Abstract

In our search for multiplexed assays that increase both assay speed and sensitivity we have developed LiveResponse™ a panel of 5 secreted ultra-sensitive luciferase reporters- Gaussia luciferase (emission max 482 nm), Cypridina luciferase (emission max 463 nm), Blue-shifted Renilla luciferase emission max 467 nm, Green Renilla luciferase (emission max 527 nm) and a Red Italica firefly luciferase (emission max 613 nm). These significantly brighter luciferases (Gaussia luciferase is over a 1000-fold brighter than the Photinus firefly luciferase and the red-emitting Luciola luciferase is a 1000 times brighter than it's native counterpart) offer increased sensitivity in screening applications involving analysis of weak promoters or hard-to-transfect cells. The green variant of Renilla luciferase that is a component of the LiveResponse™ system has 100-fold greater signal intensity and offers improved stability of the luminescent signal both in vitro and in vivo. This study demonstrates the usefulness of these reporters to study multiple pathways within the same cell and profile responsiveness at different times (without cell lysis) using NF-kB and CREB response elements to follow apoptosis and GPCR profiling in the same group of transfected cells. The LiveResponse™ system (a collection of 5 luciferase reporters) and assay reagents for the same) offers flexibility in assay format i.e.. different reporters can be assayed individually using separate aliquots of the supernatant or up to three luciferases can be assayed in the same sample of supernatant and the relative activities of the different luciferases can be spectrally resolved using appropriate filters.

LiveResponse™ - A Panel of 5 Novel Ultra-sensitive Secreted Luciferase Reporters for Cell-based HTS Applications

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Rationale:

A panel of improved ultrasensitive secreted luciferase reporters was developed in an effort to enable analysis of different promoter activities in the same group of transfected cells. This approach would not only enable analysis of three or more pathways (responses) in the same group of cells but also enable one to study the response in real time without killing the cells since the reporters are secreted. By choosing luciferases with different emission maxima we provide an additional advantage in that multiple luciferases can be assayed using a single luciferase assay reagent.

Methods:

HEK-293 cells obtained from ATCC and maintained in DMEM medium supplemented with 5% serum were used in these studies. The LiveResponse™ plasmids are commercially available from Targeting Systems. All luciferase reporters were expressed under control of the CMV promoter for comparative studies between the different luciferases. An expression vector expressing Gaussia luciferase under control of the NF-kB response element was kindly provided by Dr Bakhos Tannous for these studies. The pSV40 TAT plasmid was a gift from Dr Jeff Kudlow, University of Alabama at Birmingham, AL USA. The HIV-Luc plasmid was constructed by subcloning the HIV1 promoter (including the TAR element) upstream of the luciferase gene in the pBasic-VLuc vector. All luciferase assay reagents used were ones commercially available from Targeting Systems, El Cajon, CA. A Lentivirus expressing Gaussia luciferase under control of the CMV promoter and co-expressing EGFP under control of an IRES (LentiGlo™, Targeting Systems) was used for the in vivo studies in a mouse model.

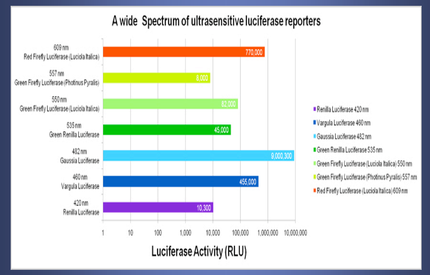
Results:

As shown in Figure 1, a panel of luciferase reporters with robust luciferase activities and different emission maxima has been developed. Four of the 5 reporters that comprise the LiveResponse system show excellent stability of the bioluminescent signal (Figure 2) indicating their suitability for high throughput screening (HTS) applications. These novel luciferase reporters enable analysis of gene expression in real time by assaying luciferase activity in the supernatant media without lysing the cells. These results also suggest that Cypridina luciferase, Green renilla luciferse ans Red Italica luciferases are excellent candidates for tltrasensitive triple luciferase reporter assays based on three different luciferase substrates (vargulin, coelenterazine and firefly luciferin). Gaussia luciferase and Cypridina luciferase are excellent complements for a dual secreted luciferase-based assay which uses two different substrates (colenterazine for Gaussia luciferase) and Vargulin (Cypridina luciferin) for Cypridina luciferase.

Further, the differences in emission maxima of many of these reporters could be successfully exploited to develop multiplexed dual or triple luciferase assays that permit analysis of two or three different luciferase activities in the same sample using a single assay solution by spectrally resolving the different luciferase activities (Figures 3A and 3B). The applicability of the LiveResponse reporters extends far beyond in vitro assays for studying gene expression. The high activity of the secreted Gaussia luciferase reporter makes it an excellent reporter for in vivo studies for tracking small numbers of implanted cells transduced with lentiviral vectors expressing Gaussia luciferase. Figure 4 shows the applicability of LentiGlo (a ready to use lentivirus expressing Gaussia luciferase and EGFP) in tracking a small numbers of human mesenchymal stem cells subcutaneously implanted into mice. The transfection efficiency of mesenchymal stem cells with the LentiGlo vectors (Fig 4) is almost 100%. Even a small number of transduced cells implanted subcutaneously could be easily visualized by bioluminescent imaging.

The applicability of the LiveResponse reporters to analyze multiple promoter activities in the same group of transfected cells was demonstrated by efficient induction of HIV promoter-driven Cypridina luciferase expression with the HIV-TAT protein (Figure 5A), and induction of NF-kB driven Gaussia luciferase expression with tumor necrosis factor (TNF-alpha) , Figure 5B.

Figure 1: Luciferase activities and emission max of different luciferase reporters in the LiveResponse reporter panel



Substrate specificities of different luciferases

Figure 2: Kinetics of luciferase activity of different luciferase reporters using luciferase assay reagents in the LiveResponse panel

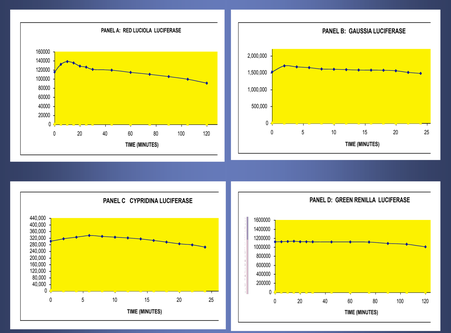


Figure 2: Reactions were set up to measure the kinetics of the luciferase activities of different luciferases in samples of transfected cells. Luciferase activities were assayed using the luciferase assay reagents supplied with the LiveResponse assay kit. Panel A: Red Luciola (firefly) luciferase, Panel B : Gaussia Princeps luciferase, Panel C: Cypridina luciferase and Panel D: Green Renilla luciferase. Data represents mean of triplicate deterrminations.

Figure 3A: Dual Luciferase assays based on the LiveResponse reporters

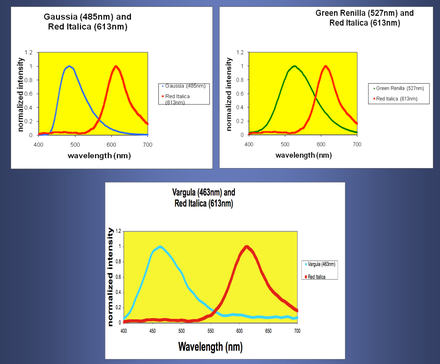


Figure 3A: Emission spectra of different luciferases in samples of transfected cells (lysates or supernatants). The luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. Emission max of Gaussia luciferase is 482 nm; Green Renilla is 527 nm; Cypridina Luciferase is 463 nm; Red italica 617 nm

Figure 3B: A Triple Luciferase assay based on Cyridina luciferase, Green Renilla luciferase, and Red Luciola luciferase

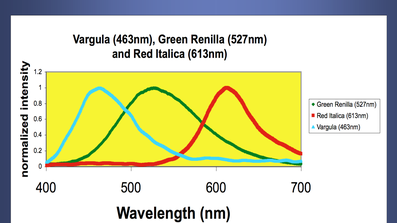


Figure 3B: Emission spectra of different luciferases in samples of transfected cell lysates. Relative luciferase activities of Cypridina, Renilla and Red Luciola luciferases were assayed with the appropriate luciferase assay reagent to obtain spectral profiles. The emission max of Vargula luciferase is 463 nm; Green Renilla luciferase is 527 nm and Red Luciola luciferase is 617 nm

Figure 4: Gaussia luciferase as an in vivo reporter to monitor cell survival or gene expression in vivo

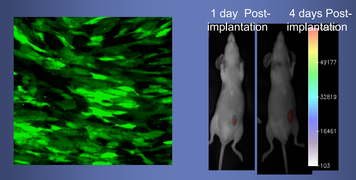
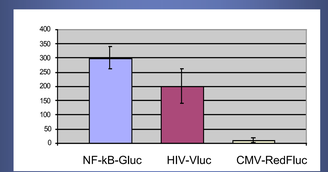


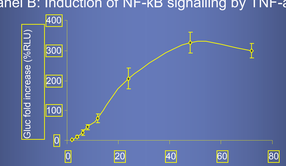
Figure 4 : Primary human bone marrow-derived mesenchymal stem cells were transduced with a lentivirus vector carrying the expression cassette of Gaussia luciferase and GFP, separated by an IRES element, under control of the CMV promoter (VMV-Gluc-IRES-CFP) at an MOI of 30. The results indicate that the transduction efficiency was nearly 100% (left). One million of these cells were mixed with matrigel and implanted subcutaneously in nude mice. At different time points, mice were injected with coelenterazine and imaged using a CCD camera (right). The signal increased over time showing that these cells proliferated in vivo.

Figure 5: Induction of HIV promoter-driven Cyrpridina luciferase expression by the TAT protein (Panel A) and induction of NF-kB –driven Gaussia luciferase expression by TNF-alpha (Panel B) in HEK-293 cells

Panel A: Induction of HIV promoter by the TAT protein



Panel B: Induction of NF-kB signalling by TNF-alpha



(Figure 5: HEK 293 were transduced with 3 vectors carrying expression cassette for: (1) *Gaussia* luciferase under control of NF-kB responsive promoter (NF-kB-Gluc); (2) Cypridina-luciferase under control of HIV1-promoter (HIV-Vluc); (3) red-emitting firefly luciferase under control of CMV promoter (CMV-RedFluc). These cells were then transfected with the SV40-pTAT plasmid (which activates HIV promoter) and treated with 15 ng/ml TNFα (which activates NFkB). 24 hrs post-treatment, aliquots of conditioned medium were assayed for Gaussia and Cypridina luciferase activity. Cell lysates were also assayed for RedFluc activity which was used as a viability marker. Signals were normalized to RedFluc activity and plotted as % fold increase in which the untreated samples was set to 1%. (B) real-time monitoring of NFkB activation using Gaussia luciferase. Cells expressing NFkB-Gluc were treated with 2.5 ng/ml of TNFα. Aliquots of conditioned medium were assayed for Gluc activity at different time points.

♣Conclusion:

- **The LiveResponse™ luciferase reporters can be effectively used in HTS applications for profiling multiple promoter activities in the same group of transfected cells.**
- **At least three different promoter activities can be efficiently analyzed in the same sample of cell lysate or supernatant with a fourth reporter serving as a denominator plasmid.**
- **It is possible to resolve spectrally the different luciferase activities so that dual or triple luciferase assays can be performed using a single assay solution.**
- **Differences in the substrate specificities of different luciferases have been exploited to develop novel secreted dual luciferase assays such as Gaussia luciferase/Cypridina luciferase, Green Renilla luciferase/Cypridina luciferse .**
- **Both Gaussia luciferase as well as the Green Renilla luciferase can be multiplexed with Luciola luciferase for an intracellular dual luciferase assay.**
- **A novel efficient triple luciferase assay has been developed based on Cypridina luciferase, Green Renilla luciferase and Red Luciola luciferase. This assay is applicable in a format wherein the three luciferase activities can be assayed using three different substrates or spectrally resolved using a single assay solution and appropriate filters.**
- **The LiveResponse™ luciferase panel contains several luciferases with improved properties. The green secreted Renilla luciferase is about 100-fold brighter than the native luciferase, shows improved stability of the bioluminescent signal and improved stability in vivo (in vivo data not shown). Gaussia luciferase, the brightest known luciferase offers much greater sensitivity than the commonly used versions of the firefly//Renilla luciferase reporters. The red-emitting firefly luciiferase from Luciola Italica shows a 1000-fold improvement in luciferase activity compared to the native enzyme.**

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