Method for quantification of porcine type I interferon activity using luminescence, by direct and indirect means

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Abstract

Background: Type I interferons are widely used in research applications and as biotherapeutics. Current assays used to measure interferon concentrations, such as plaque reduction assays and ELISA, are expensive, technically challenging, and may take days to provide results. We sought to develop a robust and rapid assay to determine interferon concentrations produced from transiently transfected cell cultures.

Method: Indirect quantification of recombinant interferon was evaluated using a novel bi-cistronic construct encoding the Foot-and-mouth disease virus 2A translational interrupter sequence to yield equimolar expression of Gaussia princeps luciferase and porcine interferon α. Direct quantification was evaluated by expression of a novel fusion protein comprised of Gaussia princeps luciferase and porcine type I interferon. Plasmids encoding constructs are transiently transfected into cell cultures and supernatant harvested for testing of luminescence, ELISA determined concentration, and anti-viral activity against vesicular stomatitis virus.

Results: Bi-cistronic constructs, utilized for indirect quantification, demonstrate both luciferase activity and anti-viral activity. Fusion proteins, utilized for direct quantification, retained secretion and luminescence however only the interferon α fusion protein had antiviral activity comparable to wildtype porcine interferon α. A strong linear correlation was observed between dilution and luminescence for all compounds over a dynamic range of concentrations.

Conclusion: The correlation of antiviral and luciferase activities demonstrated the utility of this approach, both direct and indirect, to rapidly determine recombinant interferon concentrations. Concentration can be determined over a more dynamic concentration range than available ELISA based assays using this methodology.

Keywords: Gaussia luciferase, Interferon α, Interferon β, Fusion protein, VSV, FMDV, Luciferase, Assay, Anti-viral

Background

Type I interferons, IFNα and IFNβ, are used as biotherapeutics to treat a number of medical conditions including, leukemia, melanoma, human papillomavirus, chronic hepatitis B and C, and multiple sclerosis. Porcine IFNα and IFNβ have been used to inhibit Vesicular Stomatitis Virus (VSV), Porcine Reproductive and Respiratory Syndrome Virus, and Foot-and-Mouth Disease Virus (FMDV) in livestock [1–3].

Interferons are typically quantified through antibody-capture assays, or through assays that measure anti-viral biological activity, such as plaque reduction assays [4–6]. Comparing interferon levels among samples with these assays can be problematic, especially when conducting mutational analyses that may disrupt target epitopes or interferon activity [7]. Rapid quantification of interferon levels, independent of antibody-capture or anti-viral

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activity, would aid interferon research and development, especially through increased screening of large sample numbers.

Previously we demonstrated that the addition of a 30 amino acid sequence comprising the FMDV translational interrupter sequence (Δ1D2A) to either the N- or C-terminus of *Gaussia* luciferase (GLuc), a naturally secreted luciferase isolated from *Gaussia princeps* [8–10], does not prevent either GLuc secretion or luminescence [11]. Furthermore, GLuc activity can be measured directly in biological samples, including blood, serum, and urine [9, 10]. The addition of the Δ1D2A sequence to a GLuc 8990 mutant (SGLuc), which enhances luciferase output in the presence of cell lysis buffers [11, 12], also has no effect on SGLuc secretion or luminescence.

To determine if SGLuc- and Δ1D2A-containing constructs could be used to accurately quantify recombinant IFNα expression, we used the SGLuc-Δ1D2A and Δ1D2A-SGLucΔ1M variants to produce bicistronic single open reading frame vectors expressing SGLuc and porcine IFN proteins. These constructs represented an indirect interferon concentration assay because the luciferase and IFN proteins are separated upon translation,

**Fig. 1A.** Supernatant from transiently transfected cell cultures are subsequently evaluated for luciferase activity, derived from SGLuc, and anti-viral activity, derived from IFNα.

Interferon fusion proteins have been used for: (1) incorporation of reporter molecules [13, 14]; (2) immuno-therapy [15–18]; (3) enhancement of half-life [19–21]; (4) enhancement of activity [22], and (5) facilitation of secretion in non-mammalian systems [23]. To measure the interferon concentration directly via luciferase activity we constructed fusion proteins of SGLuc and porcine IFNα or IFNβ, identified as SGLuc-IFNα and SGLuc-IFNβ, respectively. In these SGLuc-IFN fusion proteins, the IFNα and IFNβ secretion signal peptides, normally removed during secretion by membrane-bound peptidases [24–29], were replaced with SGLuc. Because these constructs lack the Δ1D2A they remain a single expressed protein after translation which is capable of both luciferase and anti-viral activity, Fig. 1B.

This study evaluates both bicistronic and fusion protein constructs expressing SGLuc and type I interferons and utilizes luciferase activity to quantify interferon produced from transiently transfected cell lines.

**Fig. 1** Schematic diagram of methodology utilizing luminescence to quantify IFNα. **A** Indirect quantification is achieved through the FMDV 2A translational interrupter sequence, blue, that results in expression of luciferase and IFNα as separate proteins in an equimolar ratio in cell culture media. **B** Direct quantification is achieved utilizing a fusion protein consisting of SGLuc and IFNα that is secreted into cell culture media. The SGLuc-IFNα fusion protein retains both luminescence and anti-viral activity.
Methods

Construction of expression plasmids
Synthetically synthesized sequences for porcine IFNα, IFNB, SGLuc-IFNα and SGLuc-IFNβ were inserted into the pUC57kan vector (Genscript USA Inc) and subsequently cloned into a modified pTARGET™ vector (mpTarget) for mammalian expression. The sequence for IFNα was inserted into previously constructed Δ1D2A-SGLucΔ1M and SGLuc-Δ1D2A constructs for bi-cis-tronic vectors [11]. These constructs differ in whether the luciferase is on the N- or C- terminus of the Δ1D2A Foot-and-Mouth Disease Virus derived translational “skipping” mechanism. For the Δ1D2A-SGLucΔ1M construct the first methionine of SGLuc is also deleted. Plasmids were transformed into NEB® 5-α Competent E. coli (New England Biolabs) and plated on LB Agar plates with 100 μg/mL carbenicillin (Teknova, L1010). Selected colonies were grown in 4 mL of Terrific Broth with 100 μg/mL carbenicillin (Teknova, T7030) overnight at 37 °C, and plasmid purification was performed using QIAprep® Spin Miniprep kit (Qiagen, 27,106). Insertion was validated by sequence analysis using primers mpTarget-F (CTC ATC AAT GTA TCT TAT CAT GTC ) and mpTarget-R (CTCATCAAGTATCTTATCA GTC). Recombinant plasmid DNAs were purified utilizing a EndoFree Plasmid Maxi kit (Qiagen, 12,362).

Transfection of HEK293-T cells
Purified plasmid DNA was used to transfect HEK293-T cells (ATCC, CRL-3216), passage 48, at roughly 80% confluence in 6-well plates (Costar, 3516). Prior to transfection, growth medium, composed of 1X DMEM, 10% fetal bovine serum, 1X antibiotic–antimycotic, and 1X non-essential amino acids, was removed, and cells were rinsed with 1X DPBS (Gibco™, 14,190,250). Fresh media, 1 mL, was applied and transfections were performed using 4 μg of plasmid DNA and Lipofectamine™ 2000 (Thermo Fisher, 11,668,027). Cells were incubated overnight at 37 °C in a 5% CO₂ incubator. Media from transfected cells was harvested and stored in aliquots of 200 μL at -70 °C.

After removal of media, cells were washed with 1 mL of 1X DPBS (Gibco™, 14,190,250) and lysed by adding 1 mL of Mammalian Protein Extraction Reagent (M-PER™; Thermo Fisher, 78,501) with repeated pipetting. Lysates were stored at −70 °C.

Quantification of luminescence
Luminescence was assayed by injecting 100 μL of 50 μM water soluble coelenterazine (Nanolight, 3031–10) into 100 μL of harvested media diluted with fresh cell culture media. Luminescence was quantified from samples in 96-well white LUMITRAC™ 200 polystyrene microplates (Greiner Bio-one, 781075) analyzed in a 96-well Veritas™ microplate luminometer (Turner Biosystems) with an integration time of 0.5 s. Data is represented in relative luciferase units per half second (RLUs/0.5 s).

Western blotting of cell culture supernatant
Western blotting of cell culture supernatant was performed utilizing 60 μL of sample mixed with 30 μL of 4× NuPage LDS Sample Buffer (Invitrogen, NP0007), heated at 97°C for 10 min and 15 μL loaded into wells of 10-well NuPage 4–12% Bis–Tris gel (Invitrogen, NP0321Box). Gels were electrophoresed in 1× MES buffer (Invitrogen, NP0002) at 200 V for 35 min followed by transfer onto PVDF Pre-cut blotting membranes (Invitrogen, LC2002) utilizing the iBlot2 system (Invitrogen).

Membranes were incubated in a blocking buffer of 5% milk for 1 h at room temperature, then washed three times with 1X PBS-T (EMD Millipore, 524653-1EA) for 5 min each. Primary antibodies, polyclonal Antibera GLuc (Nanolight Technology, 401P), anti-IFNα (pbl Assay Science, 27100–1 Lot: 5795), and anti-IFNβ (ATCC, ab136385 Lot: GR142674–6), were added at 1:1000 dilutions of the secondary antibodies, goat anti-mouse-HRP (LGC Seracare, 5220-0338) or goat anti-rabbit-HRP (LGC Seracare, 5220-0335) applied to membranes for 1 h at room temperature. After three washes of 1× PBS-T membranes were incubated using the SIGMAFAST 3,3’-diaminobenzidine tablets (Sigma, D4293-50SET) as suggested by the manufacturer.

Cytopathic effect inhibition assay
Interferon induced inhibition of infection by VSV was used to evaluate interferon biological activity due to well-characterized acute VSV sensitivity to interferon [6, 30–32]. Cytopathic effect inhibition assays (CEI) were performed on samples as described previously [33]. Bovine derived MDBK cells (ATCC, CCL-22), passage 134, were infected with VSV-New Jersey (VSV-NJ) at a 0.0028 multiplicity of infection (MOI). Antiviral activity was reported as Interferon Antiviral Activity, the absence of cytopathogenic effect in 50% of tested wells, per 100 μL (IFNAA50/100 μL) or as the number of samples containing plaques.

Determination of IFN and SGLuc-IFN interferon concentrations by ELISA
Concentrations of porcine IFNα and SGLuc-IFNα samples were determined using a Porcine IFNα ELISA kit (Millipore Sigma, RAB1131-1KT), and a Porcine Interferon Beta ELISA kit (Novateinbio, NB-E50024) was used for IFNβ and SGLuc-IFNβ samples. Absorbance was
recorded at OD_{450} nm using an ELx 808 ultra microplate reader (BIO-TEK Instruments). Samples of IFNα and SGLuc-IFNα were assayed with two separate lots of the IFNα ELISA kit (Millipore Sigma, RAB1131-1KT) using at least three dilutions of each sample. Results were averaged to calculate the initial sample concentration.

**VSV plaque assays**

Plaque assays were performed using MDBK cells (ATCC, CCL-22), passage 134, plated on 6-well plates (Costar, 3516). Samples were tested in triplicate and results were averaged. MDBK cells, grown to full confluence, were treated with select dilutions of porcine IFNα, porcine IFNβ, SGLuc-IFNα, or SGLuc-IFNβ media. Commercially available porcine IFNα and porcine IFNβ were used as positive controls. After cells were incubated at 37 °C overnight with 5% CO₂, media was removed, and cells were gently washed with dPBS. Fresh media was applied, and cells were infected with VSV-NJ at a MOI of 0.0002 and incubated overnight at 37 °C with 5% CO₂. After incubation, media was removed, and cells were stained with 500 µL of 0.5% crystal violet in 20% Methanol to aid in visualizing plaques.

**FMDV plaque assays**

Plaque assays using FMDV were performed on FMDV permissive LFBK-αvβ6 cells, a porcine derived cell line transformed to stably express the bovine αvβ6 integrin, [34, 35], cultured in 6-well plates (Costar, 3516) to full confluence, treated with SGLuc-IFNα at appropriate dilution, and incubated overnight at 37 °C with 5% CO₂. After incubation, media was removed and 1 mL of supplemented DMEM (+2% FBS, +1% Anti-Anti, +1% L-Glut, +1% NEAA) media was applied gently to avoid disrupting the cell monolayer. The supplemented media was reduced to 100 µL followed by adding of 100 PFUs of FMDV serotype O1 Manisa to each well. After 1 h of incubation at 37 °C in 5% CO₂, 2 mL of an overlay (50% gum, 50% 2× MEM supplemented with 2% FBS, 1% anti-anti, 1% NEAA, 1% L-Glut) was added to each well, and plates were incubated overnight. The next day plates were stained with 1 mL of crystal violet and plaques were counted.

**Results and discussion**

**Indirect assay of IFNα levels using luminescence**

Cell culture media containing recombinant IFNα and SGLuc expressed from bi-cistronic vectors SGLuc-Δ1D2A-IFNα or IFNα-Δ1D2A-SGLucΔ1M, Fig. 2A, was assayed for luminescence and antiviral activity and compared to porcine IFNα expressed alone. Western blotting with anti-IFNα and anti-GLuc antibodies confirmed expression, secretion, and separation of the individual luciferase and interferon proteins from bi-cistronic vectors, Fig. 2B. Media from positive cell cultures was serially diluted, and luciferase activity was quantified for both IFNα-Δ1D2A-SGLucΔ1M and SGLuc-Δ1D2A-IFNα samples. For samples produced by both constructs, a strong correlation (R² > 0.99) was seen between relative luciferase units per half second (RLUs/0.5 s) and dilutions, Fig. 2C.

To further characterize the parameters of ELISA determined concentration, antiviral activity, and luciferase activity for the bi-cistronic constructs, we analyzed three independent batches produced by transfection with different concentrations of plasmid DNA, Table 1. The correlations among the three parameters was ≥ 0.96 for samples produced with the IFNα-Δ1D2A-SGLucΔ1M construct. Concentrations for the three batches of SGLuc-Δ1D2A-IFNα were not divergent with ELISA determined concentrations for all samples being within obtained standard deviations, complicating any attempt at validating correlations among tested parameters.
Expression of SGLuc and type I interferon fusion proteins

Fusion proteins of SGLuc and IFNα, Fig. 3A, were developed to measure interferon activity using luminescence as a proxy. For these fusion proteins, the first 22 amino acids of IFNα and IFNβ, encoding the native IFN secretion domains, were removed so secretion would be dependent upon SGLuc, Fig. 3A.

Expression and secretion of both fusion proteins, SGLuc-IFNα and SGLuc-IFNβ, was compared to native IFNα and IFNβ respectively. IFNα constructs produced bands correlating in size to monomers and probable aggregates, while IFNβ constructs produced a series of bands ranging in size from 15 to 28 kDa, suggesting post-translational modifications such as glycosylation, Fig. 3B. SGLuc-IFNα produced three bands at approximately 38 kDa, 70 kDa, and 80 kDa, Fig. 3B. The 38 kDa band is consistent with the predicted size of a monomer of SGLuc-IFNα with additional bands likely IFNα aggregate. SGLuc-IFNβ produced bands roughly 40 kDa and 80 kDa in size, consistent with the predicted size of a monomer and a probable aggregate, Fig. 3B. The lack of multiple bands in fusion-protein samples, as seen with similarly expressed control IFNβ, suggested that SGLuc-IFNβ does not undergo the same degree of post-translational modification as IFNβ.

Correlation of concentration, luciferase, and antiviral activity of SGLuc-IFN

Anti-viral activity of SGLuc-IFNα

The luminescence of media containing SGLuc-IFNα was linear over a dynamic range of dilutions, \( R^2 = 0.99 \), Fig. 4A. To correlate luciferase activity with antiviral activity, production cells were transfected with three different concentrations of SGLuc-IFNα encoding plasmids to produce independent batches. The resulting samples were used to compare luciferase activity with anti-viral activity as determined by plaque reduction assay.

The CEI assay was used to determine dilutions at which plaques could be found, data not shown. A 1/12800 dilution was selected for all three SGLuc-IFNα batches for plaque assays, and luciferase activity was determined using a 1/50 dilution, Fig. 4B. Luminescence and the mean number of plaque forming units were found to be inversely correlated (\( r = -0.90 \)) utilizing Pearson correlation, indicating a correlation between luminescence and the anti-viral activity of SGLuc-IFNα.

A single SGLuc-IFNα batch was used to confirm anti-viral activity against FMDV. Dilutions of 1/1600 and 1/3200 were selected based on CEI assay results, data not shown, and FMDV plaque production confirmed that the anti-viral effect of SGLuc-IFNα was not limited to VSV, Fig. 4C.

Anti-viral activity of SGLuc-IFNβ

The luminescence of media containing SGLuc-IFNβ was linear over a dynamic range of dilutions, \( R^2 = 0.99 \), Table 1. Activities of SGLuc-Δ1D2A-IFNα and IFNα-Δ1D2A-SGLuc Δ1M. Concentration was determined by porcine IFNα ELISA, luminescence by reactivity with coelenterazine (\( N = 3 \) replicates per sample), and anti-vSV activity by CEI (\( N = 4 \) replicates per sample). RLU/0.5 s, relative luciferase units per half second; CEI, Cytopathic effect inhibition assay; IFNAA50/100 μL, Interferon Antiviral Activity per 100 μL.

| Concentration measured by ELISA (μg/mL; ± SD) | Luminescence (RLUs/0.5 s) | CEI Assay IFNAA50 (log10)/100 μL |
|-----------------------------------------------|--------------------------|----------------------------------|
| SGLuc-Δ1D2A-IFNα                             |                          |                                  |
| Batch 1                                       | 5.58±1.39                | 2.99×10^10 1.14×10^9 4.85 4.78  |
| Batch 2                                       | 6.93±1.97                | 3.23×10^10 1.14×10^9 5.00 4.93  |
| Batch 3                                       | 2.27±0.84                | 1.31×10^10 5.81×10^8 4.55 4.55  |
| SGLuc-Δ1D2A-IFNβ                             |                          |                                  |
| Batch 1                                       | 9.89±1.15                | 2.61×10^10 5.78×10^9 5.15 5.08  |
| Batch 2                                       | 9.56±1.20                | 2.13×10^10 6.05×10^8 5.30 5.15  |
| Batch 3                                       | 11.68±3.98               | 2.40×10^10 7.30×10^8 5.30 5.15  |

Fig. 3 A Diagrams of mono-cistronic constructs expressing SGLuc-IFNα, SGLuc-IFNβ, IFNα, and IFNβ. In SGLuc-IFN constructs, the native secretion domains of porcine IFNα and IFNβ, pale red and pale green respectively, are replaced with the amino acid sequence for SGLuc. B Western blots of media from transfected cell cultures expressing native porcine IFN and chimeric SGLuc-IFNs using anti-IFNα, anti-IFNβ, and anti-GLuc antibodies demonstrate expression and secretion into cell culture media. Blots are cropped to focus on results and representative of \( N = 3 \) replicates; uncropped full-length blots are included in Additional file 1: Fig. S2.
Fig. 4A. A 1:100 dilution of media containing SGLuc-IFNβ was required to completely inhibit VSV plaque formation in the CEI assay compared to a 1:1600 dilution of IFNβ, Table 2. The decreased potency of SGLuc-IFNβ may be due to the lack of post-translational modifications seen in Fig. 3B. Because of the reduced antiviral activity, SGLuc-IFNβ samples were not tested further.

Comparison of SGLuc-IFNα and IFNα antiviral activity with concentrations determined by ELISA
Using Pearson correlation, we determined that there was a correlation, $r = -0.85$, between the antiviral activity and the interferon concentration in the IFNα and SGLuc-IFNα preparations, Table 3. Among the three preparations of SGLuc-IFNα alone, the antiviral activity was also correlated with concentration as determined by ELISA, $r = -0.76$. While comparisons of anti-viral

![Graph showing correlation between dilution and RLU/0.5s](image)

![Bar chart showing comparison of SGLuc-IFNα and IFNα at 1:12800 dilution](image)

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Table 2 Cytopathic effect inhibition assay with IFNβ and SGLuc-IFNβ samples

| Sample     | No. samples with vesicular stomatitis virus plaques in diluted growth media |
|------------|--------------------------------------------------------------------------|
|            | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12,800 |
| IFNβ       | 0/4   | 0/4   | 0/4   | 0/4   | 0/4    | 2/4    | 4/4    | 4/4      |
| SGLuc-IFNβ | 0/4   | 4/4   | 4/4   | 4/4   | 4/4    | 4/4    | 4/4    | 4/4      |

For determination of anti-viral activity for IFNβ and SGLuc-IFNβ samples, four wells for each dilution were evaluated for the presence of VSV plaques at 24 h post-infection. Wells were scored positive when plaques were observed.
activity between ELISA standardized IFNα and SGLuc-IFNα batches was not as linear as desired, this data demonstrated that fusion of SGLuc and IFNα to produce SGLuc-IFNα retained anti-viral activity when using equivalent concentrations. Future research may improve the understanding of this relationship.

Conclusion
This study sought to develop a method to rapidly determine cell culture-produced recombinant interferon concentrations through use of luminescence. We evaluated both direct and indirect means of measuring interferon activity. Direct covalent linking of SGLuc and IFNα into a single fusion-protein resulted in a high correlation between luminescence and anti-viral activity. Compared to current assays, the use of luminescence to quantify interferon concentrations enabled linear correlations across a dynamic range of concentrations, was faster, required less sample input, and was more compatible with high-throughput screening. Similar fusion proteins may be valuable research tools as fast and efficient means to analyze recombinant interferon concentrations. Further, the ability to detect GLuc activity in biological samples such as blood, serum, and urine opens the possibility of quick quantification of SGLuc-type molecules in animal models of disease or in ex vivo clinical samples.

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Author contributions
MP performed cloning and expression of proteins, western blotting, luciferase assay, ELISAs, and wrote manuscript. JB performed CEI and plaque assays for VSV. MS performed CEI and plaque assays for FMDV. MR contributed to experimental design and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Sequences of all constructs are available in U.S. patents 10,435,695 and 10,829,770.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
M. Puckette and M. Rasmussen are co-inventors on two U.S. patents, 10,435,695 and 10,829,770, as well as additional patent applications describing the fusion of interferon with luciferase used in these studies. J. Barrera and M. Schwarz have no competing interests.

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Supplementary Information
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Additional file 1: Source image files for western blots.

Table 3  Comparison of anti-viral activity for different concentrations of IFNs and SGLuc-IFNα

| Source     | Dilution | Concentration (pg/mL ± SD) | PFU (± SD) |
|------------|----------|---------------------------|------------|
| IFNα       | Batch 1  | 1/102400                   | 404 ± 151  | 80 ± 9.0  |
| IFNα       | Batch 1  | 1/51200                    | 807 ± 301  | 33 ± 3.2  |
| SGLuc-IFNα | Batch 1  | 1/12800                    | 738 ± 114  | 27 ± 9.6  |
| SGLuc-IFNα | Batch 2  | 1/12800                    | 948 ± 114  | 50 ± 1.7  |
| SGLuc-IFNα | Batch 3  | 1/12800                    | 1449 ± 214 | 3.7 ± 1.5 |

Concentration, as determined by ELISA, and SVV plaque forming units (PFU) in triplicate wells from select dilutions of IFNα and SGLuc-IFNα. Three preparations of SGLuc-IFNα were diluted to 1/12,800 for comparison across batches.
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