	20.0	30.0	5.0	5.0	8.0	98.8
3	60.0	45.0	60.0	30.0	20.0	5.0	5.0	10.0	97.0
4	45.0	60.0	45.0	30.0	30.0	3.0	5.0	10.0	107.9
5	45.0	45.0	60.0	20.0	30.0	5.0	3.0	10.0	108.2
6	45.0	45.0	45.0	30.0	20.0	5.0	5.0	8.0	86.8
7	60.0	45.0	45.0	20.0	30.0	3.0	5.0	10.0	125.3
8	60.0	60.0	45.0	20.0	20.0	5.0	3.0	10.0	123.4
9	60.0	60.0	60.0	20.0	20.0	3.0	5.0	8.0	84.7
10	45.0	60.0	60.0	30.0	20.0	3.0	3.0	10.0	85.7
11	60.0	45.0	60.0	30.0	30.0	3.0	3.0	8.0	155.5
12	45.0	45.0	45.0	20.0	20.0	3.0	3.0	8.0	73.2
13	52.5	52.0	52.5	25.0	25.0	4.0	4.0	9.0	72.5
14	52.5	52.0	52.5	25.0	25.0	4.0	4.0	9.0	66.4
15	52.5	52.0	52.5	25.0	25.0	4.0	4.0	9.0	75.6

Table 3. ANOVA for the Plackett–Burman design.

Source	Sum of squares	Df	Mean square	F-value	Prob >F‡	Coefficient estimate	Standard error
Model	7268.91	4	1817.23	26.82	<0.0001		
A	2685.77	1	2685.77	39.64	0.0001	14.96	2.38
D	450.98	1	450.98	6.66	0.0297	6.13	2.38
E	3306.22	1	3306.22	48.80	<0.0001	16.60	2.38
G	825.93	1	825.93	12.19	0.0068	-8.30	2.38
Residual	609.74	9	67.75				
Lack of fit	566.09	7	80.87	3.70	0.2290		
Pure error	43.66	2	21.83				
Total	11,140.69	14					

‡$p < 0.05$ is significant.
Df: Degrees of freedom.

of analytical methods. The classical univariate methods (OVAT) only provide a local maximum, require a high number of experiments and do not consider the interaction effect between factors [31].

During robustness, analysis of simpler experimental design is usually applied, using lineal models as two-level full factorial or P–B [31].

In the current work, a P–B design was performed. It is a resolution III design, as only assumes that only the main effect is significant, ignoring the interaction effect between factors. P–B designs are used to investigate n-1 variables in n experiments. Thus, up to 11 factors could be studied in 12 experiments [29]. When the number of experiments is <11, the matrix can be completed with imaginary factors denominated 'dummy'. Sometimes, replicates of the central point allow determining the SD of the effect and studying the presence of curvature in the design.

Considering the iPCR critical parameters, the analyzed factors were: blocking time; sample incubation; biotinylated antibody incubation; Neutravidin incubation; DNA probe incubation; number of washes post sample incubation; number of washes post DNA probe incubation; and number of washes pre-qPCR assay. The levels of these factors were modified considering variations that may occur in an iPCR experiment due to the uncertainty of the approach, as indicated in Table 2. The Pareto chart was studied to determine the influencing factors (Figure 2A). Thereafter, an ANOVA test was applied to study the effects on the response and to confirm the significant effect of the factors. The obtained model (Table 3) satisfied the assumptions of normality, homoscedasticity and independence of the variables (Figure 2B–D, respectively). Further, the adjusted R-squared (0.8882) indicates a

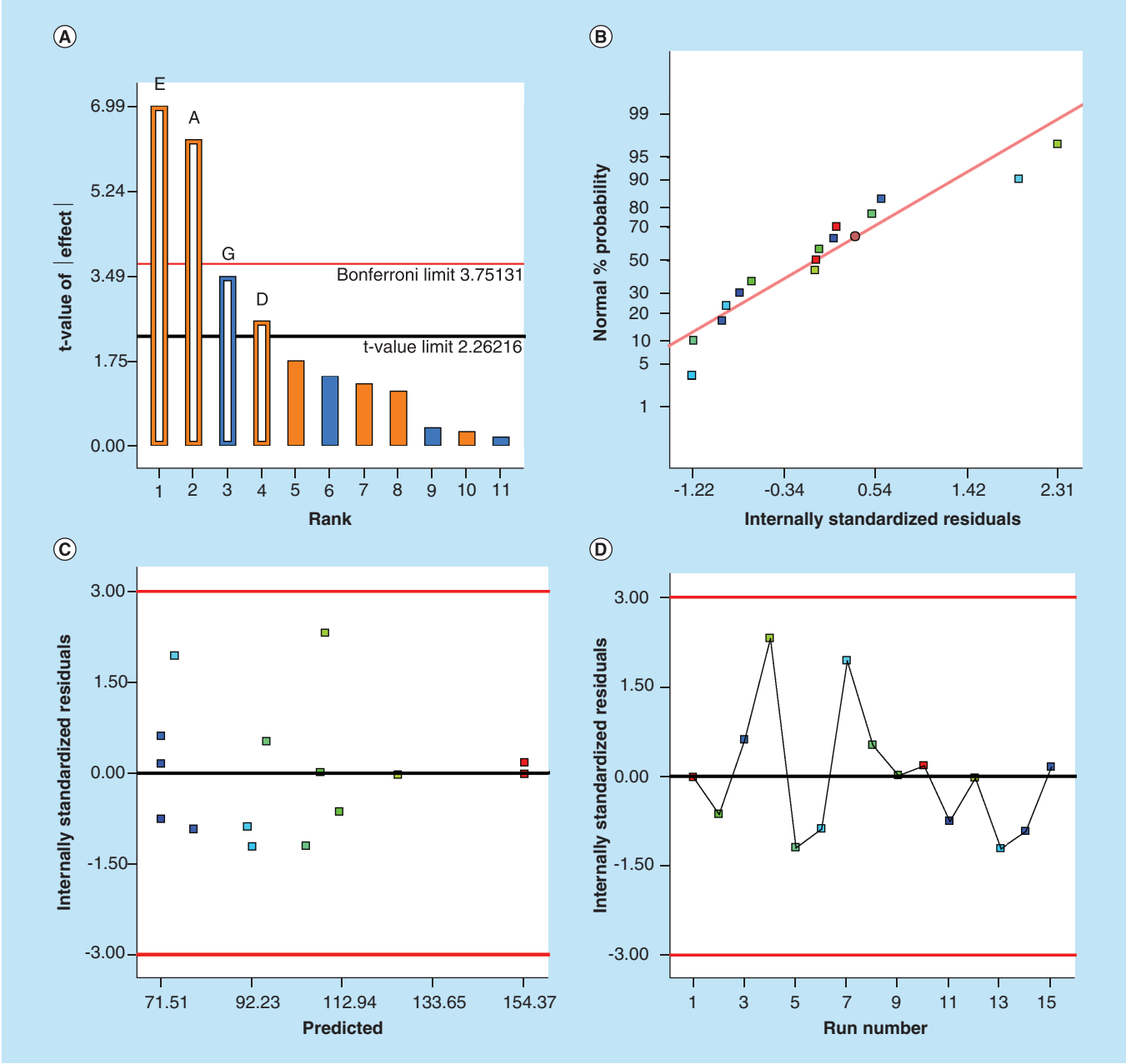

Figure 2. Statistical paremeters of the obtained P–B model. (A) Pareto graph to study the significant effects. The effects exceeding the statistical limit of Bonferroni are significant, whereas the effects between Bonferroni limit and *t*-value could be significant. On the other hand, effects below the *t*-value limit are not likely to be significant. E: DNA probe incubation, A: blocking time, G: number of washes post-DNA probe incubation, D: neutravidin incubation. **(B)** Normal probability plot of the residues. No deviation from the straight line was found. **(C)** Homoscedasticity plot. Residues versus predicted value were plotted, observing a random distribution. **(D)** Independency analysis. Residues versus experiment run number were plotted, obtaining an aleatory distribution of the residues.

good relationship between the experimental data and the fitted data. In addition, the triplicate in the central point can be used as a good estimator of the method repeatability, obtaining a value of 8.2%, that is adequate and in concordance with the previously interassay CV (%) calculated values (Table 1).

As indicated in Table 3, and similar to that obtained by Pareto analysis, the critical factors in the rhIFN-α2b quantitation are A, D, E and G. The blocking time (A) is one of the iPCR critical parameters because of it allows solving the nonspecific binding of components and decreasing the background signal. In our experience, when the iPCR assay was made in a nonsequential way, meaning that the blocking step, sample and biotinylated antibody

Table 4. IFN-α2 concentrations in plasma from patients (n = 2).

Patient	IFN-α2 (pg/ml)
1	1500 ± 100
2	960 ± 60
3	2400 ± 200
4	1400 ± 100
5	1600 ± 100
6	2000 ± 200
7	1240 ± 80
8	1120 ± 40
9	840 ± 80
10	1300 ± 200
11	1100 ± 100
12	1500 ± 80
13	1030 ± 20
14	1280 ± 20
15	1300 ± 100
16	3800 ± 300
17	320 ± 20

incubation steps were made in an single procedure, the nonparallelism between real samples (plasma) was significant ($p < 0.05$), altering also the accuracy of the assay (data not shown).

An iPCR assay is the result of a combination of ELISA and PCR assay. Thus, the manner in which the two methods are linked becomes a pivotal step [1]. Consequently, the factors D, E and G are critical steps, which is in agreement with the results obtained by the predicted model. Neutravidin can be used to bridge two biotinylated molecules (DNA probe and biotinylated antibody). Hence, it is necessary to establish a fix and adequate time allowing the interaction between neutravidin-biotin systems and avoiding major changes in the assay reproducibility. Similarly, the number of washes post-DNA probe incubation is a critical step.

In summary, the blocking time, neutravidin and DNA probe incubation, and the number of washes post-DNA probe incubation are important parameters to be considered when plasma hIFN-α2b concentration need to be quantified by the iPCR assay.

iPCR & sandwich ELISA comparison. iPCR application to test IFN-α2b in human plasma

As it was previously described, a sandwich ELISA using peroxidase-labeled streptavidin as the detection reactive was available in our laboratory. Indeed, the figures of merits of this assay were calculated. The LOD of this assay was 347 pg/ml and the analytical SEN was 8×10^{-3} ml/pg. In this way, this iPCR method can detect 1100-times less amount of IFN-α2b in plasma than the sandwich ELISA approach.

Until now, no other analytical method published the possibility to detect low IFN-α concentrations as it was herein reported, except by Llibre *et al.* [35], who informed IFN-α concentrations in the order of femtogram/milliliter. However, our method is more flexible and versatile, as only a real-time PCR equipment is required. Also, if the adequate antibodies are available this system can be used to detect and quantify other cytokines in human plasma samples because a biotinylated antibody is employed. The use of the biotinylated reagent enhances the versatility of this assay.

Taking into account that IFN levels could be related with several human pathologies such as SLE and another autoimmune diseases [36], the ability to measure IFN-α2b in human plasma samples enables improving the diagnosis of this syndromes and helps to understand the diseases' etiology and mechanism. To test the iPCR ability to detect IFN-α2b in human plasma, a panel of 17 human volunteers' samples was evaluated. The cytokine concentrations ranged from 300 to 3400 pg/ml (Table 4). This large spectrum of IFN-α2b levels could be correlated with differences among pathologies. These results are in correlation with previous reports, reflecting that different IFN amounts are in correlation with different patients' phenotypes, for example, higher IFN amounts (>0.3 pg/ml) could be associated with increased lupus biomarkers, as erythrocyte sedimentation rate or number of autoantibodies [37].

Conclusion

A sensitive, accurate, versatile, selective and validated iPCR assay to quantify IFN-α2b in human plasma was designed and developed. Considering the analytical SEN parameter, the iPCR was able to detect 1100-times lower IFN-α2b concentrations in plasma samples than a sandwich ELISA approach, showing an LOD of 0.30 pg/ml compared with 347 pg/ml of the ELISA assay. Also, the absence of human plasma matrix effect and the method accuracy was confirmed in the proposed working range. In addition, results showed acceptable repeatability and intermediate precision. Although this iPCR fulfilled the robustness criteria, the procedural steps corresponding to blocking time, neutravidin and DNA probe incubation, and the number of washes post-DNA probe incubation are the most important and key parameters to be considered when quantifying IFN-α2b in human plasma by this assay.

In summary, this iPCR assay can potentially be used as an alternative method for quantitating and detecting low amounts of IFN-α2b in human plasma, constituting a reliable analytical method for clinical use, and a straightforward method adaptable to simple complexity laboratories.

Future perspective

The results shown herein demonstrated that this iPCR assay is an accurate, sensitive, selective and versatile method to detect and quantify low amounts of IFN-α in human plasma and constitutes a reliable analytical method for clinical use. Indeed, we will use this assay in upcoming studies, trying to explain the role of IFN-α in autoimmune diseases such as SLE.

Interesting, if the adequate antibodies are available, this system can be used to detect and quantify other cytokines in human plasma samples because a biotinylated antibody is employed, improving its versatility.

Considering the complex cytokine network that participates in autoimmune diseases, the analysis of other key cytokines, that may positively or negatively interact with IFN-α, will be able to carry out using this highly sensitive and completely validated method.

Summary points

- An immuno-PCR (iPCR) method to quantify IFN-α2b in human plasma was designed and validated.
- It detects 1100-times less IFN-α2b in plasma than a sandwich ELISA, obtaining an LOD of 0.3 pg/ml.
- The method is sensitive, accurate, versatile and selective.
- Throughout a quality by design approach, the critical assay steps were identified.
- This iPCR assay can potentially be used as an alternative method for quantitating and detecting low amounts of IFN-α2b.
- The method is versatile since it can be adapted to detect and quantify other cytokines in human plasma.
- The iPCR assay can be used to research and for clinical purposes.

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Ethical conduct of research

Informed consent was obtained from all volunteers according to the ethical guidelines for human subject research. The project was approved by the ethical committee from Facultad de Bioquímica y Ciencias Biológicas of the Universidad Nacional del Litoral and the 'José María Iturraspe' Hospital, both institutions from Santa Fe, Argentina.

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