Validation of a cell-based colorimetric reporter gene assay for the evaluation of Type I Interferons

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A B S T R A C T

The biotherapeutic type I interferons (IFN-I) are indicated to treat several diseases. These products are regulated to guarantee safety and efficacy through critical quality attributes. For this purpose, the development of robust assays is required, followed by its validation to demonstrate their suitability for its intended purpose. Despite there are some commercial kits to evaluate IFN-I signaling, these are focused on measuring *in vitro* biological response instead of their validation, which is a pharmaceutical industry requirement. The aim of this work was to validate the HEK-Blue IFN-α/β system evaluating the biological activity of IFN-α/β under good laboratory practices, according to international standards. Our results demonstrated that HEK-Blue IFN-α/β system comply with accuracy ($r^2>0.95$) precision (CV < 20%) and specificity for both IFN-α/β; confirming that this assay is robust for this biotherapeutics' evaluation. Thereby, this bioassay could be implemented as a complementary method to the classical anti-proliferative and anti-viral assays under quality control environments.

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1. Introduction

Type I Interferons (IFN-I) are cytokines composed mainly by IFN-α and β [1], which have important roles in innate and adaptive immune responses with effects over a broad range of mammalian cells [2,3]. The type I IFN display broad biological activity, including control of cell proliferation, induction of genes responsible for protecting cells against the effects of viral infection, regulation of the differentiation state of immune cells and modulation of their function, accordingly these cytokines have been used in the treatment of viral and proliferative disease [4–9].

Type I IFN for therapeutic use are produced by recombinant DNA technology using either eukaryotic or prokaryotic expression systems. For instance, recombinant IFN-β1a is produced in Chinese hamster ovary (CHO) cells containing a glycosylation pattern like human IFN-β, while recombinant IFN-β1b and IFN-α are produced in *Escherichia coli* as a non-glycosylated form with minor modifications in its original amino acid sequence [10,11]. Despite the physicochemical differences between the type I IFN, these proteins exhibit the same function as the native [12] IFN. However, it is well known that the glycosylated isoforms have a longer half-life than the non-glycosylated [10,13].

Biological characterization of recombinant type I IFN is required to confirm their identity and evaluate their strength. These attributes can be assessed by any of the following *in vitro* methodologies: 1) receptor binding assays, 2) antiviral activity in cell culture and/or 3) antiproliferative effects in tumor cell lines [14]. This characterization must be performed throughout different stages of their life cycle (i.e. development, stability tests, and batch release).

Several assays for the evaluation of IFN biological activity require the use of viruses to measure antiviral activity [15]. This implies the compliance of special biosafety measures to handle

these pathogens. As an alternative, reporter gene assays is an indirect approach to evaluate the activation of signal transduction to measure the function of target genes using fluorescent proteins or colorimetric enzymatic reactions as detection systems [16]. Assays that employ reporter genes have demonstrated to be highly robust in terms of their specificity, sensitivity, precision, and easiness to perform [17–22] than the classical antiviral assays used to evaluate the strength of type I IFN [20,18–22].

HEK-Blue IFN-α/β cells system from InvivoGen® is a commercial gene reporter-based bioassay kit designed to evaluate the activation of the type I IFN pathway. This system is based on stable transfection of human embryonic kidney 293 (HEK293) cells that express signal transducer and activator of transcription 2 (STAT2) and IFN regulatory factor (IRF9) human genes to enrich a fully type I IFN signaling pathway. Once IFN-α or IFN-β bind to common receptor IFNAR1/2, allow the recruitment of JAK1 (Janus kinases 1) and Tyk2 (Tyrosine kinase 2), which induce the phosphorylation and dimerization of STAT (Signal Transducer activator of transcription) 1 and STAT 2. This dimer interacts with IRF9 forming the ISGF3 (IFN stimulated gene factor 3) complex. Finally, this transcriptional complex binds to the promoter of ISG54 (IFN-stimulated gene 54) and induce the production of SEAP (secreted embryonic alkaline phosphatase) as a reporter gene [23].

The HEK-Blue IFN-α/β system has been employed for research purposes using *in vitro* infection models, mainly to detect or quantify the production of bioactive IFN-I during viral or bacterial infections [24–30]. This system has also been proposed as a diagnostic tool for the indirect identification of neutralizing autoantibodies against IFN-I in some autoimmune diseases [31].

In this work, we validated the HEK-Blue IFN-α/β gene reporter cell-based bioassay according to the international standard guidelines; International Conference for Harmonization (ICH) Q2 (R1) [32] and the <1033> United States Pharmacopeia (USP) chapter [33] that establish the types and characteristics that should be evaluated during validation execution. This validation was intended to demonstrate that the assay is appropriate to be used under a pharmaceutical quality control environment for the *in vitro* biological activity assessment of biopharmaceutical products containing recombinant IFN-α and IFN-β.

2. Materials and methods

2.1. Materials

HEK-Blue[TM] IFN-α/β cells, selected antibiotics (Blasticidin, Normocin, and Zeocin) and SEAP substrate (QUANTI-Blue[TM]) were purchased from InvivoGen® (San Diego, CA, USA). Fetal Bovine Serum (FBS), trypsin TryPLE Select 1X and Phosphates Buffer Solution (PBS) were purchased from Gibco (GI, NY, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from ATCC (Manassas, VA, USA).

Human recombinant type I IFNα-2b and IFNβ-1a Chemical Reference Substances (CRS) were purchased from European Directorate for the Quality of Medicines (EDQM) CAS number 99210-65-8 and 145258-61-3, respectively, (Strasbourg, France). IFN-γ was purchased from Thermo Fisher (Waltham, MA, USA).

Human recombinant type I IFN were purchased: IFNα-2b Intron A® from Merck (New Jersey USA), IFNβ-1b Betaferon® from Bayer (Berlin Germany), IFNβ-1a Avonex® from Biogen Idec (Cambridge, Massachusetts) and IFNβ-1a Rebif® from Merck.

2.2. Cell culture

HEK-Blue[TM] IFNα/β cells were grown in DMEM medium with 10% FBS and selective antibiotics: blasticidin, zeocin and normocin (supplemented medium) and incubated at 37 °C, 5% CO_2. The cells

were harvested with trypsin when confluence reached 80%. Then cells were centrifuged, washed and resuspended in PBS. Viable cells were counted by the trypan blue exclusion method and cell concentration was adjusted to 2.8×10^5 cells/mL in a supplemented medium.

2.3. Evaluation of type I IFN response by SEAP activity

HEK-Blue cells were seeded in 96 well plates at a concentration of 50,400 cells/well. Aliquots of IFN-α and IFN-β were prepared independently at different concentrations (from 5×10^{-2} to 5×10^5 IU and 1×10^{-1} to 1×10^6 IU, respectively). Twenty μL of each IFN at different concentrations were added to corresponding wells and incubated at 37 °C and 5% CO_2 for 24 h. After the incubation period, 20μL of supernatant was transferred into a 96-well plate with 180 μL of QUANTI-Blue[TM] ready to use solution (prepared as indicated by manufacturer).

QUANTI-Blue[TM] and supernatants were incubated at 37 °C, 5% CO_2 for 3 h. Finally, O.D. at 655 nm was measured in an EPOCH spectrophotometer (BioTek, Winooski, VT, USA).

2.4. Validation

Parameters for validation were established according to the ICH guideline Q2 (R1) and the USP <1033> Biological Assay Validation Chapter [32,33] considering the parameters described below in each characteristic evaluated: dose-response curve (4 PL model fitting), specificity, precision, accuracy (dilutional linearity), and system suitability. The acceptance criteria for each evaluated parameter of validation exercise were established according to its intended use that evaluates the biological activity of IFN-α/β *in vitro* for quality control (QC) and biosimilarity assays using IFNα-2b and IFNβ-1a international standards. In this sense, it is expected a relative potency specification for batch release analysis establish in the biopharmaceutical industry between 80–125%. Therefore, the %CV should be established at $\leq$20%.

2.4.1. Dose-response curve (4 PL model fitting)
Independent triplicates were prepared at 12 dilution levels in a range from 5×10^{-2} to 5×10^5 IU and 1×10^{-1} to 1×10^6 IU for IFN-α2b and IFN-β1a respectively, in order to obtain the sigmoidal curve from the biological activity of these molecules fitted to the four parameters logistic (4 PL) model: bottom and top plateaus, the EC_{50}, and the slope factor (Hill's slope). The acceptance criteria were established by the correlation coefficient (r^2) >0.90.

2.4.2. Specificity
The specific response was given by the fitting of data curve through a non-linear regression model (4 PL). As specificity negative control, we used IFN-γ

2.4.3. Precision
Repeatability was estimated through the percentage of coefficient of variation (CV) from three independent replicates at the slope factor concentrations in the dose-response curve and intermediate precision as the %CV of three independent assays; acceptance criteria were CV $\leq$ 20% among replicates at all the evaluated levels.

2.4.4. Accuracy (dilutional linearity)
Accuracy was evaluated as dilutional linearity at all dilution levels of the dose-response curve in a concentration range from 70% to 130% for IFN-α2b and 40% to 120% for IFN-β-1b. Acceptance criteria for linearity were $r^2 \geq 0.90$ and slope in a range from 0.80 to 1.25. The nominal value is theoretical according to the dilution of the sample at 70–130% for IFN-α2b whereas 40–120% for IFN-β1a.
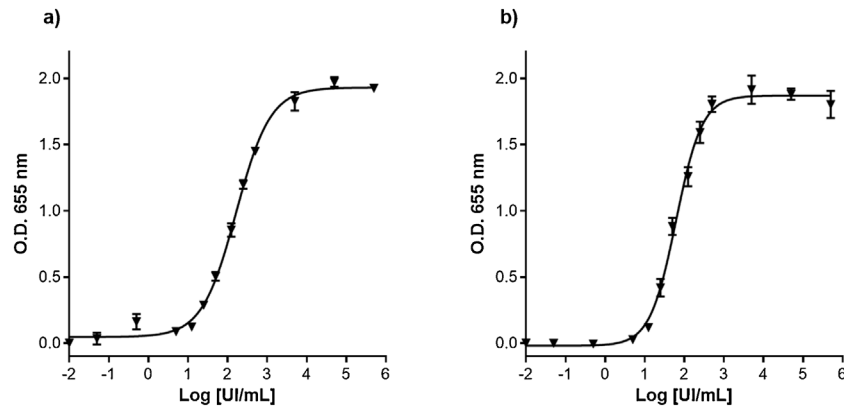
Fig. 1. Four parameters mathematic model fitting. HEK-blue α/β cells were stimulated with increasing concentrations of IFN-α2b or IFN-β1a, production of SEAP in culture medium was determined and measured at 655 nm optical density (O.D.). The dose-response curve and 4 PL mathematic model fitting for a) IFN-α2b ($r^2 = 0.99$) and b) IFN-β1a ($r^2 = 0.99$) are shown.

While the measured potency was estimated as percentages of relative potency were obtained from EC_{50} values.

2.4.5. System suitability

System suitability was determined considering the capacity to get a sigmoidal dose-response curve. The evaluated parameters were the %CV (from precision), r^2 of the curve within the concentration levels range, the relationship between the upper and lower asymptotes and the distribution of experimental data.

2.5. Performance of HEK–Blue system with commercial therapeutic IFN

The validated HEK-Blue system was tested with medical use type I IFN: Intron A®, Betaferon®, Avonex®, and Rebif®. The assay was performed using the established IFN-α/β concentrations and the methodology described.

2.6. Statistical analysis

Data were analyzed with Graph Pad Prism 6.0 software (La Jolla, USA) to evaluate the fit to the 4 PL model and the %CV for replicates.

3. Results

3.1. Optimization of the HEK-Blue IFN-α/β cell system

To optimize the HEK-Blue IFN-α/β cell system we identified the critical steps of the assay and divided into the following three: 1) cell preparation, 2) IFN-α/β binding to its receptor and 3) detection of SEAP activity.

Under our laboratory conditions, the cell preparation considered the number of passages below 12 to guarantee the reproducibility of response, also the adjustment of cell concentration.

In the next step, the type I IFN concentration range was established: from 5×10^{-2} to 5×10^5 IU and 1×10^{-1} to 1×10^6 IU for IFN-α and IFN-β, respectively. The kit's manufacturer established a range between 20–24 hours as incubation of the reaction system; we set 24 h for our assay. To measure the SEAP activity the incubation time was set on 3 h for type I IFN, after testing 0.5, 1.0, 2.0, 3.0 h, it allowed to determine the signal in the low concentration levels and a suitable correlation coefficient with the 4 PL model.

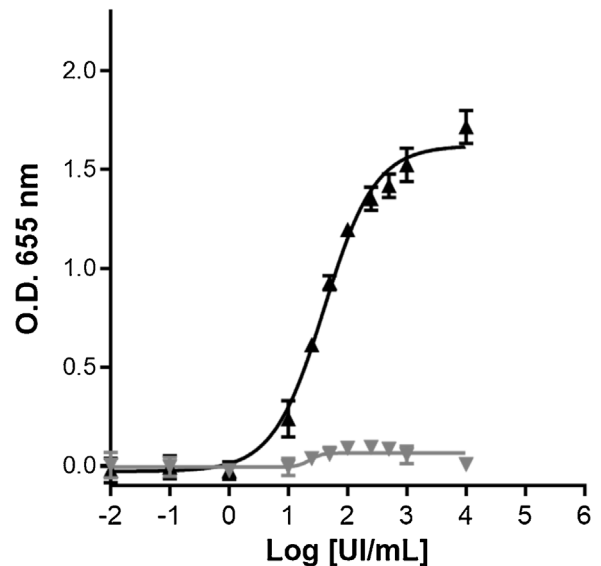Following these steps, we found that the assay was reproducible to evaluate the response of bioactive IFN-α/β form.

Fig. 2. Specificity. HEK-blue α/β cells were stimulated with increasing concentrations of IFN-α2b or IFN-γ, the SEAP production in culture medium was determined and measured at 655 nm optical density (O.D.). The response obtained with IFN-α2b (black line) was fit to 4 PL ($r^2 = 0.99$) but the IFN-γ stimuli (grey line) did not fit to the 4 PL ($r^2 = 0.40$). The results demonstrate the specificity parameter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Repeatability and intermediate precision IFN-α2b.

Concentration of each level	Acceptance criteria	Repeatability %CV	Intermediate precision		
			Log EC_{50}		%CV
5×10^{-2}	–	ND	–		ND
5×10^{-1}					
5	CV $\leq$ 20%	14.6	Day 1	1.80	7.9
12.5		16.2			
25		5.5			
50		9.4	Day 2	1.98	
125		12.2			
250		0.7			
500		3.1			
5×10^3		6.2	Day 3	2.11	
5×10^4		7.9			
5×10^5		2.7			

Table 2
Repeatability and intermediate precision IFN- β1a.

Concentration of each level	Acceptance criteria	Repeatability % CV	Intermediate precision Log EC$_{50}$	% CV
1×10^{-1}	–	ND	–	ND
1				
10	CV $\leq$ 20%	3.0	Day 1 1.56	16.3
25		8.7		
50		7.7		
100		5.9	Day 2 1.59	
250		6.6		
500		4.2		
1×10^3		10.2		
1×10^4		2.5	Day 3 1.17	
1×10^5		1.8		
1×10^6		2.0		

Once the assay was optimized in our laboratory, the parameters for its validation were established.

3.2. Validation assay

3.2.1. Dose-response curve fitting

As shown in Fig. 1, the biological assay presented a sigmoidal dose-response curve, which fit 4 PL showing an $r^2 = 0.97$ for IFN-α2b and $r^2 = 0.98$ for IFN-β1a. The curve showed at least two points in each asymptote and three points in the slope for both IFN-α/β that is a pharmacopeical suggestion to make a suitable fitting for 4 PL model according to obtained results of the correlation coefficient (r^2). We included two concentration levels higher than recommended by the manufacturer, these two added points allowed to obtain a better sigmoidal behavior in the curve.

3.2.2. Specificity

In order to test the specificity of the HEK-Blue cell system, we use IFN-γ as a negative control as was previously reported [31]. We are shown that the responses of IFN-α2b and IFN-β1a were fitted to 4 PL, in contrast, IFN-γ at the same concentration range did not fit to 4 PL ($r^2 = 0.4047$) demonstrating the characteristic response of these cells to type I IFN, complying with specificity parameter (Fig. 2). Despite IFN-γ is a cytokine related to IFN-α and IFN-β do not share the same signaling pathway [31]. Although HEK-Blue cells are able to respond to different stimulus included type II IFN such as IFN-γ, the reporter gene under control of ISG54 promoter is induced by type I IFN only, therefore it requires a different transcriptional factor complex, and this explains the high specificity of the assay.

3.2.3. Precision

The precision of the HEK-Blue IFN-α/β cell system showed CV lower than 20% obtained from triplicates of each level of the curve (intra-assay) and EC$_{50}$ at different days (inter-assay) (Table 1 and 2). Both assays fulfill repeatability and reproducibility.
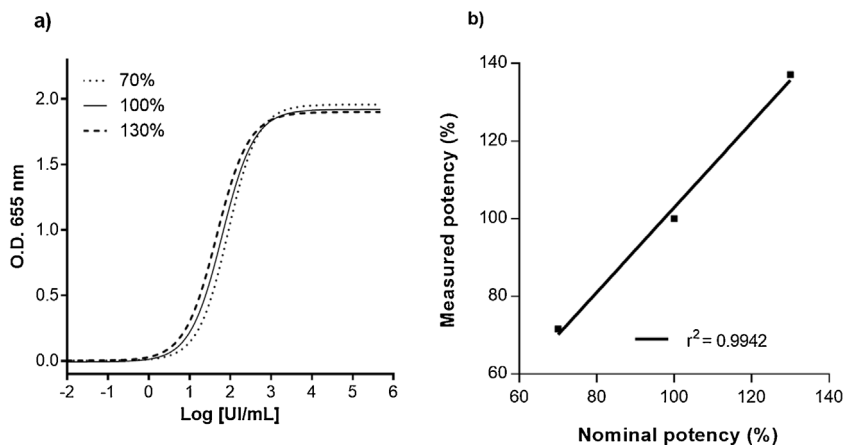

Fig. 3. Accuracy (dilutional linearity) IFN-α2b. Graphic a) shows dilutional linearity and b) depicts the relationship between nominal and measured potency in a range of 70–130%. The results demonstrate that the bioassay is accurate for IFN-α2b.
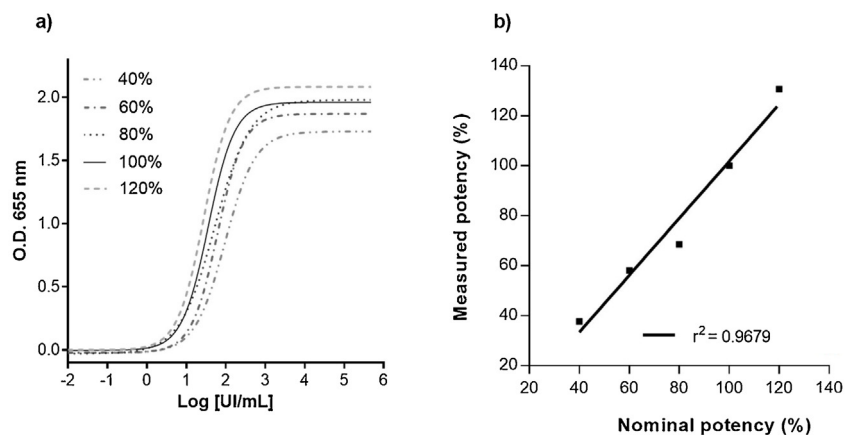

Fig. 4. Accuracy (dilutional linearity) IFN-β1a. Graphic a) shows dilutional linearity and b) depicts the relationship between nominal and measured potency in a range of 40–120%. The results demonstrate that the bioassay is accurate for IFN-β1a.

Table 3
Summary of validation results for IFN-α2b and IFN-β1a.

Characteristic	Parameter	Acceptance criteria	IFN-α2b results	IFN-β1a results
4 PL mathematic model fitting	Curve fitting	Fitting of biotherapeutic samples to the 4 PL : $r^2 > 0.90$	0.97	0.98
Specificity	Fit 4 PL with international standards	$r^2 > 0.90$	0.97	0.98
	Do not fit 4 PL without international standards	Curve profile	Do Not fit	Do not fit
Precision	Coefficient of variation percentage (%CV) among independent triplicates at each concentration level of the dose-response curve	$\leq 20.0\,\%$	0.5-18.2%	0.4 – 12.4%
Accuracy	Correspondence between nominal potency and measured potency obtained from dilutional linearity	$r^2 > 0.95$ Slope 0.80-1.25	0.99 1.09	0.97 1.14
System suitability	Ratio between maximum response / minimum response of international standards	> 1.50	1.88	1.89
	Differential dose-response between international standards in a determined concentration range	The dose-response curve fitting to 4 PL	$r^2 = 0.97$ of fitting to 4 PL in the range of 5×10^{-2} - 5×10^5 μg/mL	$r^2 = 0.98$ of fitting to 4 PL in the range of 1×10^{-1} - 1×10^6 μg/mL
	Precision	%CV $\leq 20.0\,\%$	0.5-18.2%	0.4– 12.4%

The bioassays have intrinsic variability however, the HEK-Blue IFN-α/β cell system showed a CV lower than 20% among replicates in a range of concentrations 5 - 5×10^5 and 10 - 1×10^6 UI/mL for IFN-α2b and IFN-β1a respectively (intra-assay), meeting with established criteria validation (Table 1 and 2). As indicated in the sample preparation, we tested two lower concentrations than recommended by InvivoGen®, those not fulfilled with CV due to the low detection level; our results demonstrated that if these two points were or not considered, the sigmoidal curve was preserved.

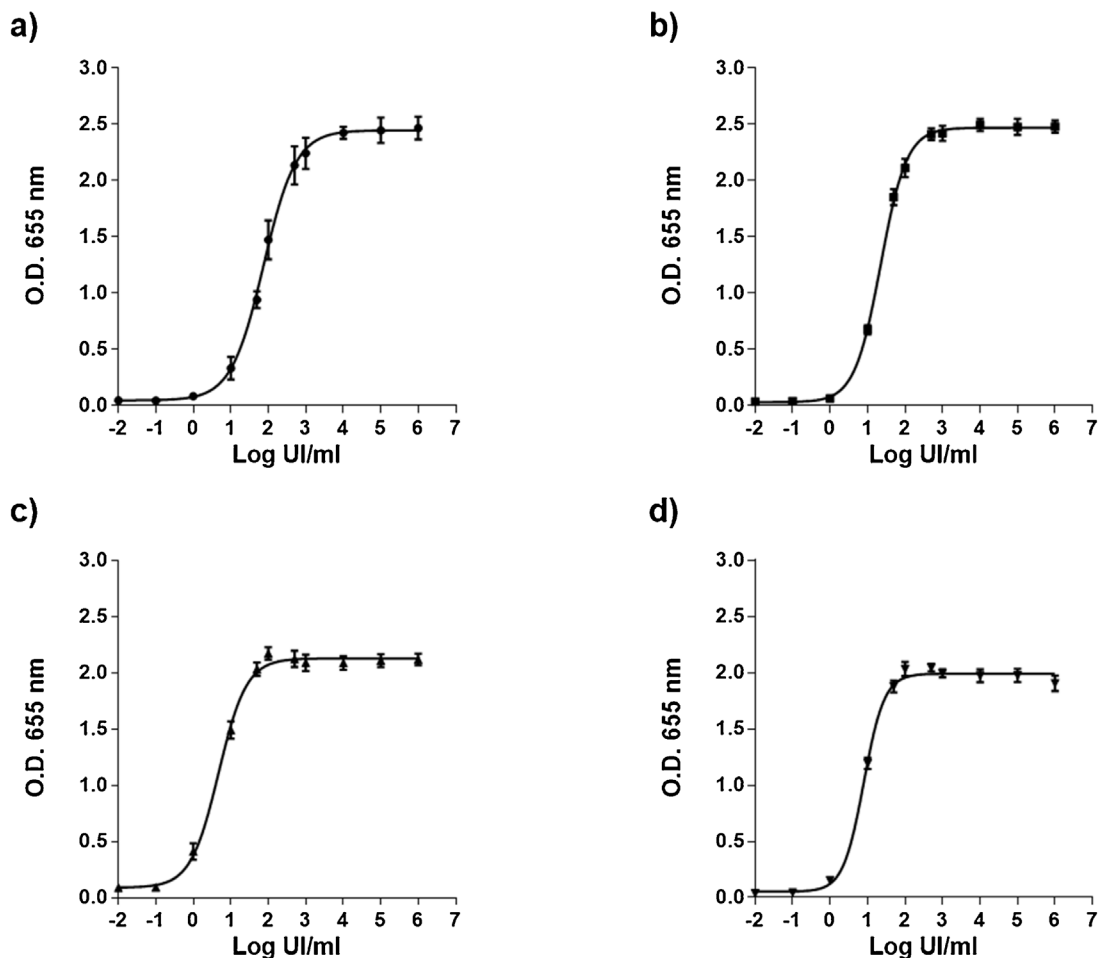The CV of EC$_{50}$ at three different days (inter-assay) was precise with a CV lower than 20%.

Fig. 5. Evaluation of response induced by commercial type I IFNs. a) Responses obtained from the stimuli with commercial type I IFN: a) Intron A® (IFN-α2b), b) Betaferon® (IFN-β1b), c) Avonex® (IFN-β1a) and d) Rebif® (IFN-β1a). The production of SEAP in the culture medium was determined and measured at 655 nm optical density (O.D). Dose-response curves showed 4 PL fitting: Intron A® $r^2 = 0.98$, Betaferon® $r^2 = 0.98$, Avonex® $r^2 = 0.96$ and Rebif® $r^2 = 0.98$.

In summary, this assay satisfies repeatability and intermediate precision for its validation.

3.2.4. Accuracy

In Fig. 3 we demonstrated the accuracy of IFN-α2b in a dilution range from 70 to 130% with a linear correlation between nominal and measured potencies, $r^2 = 0.99$, and a slope value of 1.09. In the same way in Fig. 4, IFN-β1a demonstrated dilutional linearity ($r^2 = 0.97$ and slope = 1.140) in a range from 40 to 120% and was able to distinguish two more dilution levels than IFN-α. Both parameters showed to be within the established acceptance criteria.

3.2.5. System suitability

The system suitability was predefined according to 4 L P model fitting and precision parameters in order to verify equipment, analytical operations and samples in a routine analysis to guarantee the reliability of results as recommended the ICH Q2R1 [32]. For system suitability, we defined the same rigor in the acceptance criteria mentioned in the summary of the validation of assay (Table 3).

3.3. HEK –Blue system performance with commercial therapeutic IFN

At present, there are several commercially available IFNα/β for medical uses. Here we proved if the validated HEK-Blue IFN-α/β system is useful to determinate bioactive form of some of this commercial type I IFN through IFNAR signal transduction. Once the assay was validated with international standards, we tested this system with IFN-α2b (Intron A®), IFN-β-1a (Avonex® and Rebif®) and IFN-β-1b (Betaferon®). As it was shown in Fig. 5, the HEK-Blue IFN-α/β system was suitable to detect the bioactive IFNα/β contained in these biotherapeutics, complying with the fit to 4 PL.

In summary, the HEK-Blue IFN-α/β system could be useful to evaluate the bioactive type I IFN inside biotherapeutic products despite their origin.

4. Discussion

In this work, we optimized and validated the commercial HEK-Blue IFN-α/β system, which complies with precision, accuracy, specificity and system suitability according to established parameters. As expected, being a biological system, it shows a classical dose-response sigmoidal curve, besides our IFN-α/β results fitted 4 PL model.

Both IFN-α/β are recognized by the same receptor IFNAR, differences in their structure might affect cell response [7]. In our results, we observed similar sigmoidal behavior by adjusting concentration range of IFN-α/β however, IFN-β shows higher potency than IFN-α as previously reported [24]. Potency differences could be explained by different molecular mechanisms as the magnitude of the signal by ligand affinity to each IFNAR subunits, response to different type I IFN concentrations and membrane receptor exchange [25–29].

In addition, we proved that the HEK-Blue IFN-α/β system could be used to evaluate bioactive IFNα/β of commercial biotherapeutics: IFN-α2b, IFN-β-1a, and IFN-β-1b. We found a similar response that fit to 4 PL, despite the different source of type I IFN.

Moreover, the recommended use of the HEK-Blue IFN-α/β system in the research field, our results supported that this could be extended for pharmaceutical industry use because it showed to be robust evaluating identity and potency of therapeutic type I IFN, regardless their source.

5. Conclusions

We validated the commercial HEK-Blue IFN-α/β system complying with precision, accuracy, specificity and system suitability according to established parameters. Our results suggest that this bioassay could be implemented as a complementary method to the classical anti-proliferative and anti-viral assays under a quality control environment because it was robust and easy to perform to evaluate type I IFN biological activity.

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