Bioluminescence monitoring of promoter activity *in vitro* and *in vivo*

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Summary

The application of luciferase reporter genes to provide quantitative outputs for the activation of promoters is a well-established technique in molecular biology. Luciferase catalyses an enzymatic reaction, that in the presence of the substrate luciferin, produces photons of light relative to its molar concentration. The luciferase transgene can be genetically inserted at the first intron of a target gene to act as a surrogate for the gene's endogenous expression in cells and transgenic mice. Alternatively, promoter sequences can be excised and/or amplified from genomic sources or constructed *de novo* and cloned upstream of luciferase in an expression cassette transfected into cells. More recently, the development of synthetic promoters where the essential components of an RNA polymerase binding site and transcriptional start site are fused with various upstream regulatory sequences are being applied to drive reporter gene expression. We have developed a high-throughput cloning strategy to develop lentiviral luciferase reporters driven by transcription factor activated synthetic promoters. Lentiviruses integrate their payload cassette into the host cell genome, thereby facilitating the study of gene expression not only in the transduced cells, but also within all subsequent daughter cells. In this manuscript we describe the design, vector construction, lentiviral transduction and luciferase quantitation of transcription factor activated reporters (TFARs) *in vitro* and *in vivo*.

Key Words

Luciferase, Lentivirus, Promoter, Transcription Factor, Bioluminescence

1. Introduction

The broad concept of exploiting reporter genes to mark or even quantify gene promoter activity in cells and tissues has traditionally employed bacterially-derived enzymes (β-galactosidase and chloramphenicol acetyltransferase –CAT) or an array of fluorescent proteins originating from a myriad of exotic sources. More recently, the ability to quantify promoter activation with greater fidelity and linear range has been made possible by quantifying photonic emission from luciferase activity.

Luciferase reporter genes

The North American firefly luciferase, *Photinus pyralis*, was the first to be cloned and characterized [1]. Firefly luciferase (FLuc) catalyzes an oxidative reaction in the presence of its substrate D-luciferin and adenosine triphosphate (ATP), Mg^{2+} and O_2. The reactive production of the intermediate oxyluciferin releases a photon of light with a quantum yield of 0.88 photons per molecule of luciferin [2]. FLuc in particular is a good biomarker as it requires no post-translational modifications, and has a half-life of approximately 3 hours *in vitro* and *in vivo* allowing adequate timing for analysis [3]. The emission wavelength of FLuc ranges between 530-640 nm, with a peak at 560 nm [4]. Since molecular methods which employ luciferase as a tool occur outside of its natural firefly host, many luciferase constructs have undergone a process of molecular evolution known as codon optimisation.

Species-specific codon usage bias results in higher levels of heterologous protein expression due to the enhanced translation processes related to the relative abundance of particular tRNA isoforms [5]. For example, a high GC content at the 3rd position of the codon, also known as the silent-site, correlates with an increase in gene expression efficiency within mammalian cells [6]. FLuc DNA has been further improved through the removal of sequence repeats, cryptic splice sites, and local hairpin structures to increase mRNA stability [7].

Many different luciferase enzymes have been cloned, including those from the sea pansy (*Renilla*) [8], the railroad-worm (*Phrixothrix vivianii)* [9], the click beetle (*Pyrophorus plagiophthalamus*) [9, 10], and the jellyfish (*Aequorea victoria*) [11]. Luciferases originating from the sea pansy *Renilla reniformis* (Rluc), and numerous other ocean-derived organisms oxidize an imidazopyrazinone-based luciferin, coelenterazine, which releases light mostly within the blue-green range at a wavelength of 480 nm [12] in an ATP-independent reaction. Vargula luciferase is derived from the nocturnal sea ostracod, *Vargula hilgendorffii,* which naturally inhabits the waters of Japan where it is more commonly known as the “sea firefly”.

Historically, vargula has also been named cypridina, owing to the fact that it belongs in the family Cypridinidae [13]. In the presence of molecular oxygen and the absence of ATP, vargulin, its substrate, is oxidized and emits light with a peak emission of around 452 nm. This peak emission, however, can shift between 448 and 463 nm depending on the pH and salinity of the environment in which the reaction takes place. Interestingly, Thompson *et al*. showed through sequencing of the vargula gene, that it contains a signal sequence for secretion, and went on to show that the vargula luciferase, secreted from mammalian cells into culture medium, is well suited as a specific and highly sensitive reporter [14].

NanoLuc® (NLuc) luciferase is a small luciferase of only 19 kDa, isolated from the deep sea shrimp *Oplophorus gracilirostris* within which it is used as a defense mechanism against predation. The sequence has been isolated and cloned, with the aim of producing a luciferase capable of producing a sustained signal with high sensitivity and low background interference. Following multiple rounds of mutagenesis, this enzyme was engineered to have improved luminescence and stability, and has been codon optimized for mammalian expression. NLuc utilizes the substrate coelenterazine, or ideally its more stable derivative, furimazine, in an ATP-independent reaction. The reaction produces luminescence with an emission peak at 454 nm and a signal half-life of >2h. In contrast to the flash luminescence produced by other luciferases NLuc produces a glow-type luminescence that is not as bright, but can be sustained for hours. Using a secretion signal from the human IL-6 protein, a signal sequence was appended to the N-terminus of the reporter, allowing it to be secreted out of the cell. The secreted NLuc has also been designed to have increased thermal stability, maintaining its enzymatic activity up to 55°C and for >15 hours at 37°C in culture medium [15].

Transcription factor activated luciferase reporters

Luciferase reporter vectors have been widely used in the study of promoter activity after transfection into cell lines. Most often promoters or elements of promoters are cloned upstream of the reporter in order to measure its activity either in steady state or after stimulation by an agonist. More recently the design of synthetic promoters incorporating multiple regulatory sequence motifs has enabled the targeted interrogation of gene enhancer and transcription factor inter-relationships. These experiments are often carried out by plasmid transfection and are thus transient. Synthetic promoters whereby serial minimal transcription factor binding consensus sequences upstream of a minimal polymerase initiating sequence drive reporter activity have been variously described [16]. For

example, Pessara *et al*. first described the application of an NFκB responsive promoter driven reporter to assay TNFα mediated inflammatory responses in a cell model [17]. The canonical NFκB (p50/p65) genomic binding has been further defined as 5'-GGGACTTTC-3' and synthetic promoters containing serial repeats of this sequence are dose responsive to NFκB activators. In certain circumstances it may be required to measure transcription factor activity in the long-term or in phenotypically dynamic cells such as stem cells. We recently developed a library of transcription factor activated luciferase reporters cloned into lentiviral vectors [18]. We utilised these in the study of cell signalling networks in human dermal fibroblasts as they reprogram to induced pluripotent stem cells [19]. This new innovation means that transcription factor activity can be assayed in the long-term in living, changing cells.

Building upon these *in vitro* tools, transgenic mouse strains have been generated in which luciferase expression was controlled by estrogen responsive elements [20] and NF-kB response elements [21], amongst others. However, luciferase activity in the early mouse experiments could only be assayed *post-mortem* by *ex vivo* luminometry. The development of highly sensitive charge-coupled device (CCD) cameras has enabled the quantification of luciferase activity in living rodents in a continual and non-invasive manner [22]. This has permitted continual measurement of transcription factor activity in live rodents, in numerous models of inflammation [23]. However, in germ line transgenic strains it is difficult to distinguish transcription factor activity in specific organs due to the whole-body nature of the transgenesis. Following gene transfer to neonatal mice, using viral vectors, we have demonstrated immune tolerisation [24] and long-term gene expression [25]. By exploiting these advantages of neonatal administration, we have been able to apply our library of lentiviral reporter constructs to generate somatic transgenic rodents in which transcriptional activity can be monitored continually and in targeted organs and tissues [18] (See Figure 1).

2. Materials

2.1 Construction of lentiviral reporter gene cassette

1. pLNT-Gateway-MCS was developed as a parental vector by Dr. Steven Howe, UCL and provided as a kind gift.
2. Primers and template to amplify desired reporters. We used both a fluorescent and a luminescent reporter.

Primer	Sequence (5' --> 3')
3xFLAG (F1)	CTGGGGCCACGA**G▼GATCC**GCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATC
3xFLAG (R1)	TTCTTGGCGTCCTCCATGCTGCCGCCGCCGCTCTTG
Fluc (F2)	GCAGCATGGAGGACGCCAAGAACATCAAGAAGGG
Fluc (R2)	CTGCGCGGATCTTGCCGCCCTTCTTGGCC
2A-eGFP (F3)	AAGGGCGGCAAGATCCGCGCAGAGGGCCGGGGCTCAT
2A-eGFP (R3)	GTCAGCTGGGCA**ATGCA▲T**ACTAGTTTGTTGAGTCAAAACTAGAGCCTGGACCA

3. High-fidelity polymerase
4. dNTPs
5. PCR cleanup kit
6. 1.5% agarose gel
7. Gel extraction kit
8. *Xho* I and *Mlu* I restriction enzymes
9. Quick Ligase
10. SOC outgrowth media
11. ccdB resistant competent cells such as DB3.1 or One Shot® ccdB Survival™ 2 T1R Competent Cells (*see* **Note 1**)
12. Luria broth (LB)
13. Ampicillin (100 µg/ml)
14. Plasmid DNA mini-prep kit
15. WPRE reverse primer for sequencing

2.2 Design and construction of transcription factor activated synthetic promoter

1. *De novo* synthesis of minimal promoter and synthetic promoter DNA was performed by Aldevron, Fargo ND, USA

Primer	Sequence (5' --> 3')
Minimal promoter (Forward)	C▼TCGAGGGGCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTCACTCTCTTCCC▼TCGAG
Minimal promoter (Reverse)	GAGCT▲CCCCGATATTTTCCCCCACCCCCGCGCAAGCAGGAGTGAGAGAAGGGAGCT▲C

2. TOP10, DH5α or similar regular cloning competent cells
3. LB containing kanamycin antibiotic (50 µg/ml)
4. Plasmid DNA mini-prep kit
5. T7 primer for sequencing

2.3 Cloning synthetic promoter into the lentiviral reporter gene cassette

1. LNT-GW-Luc/eGFP destination vector
2. pENTR-MP vector containing choice of synthetic promoter
3. TE Buffer (pH 8.0)
4. LR clonase II (Invitrogen) (*see* **Note 2**)
5. Proteinase K
6. Stbl3 competent cells (*see* **Note 3**)
7. SOC outgrowth media
8. 2x ampicillin laden broth and plates (100 µg/ml)
9. Thermocycler or waterbath to be used at 25°C and 37°C respectively
10. Sterile glass spreader
11. Plasmid DNA mini-prep kit
12. *Bam*HI restriction enzyme

2.4 Generation of high-titer TFAR lentivirus

1. Highly proliferating, mycoplasma-free HEK293T cells (*see* **Note 4**)
2. OptiMem® I reduced-serum medium (Gibco)
3. Phosphate buffered saline (PBS) containing calcium and magnesium (*see* **Note 5**)
4. Vasicular Stomatitis Virus glycoprotein (VSV-g) envelope plasmid (pMD2.G)
5. Packaging plasmid containing gag, pol, tat, rev viral genes (pCMVΔR8.74)
6. Transfer plasmid containing the transgene (pLNT-TFAR-Luc/eGFP)
7. Polyethylenimine transfection reagent (10 mM): 10 ml branched PEI made up to 41.2 ml with dH_2O (pH 7.0) (*see* **Note 6**)
8. 0.45 µM PVDF sterile filter cups (*see* **Note 7**)
9. Complete Media: Dulbecco's Modified Eagles Media, 10% FBS, 1% penicillin/streptomycin
10. Virkon™

2.5 Lentiviral TFAR transduction of cells

1. Target cell line to be transduced
2. Concentrated lentivirus containing LNT-TFAR-Luc/eGFP
3. Appropriate target cell media
4. Polybrene or alternative cationic polymer for increased transduction

2.6 *In vitro* luciferase quantitation to measure TFAR activity.

1. Opaque, white 96-well plate
2. Luciferase lysis buffer: (0.5M Tris, 0.5M Ethylenediaminetetraacetic acid (EDTA), 0.5M NaCl, 0.65% NP-40)
3. Luciferase assay buffer: (25 mM Tris Phosphate (pH7.8), 1% Triton X-100, 1 mM EDTA, 1 mM Dithiothreitol (DTT), 8 mM MgCl, 5% bovine serum albumin, 30% glycerol.
4. D-luciferin substrate: (Gold Biotechnology) reconstituted in PBS to a working concentration of 3 mM
5. GloMax luminometer (Promega) or alternative luminometer preferably with injectors
6. Bradford assay reagent
7. Transparent 96-well plate
8. Absorbance reader

2.7 Neonatal administration of lentiviral TFAR

1. P0 (day of birth) neonatal mice (see **Notes 8 and 9**)
2. 33 gauge Hamilton needle and 100 µl syringe (Fisher Scientific) (see **Note 10**)
3. Wet ice
4. High titred VSV-G TFAR lentivirus vector

2.8 Continued monitoring of TFAR activity in living mice

1. D-luciferin (Gold Biotechnology) reconstituted in sterile PBS to a working concentration of 15 mg/ml
2. 27 gauge needle (see **Note 11**)
3. 1 ml syringe
4. Anesthetic, 100% Isoflurane inhalation liquid vapor liquid (Abbott)
5. Oxygen - 5.1%, flow rate 1.5 L/min

6. IVIS machine (Perkin-Elmer)

3. Methods

3.1 Construction of lentiviral reporter gene cassette

1. Primary PCR amplification of individual 3xFLAG-FLuc and 2A-GFP sequences.
2. Secondary, overlap extension PCR performed to anneal and create Fluc-2A-eGFP insert using the forward primer of FLAG (F1) and the reverse primer of GFP (R3).
3. Gel extract fused product, restriction enzyme digest, and heat inactive enzymes by incubating at 80°C for 20 minutes.
4. Digest LNT-Gateway-MCS vector using compatible ends to those generated for the insert (we employed *Xho* I / *Mlu* I), electrophorese and gel extract the correct sized band.
5. Ligate the Fluc-2A-eGFP insert into the digested pLNT-Gateway-MCS vector.
6. Transform ligated plasmid into chemically competent DH5α *E.Coli* bacteria, preferably a recA strain to prevent plasmid recombination due to the presence of lentiviral LTRs. Use a standard heat shock protocol.
7. Resuspend the transformation solution in 500 µl SOC medium and plate out 50µl on LB agar plates containing ampicillin (30 µg/ml) and incubate inverted plates at 37°C overnight.
8. Screen colonies for the presence of insert using appropriate restriction digest.
9. Confirm sequence integrity by sequencing using a WPRE reverse sequencing primer.

3.2 Design and construction of transcription factor activated synthetic promoter

1. *De novo* synthesise minimal promoter (MP) sequence flanked by *Xho*I sites and clone into unique *Xho*I site in the multi-cloning site of the pENTR-1A Gateway cloning vector to produce pENTR-MP. We employed the adenoviral E1A minimal promoter.
2. Transform ligated plasmid into chemically competent DH5α *E.Coli* bacteria by a standard heat shock protocol.
3. Resuspend the transformation solution in 500 µl SOC medium and plate out 50µl on LB agar plates containing ampicillin (30 µg/ml) and incubate inverted plates at 37°C overnight.
4. Sequence clones to select correctly oriented MP.
5. Derive minimal consensus binding sequence for the candidate transcription factor

from the literature.

6. Design serial transcription factor binding sequence (TFBS) by interspersing 4-10 binding sequences with 10 random nucleotides.
7. *De novo* synthesize the resultant sequence with restriction enzyme sites at the 5'- and 3'- termini. We employ *Bam*HI and *Eco*RI for pENTR-MP which removes the ccdB and chloramphenicol resistance genes.
8. Directionally clone TFBS into pENTR-MP using TOP10 or DH5α competent cells to produce pENTR-Prom and grow on kanamycin-laden plates and LB.
9. Confirm clones by sequencing using T7 primer.

3.3 Cloning synthetic promoter into the lentiviral reporter gene cassette

1. Set up a recombination reaction by incubating 80 ng of pENTR-MP vector containing the desired synthetic promoter with 80 ng of the LNT-GW-Luc vector. (*see* **Note 12**)
2. Make up to 4.5 µl with TE buffer.
3. Mix the Gateway® LR Clonase Enzyme Mix twice for 2 seconds each time and immediately return to -20°C to maintain enzymatic stability.
4. Add 0.5 µl of the Gateway® LR Clonase Enzyme Mix to the reaction and incubate at 25°C for 1 hour. (*see* **Note 13**)
5. Add 1.0 µl proteinase K, vortex, incubate at 37°C for 15 minutes.
6. Transform 1 – 1.5 µl of this reaction into Stbl3 competent cells (or derivative of).
7. Add 250 µl of SOC and allow transformed cells to recover.
8. Plate 50 µl of transformed cells on one agar plate (30µg/ml ampicillin) and use sterile spreader until all the media has been absorbed into the plate.
9. Spin the remaining competent cells for 30 seconds at 5500 rpm.
10. Decant all but 100 µl of media.
11. Resuspend bacterial pellet and plate onto second ampicillin-containing plate (100 µg/ml). (*see* **Note 14**)
12. Invert plates and place at 37°C overnight.
13. Select and screen colonies for positive clones using the *Bam*HI restriction enzyme and gel electrophoresis. Correct clone contains 5 fragments with the following sizes: 10, 928 bp, 857 bp, 702 bp, 228 bp, 12 bp (not seen on gel).

3.4 Generation of high-titer TFAR lentivirus

1. Seed HEK293T cells at approximately $2x10^7$ cells per T175 cm^2 flask and incubate at 37°C, 5% CO_2 overnight to achieve up to 90% confluence.
2. Mix 50 µg transgene vector, 17.5 µg VSV-g envelope vector (pMD2.G), and 32.5 µg gag-pol packaging vector (pCMVΔR8.74) and incubate for 5 minutes at room temperature in 6 ml OptiMem® I (Gibco).
3. Add another 6 ml OptiMem® I containing 1 µl polyethylenimine (PEI) (10 mM) and incubate for a further 20 minutes at room temperature.
4. Wash cells with PBS and add 12 mls of PEI/DNA/OptiMem® I solution.
5. Incubate at 37°C, 5% CO_2 for 3 hours.
6. Remove PEI/DNA/OptiMem® I solution re-feed cells with DMEM containing 1% penicillin/ streptomycin and 10% FCS.
7. After 24 hours, refresh the culture medium.
8. After a further 48 hours collect the virus containing medium and filter through a 0.45 µm PVDF filter.
9. Subject virus-containing medium to overnight centrifugation at 4500 x g at 4°C.
10. Repeat harvest and overnight centrifugation for 72 hour post-transfection supernatant.
11. As quickly as possible after centrifugation, invert viral supernatant into Virkon™ to decontaminate the media.
12. Resuspend the viral pellet in 50 µl OptiMem® I and gently mix every 20 minutes for 1 hour at 4°C. (*see* **Note 15**)
13. Aliquot viral supernatant into 5- 10 µl aliquots and transfer to the -80°C freezer as quickly as possible.
14. Lentiviral titer is obtained using a p24 antigen ELISA (Zeptometrix) as per manufacturer's protocol. (*see* **Note 16**)

3.5 Lentiviral TFAR transduction of cells

1. Passage target cells as per normal maintenance protocol.
2. Transduction is usually carried out once the cells have attached, usually 12-24 hours post-passaging. (*see* **Note 17**)

3. Remove media, wash cells in PBS. (*see* **Note 18**)
4. To a Falcon tube, add the lowest possible volume of target cell growth media required to cover cells.
5. Add TFAR lentivirus at a multiplicity of infection (MOI) of 10 (see **Note 19**).
6. Incubate cells overnight to allow transduction to take place.
7. Replenish media and continue feeding and passaging processes required for appropriate maintenance of cells.
8. If a fluorescent marker gene has been used as a control to test the transduction efficiency of the particular target cell line, wait 3 days after transduction before ascertaining the levels of transduction. (*see* **Note 20**)
9. If an antibiotic resistance gene has been added to the lentiviral TFAR cassette, give the cells 3-5 days following transduction before selection to ensure adequate expression of the resistance gene.
10. Difficult to transduce cells may require the use of additives or alternative transduction techniques in order to improve transduction efficiency. (*see* **Note 21**)
11. Long term storage of stable cell lines can be achieved by placing passaged cells into freezing media and stored in liquid nitrogen.

3.6 *In vitro* luciferase quantitation to measure TFAR activity.

1. Lyse approximately $5x10^5$ cells in 300 µl luciferase lysis buffer and pellet the soluble lysate by high speed centrifugation (13,000 xg for 30 seconds).
2. In triplicate, aliquot 20 µl of each cell lysate into a white opaque 96-well plate trying to avoid the pelleted debris.
3. Add 20 µl of luciferase assay buffer, mix and serially inject luciferin substrate into each well to a final concentration of 1.5 mM. Luminescence output is measured using an appropriate luminometer with detection parameters in the range of 530-640 nm.
4. Relative photonic light units are normalized relative to total protein as determined by Bradford assay.
5. Statistical analysis using a student's t-test to compare activated vs non-activated samples is used to determine statistical significance between the two groups.

3.7 Neonatal administration of lentiviral TFAR

1. Perform all *in vivo* injections within twenty-four hours after birth of neonatal mice (see **Note 22**).
2. Anaesthetize neonatal (P0) CD1 mice on ice.
3. Inject the neonatal mice by the following routes and volumes: intracranially (5 µl), intravenously (20 µl), subcutaneously (10 µl), intranasally (20 µl), or ventral subcutaneously (5 µl) with high-titre lentivirus.

3.8 Continued monitoring of TFAR activity in living mice

1. Anesthetize mice with 4% isofluorane in 100% O_2 (see **Note 23)**, Inject 300 µl D-luciferin solution at a concentration of 15 mg/ml (a dose of approximately 150 mg/kg) into the intraperitoneal cavity (see **Note 24**).
2. Image the unconscious mice in the warmed light-proof detection chamber of the IVIS *in vivo* imaging system. Commence imaging 5 minutes after D-luciferin administration (see **Note 25**). An overlay of the two images is generated using Living Image software (Perkin Elmer) to create a pseudo-colored image to depict luminescent intensities over each animal (see **Note 26**).
3. Define regions of interest (ROIs) manually using a standard area for each organ.
4. Prior to agonist-mediated activation or surgical induction of disease, each of the animals is imaged three times within 72 hours in order to ascertain a robust median baseline measurement of bioluminescent imaging which can subsequently be used to express all future data points as a fold-change over this baseline value.
5. The type of statistical test depends upon the nature of the biosensor and the kinetics of response. Two possible approaches are A) For each animal in the two experimental groups, obtain the area under the curve using the parallelogram method. Compare using a Student's t-test if data is normally distributed. Otherwise use Mann-Whitney U-test. B) Compare two or more experimental groups over time using analysis of variance (ANOVA) with repeated measures. If ANOVA shows a significant difference between groups, perform a post-hoc test (e.g. Tukey, Bonferroni or Sidak) to test which time points might be significantly different.

4. Notes

1. *Ccd*B expression results in gyrase-mediated double-stranded DNA breakage, thereby inhibiting bacterial growth. An *E.coli* mutant strain containing an Arg462 to Cys substitution within the GyrA gene has shown to be resistant to the cytotoxic activity of *ccd*B, and is therefore required for propagation of any clones containing the Gateway cassette.
2. The LR Clonase enzyme is unstable even at -20°C for extended periods. Thus it is recommended that small aliquots of 5 µl are made and preferably stored at -80°C to reduce the number of freeze/thaw cycles and retain as much enzyme activity.
3. One Shot® Stbl3™ competent cells have been designed specifically for the propagation of unstable DNA sequence such as those found within the lentiviral backbone which contains direct repeats.
4. Mycoplasma infections have the capacity to reduce lentiviral titers. Therefore, perform a mycoplasma test using a PCR method with the following primers: Forward (5'- gggagcaaacaggattagataccct - 3') and Reverse (5'- tgcaccatctgtcactctgttaacctc -3'), or alternatively use a fluorescence based method using a kit such as MycoAlert™ Mycoplasma Detection kit (Lonza).
5. PBS containing calcium and magnesium improves the adherence of the HEK293T cells during the washing process.
6. It is best to add 5 ml dH_2O to 5 ml PEI. The PEI is extremely viscous, and so it is best to place the solution in a beaker with a magnetic stirrer. To pH, slowly add HCL in a drop-wise fashion. This releases fumes from the HCL, and so this step should be carried out in a well-ventilated fume hood. Keep stirring for 2-3 hours in the fume hood. Make up to 20.6 ml final volume with dH_2O, sterilize through a 0.22 µM filter and store in 500 µl aliquots in the -80°C. We have found this concentrated stock to be stable in the fridge for at least 6 months, after which it should be discarded and another aliquot used.
7. Both PES and PVDF filters are suitable but PVDF has been shown to be lower protein binding. If using PES, a 0.22 µM filter can be used.
8. Adult female mice will require mating ≈20 days before neonatal mice are required. Ideally this should be timed-mating, where males and females are housed together for one night only (per week).
9. To achieve optimal luciferase expression from the mice, it is best to use white furred

mice as the black furred mice prevent the bioluminescence from penetrating through.

10. Mice can be injected without anesthesia. However, anesthesia reduces mobility and improves injection accuracy. Mice should only be kept on wet ice until they are immobile. Inhalation or injection anesthetics are avoided as they are associated with a relatively high degree of mortality whereas death from hypothermic anesthesia is very rare.

11. The 33 gauge Hamilton needle should be kept moist at the tip by placing a wet paper towel around the needle. This helps reducing the friction against the new born mouse skin.

12. The site-specific recombination reaction occurs between regions of sequence homology, e.g. the *att*L sites found flanking the donor sequence within the pENTR-Prom vector and the *att*R sites found flanking the Gateway cassette in the acceptor pLNT-FLuc/eGFP vector.

13. It is uncommon, however, if no recombinants have been detected after the first round of recombination, it may be helpful to carry out the recombination reaction using the LR Clonase Enzyme mix for longer than the 1 hour at 25°C. Instead, the LR reaction can be incubated up to 18 hours (overnight). If the destination vector is >10 kb and no recombinants are found following recombination, it is suggested that the destination vector is linearized or a topoisomerase I used to relax the supercoiled DNA prior to incubation with the pENTR and LR Clonase mix.

14. Non-recombinants are dually selected against. Bacteria transformed with the non-recombined parental vector containing *ccd*B are killed due to its cytotoxicity in *ccd*B-sensitive, Stbl3 competent cells, while any non-recombined pENTR vectors are selected against through antibiotic selection as the pENTR-Prom vectors contains Kan^R, while the destination vector contains Amp^R, leading to the growth of bacteria transformed only with recombinants on the ampicillin containing agar plates.

15. Viral pellet may not be visible. The pellet is at the bottom of the Falcon tube and should still be gently resuspended in 50 µl of OptiMem even if it cannot be visually seen.

16. Using this method, make a dilution of 10^{-5} or 10^{-6} of the concentrated lentiviral supernatant. Dilutions can be made in dH_2O, with 450 µl of the final dilution lysed with 50 µl of the supplied lysis buffer.

17. Cells that proliferate quickly and form colonies might be best to transduce directly

into media following passage. This will allow the cells to be single celled at the time of transduction.

18. Cells may have to be washed more than once if there are a lot of floating cells within the culture. These cells will also be transduced, and therefore will result in lower transduction of the target cells.

19. Cells in suspension or sensitive to cationic polymers can be transduced using centrifugal inoculation (spinoculation) which uses centrifugal force to enhance lentiviral interactions with the cells. This can be performed by spinning the cells in virus-containing media at 800-1200 x g for 30 minutes at 32°C (varies for each cell line) after which the media is aspirated and the cells are resuspended in an appropriate volume of media for their maintenance.

20. If the infection potential of your cell line is not known, use a vector which constitutively expresses the GFP marker gene to ensure that an MOI of 10 is sufficient to transduce almost all of your cells.

21. VSV-g pseudotyped lentivirus has broad targeting tropism, if however, the cells are difficult to transduce, the addition of a cationic polymer to the media could enhance transduction. Polybrene (1-8 μg/ml) is the most commonly used transduction additive but protamine sulfate (4-10 μg/ml) or poly-L-lysine (10 μg/ml) can also be used.

22. When administering the D-luciferin via intraperitoneal injection, make sure that the bladder or other internal organs are not penetrated by the needle. This can be achieved by "tenting" the skin for injection. Similarly, ensure penetration into the peritoneal cavity by watching for, and avoiding formation of a subcutaneous bleb.

23. Waiting 5 minutes after luciferin administration permits time for entry of the luciferin into the bloodstream. It is worth performing a preliminary experiment to determine kinetics of bioluminescence for different cell and tissue targets. In addition, alternative routes of luciferin administration (e.g. intranasal) may be used [26].

24. Mice may also be anaesthetized using air or air and a nitrous oxide mix. The choice of carrier gas may affect the chosen biosensor as well as firefly luciferase activity (since this is an oxygen-dependent reaction).

25. Image acquisition performed using a 24 cm field-of-view for greyscale photographic images. Luminescent images are subsequently acquired using a binning factor of 4, with an aperture of f/1.2 and expressed as photons per second per cm^2 per

steradian. Larger binning values and longer exposures may be necessary if bioluminescence is weak.

26. It is good practice to perform a preliminary experiment to gauge the kinetics of the biosensor response and to identify timeframes of these responses in order to refine subsequent statistical tests. The statistical test and the time points of analysis should be decided before the experiment is performed, not afterwards.

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