Evaluation of *Gaussia* luciferase and foot-and-mouth disease virus 2A translational interrupter chimeras as polycistronic reporters for transgene expression

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**Abstract**

**Background:** The *Gaussia princeps* luciferase is used as a stand-alone reporter of transgene expression for in vitro and in vivo expression systems due to the rapid and easy monitoring of luciferase activity. We sought to simultaneously quantitate production of other recombinant proteins by transcriptionally linking the *Gaussia princeps* luciferase gene to other genes of interest through the foot-and-mouth disease virus 2A translational interrupter sequence.

**Results:** We produced six plasmids, each encoding a single open reading frame, with the foot-and-mouth disease virus 2A sequence placed either N-terminal or C-terminal to the *Gaussia princeps* luciferase gene. Two plasmids included novel *Gaussia princeps* luciferase variants with the position 1 methionine deleted. Placing a foot-and-mouth disease virus 2A translational interrupter sequence on either the N- or C-terminus of the *Gaussia princeps* luciferase gene did not prevent the secretion or luminescence of resulting chimeric luciferase proteins. We also measured the ability of another polycistronic plasmid vector with a 2A-luciferase sequence placed downstream of the foot-and-mouth disease virus P1 and 3C protease genes to produce foot-and-mouth disease virus-like particles and luciferase activity from transfected cells. Incorporation of the 2A-luciferase sequence into a transgene encoding foot-and-mouth disease virus structural proteins retained luciferase activity and the ability to form virus-like particles.

**Conclusions:** We demonstrated a mechanism for the near real-time, sequential, non-destructive quantitative monitoring of transcriptionally-linked recombinant proteins and a valuable method for monitoring transgene expression in recombinant vaccine constructs.

**Keywords:** *Gaussia* luciferase, Foot-and-mouth disease virus, 2A, Bicistronic, Polycistronic, Biomarker, Virus-like particles

**Background**

Real-time sequential monitoring of recombinant protein production is advantageous over single-event, terminal monitoring that requires destruction of expressing cells in vitro or the *ex vivo* analysis of clinical samples. For example, transfected cell cultures may require lysis for detection of recombinant proteins of interest through polyacrylamide gel electrophoresis, western blots, ELISA, indirect fluorescent antibody assay or other methods. These detection methods are time-consuming, costly and often require protein-specific antibody reagents. Monitoring in vivo expression of recombinant proteins is more problematic. It requires invasive sampling at fewer time points, or terminal procedures, as well as protein-specific reagents.

Linking expression of a recombinant protein of interest to an easily detectable, secreted biomarker in a single open reading frame would allow for rapid, quantitative, and sequential monitoring of all proteins within the transcriptional unit. Moreover, using a secreted biomarker would be a useful tool for quantitating in vivo recombinant protein expression independent from host immune responses.
The *Gaussia princeps* luciferase (GLuc) is a naturally secreted luciferase that catalyzes oxidation of the substrate coelenterazine to produce an intense luminescent burst [1, 2]. GLuc is readily quantifiable in clinical samples (i.e. blood, plasma, and urine) within a linear detection range [3–8]. The luminescent output of wild-type GLuc is enhanced by mutation of two amino acid residues, F89W and I90L, resulting in a super-luminescent GLuc variant (SGLuc) with a peak emission wavelength of 481 nm [9]. We sought to use secreted GLuc (and GLuc variants) as a general biomarker to monitor overall expression of recombinant proteins from a single transcriptional unit. GLuc is non-native to the mammalian system. This allows for more definitive quantification than other enzymatic biomarkers, such as secreted embryonic alkaline phosphatase, which can have innate levels in vivo [10].

Production of multiple recombinant proteins from a single open-reading frame has been previously accomplished through use of proteolytic cleavage, self-processing peptides, multiple internal ribosome entry sites (IRESs), and other mechanisms [11, 12]. Foot-and-mouth disease virus (FMDV) encodes a nonstructural 2A translational interrupter which induces ribosome skipping causing the separation of the FMDV P1 and P2 polyproteins in a non-proteolytic manner [13, 14]. The efficiency of FMDV 2A-mediated translational interruption is amino acid sequence dependent, and its activity is enhanced when the additional sequence derived from the C-terminus of the FMDV 1D (VP1) protein is included [15, 16]. Four bicistronic constructs had the Δ1D2A sequence inserted on either the N- or C-terminus of either GLuc/SGLuc. We found that the methionine normally at position 1 was dispensable for translation initiation when the Δ1D2A was positioned on the N-terminus of GLuc/SGLuc (Fig. 2, results discussed below). Consequently, the final two bicistronic constructs were composed of GLuc/SGLuc templates with an N-terminal Δ1D2A sequence and a deletion of the position 1 methionine within the luciferase (Δ1M) (Fig. 1a).

**Results**

**Design of six bicistronic GLuc/SGLuc constructs**

A total of six bicistronic GLuc/SGLuc constructs were evaluated for retention of secretion and ability to luminesce (Fig. 1a). To facilitate efficient translational interruption in bicistronic constructs, we used a modified FMDV 2A sequence identified as Δ1Δ2A [17] consisting of the 11 C-terminal amino acids of VP1 (Δ1D), and the defined 18 amino acid 2A sequence with a C-terminal proline (Fig. 1b) [16]. Four bicistronic constructs had the Δ1Δ2A sequence inserted on either the N- or C-terminus of either GLuc/SGLuc. We found that the methionine normally at position 1 was dispensable for translation initiation when the Δ1Δ2A was positioned on the N-terminus of GLuc/SGLuc (Fig. 2, results discussed below). Consequently, the final two bicistronic constructs were composed of GLuc/SGLuc templates with an N-terminal Δ1Δ2A sequence and a deletion of the position 1 methionine within the luciferase (Δ1M) (Fig. 1a).

**Luciferase activity in cell culture**

Addition of the C-terminal Δ1Δ2A sequence in GLuc-Δ1Δ2A and SGLuc-Δ1Δ2A constructs resulted in a molecular weight increase of resulting proteins as visualized in western blots used to establish relative sample loading prior to quantifying luciferase activities (Fig. 2a). Luciferase activity was present in all samples except the histone negative control confirming that addition of the Δ1Δ2A sequence on either the N- or C-terminus of the luciferase did not prevent secretion or luminescence (Fig. 2b). Relative sample loading was used to account for the possibility that the addition of the Δ1Δ2A to GLuc/SGLuc may alter luminescent intensities. By establishing relative sample loading using an anti-GLuc polyclonal
antibody we were able to observe that the GLuc-Δ1D2A construct displayed 45 and 48% reductions in luminescent output versus the unmodified GLuc and Δ1D2A-GLuc constructs (Fig. 2b). The SGLuc-Δ1D2A construct exhibited a 59 and 68% reduction relative to Δ1D2A-SGLuc and SGLuc constructs (Fig. 2b). Deletion of the GLuc/SGLuc start codon, Δ1M, from N-terminal chimeras did not prevent SGLuc secretion and resulted in an 18% reduction compared to Δ1D2A-GLuc Δ1M and a 6% increase when compared to Δ1D2A-SGLuc.

**Luciferase activity in cell lysis buffer**

Despite previous reports identifying the SGLuc variant as emitting approximately 10-fold higher luminescence than GLuc from lysed cells [9], we measured a 36% average increase in luminescence in cell culture media (Fig. 2b). To determine if the choice of cell lysis buffer affected the luminescence output between GLuc and SGLuc, we combined harvested cell media from each variant with one of two common cell lysis buffers (2× Luciferase Cell Lysis Buffer [LCLB] and Mammalian Protein Extraction Reagent [M-PER]), and measured luciferase levels (Fig. 3). The SGLuc and GLuc constructs lost >30 and >80% activity when diluted in either cell lysis buffer as compared to control cell culture media, but the SGLuc construct retained approximately four-fold more luciferase activity than the GLuc construct in either cell lysis buffer.

**A biomarker for expression in FMDV VLP producing constructs**

To determine if the transcriptionally-linked luciferase affected the ability of a FMDV transgene to produce virus-like particles (VLPs), we added the Δ1D2A-SGLuc chimera to the C-terminus of a plasmid vector, P1-2A-3C, capable of producing FMDV VLPs in transfected cell cultures (Fig. 4). Luciferase activity was readily detected in media from LFBK-αvβ6 cells transfected with the FMDV P1-2A-3C-Δ1D2A-SGLuc construct (Fig. 5).

Using immunoelectron microscopy (IEM) we observed electron-lucent arrays of 25–30 nm diameter FMDV VLPs in the cytoplasm of LFBK-αvβ6 cells transfected with either P1-2A-3C or P1-2A-3C-Δ1D2A-SGLuc plasmids (Fig. 6). Immunogold labeled FMDV conformation dependent monoclonal antibody (Mab) (F21) localized to these array structures (Fig. 6). Immunofluorescent assays with two additional non-conformation dependent FMDV-specific Mabs, 6HC4 and 12FE9, further validated serotype-specific expression of FMDV proteins in transfected cells (Fig. 7).

**Discussion**

We produced GLuc/SGLuc Δ1D2A luciferase chimeras that retained both secretory and luciferase activity, and demonstrated use of these chimeras as tools to quantitate transgene expression through sequential, non-destructive sampling of all culture media.
In our system, the SGLuc variant showed enhanced luminescence over GLuc, but at lower levels than previously reported [9]. We showed that this difference is influenced by the choice of cell lysis buffer, and may explain the differences in luciferase intensities observed between our results and those previously reported utilizing secretion-deficient mutants harvested from lysed cells [9]. The reduction of luciferase activity in cell lysis buffer may be due to the presence of denaturing conditions which may have more impact on GLuc than on SGLuc. Constructs containing a C-terminal Δ1D2A showed less luminescence despite having similar levels of protein tested, as determined by western blot. These data suggest that the luminescent activity of these chimeras was inhibited compared to N-terminal and GLuc/SGLuc constructs.

We produced a plasmid encoding the FMDV P1, 2A, 3C genes in-frame with the Δ1D2A-SGLuc sequence on the 3′ terminus. This marker-vaccine construct retained the ability to produce assembled FMDV VLPs along with a concomitant readout of luciferase activity in culture media as an easily quantifiable marker of FMDV protein production. Notably, the VLP crystalline arrays produced in this system appeared to be identical to those previously reported from cells infected with wildtype FMDV [18, 19]. The conformation dependent F21 Mab will only interact with VP2 in the proper confirmation, such as when it is contained in a VLP. As FMDV VLPs form and become localized within arrays it results in the localization of F21 binding to these arrays as observed in IEM.

Rapid quantitation of recombinant protein levels is a critical tool for molecular biology. The system described here provides a direct correlate to quantitate recombinant proteins produced in various matrices, including clinical samples, without the need for antigen-specific reagents. For development of recombinant vaccines, this system may provide a mechanism to monitor recombinant antigen production in vivo following vaccination, determine vaccine vector expression kinetics, optimize delivery methods, and correlate the amount of recombinant antigen produced with host immune response, and protection from infection. Lastly, this reporter system may be widely applicable to monitor transgene expression for a variety of biomedical purposes including gene therapy, drug screening, vaccine production, and bio-manufacturing.

**Conclusions**

We describe the use of Gaussia luciferase chimeras as a reporter system for quantifying recombinant gene expression. GLuc/SGLuc variants with the FMDV Δ1D2A sequence placed on either the N- or C-terminus retained secretory and luciferase activity that enabled rapid, sequential quantitation of transgene expression through non-destructive sampling without need for protein-specific detection reagents. Placing the Δ1D2A-SGLuc sequence downstream of an FMDV VLP
Fig. 6 Immunoelectron microscopy images using immunogold coated with monoclonal antibody F21 to localize FMDV VP2 in the virus-like particle arrays of LFBK-αVβ6 cells transfected with construct P1-2A-3C-Δ1D2A-SGLuc at a 5000× (bar, 500 nm) and b 25,000× (bar, 100 nm) as well as construct P1-2A-3C at c 5000× (bar, 500 nm) and d 25,000× (bar, 100 nm).

Fig. 7 Immunofluorescent assay with FMDV specific monoclonal antibodies 6HC4, 12FE9 and F21 on LFBK-αVβ6 cells transfected with constructs encoding either SGLuc, P1-2A-3C, or P1-2A-3C-Δ1D2A-SGLuc (bar, 200 μm).
production cassette resulted in both VLP production and luciferase activity, thereby demonstrating the practical application of this technology to recombinant vaccine development.

**Methods**

**Insertions into pTarget plasmid**

GLuc template sequence was obtained from the pMCS-Gaussia Luc plasmid (Thermo Fisher Scientific). PCR was performed using OneTaq 2× Master Mix with Standard Buffer (New England Biolabs) and primers GLuc-F and GLuc-R (Table 1). Insertion into the pTarget vector (Promega) followed manufacturer's instructions for T/A cloning. Plasmids were sequenced using primers T7 and Seq-R (Table 1), and analyzed using the Sequencher 4.8 program (GeneCodes).

PCR of bovine Histone H3 was performed using OneTaq 2× Master Mix with Standard Buffer (New England Biolabs) with primers H3-F and H3-R (Table 1), using cDNA synthesized from isolated bovine mRNA. Sequencing and analysis were performed as described above.

**Mutation to SGLuc**

SGLuc was generated using the pTarget GLuc template and site-directed mutagenesis using the GENEART Site-Directed Mutagenesis System (Invitrogen) with primers SGLuc8990–MF and SGLuc8990–MR (Table 1).

| Primer name | Sequence |
|-------------|----------|
| GLuc-F | TGGCCGCCGCCACCATGGGAGATCAA |
| GLuc-R | GAGGCTGATTTTGCGTCTAGA |
| T7 | TATACGACTCACTATAGGG |
| Seq-R | TTACGCCAAGTTATTTAGGTGACA |
| H3-F | AAGGAGCTGAGCCACCATGGCTCGT |
| H3-R | GTTACCTTAAGCAGTTCCTCA |
| SGLuc8990–MF | CCAAGATGAAAGGTGGCTCCACGAGAAGCTG |
| SGLuc8990–MR | GCCAGGCTCTGGAGCCAATCTTCATCTTGG |
| GLuc-NS-Nhel-R | CGGCAAGCTGACCAACCCGGCCGCCC |
| Ascl-Kzk-GLuc-F | TTGCGGCGCCGGCACTATGGGAGTCAA |
| 2A-Xmrl-R | TATACCGGTTGACCTTCAGAAGT |
| GLuc-R-NotI | GGCGCCGCTTAGTCACCCAGCAG |
| GLuc8990–MF | CCAAGATGAAAGGTGGCTCCACGAGAAGCTG |
| GLuc8990–MR | GCCAGGCTCTGGAGCCAATCTTCATCTTGG |
| No Met GLuc-MF | TCCACCGTGGCGGAGATCTTCGAC |
| No Met GLuc-MR | CAGAATTTGCT GCCGGGAGGAAGTTG |
| NotI-3CLeb89–F | CAGCGGCCGATAGGGCAGCCAC |
| 3CLeb89–EcoRI-R | GAATTTCTAGGGTGGTGGT |
| 3CLeb89–ns-EcoRI-R | GAATTTCTAGGGTGGTGGT |

**Creation of GLuc/SGLuc-Δ1D2A chimeras**

For construction of GLuc/SGLuc-Δ1D2A chimeras, the Δ1D2A-GLuc/SGLuc chimera was used as a template for site-directed mutagenesis using the GENEART Site-Directed Mutagenesis System (Invitrogen) with primers GLuc8990–MF and GLuc8990–MR (Table 1). Confirmation of mutagenesis was performed by sequencing with primers T7 and Seq-R (Table 1), as described above.
of mutation was performed by sequencing with primers T7 and Seq-R as described above.

Creation of Δ1D2A-GLuc/SGLuc Δ1M chimeras
To construct Δ1D2A-GLuc/SGLuc Δ1M chimeras, pTarget Δ1D2A-GLuc and pTarget Δ1D2A-SGLuc template were used for site-directed mutagenesis using the GENEWANG Site-Directed Mutagenesis System (Invitrogen) with primers No Met GLuc-MF and No Met SGLuc-MR (Table 1). Sequencing confirmation used primers T7 and Seq-R (Table 1), as described above.

Creation of FMDV VLP producing constructs
Nucleotide sequence derived from FMDV O1 Manisa serotype and coding for the P1 polyprotein was synthesized by GenScript and cloned into a modified pTarget vector using BamHI-HF and NotI-HF restriction enzymes (New England Biolabs). For the P1-2A-3C construct, insertion of FMDV 3C was performed by using PCR amplification with primer NotI-3CLEb89-F and primer 3CLEb89-EcoRI-R (Table 1). For the creation of the P1-2A-3C-Δ1D2A-SGLuc construct, insertion of 3C was performed by using PCR amplification with primer NotI-3CLEb89-F and primer 3CLEb89-ns-EcoRI-R (Table 1). The sequence for Δ1D2A-SGLuc was inserted 3’ of the 3C sequence by digestion of the template with EcoRI-HF and XmnI restriction enzymes (New England Biolabs). All ligation were performed as described above. Confirmation of insertion was performed by sequencing with primers NotI-3CLEb89-F, 3CLEb89-ns-EcoRI-R (Table 1), as described above.

Transfection and harvesting of HEK293-T cells
HEK293-T cells (ATCC® CRL-11268®) were maintained in 1× MEM media (Gibco) with 10% Fetal Bovine Serum defined (HyClone), 1× Antibiotic-Antimycotic (Gibco), and 1× Glutamax (Gibco). At passage 58, HEK293-T cells were transfected with each of the six bicistronic chimeras, a negative control plasmid (Histone), or one of two positive control plasmids (GLuc and SGLuc) in a six-well plate using Lipofectamine 2000 (Invitrogen). After incubating for 24 h in a 37 °C CO2 incubator, media was removed and stored at 4 °C. Attached cells were removed from plates with 200 μl of 2× Luciferase Cell Lysis Buffer (Thermo Fisher Scientific) and stored at -70 °C.

Luciferase assay
To adjust for differential expression of transgene among samples, harvested media was mixed with 4× NuPage LDS Sample Buffer (Invitrogen), heated at 97 °C for 10 min, and loaded into wells of a 10-well NuPage 4-12% Bis-Tris gels (Invitrogen). Gels were electrophoresed in 1× MES buffer (Invitrogen) at 200 V for 35 min. Samples were transferred onto membranes using the i-Blot system (Invitrogen). Membranes were incubated in 5% milk blocking buffer for 40 min and washed 3 times with 1× phosphate buffered saline (PBS)-Tween (PBS-T) buffer (EMD Millipore) for 5 min each. A 1:1000 dilution of rabbit polyclonal Antiseras-GLuc (NanoLight Technologies) was used for primary antibody incubation with shaking at room temperature for 1 h. Membranes were washed three times with 1× PBS-T for 5 min each after primary antibody incubation. A 1:500 dilution of goat anti-rabbit-HRP secondary antibody (KPL) was applied and incubated with shaking at room temperature for 1 h, followed by three washes with 1× PBS-T for 5 min each. DAB staining was performed using SIGMAFAST 3,3’-Diaminobenzidine tablets (Sigma) dissolved in 15 μl of ddH2O for 1 h followed by de-staining with two rounds of washing with 1× PBS-T for 5 min. Values of media loaded onto the gel were adjusted until roughly equal loading was obtained for each sample as shown by similar staining intensity.

Luciferase activity was measured using a 96-well BioSystems Veritas luminometer (Turner Biosystems). For unadjusted samples, 20 μl of harvested media was used and readings taken with no delay after an injection of 25 μl of 100 μM water soluble coelenterazine solution (NanoLight Technologies). An integration time of 0.5 s was used for data collection both before and after injection of coelenterazine. Readings for pre-injection were used to establish a baseline of light emission at the time of injection and subtracted from the post-injection values during data analysis. Replicates were averaged together to give relative luciferase units per half second (RLU/0.5 s). Media from non-transfected HEK293-T cells was used to dilute samples as needed to maintain a constant volume.

To measure the effects of cell lysis buffers 10 μl harvested GLuc/SGLuc media was mixed with 90 μl of either cell culture media, 2× Luciferase Cell Lysis Buffer (LCLB, ThermoFisher), or Mammalian Protein Extraction Reagent (M-PER, ThermoFisher Scientific). LCLB and M-PER were selected as test buffers to represent a buffer that is supplied with luciferase reporter assays, LCLB, and a commonly used mammalian cell lysis buffer, M-PER. A total of 100 μl was used in each well with no delay after an injection of 25 μl of 50 μM water soluble coelenterazine solution (NanoLight Technologies). Readings were processed as above and used to calculate percentage of luciferase activity retained in added lysis buffer compared to cell culture media.

Transfection of LFBB-αVβ6 cells
LFBB-αVβ6 cell line [20, 21], was grown in 1× DMEM media (Gibco) with 10% Fetal Bovine Serum defined (HyClone), 1× Antibiotic-Antimycotic (Gibco), 1×
MEM-NEAA (Gibco), and 1x Glutamax (Gibco). Cells were transfected at passage 44 using Lipofectamine 2000 (Invitrogen). After incubating for 24 h in a 37 °C CO₂ incubator, media from transfected cells were removed and used for luciferase assays as previously described while cells were harvested and used for immunoassays.

**IEM and IFA**

To provide the volume of cells necessary for immunoelectron microscopy and for immunofluorescent assays (IFA), LFBK αVβ6 cells were grown and transfected in T-75 flasks. Immunoassays were performed using three different FMDV Mabs supernatants 6HC4, 12FE9, and F21 [22, 23]. The 6HC4 antibody does not react with FMDV Type O and was used as a negative control. Antibodies 12FE9 and F21 are reactive to FMDV type O VP1 and VP2 proteins respectively. For IEM, samples were fixed with a solution containing 4% paraformaldehyde (Electron Microscopy Sciences) with 1% periodate and 1% lysine in 0.1 M sodium cacodylate buffer (pH 7.4), embedded in 2% agarose, dehydrated to 70% ethanol, and embedded in medium grade LR White resin (Electron Microscopy Sciences). Ultrathin (80 nm) sections were cut on a Leica UC6 with a diamond knife, collected on formvar-carbon coated nickel grids (Electron Microscopy Sciences), stained with 1% uranyl acetate and lead citrate for 2 mins, mounted with PolyPrep™ Gold (Electron Microscopy Sciences), and 2% uranyl acetate and lead citrate were contrasted with 2% uranyl acetate and lead citrate and lead citrate and imaged with a Hitachi 7600 at 80 kV with a digital camera (Advanced Microscopy Techniques).

For IFA, suspensions of transfected LFBK-αVβ6 cells were attached (CytoSpin) onto Superfrost Plus Gold Slides (EMS) and fixed with acetone:methanol at 4 °C for 10 min. Slides were blocked with 10% fetal bovine serum (FBS) and incubated with Mabs diluted 1:10, washed. Antibody binding was localized using Fab goat anti-mouse fragment conjugated to 1.4 nm nanogold (Nanoprobes) and enhanced with GoldEnhance EM (Nanoprobes). Labeled sections were contrasted with 2% uranyl acetate and lead citrate and imaged with a Hitachi 7600 at 80 kV with a digital camera (Advanced Microscopy Techniques).

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**Availability of data and materials**

The datasets used and/or analyzed during the current study available from the corresponding author upon reasonable request.

**Authors’ contributions**

MP designed experiments, produced chimeras, performed transfections, western blots, and luciferase assays, and drafted the manuscript. TB performed IEM and IFA experiments. JN and MR assisted in experimental design. All authors read and approved the final manuscript.

**Competing interests**

MP and MR are co-inventors on a U.S. patent application.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

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