Title: Validation of an antiviral assay method for quantifying IFN-α5 activity in macaque and human serum

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Defined key terms:

-Antiviral: An agent that kills a virus or that suppresses its ability to replicate and, hence, inhibits its capability to multiply and reproduce.

-Method validation: The process of verifying that a method is fit for purpose.

-Selectivity: Selectivity is the ability of the bioanalytical method to measure and differentiate the analytes of interest and internal standard in the presence of components which may be expected to be present in the sample.

-Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained under the stipulated conditions.

-Accuracy: The accuracy of an analytical procedure expresses the closeness of the determined value to the value which is accepted either as a conventional true value or an accepted reference value.

-Robustness: Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Abstract

Background: IFN-α5 has demonstrated that induces stronger signaling and higher expression of antiviral genes than IFN-α2, the current treatment in chronic viral hepatitis. However, there is no specific and validated quantification method in order to conduct kinetic studies as part of the preclinical and clinical evaluation for regulatory purposes.

Results: A novel integration of an antiviral assay against the cytopathic effect of the encephalomyocarditis virus in HeLa cells with a very sensitive method for assay processing, Vialight® Plus assay, is presented for IFN-α5 activity quantification. The bioassay has been validated in macaque and human serum and it has demonstrated to be selective, precise and accurate.

Conclusion: The validated bioassay meets suitable acceptance criteria for these types of biological assays.

Introduction

Interferons (IFNs) are the first line of defense against viral infections in mammals. In addition to their antiviral activity, they have also shown several antiproliferative and immunomodulatory effects [1]. Human type I IFNs includes a multigene family of different IFN-α subtypes (13 subtypes) and a single IFN-β. The IFN-α subtypes are very similar at the structural level and all of them interact with the same receptor, but differences have been found in their biological activity [2].

Hepatitis C virus (HCV) infection is notably by the tendency to develop chronicity, being the main cause of hepatic disease. 130 million people are chronically infected by the virus, which resembles that the virus has developed mechanisms particularly efficient to avoid the antiviral system. Six main genotypes (1-6) distinguish the global geographic diversity of HCV and a multitude of closely related subtypes (a-j) within these genotypes are also defined and may influence clinical outcome and treatment options [3]. Current treatment consists in the combined administration of pegylated IFN-α2 and Rivabirin. Although main of the patients infected with genotype 2 or 3 show a sustained virological response (SVR) to the current standard of care, only 50% of those infected with genotype 1 achieve SVR with this therapeutic strategy. As more than 80% HCV infected patients in Western world and Asia correspond to genotype 1, an increase in the efficacy of IFN-α treatment is urgently needed.

IFN-α5 has been found to be the main IFN-α subtype expressed in liver of healthy people and is markedly decreased in the livers of patients with chronic HCV infection as compared with patients with other liver diseases (including hepatitis B virus chronic infection) [4]. It has also been demonstrated that it induces stronger signaling and higher expression of antiviral genes than IFN-α2 [5]. This data suggests that exogenous

administration of IFN-α5 to HCV chronically infected patients might be very useful for the effective virus clearance over the current use of IFN-α2. These findings warrant preclinical studies in order to evaluate its safety and efficacy in animal models, and its subsequent clinical development.

In order to conduct successful preclinical and clinical studies for regulatory purposes, IFN-α5 has to be quantified in biological fluids in order to obtain pharmacokinetic and toxicokinetic data to support and interpret the pharmacological and toxicological findings. Moreover, the quality of these pharmacokinetic and toxicokinetic studies is directly related to the quality of the underlying bioanalytical data; so, the validation of the bioanalytical method is a critical requirement [6].

IFN-α5 is a very active substance, so the dose administered to animals or humans is very low, leading to very low concentration levels in biological samples. The aforementioned, together with the fact that low volumes of biological samples could be extracted at each time in pharmacokinetic studies so as to avoid altering the individual volemia, explains the need to use very sensitive analytical methods in the determination of IFN-α5. Attempts of our research group to quantify it using chromatographic methods (HPLC, LC-MS, etc.) have not given good results regarding sensitivity. In addition, there are no ligand-binding assays capable of specifically detecting IFN-α5 because there are no IFN-α5-specific antibodies. Therefore, the idea of quantifying IFN-α5 through its antiviral activity by means of an antiviral assay (AVA) arose.

Historically, antiviral assays have been the first type of biological assays developed for the quantification of IFN activity, but no validation data has been reported for any of them. Some combinations of suitable cell lines with viruses have demonstrated to be

highly sensitive, although they showed considerable inter-assay variations, mostly because of the staining and fixing procedures needed to end the AVA.

Due to the aforementioned, the aim of this study was to develop a very reproducible, sensitive and rapid method in order to provide robust dose-response data for IFN-α5 activity. For this purpose, an AVA, with the combination of the HeLa cell line and the encephalomyocarditis virus (EMCV), has been integrated for the first time with the Vialight® Plus assay. This method has been validated, under GLP conditions, in order to quantify IFN-α5 activity in macaque (*Macaca fascicularis*) and human serum. Human serum has been chosen due to the fact that it will be the main matrix in clinical studies. The macaque was chosen because it is the most adequate animal model for the preclinical evaluation of IFN-α5, given the fact that it is an interferon of human origin and that its pharmacological activity and interaction with the receptor is known to be species-specific [7].

Therefore, the novelty of this study consists on the integration for the first time of an AVA with EMCV in HeLa cells, with Vialight® Plus assay for processing of AVA, and on its validation under GLP conditions to a level which ensures precise and accurate measurements of IFN-α5 activity in serum samples. All the expected criteria for these most widely available biological assays for IFNs have been achieved.

Materials and methods

Reagents

Dulbecco´s Modified Eagle´s Medium (DMEM) + GlutaMAX™ ([+] 4.5 g/L glucose, [-] pyruvate), PBS with and without Ca^{2+}/Mg^{2+} and fetal bovine serum were purchased from Gibco; Penicilin-Streptomycin, Tripsin/EDTA and Vialight® Plus kit from Lonza;

trypan blue dye was purchased from Sigma; and finally, EMCV virus was provided by Digna Biotech.

Cell culture

HeLa cells (CCL-2), which are human epithelial cervix adenocarcinoma cells, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were incubated in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic (Penicillin-Streptomycin) (complete DMEM medium).

Test item (IFN-α5) and serum samples

IFN-α5 was provided by Sicor Biotech UAB (Vilnius, Lithuania). IFN-α5 was produced through recombinant DNA technology in *E. Coli* and a structural characterization and evaluation of physicochemical properties of purified IFN-α5 protein were carried out [8]. The original test item was used to obtain a stock solution (SS) of IFN-α5 at 1×10^8 IU/mL in culture medium (complete DMEM medium). This SS was aliquoted and stored at -80 ºC until its use in the preparation of the standard curves and for the spiking of the serum samples.

The serum was obtained from six *Macaca fascicularis* macaques at the animal care facility of the University of Navarra and also from ten healthy volunteers at the Clínica Universidad de Navarra (CUN) blood extraction service. Blood was collected in BD Vacutainer® SST extraction tubes with serum separator gel, maintained 1 hour at ambient temperature in a vertical position and centrifugated following the manufacturer's instructions. The obtained serum was aliquoted and stored at -80 ºC. Extraction of blood from the animals and volunteers was approved by the Animal

Experimentation Ethics Committee and Research Ethics Committee of the University of Navarra, respectively.

The serum used for the spiking process, with the exception of that used in the study of the selectivity parameter, was a pool of blank macaque or human serum.

Antiviral assay procedure

The bioassay was conducted on a 96-well cell culture plate compatible with luminometry (opaque white plate with clear bottom) (figure 1). The inner 60 wells were only used due to the uneven response of the outer wells. The standards (standard curve), the controls (growth control and viral effect control) and the samples (blank and spiked, or the real samples of a study) were included in the plate, each in triplicate (figure 2). The growth control (nonvirus-treated HeLa cells) was used for evaluating the normal growth of the cells, and the viral effect control (HeLa cells treated with virus) was necessary in order to evaluate the viral cytopathic effect in the absence of IFN-α5.

First, 50 µL of complete DMEM medium was added to the control wells (growth control and viral effect control wells). 50 µL of each point of the standard curve and 50 µL of each sample (blank and spiked, or the real samples of a study) were then added to the corresponding wells.

Next, 20,000 HeLa cells per well were seeded, adding 100 µL of 2×10^5 cell/mL cell suspension to all the wells of the plate. The plate was incubated in an incubator at 37ºC and with 5% CO_2 for at least 12 hours.

After that, an EMCV virus dilution was prepared in order to produce a complete cytopathic effect (in the viral effect control) starting with the stock concentration of the EMCV virus in complete DMEM medium (about 2×10^7 pfu/mL). The dilution was

agitated with a vortex and 50 µL were added to all the wells except to the growth control wells. Then, the plate was incubated in an incubator at 37ºC and with 5% CO_2 for 16 hours.

Before proceeding to the plate washing and development process, the cells are observed in order to ensure that the cytopathic effect of the virus is complete. This is accomplished by observing the viral effect control wells under a microscope (x4, Eclipse TS100, Nikon), in which cell lysis should have occurred as a result of the viral effect, and by comparing the wells with the growth control wells in which the cells ought to be at confluence. Next, the luminescence reading was carried out using the Vialight® Plus kit following the manufacturer's protocol. Briefly, the plate was removed from the incubator and left for 5 minutes at room temperature (RT). A vacuum pump was used to remove the medium from all the wells and they were washed with 200 µL of PBS with Ca^{2+} and Mg^{2+}. Then, 100 µL of PBS with Ca^{2+} and Mg^{2+} and 50 µL of *Cell Lysis Reagent* were added to each well and left for 20 minutes at RT with orbital agitation. Finally, 100 µL of the *ATP Monitoring Reagent* was added to each well and after 2 minutes at RT, the luminescence was read using a luminometer (Mutimode detector DTX-800, Beckman Coulter).

Quantification procedure

The final step of the method was to calculate the IFN-α5 activity of each sample. The mean value of luminescence obtained for the three replicas corresponding to the viral effect control was subtracted from the values of luminescence obtained for each point and replica corresponding to the standard curve, thereby obtaining the corrected luminescence values of each point assayed.

Next, the mean value of the corrected luminescence values for each activity of IFN-α5 of the standard curve analyzed in triplicate was obtained. Then a graphic representation was made of the standard curve (dispersion graph: IFN-α5 activity (IU/mL) on the x axis versus mean value of corrected luminescence (RLU: Relative Light Units) on the y axis).

And finally, the mean value of luminescence obtained for the three replicas corresponding to the virus control was subtracted from the values of luminescence obtained for each blank and spiked sample and corresponding replicas. Then, the activities of IFN-α5 were calculated in each blank and spiked sample evaluated by means of extrapolation of each corrected luminescence in the equation obtained in the standard curve.

Assay optimization

In order to develop an adequate AVA, different aspects were optimized. The manufacturer´s protocol for the Vialight® Plus kit allows for the two reagents (*Cell Lysis Reagent* and *ATP Monitoring Reagent*) to be added directly to the culture well without the requirement of cell washing or medium removal. In order to evaluate the lack of sensitivity loss in our assay conditions, the influence of the culture medium and matrix (serum) on the reagents from the kit was studied. Therefore, cell viability was measured following the kit protocol on cells grown in culture medium or at different proportions of serum in culture medium. An evaluation was also carried out in order to determine the influence of the matrix on the accuracy and precision parameters of the standard curves, and the adequate quantification interval. Therefore, comparisons were made between standard curves

obtained with IFN-α5 in culture medium and IFN-α5 in different proportions of serum in culture medium.

Assay validation

Selectivity

The capability of the bioassay to quantify IFN-α5 in the presence of other matrix (macaque *Macaca fascicularis* and human serum) components was evaluated. In order to do so, a study was carried out with serum obtained from five animals and ten volunteers. For each animal or volunteer, blank serum samples (basal) and serum samples spiked with two different IFN-α5 activities were compared. Each sample was analyzed in triplicate.

The bioassay was considered to be selective if the responses of the basal serum samples were not higher than those obtained with the viral effect control taking an error of 30% into account. In addition, an increase in the signal of spiked serum samples for each individual should be observed when compared to the blank serum samples. The coefficients of variation (CV) and relative errors (RE) for each spike serum sample and individual must not be higher than the CV expected for the bioassay (30%).

Standard curves

Standard curves were prepared in different proportions of serum in the culture medium ranging from 0.7 to 25 IU/mL of IFN-α5. In order to obtain a large quantification range, different dilutions of serum with culture medium were prepared: 1/10, 1/100, 1/300, 1/5000 and 1/10000 of macaque serum in complete DMEM medium; and 1/10 and 1/100 of human serum in medium. An intermediate dilution of 41.66 IU/mL was obtained from

the SS in each of the aforementioned proportions of serum in complete DMEM medium. Each of these intermediate dilutions was used to make 3/5 serial dilutions in the plate, taking 75 µL from the previous well and adding 50 µL of the corresponding proportion of serum in complete DMEM medium. In this way, the activities 0.7, 1.2, 1.9, 3.2, 5.4, 9, 15 and 25 IU/mL of IFN-α5 were obtained for each standard curve point, maintaining the desired proportion of serum in culture medium. The standard curves were performed according to the AVA procedure in triplicate on six different days. Precision and accuracy of the standard curves were studied.

The acceptance criteria for the standard curves was to have a minimum of six points, an $R^2 \geq 0.98$, with a CV and a RE of the three replicates and on six different days (3 x 6 = 18 replicates), ≤ 30%.

Precision and accuracy

The precision expresses the capability of an assay to provide closeness of agreement between results. Accuracy shows the capability of an assay to provide results that are as close as possible to the theoretical value. In order to assess these two parameters on the spiked serum samples, they were analyzed in the AVA on six different days. The activity corresponding to the spiked serum samples analyzed and the dilution used are described in table 1. The spiked samples are 100% serum. The acceptance criteria were that the CV and the RE of the three replicates, on six different days, must be ≤ 30% in at least 75% of the spiked samples. In addition, the sum of the % of the CVs plus the % of the RE for the six repetitions made (3 x 6 = 18 replicates) must be ≤ 40%.

Robustness

The test item batch and the development kit reagent batch may vary in subsequent studies using this quantification method. In order to evaluate the robustness of the AVA in these terms, a study was made with two batches of IFN-α5 and two batches of the reagents from the Vialight® Plus kit.

Standard curves were obtained with each batch of IFN-α5. In addition, the same IFN-α5 was used to obtain standard curves but processed with two batches of the reagents from the Vialight® Plus kit. For each IFN-α5 or kit batch change, each standard curve must comply with the repeatability and accuracy criteria established (CV $\leq$ 30%; RE $\leq$ 30%) when extrapolated within the other standard curve.

Sample stability

The stability of IFN-α5 in macaque and human serum in storage at -80 ºC was determined. For the macaque serum samples, an analysis was carried out on serum samples spiked with six activities within the quantification interval (12.5, 125, 500, 2000, 50000 and 200000 IU/mL), at the following time intervals after been maintained at -80ºC: time 0, 24 and 48 hours, one and two weeks. For the human serum samples, an analysis was carried out on serum samples spiked with two activities within the quantification interval (10 and 2000 IU/mL), at time 0 and after 72 hours, 1 week, 1, 2, 3, 4, 5 and 6 months after having been frozen. A frozen blank serum sample was also analyzed for each time interval. For each stability condition, IFN- α5 levels versus time were plotted. It was assumed that IFN-α5 remained stable if a straight line was obtained in which the confidence interval of the slope included 0 with a $p<0.05$.

The stability of IFN-α5 in human serum (10 and 2000 IU/mL) after 1 and 3 consecutive freeze-thaw cycles, and after 2 hours at RT and on ice before its quantification, was also studied. As in the rest of the parameters studied, each sample was analyzed in triplicate. IFN-α5 was considered to be stable following 1 or 3 consecutive freeze-thaw cycles or after 2 hours at RT and on ice before its quantification if the mean of the activity levels measured for each condition was within the interval formed by the nominal value (activity value at which the sample was spiked: 10 or 2000 IU/mL), taking into account the assay error (RE $\leq$ 30%).

Finally, the stability of the SS of IFN-α5 in culture medium (1 x 10^8 IU/mL) was also evaluated after approximately 1 month in storage at -80 ºC, comparing it with a SS prepared on the day of use. Standard curves were obtained with both SS on the same plate, and the mean luminescence values obtained with the IFN-α5 SS frozen for 1 month were plotted against the mean luminescence values obtained with the SS prepared at that moment. The IFN-α5 SS frozen at -80 ºC was considered to be stable if a line was obtained with a value of $R^2 \geq 0.99$ whose ordinate at the point of intersection includes 0, with a probability of 95%, and whose slope includes 1 with a probability of 95%.

Quantification limit

The lowest level of IFN-α5 that reached a good quantification, with adequate precision and accuracy values, was defined as the limit of quantification and it was included as the lowest level in the standard curve.

Results

Optimization

It was observed that the culture medium and the matrix (serum) interfere with the lysis reagent of the Vialight® Plus kit. A much lower response was observed in those wells in which the lysis reagent was added directly to the culture medium, the serum, or a mixture of both. Therefore, it was decided to introduce a washing step at the plate development stage in the manufacturer's protocol.

The serum interfered in the AVA, promoting cell growth which, in turn, gave rise to a different response between the IFN-α5 and the virus. It was observed that the greater the proportion of serum in the well, the greater the response, meaning the greater the cell growth. Inter-plate cell growth variability was also observed. Due to these observations, both the samples (blank, spiked or the real samples of a study) and the standard curve need to be tested in the same proportion of serum and culture medium, and also on the same plate. The results of this study suggest also that the lowest proportion of serum in complete DMEM medium at which the AVA must be validated is 1/10. It was confirmed that lower dilutions of serum in complete DMEM medium, for example 1/2 or 1/5, did not meet the validation established criteria.

Finally, after evaluating different concentrations in order to determine the quantification interval (from 0.32 to 83.32 IU/mL), a range of 0.7 to 25 IU/mL of IFN-α5 was considered to be adequate because quantification of the analyte with good values of precision and accuracy was achieved.

Selectivity

The bioassay was confirmed to be selective, by demonstrating that the AVA was capable of quantifying IFN-α5 in the presence of other components in the serum of five different macaques and ten volunteers. The responses of the basal serum samples were

not higher than those obtained with the viral effect control taking the method error into account (table 2). In addition, the signal was observed to increase when comparing the blank serum samples with the spiked serum samples for each individual. After calculating the CV and RE for the results obtained for each spiked sample and all the individuals, this was ≤ 30% (tables 3-4). The noninfluence of each individual on the reproducibility of the results was therefore also demonstrated.

Standard curves

Standard curves were obtained with a quantification interval from 0.7 to 25 IU/mL for the different proportions of serum in culture medium (1/10, 1/100, 1/300, 1/5000 and 1/10000 for macaque serum; 1/10 and 1/100 for human serum), with a minimum of six points and with a value for $R^2 \geq 0.98$ (figure 3); for the triplicate analysis, both CV and RE were ≤ 30% on six different days. Taking into account the dilution factor to be applied in the problem serum samples (1/10, 1/100, 1/300, 1/5000 and 1/10000), this quantification interval will allow samples to be quantified within an interval from 7 to 250000 IU/mL in macaque serum, and from 7 to 2500 IU/mL in human serum.

Precision and accuracy

In the repeatability and intermediate precision study with serum samples spiked with IFN-α5, precise measurements were obtained, where 75% of the points gave a value of CV ≤ 30% (table 5). In the within- and between-day accuracy studies, the measurements gave an RE ≤ 30% (table 6). In addition, the sum of the % of the CV plus the % of the RE in the six repetitions carried out was ≤ 40% (table 7).

Robustness

The robustness of the bioassay was demonstrated by changing the IFN-α5 batch and the batch of reagents of the development kit (Vialight® Plus). The measurements complied with the established criteria of repeatability and accuracy (tables 8-9).

Sample stability

In all the conditions evaluated, IFN-α5 remained stable. IFN-α5 in macaque serum was stable at -80 ºC for a two week time interval, and up to 6 months in human serum, as the confidence interval of the slopes with a p=0.05 included 0 (tables 10-13). Moreover, IFN-α5 was also stable in human serum after 1 and 3 consecutive freeze-thaw cycles and after 2 hours at RT and on ice before its quantification.

IFN-α5 SS was stable for 39 days frozen at -80 ºC given the fact that a line was obtained (y=0.9604x – 534.98) with $R^2 \geq 0.99$ whose ordinate at the point of intersection included 0 (-1518.2413, 448.2736) with a probability of 95% and whose slope included 1 (0.8998, 1.0209) with a probability of 95%.

Discussion

The regulatory agencies demand that the validation of the bioanalytical methods be developed according to the guidelines currently in force [9-11]. However, no specific guidelines regarding these particular bioassays exist. Furthermore, the available guidelines, where the acceptance criteria of the essential parameters to be studied are described, are being discussed because they focus more on chromatographic assays for small molecules. The increasing interest in macromolecular therapies (recombinant

proteins, cytokines, monoclonal antibodies, etc.) has highlighted the need for new different assays for quantifying these molecules. Over the past few years, many efforts have been made and many aspects have been discussed at different workshops and conferences in order to set up the minimum requirements for bioanalytical methods [12]. The approach and the validation criteria we selected for the AVA to quantify IFN-α5 activity are based on current guidelines, and also on recent publications and reports regarding this topic, especially applicable to ligand-binding assays [13-16].

There are many cell/virus combinations that can be used to construct AVA. EMCV virus was selected because it is stable, has low pathogenicity for humans, and is sensitive to interferon [17]. Furthermore, the combination of the EMCV virus with HeLa cell line has shown high sensitivity and good dose-response relationship in different studies [18,19]. This, together with a very sensitive method at the point of termination of AVA used for evaluating cell viability (Vialight® Plus kit), capable of discriminating as few as ten cells, leads to the results showed in this work. Vialight® Plus kit is based upon the bioluminescent measurement of ATP in metabolically active cells. The washing step that has been included in the assay contributes to the elimination of the dead adherent cells that remain attached to the plastic bottom of the wells giving a low background in the viral effect control wells. This fact is important because high backgrounds may affect the slope of the dose response lines and therefore, adversely impact on the outcome of AVA [1]. Therefore, the novelty of the integration of AVA with the HeLa cells, EMCV and Vialight® Plus assay is highlighted, showing robust dose-response data in serum samples.

This bioassay is not a ligand specific assay, but it allows the quantification of IFN-α5 present in the samples through its activity. Antiviral efficacy was determined by measuring the ATP level in cells that were protected from the viral cytopathic effect by

the presence of IFN-α5. This is a very important aspect that the methods that are specific have to resolve, thereby needing a correlation between the product concentration measured and the biological response evaluated on a bioassay [14]. Although, it is not specific for IFN-α5, the AVA was confirmed as being selective because it demonstrated that it was capable of quantifying IFN-α5 activity in the presence of other components in the serum. The responses of the macaque and volunteers´ basal serum samples were not higher than those obtained with the viral effect control. These samples did not show any antiviral activity in our assay conditions. Only when the samples were spiked with IFN-α5, antiviral activity was measured, and furthermore, with good precision and accuracy. The analysis of basal serum samples from five animals and ten volunteers, also demonstrated the noninfluence of each individual on the reproducibility of the results.

Standard curves were obtained with a quantification interval from 0.7 to 25 IU/mL for the different selected proportions of serum in culture medium, allowing samples to be quantified within an interval from 7 to 250000 IU/mL in macaque serum, and from 7 to 2500 IU/mL in human serum. This interval permits quantification from very low levels, necessary for human IFN-α5 pharmacokinetic profile, to very high levels that could be achieved in repeated dose toxicokinetic studies in animals. Taking into account the data of the antiviral activity per mg of protein and protein content of the IFN-α5 provided by the manufacturer, the bioassay was capable of measuring the activity of IFN-α5 in the range of pg/mL. This limit of detection is quite low and difficult to obtain with other analytical methods.

Assay precision and accuracy has been set at 30% for both inter- and intra-assay variations (within and between days). This error percentage is higher than those permitted in the guidelines [9,11]. For the ligand-binding assays, the acceptance criteria for calibration standards (standard curve) and spiked samples is that a minimum of 75%

and 67% of the samples, respectively, should have an error no greater than 20%, or 25% for the low and high points [15]. In this type of biological activity assays, it is usual to have larger variations, more than with chromatographic or ligand-binding assays, due to inherent biological sources of variation that are not completely controllable (cell growth, virus activity, larger assay times, etc.). Our validation data has shown that if these variations are known, assumed and reflected in the assay performance acceptance criteria, the assay can be appropriate for its intended use. Recent publications regarding bioanalytical method validation for macromolecules and biomarkers are also supporting the idea that the levels of precision and accuracy do not necessarily have to be linked to any particular threshold, but should be scientifically justified so that the variability and accuracy of the assay are acceptable for its purpose [14].

Another advantage is that the same bioassay with the same procedure has shown to be valid for both macaque and human serum. Furthermore, the bioassay has demonstrated the stability of the IFN-α5 in macaque and human serum stored at -80 ºC, thereby allowing sample conservation without loss of its properties for several days or months. It was assumed that a 15-day interval was enough for measuring all the samples of the preclinical studies. However, for human samples, stability for up to 6 months was studied because the patient recruitment for clinical trials is not always easy and can often involve long time periods. This stability allows measuring human serum samples extracted during a 6 months period at the same time. Furthermore, the bioassay showed the stability of IFN-α5 in human serum after 1 and 3 consecutive freeze-thaw cycles, a great advantage for repeating the measurements of these valuable human samples.

In the regulatory field, these results can also contribute to the improvement of the specific requirements and recommendations established for different assays and validation procedures by the regulatory bodies. This work also supports the idea of

fitting the validation procedures and requirements for the different assays and purposes. In conclusion, this study presents novel integration of an AVA with EMCV in HeLa cells with Vialight® Plus assay. In view of the results obtained, this AVA is valid for quantifying IFN-α5 activity in macaque *Macaca fascicularis* and human serum in order to generate reliable pharmacokinetic and toxicokinetic data. The bioassay has been successfully applied to the study of the pharmacokinetic profile of IFN-α5 after subcutaneous and intravenous administration to macaques and to a 31-day repeated dose toxicokinetic study after subcutaneous administration of IFN-α5 to macaques. Following the positive assessment of the regulatory bodies, the bioassay is also currently being applied to a clinical trial in treatment-experienced patients with genotype-1 chronic hepatitis C.

Executive summary

- *Assay*: A novel integration of an antiviral assay (AVA) against the cytopathic effect of the encephalomyocarditis virus in HeLa cells with a very sensitive method for AVA processing, Vialight® Plus assay, is presented for IFN-α5 activity quantification.
- This bioassay is not a ligand specific assay, but it allows the quantification of IFN-α5 present in the samples through its activity.
- *Assay validation*: The validated bioassay meets suitable acceptance criteria for these types of biological assays.
- *Selectivity*: The AVA was confirmed as being selective because it demonstrated that it was capable of quantifying IFN-α5 activity in the presence of other components in the serum.

- *Standard curves*: Standard curves were obtained with a quantification interval from 0.7 to 25 IU/mL for the different selected proportions of serum in culture medium, allowing samples to be quantified within an interval from 7 to 250000 IU/mL in macaque serum, and from 7 to 2500 IU/mL in human serum.
- *Sensitivity*: The bioassay was capable of measuring the activity of IFN-α5 in the range of pg/mL showing a high sensitivity.
- *Precision and accuracy*: Assay precision and accuracy has been set at 30% for both inter- and intra-assay variations (within and between days).
- *Minimum required dilution*: the minimum required dilution for samples at which the AVA must be performed is 1/10 (serum in complete DMEM medium). Samples that need additional dilution should be diluted with complete DMEM medium and in all cases, the standard curve need to be tested in the same proportion of serum and culture medium of the samples.
- *Regulatory acceptance*: In the regulatory field, these results can contribute to the improvement of the specific requirements and recommendations established for different assays and validation procedures by the regulatory bodies.

Future perspective

Focus our research on bioanalytical assay development for therapeutic proteins.

Financial & competing interests disclosure

IG was employed by Digna Biotech when this work was performed. Digna Biotech is responsible for the preclinical and clinical development of IFN-α5. The rest of the

authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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References

1. Meager A. Biological assays for interferons. *J. Immunol. Methods 261*, 21-36 (2002). "* of interest, because it is a critical overview of the development, specificity, standardisation and present use of various biological assay methods available for the quantification of IFN activity".

2. Yamamoto S, Yano H, Sanou O, Ikegami H, Kurimoto M, Kojiro M. Different antiviral activities of IFN-alpha subtypes in human liver cell lines: synergism between IFN-alpha2 and IFN-alpha8. *Hepatol. Res. 24*(2), 99-106 (2002).

3. Newman RM, Kuntzen T, Weiner B, *et al.* Whole Genome Pyrosequencing of Rare Hepatitis C Virus Genotypes Enhances Subtype Classification and Identification of Naturally Occurring Drug Resistance Variants. *J. Infect Dis.* (2012); doi: 10.1093/infdis/jis679.

4. Castelruiz Y, Larrea E, Boya P, Civeira MP, Prieto J. Interferon alfa subtypes and levels of type I interferons in the liver and peripheral mononuclear cells in patients with chronic hepatitis C and controls. *Hepatol. 29*(6), 1900-1904 (1999).

5. Larrea E, Aldabe R, Riezu-Boj JI, *et al.* IFN-α5 mediates stronger Tyk2-Stat-dependent activation and higher expression of 2′,5′-oligoadenylate synthetase than IFN-α2 in liver cells. *J. Interferon Cytokine Res. 24*, 497-503 (2004). "** of considerable interest, because it presents data showing that IFN-α5 induces stronger signaling and higher expression of antiviral genes than IFN-α2, the current treatment used".

6. ICH Harmonised Tripartite Guideline. ICH S6 (R1): Preclinical safety evaluation of biotechnology-derived pharmaceuticals (2011).

7. Schellekens H. Animal models in interferon research: Some current trends. *Cell Mol. Life Sci. 45*(6), 558-562 (1989).

8. Certificate of Analysis of Interferon alfa-5 concentrated solution: Batch nos. P9-0060811 (November 2009), P9-0020809 (April 2009) and P9-0010709 (November 2009). Sicor Biotech UAB.

9. Food and drug administration (FDA). Guidance for industry: Bioanalytical method validation. Rockville, MD: US Department of Health and Human Services, FDA, Centre for Drug Evaluation and Research (2001). "* of interest, because it provides assistance to the validation of bioanalytical methods for preclinical studies".

10. ICH Harmonised Tripartite Guideline, ICH Q2A (R1): Validation of Analytical Procedures: Text and Methodology (2005).

11. European Medicines Agency (EMA). Committee for medicinal products for human use (EMEA/CHMP/EWP/192217/2009). Guideline on bioanalytical method validation (2011). "* of interest, because it defines key elements necessary for the validation of bioanalytical methods for pharmacokinetic and toxicokinetic parameter determinations".

12. Shah VP. The history of bioanalytical method validation and regulation: evolution of a guidance document on bioanalytical method validation. *The AAPS Journal 9*(1), E43-E47 (2007).

13. De Silva B, Smith W, Weiner R, *et al*. Recommendations for the bio-analytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res. 20*(11), 1885-1900 (2003). "** of considerable interest, because it makes recommendations for the development and validation of ligand binding assays that are intended to support kinetic assessments of macromolecules".

14. Smolec J, DeSilva B, Smith W, *et al*. Bioanalytical method validation for macromolecules in support of pharmacokinetic studies. *Pharm. Res. 22*(9), 1425-1431 (2005).

15. Viswanathan CT, Bansal S, Booth B, *et al*. Workshop/Conference report-Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *The AAPS Journal 9*(1), E30-E42 (2007). "** of considerable interest, because it addresses bioanalytical validation requirements of regulatory agencies with the purpose of clarifying expectations for regulatory submissions".

16. Kelley M, DeSilva B. Key elements of bioanalytical validation for macromolecules. *The AAPS Journal 9*(2), E156-E163 (2007). "** of considerable interest, because it addresses some of the key elements that are essential to the validation of macromolecular therapeutics using ligand binding assays".

17. WHO Report on the standardization of interferons WHO Tech Rep Ser *771* (Annex 1), 37-87 (1988).

18. Gasparian AV, Neznanov N, Jha S, *et al*. Inhibition of encephalomyocarditis virus and poliovirus replication by quinacrine: implications for the design and discovery of novel antiviral drugs. *J. Virol. 84*(18), 9390-9397 (2010).

19. Koev G, Duncan RF, Lai MM. Hepatitis C virus IRES-dependent translation is insensitive to an eIF2alpha-independent mechanism of inhibition by interferon in hepatocyte cell lines. *Virol. 297*(2), 195-202 (2002).

Tables

Table1. Spiked serum samples analyzed for IFN-α5 activity

Dilution used	IFN-α5 activities (IU/mL)
Macaque serum	
1/10	12.5, 20, 50, 100, 200
1/100	125, 200, 500, 1000, 2000
1/300	375, 600, 1500, 3000, 6000
1/5000	6250, 10000, 25000, 50000, 100000
1/10000	12500, 20000, 50000, 100000, 200000
Human serum	
1/10	20, 51, 102, 152, 203
1/100	185, 406, 841, 1219, 1523

Table 2. Selectivity: Mean luminescence signals (RLUs) obtained for the basal serum from each animal and each volunteer

Macaque serum										
Animal	1	2	3	4	5					
Mean basal serum signal	3033	2087	1199	2563	1122					
Mean viral effect control signal	3019	2627	1487	1944	1181					
Human serum										
Volunteer	1	2	3	4	5	6	7	8	9	10
Mean basal serum signal	986	620	613	525	449	1193	868	760	886	881
Mean viral effect control signal	1098					1236				

Table 3. Precision and accuracy of each macaque for the samples spiked for selectivity

Volunteer	1		2		3		4		5	
Spiked (IU/mL serum)	1.25	20	1.25	20	1.25	20	1.25	20	1.25	20
Measured (IU/mL serum)	1.48	22.75	1.46	16.19	1.41	25.57	1.03	17.19	1.05	15.11
Precision (%CV)	20.29	10.10	10.14	12.63	22.37	11.27	2.81	7.97	15.33	9.76
Accuracy (%RE)	18.13	13.73	17.07	19.07	12.53	27.86	17.87	14.05	16.00	24.43

Table 4. Precision and accuracy of each volunteer for the samples spiked for selectivity

Volunteer	1	2	3	4	5	6	7	8	9	10
Spiked (IU/mL serum)	20	52	102	152	203	20	52	102	152	203
Measured (IU/mL serum)	25.05	58.94	98.98	192.20	220.74	21.62	45.53	116.72	121.29	176.06
Precision (%CV)	21.56	8.63	6.06	27.36	23.88	20.94	29.02	9.17	27.23	15.85
Accuracy (%RE)	23.28	16.03	2.58	26.12	8.63	6.42	10.37	14.88	20.41	13.36

*Table 5. Repeatability and intermediate precision (CV) of the AVA for IFN-α5**

Spiked samples activity (IU/mL)	Dilution used	Repeatability (%) *(Within day)*	Intermediate precision (%) *(Between days)*
Macaque serum			
12.5	1/10	6.95	25.68
20		12.63	27.32
50		10.91	24.90
100		16.92	22.57
200		28.92	28.34
125	1/100	10.22	11.71
200		7.12	17.24
500		3.19	18.70
1000		23.21	28.78
2000		17.90	11.47
375	1/300	6.17	8.08
600		20.02	14.82
1500		18.25	25.87
3000		14.40	17.77
6000		16.95	28.79
6250	1/5000	4.71	13.20
10000		9.09	15.58
25000		10.55	31.46
50000		24.61	30.12
100000		28.00	21.52
12500	1/10000	14.41	13.57
20000		19.68	18.61
50000		16.33	22.48
100000		16.21	32.34
200000		10.87	29.87
Human serum			
20	1/10	21.56	30.96
51		8.63	26.20
102		6.06	21.44
152		27.36	24.16
203		23.88	18.94
185	1/100	13.65	77.40
406		9.53	24.23
841		15.57	20.79
1219		14.29	19.82
1523		11.55	14.19

**Samples were analyzed in triplicate with five different activities of IFN-α5 after different dilutions (within day); this was also done on six different days (between days)*

*Table 6. Within and between days accuracy (RE) of the AVA for IFN-α5**

Spiked samples activity (IU/mL)	Dilution used	Accuracy (%) *(Within day)*	Accuracy (%) *(Between days)*
Macaque serum			
12.5	1/10	11.26	6.48
20		24.43	8.38
50		24.90	11.93
100		16.30	15.99
200		13.99	1.66
125	1/100	3.10	3.67
200		4.60	8.07
500		21.64	1.86
1000		18.30	5.04
2000		13.56	15.47
375	1/300	25.02	20.12
600		28.66	25.23
1500		20.08	8.89
3000		0.95	2.91
6000		14.92	1.90
6250	1/5000	4.71	10.99
10000		9.09	23.44
25000		10.55	0.76
50000		24.61	2.82
100000		28.00	17.57
12500	1/10000	17.31	7.80
20000		17.39	8.86
50000		29.13	5.19
100000		19.17	6.91
200000		7.55	1.33
Human serum			
20	1/10	23.28	5.60
51		16.03	11.59
102		2.58	19.75
152		26.12	0.93
203		8.63	4.42
185	1/100	13.10	19.60
406		11.94	11.49
841		8.88	2.41
1219		14.98	1.85
1523		22.80	1.32

**Samples were analyzed in triplicate with five different activities of IFN-α5 after different dilutions (within day); this was also done on six different days (between days)*

Table 7. Sum of the CV and RE of the AVA for IFN-α5 on the six repetitions performed

Spiked samples activity (IU/mL)	Dilution used	CV+ RE (%)
Macaque serum		
12.5	1/10	32.16
20		35.71
50		36.84
100		38.57
200		29.99
125	1/100	15.38
200		25.31
500		20.56
1000		33.82
2000		26.94
375	1/300	28.19
600		40.05
1500		34.76
3000		20.69
6000		30.69
6250	1/5000	24.19
10000		39.03
25000		32.23
50000		32.94
100000		39.10
12500	1/10000	21.37
20000		27.47
50000		27.67
100000		39.25
200000		31.20
Human serum		
20	1/10	36.56
51		37.79
102		41.19
152		25.09
203		23.36
185	1/100	97.00
406		35.72
841		23.19
1219		21.68
1523		15.52

Table 8. Repeatability and within-day accuracy obtained with two different batches of IFN α5 (1 and 2), extrapolated in the curves obtained with both batches, respectively

	Standard curve Batch 1		**Standard curve Batch 2**		**Standard curve Batch 1**	**Standard curve Batch 2**			**Standard curve Batch 1**	**Standard curve Batch 2**		
	Activity measured (IU/mL)				**Repeatability (CV; %)**				**Within-day accuracy (RE; %)**			
Activity (IU/mL)	Batch 1	Batch 2	Batch 2	Batch 1	Batch 1	Batch 2	Batch 2	Batch 1	Batch 1	Batch 2	Batch 2	Batch 1
25	25.91 42.59 22.99	38.27 20.58 17.52	36.55 19.61 16.68	24.70 40.69 21.91	34.67	44.00	44.19	34.82	21.98	1.84	2.89	16.40
15	13.15 13.84 21.08	25.81 19.45 14.03	24.61 18.52 13.34	12.50 13.16 20.08	27.41	29.84	29.96	27.53	6.82	31.77	25.50	1.65
9	7.59 8.77 10.84	11.36 9.00 10.43	10.80 8.54 9.91	7.19 8.32 10.29	18.16	11.62	11.67	18.23	0.69	14.04	8.31	4.41
5.4	4.12 4.05 6.08	6.87 4.57 4.59	6.51 4.33 4.34	3.89 3.83 5.76	24.32	24.73	24.83	24.43	12.07	1.09	6.31	16.76
3.2	2.37 2.68 2.62	3.16 2.72 2.86	2.99 2.56 2.70	2.24 2.53 2.47	6.36	7.87	7.90	6.39	21.09	10.12	15.09	25.49
1.9	-- -- --	-- -- --	-- -- --	-- -- --	--	--	--	--	--	--	--	--
1.2	1.18 1.15 0.97	1.12 1.07 1.19	1.05 1.01 1.12	1.11 1.09 0.91	10.62	5.26	5.29	10.67	5.72	3.21	8.92	11.29
0.7	0.90 0.85 0.94	0.90 0.86 0.86	0.84 0.81 0.81	0.85 0.80 0.89	5.05	2.20	2.21	5.07	28.27	25.12	17.62	20.58

-- Point ruled out for non-compliance with the established criterion.

Table 9. Repeatability and within-day accuracy of the standard curves obtained with two different batches of the reagents of the development kit (Vialight® Plus) (Batch 1 and 2), extrapolated in the curves obtained with both batches, respectively

	Standard curve Batch 1		**Standard curve Batch 2**		**Standard curve Batch 1**		**Standard curve Batch 2**		**Standard curve Batch 1**		**Standard curve Batch 2**	
	Activity measured (IU/mL)				**Repeatability (CV; %)**				**Within-day accuracy (RE; %)**			
Activity (IU/mL)	Batch 1	Batch 2	Batch 2	Batch 1	Batch 1	Batch 2	Batch 2	Batch 1	Batch 1	Batch 2	Batch 2	Batch 1
25	22.31 25.42 32.44	28.03 48.66 42.52	15.46 17.35 21.55	18.92 30.87 27.39	19.42	26.66	17.20	23.88	6.89	58.95	27.52	2.91
15	-- -- --	-- -- --	17.23 15.70 16.84	16.16 17.77 23.29	--	--	4.79	19.62	--	--	10.60	27.15
9	12.05 9.79 10.04	10.26 9.63 10.45	8.95 7.45 7.61	7.76 7.34 7.89	11.64	4.24	10.29	3.77	18.06	12.40	11.09	14.85
5.4	3.72 5.26 4.81	5.63 4.10 4.29	-- -- --	-- -- --	17.28	17.85	--	--	14.85	13.40	--	--
3.2	-- -- --	-- -- --	-- -- --	-- -- --	--	--	--	--	--	--	--	--
1.9	1.52 1.55 1.55	1.58 1.55 1.57	1.43 1.45 1.45	1.47 1.45 1.47	1.10	1.02	0.98	0.91	20.65	19.56	25.64	24.74
1.2	1.21 1.09 1.06	1.18 1.10 1.16	1.17 1.06 1.04	1.14 1.07 1.12	7.19	3.67	6.37	3.26	3.92	1.77	6.67	4.81
0.7	0.92 0.85 0.87	0.94 0.92 0.88	0.91 0.86 0.87	0.93 0.91 0.88	3.79	3.31	3.36	2.94	25.70	30.32	25.51	29.60

-- Point ruled out for non-compliance with the established criterion.

Table 10. Activity measured in the macaque stability study samples

	Activity measured (IU/mL)				
Activity (IU/mL)	**0 h**	**24 h**	**48 h**	**168 h (1 week)**	**336 h (2 weeks)**
200000	173129.56 209932.59 171647.07	-- 115438.93 147350.20	103607.56 157388.16 96495.67		184047.71 184624.54 182374.28
Mean	184903.07	131394.57	130497.86		183682.18
CV (%)	11.73	17.17	25.51		0.64
50000	55156.23 44818.06 35066.98	49965.57 44744.63 29317.50	33590.89 34963.63 --		39139.64 57876.12 38902.41
Mean	45013.75	41342.57	34277.26		45306.06
CV (%)	22.32	25.97	2.83		24.03
2000	2566.45 2439.49 1807.47	1559.47 1587.25 1430.33	1645.25 -- 1520.45	1085.80 1404.52 1580.13	1962.31 2250.15 2073.80
Mean	2271.14	1525.68	1582.85	1356.82	2095.42
CV (%)	17.90	5.49	5.58	18.47	6.93
500	403.61 378.72 393.06	293.68 724.83 335.48	597.20 648.51 549.88	440.11 322.50 344.01	627.38 539.78 470.56
Mean	391.80	451.33	598.53	368.87	545.91
CV (%)	3.19	52.68	8.24	16.98	14.40
125	114.13 133.02 139.49	107.24 110.27 110.50	132.56 151.26 119.41	149.60 157.04 207.78	151.51 167.56 138.94
Mean	128.88	109.34	134.41	171.47	152.67
CV (%)	10.22	1.66	11.91	18.46	9.40
12.5	6.22 7.48 6.08	14.44 12.04 11.26	14.84 16.34 14.08	10.87 9.85 8.05	
Mean	6.59	12.58	15.09	9.59	
CV (%)	11.73	13.18	7.63	14.90	

--Point ruled out for non-compliance with the established criterion

Table 11. Activity measured in the human samples spiked with 10 IU/mL of IFN α5

Activity measured (IU/mL)							
Theoretic activity (IU/mL)	**Day 0**	**1 week**	**1 month (30 days)**	**2-3 months (79 days)**	**4 months (121 days)**	**5 months (143 days)**	**6 months (185 days)**
10	10.53 10.64 11.33	20.02 9.48 9.60	11.79 12.54 12.69	11.54 12.53 11.59	11.54 13.04 12.31	7.56 7.94 8.13	6.79 6.12 7.82
Mean	10.83	13.03	12.34	11.89	12.30	7.88	6.91
CV (%)	4.00	46.43	3.92	4.69	6.10	3.68	12.39

Table 12. Activity measured in the human samples spiked with 2000 IU/mL of IFN α5

Activity measured (IU/mL)								
Theoretic activity (IU/mL)	**Day 0**	**3 days**	**1 month (30 days)**	**2 months (63 days)**	**3 months (100 days)**	**4 months (121 days)**	**5 months (149 days)**	**6 months (189 days)**
2000	2717.55 2751.52 --	2252.97 2376.61 1782.24	1886.87 2388.49 2264.53	2123.45 1769.59 2083.63	2731.80 2713.92 2580.57	3594.61 3531.00 3696.67	2367.43 2627.98 2348.61	3302.14 3435.50 3025.31
Mean	2734.54	2137.27	2179.96	1992.22	2675.43	3607.43	2448.01	3254.32
CV (%)	0.88	14.67	11.99	9.73	3.09	2.32	6.38	6.43

--Point ruled out for non-compliance with the established criterion

Table 13. IFN-α5 stability in macaque and human serum samples stored at -80 ºC

Spiked sample activity (IU/mL)	**Dilution used**	**Line equation**	**Probability**	**Confidence interval for the slope**	**Stable up to (days)**	**Nº of time points studied**
Macaque serum						
12.5	1/10	y=0.0045x + 10.757	0.9924	-0.1496, 0.1503	15	5
125	1/100	y=0.1148x + 126.13	0.2110	-0.1156, 0.3452	15	5
500	1/100	y=0.1738x + 451.26	0.6907	-1.0880, 1.4356	15	5
2000	1/100	y=0.3499x + 1726.1	0.8430	-4.8124, 5.5121	15	5
50000	1/5000	y=12.601x + 40200	0.6139	-79.0156, 104.2185	15	5
200000	1/10000	y=92.042x + 148231	0.5302	-434.2023, 618.2869	15	5
Human serum						
10	1/10	y=-0.0251x + 12.763	0.0517	-0.0504, 0.0003	185	7
2000	1/100	y=4.7147x + 2242.6	0.1327	-1.9203, 11.3497	189	8

Figures

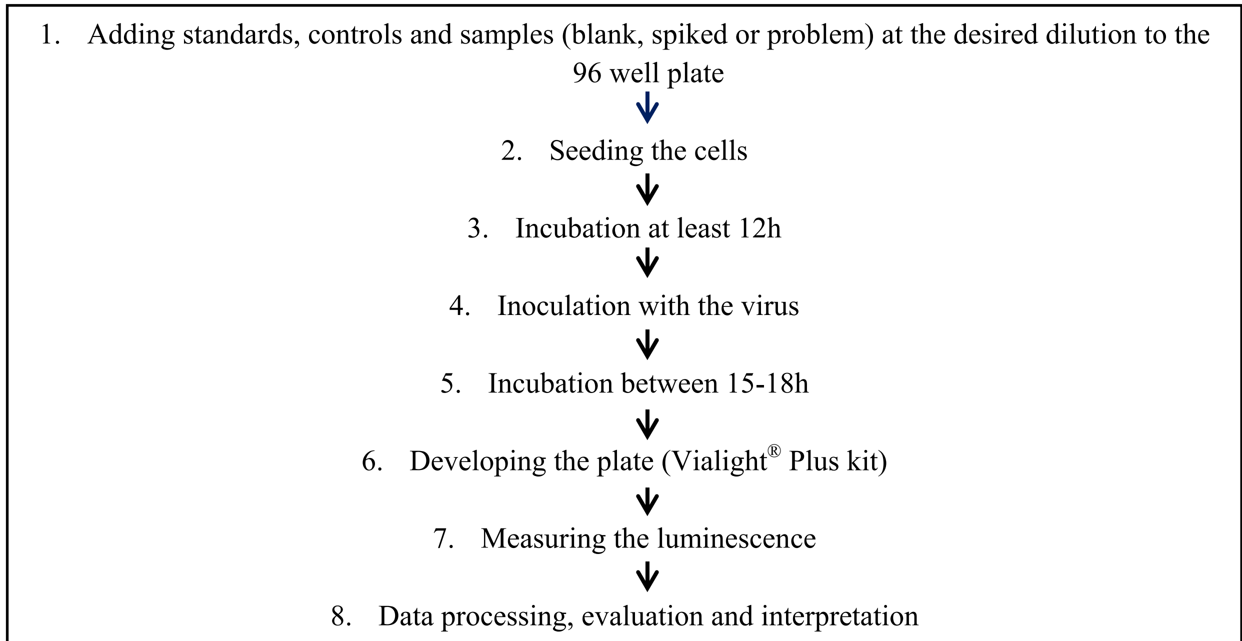

Figure 1. Flow chart for the performance of the AVA

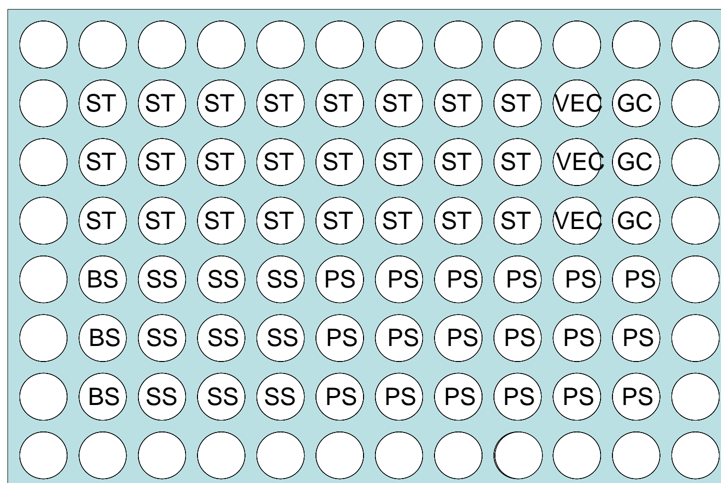

Figure 2. 96 well plate design (ST: standards; VEC: virus effect control; GC: growth control; BS: blank sample; SS: spiked sample; PS: problem sample of a study)

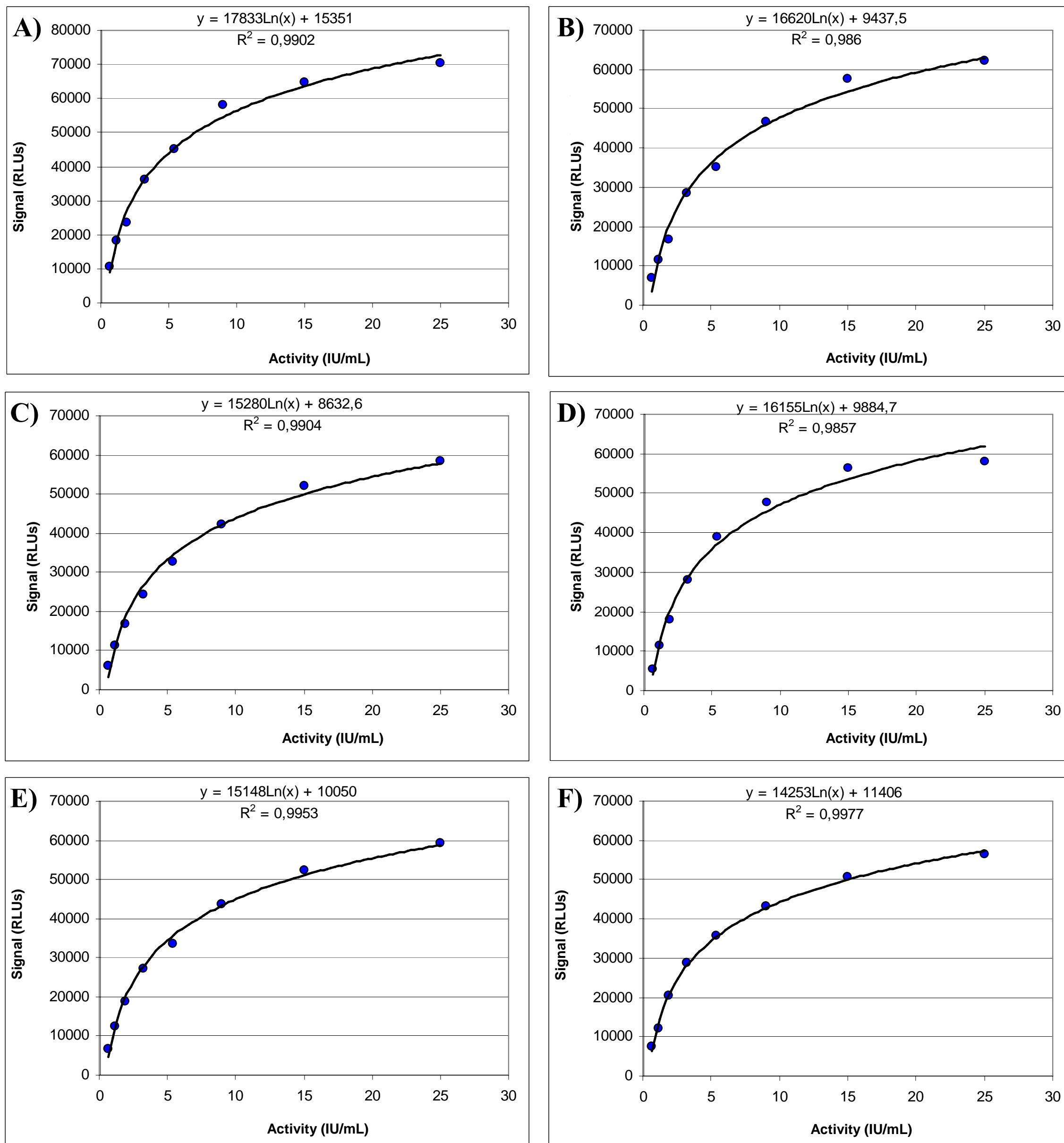

Figure 3. Examples of standard curves (luminescence vs. activity) obtained at a dilution of A)1/10, B)1/100, C)1/300 and D)1/10000 in macaque serum; and E)1/10 and F)1/100 in human serum