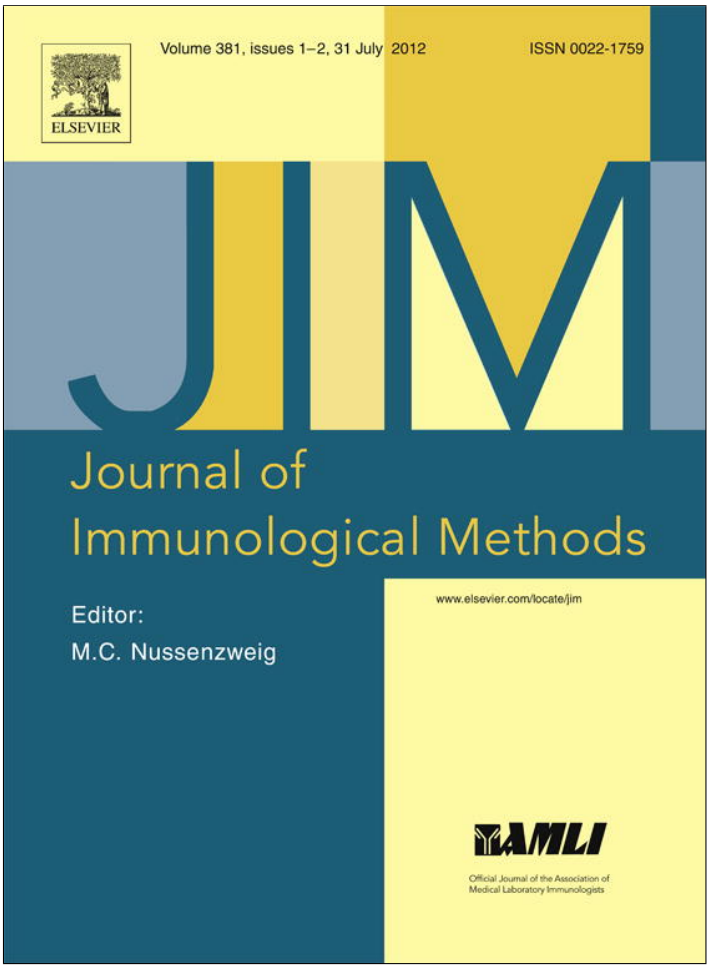

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Technical note

WISH cell line: From the antiviral system to a novel reporter gene assay to test the potency of human IFN-α and IFN-β

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ABSTRACT

Interferons (IFNs) are potent biologically active proteins that are widely used as biopharmaceuticals, so their potency must be correctly identified. Usually, the biological activity is quantified by a bioassay based on its capacity to induce an antiviral state in target cells, but this type of assays is subject to virus manipulation-related issues and they show considerable intra- and inter-assay variability.

In this work, we generated a reporter gene assay (RGA) supported on the WISH-Mx/eGFP reporter cell line to determine human type I IFN activity. WISH cells were stably transfected with the enhanced green fluorescent protein (eGFP) gene under the control of type I IFN-inducible Mx2 promoter.

This system implies the use of a standardized cell line for human IFN-potency analysis such as WISH cells and the simultaneous use of the sensitive reporter gene eGFP, having also several advantages when compared to antiviral activity assays and other RGAs: it can determine the potency of hIFN-α and hIFN-β using only one cell line showing the highest expression of eGFP after 28 h and being only observed in cells treated with type I IFNs due to the specificity of the Mx promoter. It is a sensitive assay and it represents a safe alternative when compared with the conventional antiviral tests. The cell line showed the same sensitivity along 57 generations, allowing its use during two months of successive culture. The inter- and intra-assay coefficients of variation were lower than 20%, demonstrating its reproducibility. In addition, this reporter cell line can be used for the conventional antiviral assay, either for hIFN-α or hIFN-β.

In conclusion, we have developed an alternative reporter system for the analysis of type I IFNs, in which its performance make it a suitable candidate to replace or complement conventional bioassays that are currently employed to measure IFN potency.

1. Introduction

IFNs are a family of cytokines that exert a multiplicity of actions: they play an essential role in the control of viral infections, confer potent immunomodulatory responses and show antiproliferative activities.

This family of glycoproteins has been divided into three groups: type I IFNs (IFN α, β, δ, ε, κ, τ, ω), type II IFN (IFN-γ) and type III IFNs(λ1–3). Among the human (h) type I IFNs, hIFN-α and hIFN-β have been produced in their recombinant

Abbreviations: AVA, antiviral activity assay;eGFP, enhanced green fluorescent protein;FSC, forward scatter;G-CSF, granulocyte-colony stimulating factor;GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon;IL-2, interleukin-2;ISRE, IFN sensitive respond element;IU, international unit;MEM, Minimum Essential Medium;n, sample size;NIBSC, National Institute for Biological Standards and Control;RGA, reporter gene assay;rh, recombinant human;S.D., standard deviation;SSC, side scatter.

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(r) forms with therapeutic purposes, e.g., rhIFN-α has been widely used for several cancer and viral diseases and rhIFN-β has been used for multiple sclerosis. Considering the mentioned clinical targets, their potencies have to be estimated accurately. Antiviral activity assays (AVAs) are currently recommended by the European and U.S. Pharmacopoeia to determine IFN potency by measuring their capacity to induce an antiviral state in target cells. However, AVAs are subject to high intra- and inter-test variations requiring virus manipulation under biosafety level 2 conditions (Girad and Fleischaker, 1984). An alternative to this bioassay is the use of RGAs. They are highly specific and sensitive assays (Lleonart et al., 1990) based on the use of a cell line that expresses the receptors for a given cytokine and is also transfected with a plasmid carrying a reporter gene under the control of a cytokine-inducible promoter. The product of the reporter gene is easily detected and is not naturally expressed by the cell line. After cell treatment with increased doses of the cytokine, the promoter will activate and produce increasing amounts of reporter genes (Canosi et al., 1996).

Although many reports have shown the development of several RGAs to test the potency of hIFNs, none of them took into account the simultaneous use of a human cell line that is typically applied to determine the hIFN biological activity, e.g. WISH cell line and the reporter gene eGFP, whose advantages are herein highlighted. Some examples of the former developments for human IFNs are: Vero Mx–hGH, Vero Mx–Luc, Glio GFAP/lacZ, HEK293-ISRE-SEAP, CHO-ISRE-SEAP and HeLa Mx2/Luc (Seo et al., 2009), luciferase RGA based on HT1080 cells transfected with the early IFN-inducible 6–16 promoter (Lam et al., 2008) and luciferase RGA based on U397 cells transfected with the IFN sensitive respond element (ISRE; Lallemand et al., 2010).

In the present study we describe a new reporter cell line (WISH-Mx2/eGFP) that was generated by the use of the human cell line WISH and the reporter gene eGFP under the control of the mouse Mx2 promoter taking into account that Mx genes are rapidly and specifically induced by type I IFNs (Lleonart et al., 1990; Haller et al., 2007). This new reporter cell line has many advantages from those already existing. The use of eGFP as the reporter gene shows some peculiarities, it does not need a substrate and remains stable when subjected to heat, extreme pH and chemical denaturants (Zhang et al., 1996). Additionally, WISH cell line expresses human IFN receptors and is generally used in AVAs to test the potency of human type I IFNs beta. Therefore, the robustness and sensitivity of the newly established WISH-Mx2/eGFP cell line-derived trial was compared with the AVAs that are routinely used.

2. Materials and methods

2.1. Cytokines and reagents

Recombinant hIFN-α2a and rhIFN-α2b were obtained from Zelltek S.A. (Santa Fe, Argentina), and rhIFN-β1a (Avonex) and rhIFN-β1b (Betaferon) were purchased from Biogen (USA) and Bayer (Germany), respectively. Recombinant human cytokines: interleukin-2 (IL-2), granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) were obtained from the National Institute for Biological Standards and Control (NIBSC), United Kingdom.

Chemical reagents were purchased from Sigma Aldrich (Saint Louis, USA). Media, fetal calf serum (FCS) and consumables for cell culture were obtained from Invitrogen (USA), PAA (Argentina) and Greiner (Germany).

2.2. Cell line, culture media and plasmid

WISH and MDBK cell lines were obtained from ATCC (CCL-25 and CCL-22). Minimum Essential Medium (MEM) 90% (v/v) supplemented with FCS 10% (v/v) and 2 mM glutamine was used for growth and maintenance. Stably transfected clones were selected with Neomycin 400 μg/ml in growth medium.

The pEGFP-C1 vector was purchased from Clontech (USA). The Mx2 promoter was synthesized by GENEART (USA) according to the gene sequence AB086958 (NCBI Genebank). The Mx2 promoter sequence was excised with *AseI* and *NheI* and ligated into the promoter-less pEGFP-C1, obtaining the pMx2/eGFP vector.

2.3. Transfection and selection of stable clones

One μg of DNA was transfected into WISH cells using Lipofectamine 2000 reagent (Invitrogen). Resistant colonies were selected and expanded in medium containing 400 μg/ml neomycin during 7 days. Resistant cell lines were cloned in growing media and when clones reached confluence, IFN responses were evaluated by measuring the eGFP expression by flow cytometry (Section 2.4). Clones with the best responses were amplified and cryopreserved.

2.4. Reporter gene assay conditions

For the routine assay, WISH-Mx/eGFP cells were seeded into 96-well plates (3×10^4 cells/well in 0.1 ml MEM culture medium supplemented with 10% (v/v) FCS) and incubated at 37 °C and 5% CO_2 during 24 h. The cell layer was washed with basal MEM medium, and unknown samples and standards were tested at multiple serial 1/2 dilutions in triplicate. All dilutions were performed in MEM culture medium supplemented with 2% (v/v) FCS. Plates were incubated at 37 °C in 5% CO_2 for 24 h. Supernatants were discarded and cells were trypsinized and resuspended in 0.2 ml PBS. The percentage of eGFP positive cells was evaluated by flow cytometry using a Guava® EasyCyte™ cytometer (Guava Technology, USA). Data acquisitions and analysis were performed using Guava CytoSoft™ 3.6.1 software. For each sample 5000 events were collected gating on the FSC vs SSC dot plot. Negative control was performed by adding MEM culture medium supplemented with 2% (v/v) FCS.

For the RGA optimization, IFN standards were tested on different cell densities (3×10^4, 6×10^4 and 12×10^4 cells/well) and incubation times (24, 26, 28, 30, 32 and 34 h). In both cases only the above-mentioned variable was modified, while the rest of the parameters was kept constant.

2.5. Reporter gene assay validation

RGA was validated according to the accuracy, precision, specificity, detection range, linearity, sensitivity and robustness

of the cell line. Assay samples were prepared by adding a specific amount of different type I IFNs to the culture medium. The concentrations were tested in the linear range of the standard dose–response curves. RGA and AVA were compared. Both methods were performed with the WISH-Mx/eGFP reporter cell line and different IFN preparations. Samples and standards were tested in triplicate and the obtained IFN potency was analyzed.

The specificity of the RGA was tested with 50 IU/ml of the human cytokines: IFN-α2a, IFN-α2b, IFN-β1a, IFN-β1b, IFN-γ, IL-2, GM-CSF and G-CSF on WISH-Mx/eGFP cells. The robustness of WISH-Mx/eGFP cells was determined by comparison of cells frozen and thawed after 29th, 57th and 84th generations with IFN-α2a, IFN-α2b, IFN-β1a and IFN-β1b.

2.6. In vitro AVA using WISH cell line and vesicular stomatitis virus (VSV)

The antiviral activity of both IFN subtypes was estimated by their protective effect on WISH cells infected with VSV following the recommendation of the European Pharmacopoeia, 2009. References and samples were assayed in quintuplicate, diluting them in MEM medium supplemented with 2% (v/v) FCS.

2.7. Statistical analysis

Data are presented as mean ± 3 S.D. (sample Standard Deviation) or mean ± 2 SEM (Standard Error of the Mean) where appropriate. The statistical significance between groups was assessed with the conventional parallel line assay using Origin 7.5 for Windows XP (Public software), where $p<0.05$ was considered significant.

3. Results

3.1. Generation of the Reporter Cell Line

The pMx2/eGFP construction (Fig. 1) was used to stably transfect WISH cell line. This cell line was selected because it expresses human type I IFN receptors on its surface and is susceptible to VSV virus infection. Thus, three stably transfected WISH-Mx/eGFP lines were obtained and selected during 7 days with neomycin. When untransfected cells showed total cell death, the three resistant cell lines were cloned and 50 clones were assayed for IFN-α sensitivity. L1G3 clone, showing the highest eGFP response to the cytokines (above 85%) was selected for subsequent assays (Fig. 2).

3.2. Conditions and optimization of the RGA

The Mx promoter, cloned upstream to the reporter gene, responded specifically and quantitatively to type I IFNs using the new WISH-Mx/eGFP reporter cell line (Fig. 3).

The assay was performed by testing 18 point-curves for each subtype of cytokine, starting from a potency of 5600 IU/ml to 0.04 IU/ml (two-fold serial dilutions). eGFP percentage increased as IFN in samples increased, reaching maximum values of 80% of activated cells (no eGFP expression was observed for negative controls, see Fig. 2). The detection limit

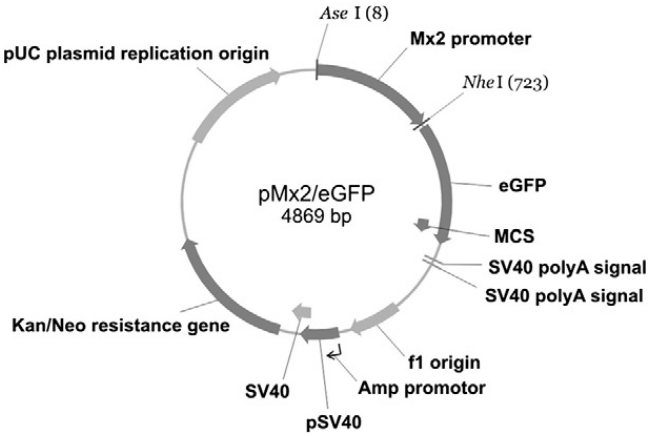

Fig. 1. Restriction map of the pMx/eGFP plasmid. The Mus musculus Mx2 promoter was cloned into *AseI*/*NheI* sites in the pEGFP-C1 promoter-less plasmid, yielding the pMx/eGFP vector.

of the assay, meaning the lowest detectable IFN potency that could be measured, was 0.04 IU/ml for all cytokines tested, and it was calculated considering the average signal value corresponding to the negative control + 3 × S.D. Taking into account the dose–response curves for hIFN-α and hIFN-β, the assay linearity was determined from the linear portion of each standard curve (Fig. 3). The linearity was obtained between 5.5 and 2800 IU/ml ($R^2>0.995$, $p<0.0001$) for hIFN-α2a or hIFN-α2b and between 0.7 and 350 IU/ml ($R^2>0.996$, $p<0.0001$) for hIFN-β1a or 0.7 and 1400 IU/ml ($R^2>0.994$, $p<0.0001$) for hIFN-β1b.

In order to optimize the assay, three different cell densities were evaluated: 3×10^4, 6×10^4 and 12×10^4 cells/well (0.1 ml) using AVA and RGA systems.

For both methods, a better response was observed when working with a cell density of 3×10^4 cells since maximum values of responses were obtained. In addition, curves showed a wider linear range and better sensitivity (data not shown).

On the other hand, both types of assays showed that as cell density increases, IFN activity decreases. This phenomenon could be attributed to a steric hindrance of receptors on the cell surface to bind the cytokines. Hence, at higher cell density, cells and their receptors overlap, preventing IFNs from interacting properly with receptors to trigger the expected response.

Aiming to achieve greater sensitivity of the assay a time-course study (from 24 to 34 h) of Mx-promoter reporter gene induction was carried out. After 28 h of induction, the eGFP expression reached its maximum value and remained without variations for 6 h. Thus, the minimum time to reach adequate assay sensitivity was 28 h with a tolerance window of up to 34 h (data not shown).

3.3. Assay specificity

The expression of eGFP reporter gene in WISH-Mx/eGFP cells was observed only in cells treated with alpha and beta hIFNs (Table 1). eGFP was not detected in cells exposed to other IFN types (IFN-γ) or cytokines: IL-2, GM-CSF and G-CSF, proving the known specificity of the Mx promoter to type I IFNs (Lleonart et al., 1990; Haller et al., 2007).

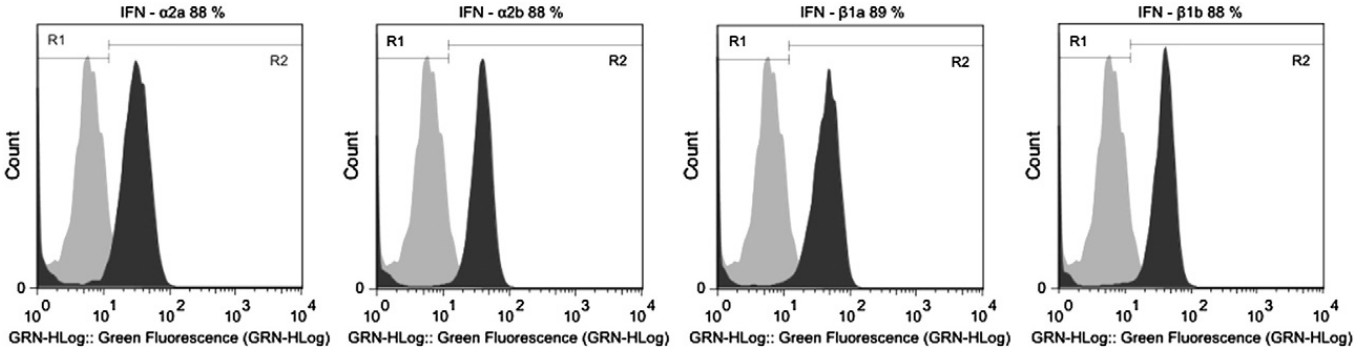

Fig. 2. L1G3 clone response to the different hIFN subtypes. Clone L1G3 was derived from WISH-Mx/eGFP reporter line and it was tested for the eGFP expression upon addition of 350 or 200 UI/ml of hIFN-α or hIFN-β, respectively. Cell population was gated using the FSC vs SSC dot plot and then represented in an eGFP histogram plot. Region 1 (R1) shows untreated cells and region 2 (R2) shows eGFP positive populations.

3.4. Validation of the reporter gene assay

In order to evaluate the reproducibility of the RGA and the AVA systems, hIFN-α and hIFN-β samples were tested against internal standards. Three independent assays of each one were performed with the aim of determining the inter-assay coefficient of variation [CV(%)] and, in each occasion, the IFN activity was measured by triplicate to determine the intra-assay CV (%). RGA showed a variable intra-assay CV from 3.3% to 20.0% with a mean CV of 10.1 ± 1.8% (n = 12). The intra-assay mean CV for the AVA was 8.5 ± 2.0% (n = 12), showing similarity to the RGA (Table 2). Nevertheless, higher fluctuations in the inter-assay CV were recorded for the AVA test with a mean CV of 30.4 ± 4.8% (n = 4) when compared to 12.6 ± 3.0% (n = 4) for the RGA (Table 2).

3.5. Stability of the reporter cell line

A drop in the maximum eGFP signal was observed after 29 generations but the parallelism between the linear portions of dose–response curves was maintained up to 57 generations (two months of successive subcultures). The assay sensitivity was not modified during the 57 generations (32.5 ± 2.2 ml/IU for 29 generations and 31.7 ± 1.2 ml/IU for 57 generations) but the detection limit increased from 3.6 IU/ml to 13.0 IU/ml, respectively (it was calculated as the potency of IFN that generates a 25% of positive eGFP cells). A significant drop in the assay sensitivity was observed (16.9 ± 0.6 ml/IU) after 84-generation cultures. Therefore, 57 generations of L1G3 culture were selected as the limit term in which it is possible to subculture this clone in order to develop an optimal RGA in which its sensitivity is not affected.

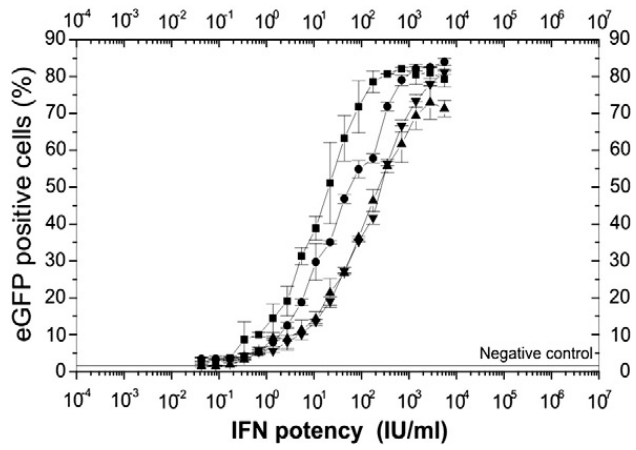

Fig. 3. Dose–response curves for hIFN-α2a (▲), -α2b (▼), -β1a (■) and -β1b (●) using the WISH-Mx/eGFP reporter line. Cells were plated (3×10^5 cell/ml) and induced with the corresponding hIFN sample. After 28 h each sample was analyzed by flow cytometry. Negative control was below 2% of eGFP positive cells.

4. Discussion

The AVA, the most common and widely used in vitro cell bioassay that determine IFN potency, suffers large variations that are inherent to cell culture, handling techniques, conditions, materials and reagents. Also, this method has certain disadvantages because it uses virus such as VSV and it is necessary to maintain a viral stock with an adequate titer to sustain its efficiency. This constitutes a drawback since viral titer decreases and the performance of the assay is reduced over time and after successive passages. Moreover, much care must be taken in handling VSV since it is necessary to guarantee biosafety level 2 conditions. The binding of IFNs to their specific receptors on the cell surface generates a rapid transcriptional activation of many cellular genes, including the Mx gene, which mediates various biological effects of

Table 1
Specificity of RGA to different cytokines.

Cytokine	eGFP (%)
IFN-α2a	32
IFN-α2b	34
IFN-β1a	53
IFN-β1b	18
IFN-γ	4
IL-2	2
GM-CSF	3
G-CSF	4
Negative control	3

A total of 3×10^4 cells were seeded in triplicate in 96-well plates and stimulated with the corresponding cytokine for 30 h. Results are expressed as the average of the percentage of eGFP positive cells.

Table 2
Reproducibility comparison between RGA and AVA. Inter- and intra-assay CVs (%) were calculated for each type I hIFN: -α2a, -α2b, -β1a and -β1b. Each sample was performed in triplicate.

IFN	Reproducibility intra-assay CV (%)			Reproducibility inter-assay CV (%)
	Assay 1	Assay 2	Assay 3	
Reporter gene assay with WISH-Mx/eGFP line				
α2a	20.0	3.3	6.4	8.3
α2b	6.5	3.8	4.2	6.7
β1a	13.9	17.0	4.1	16.7
β1b	11.4	13.6	17.4	18.5
Antiviral activity assay (AVA)				
α2a	24.2	14.7	2.7	22.2
α2b	8.4	5.6	12.5	32.7
β1a	0	12.5	0	43.0
β1b	8.7	10.7	2.4	23.8

IFNs. By inserting a reporter gene (under the control of an IFN-inducible promoter) into an IFN-responsive cell line, it is possible to develop a sensitive, quick and simple bioassay.

Although other authors have designed RGAs based on human cell lines such as Vero, Hela and HEK293, none of them has used a cell line that is commonly suggested by Pharmacopoeia (European Pharmacopoeia, 2009) to evaluate type I-IFNs. For this reason, WISH cells were engineered to obtain a novel and single platform to test type I-IFN potency using both AVA and RGA systems. Taking into account that WISH cells naturally express IFN receptors, they were selected and stably transfected with the reporter gene of the eGFP under the control of type I IFN-inducible Mx2 promoter (WISH-Mx/eGFP). Thus, the percentage of eGFP measured by flow cytometry correlated directly with the concentration of hIFN-α or hIFN-β in each sample.

As it was mentioned above, this system has several advantages: the reporter protein is highly stable when subjected to heat, extreme pH conditions and chemical denaturants (Chalfie et al., 1994; Zhang et al., 1996). It is easy to monitor and does not require a substrate for detection, helping to reduce the cost and the steps of the assay. In 28 h it is possible to reach the maximum value of eGFP which allows a higher sensitivity (data not shown). The reporter protein measurement is direct and does not require cell lysate or sample conditioning, procedures that could affect the reproducibility of the assay. RGA is easier to perform than traditional virus-dependent bioassays. Moreover, it is highly specific, as the expression of eGFP is only observed when the Mx2 promoter is activated, as a result of the binding of human IFNs to their receptors. It is a highly sensitive system with a detection limit of 0.04 IU/ml, showing a wide linear range. It fluctuates from 5.5 IU/ml to 2800 IU/ml for hIFN-α2a or hIFN-α2b and from 0.7 IU/ml to 350 IU/ml for hIFN-β1a or to 1400 IU/ml for hIFN-β1b. This is an important distinction since ranges are much wider than the corresponding to the traditional AVA, an effect also observed in systems using luciferase or SEAP as reporter protein (Smilović et al., 2008; Seo et al., 2009). Furthermore, the use of a wide linear range was not in detriment to the assay sensitivity. Thus, this RGA shows high sensitivity and wide linearity, which are necessary to quantify the IFN potency. The test proved to be accurate, since the inter- and intra-assay CVs did not exceed 20%, the threshold value for these bioassays, while the inter-assay CV calculated for AVA is substantially higher (30%).

In conclusion, this work demonstrated an upgrading of the reporter gene systems to quantify the type I IFNs. The advance of this new assay is related to the use of the human cell line WISH that is currently proposed as a standard bioassay in the mode of antiviral system. Furthermore, the simultaneous introduction of the eGFP gives an additional meaning to RGAs because of the above-mentioned features of this protein. Finally, this alternative WISH/eGFP-derived RGA has the following properties: simplicity, low cost, precision, sensitivity, safety, and the possibility of automation, turning it into a suitable candidate to replace more laborious and time consuming antiviral bioassays and non standardized cell line-derived RGAs that are employed to measure hIFN biological activity.

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References

Canosi, U., Mascia, M., Gazza, L., Serlupi-Crescenzi, S., Donini, F., Antonetti, F., Galli, G., 1996. A highly precise reporter gene bioassay for type I interferon. J. Immunol. Methods 199 (1), 69.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263 (5148), 802.

European Pharmacopoeia 6.3, 2009. Interferon Beta-1a Concentrated Solution. Council of Europe, Strasbourg, p. 4177.

Girad, D.J., Fleischaker, R.J., 1984. A study showing a high degree of interlaboratory variation in the assay of human interferon. J. Biol. Stand. 12 (3), 265.

Haller, O., Kochs, G., Weber, F., 2007. Interferon, Mx, and viral countermeasures. Cytokine Growth Factor Rev. 18 (5–6), 425.

Lallemand, C., Meritet, J.F., Blanchard, B., Lebon, P., Tovey, M.G., 2010. One-step assay for quantification of neutralizing antibodies to biopharmaceuticals. J. Immunol. Methods 356 (1–2), 18.

Lam, R., Farrell, L., Aziz, T., Gibbs, E., Giovannoni, G., Grossberg, S., Oger, J., 2008. Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFN-β in multiple sclerosis patients. J. Immunol. Methods 336 (2), 113.

Lleonart, R., Näf, D., Browning, H., Weissmann, C., 1990. A novel, quantitative bioassay for type I interferon using a recombinant indicator cell. Nat. Biotechnol. 8 (12), 1263.

Seo, Y.J., Kim, G.H., Kwak, H.J., Nam, J.S., Lee, H.J., Suh, S.K., Baek, K.M., Sohn, Y.W., Hong, S.H., 2009. Validation of a HeLa Mx2/Luc reporter cell line for the quantification of human type I interferons. Pharmacology 84 (3), 135.

Smilović, V., Caserman, S., Fonda, I., Gaberc-Porekar, V., Menart, V., 2008. A novel reporter gene assay for interferons based on CHO-K1 cells. J. Immunol. Methods 333 (1–2), 192.

Zhang, G., Gurtu, V., Kain, S.R., 1996. An enhanced green flourescent protein allows sensitive detection of gene transfer in mammalian cells. Biochem. Biophys. Res. Commun. 227 (3), 707.