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Gaussia luciferase variant for high-throughput functional screening applications

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Abstract

Gaussia luciferase (Gluc) is a sensitive reporter for studying different biological processes such as gene expression, promoter activity, protein-protein interaction, signal transduction as well as tumor cell growth and response to therapy. Since Gluc is naturally secreted, the kinetics of these processes can be monitored in real-time by measuring an aliquot of conditioned medium in culture or a few microliters of blood *in vivo* at different time points. Gluc catalyzes light emission with a short half-life which is unfavorable for certain applications. We isolated a Gluc mutant that catalyzes enhanced light stability in the presence of a detergent, in combination with high sensitivity, making it an attractive luciferase for high-throughput functional screening applications.

INTRODUCTION

Luciferases catalyze light emission in the presence of their substrates, luciferins, and this property has made them a staple in multiple applications ranging from quantitative analysis of promoter activity and cell viability in cultured cells to non-invasive imaging of biological processes such as tumor growth and response to therapy *in vivo*.[1–5] Luciferases are found in many different species including beetles, bacteria, worms, fungi, and squid with several of them cloned and expressed in bacteria and/or mammalian cells.[6–10] Each of these luciferases has a different characteristic which makes it attractive for certain applications but not optimal for others. For high-throughput assays, an optimal luciferase would display the following properties: (1) enzyme stability over a variety of conditions; (2) high light output for increased sensitivity; (3) non-invasive monitoring of enzymatic kinetic activity at different time points in real-time; and (4) the catalysis of stable light emission for minimal variability between thousands of screened wells.

The marine copepod *Gaussia princeps* secretes a luciferase (Gluc) which possesses all but the fourth characteristic. In recent years, Gluc has been shown to be the preferred luciferase for monitoring of different biological applications.[9–16] Gluc is the smallest luciferase cloned (18 kDa) with several advantages over other commonly used reporters: Gluc is over 2,000-fold more sensitive than firefly or *Renilla* luciferase and 20,000-fold more sensitive than the

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Conflict of Interest

A provisional patent was filed by the Massachusetts General Hospital with Tannous and Maguire as inventors.

secreted alkaline phosphatase;[11,17] Gluc is naturally secreted and therefore monitoring of biological processes and reaction kinetics in culture can be accomplished in real-time by measuring enzymatic activity in an aliquot of conditioned medium at different time points keeping the cells intact for confirmation analysis;[3,11] Gluc is stable over a wide pH range and in the presence of reactive compounds;[17,18] *in vivo*, Gluc can be detected in blood or urine making it a sensitive *ex vivo* tool for monitoring of *in vivo* processes.[16,19] One limitation of Gluc for high-throughput assays is the rapid decay of its bioluminescence reaction and therefore a luminometer with a built-in injector is required, making the assay time consuming.[17] In this study, we have isolated a Gluc variant that, in the presence of its substrate coelenterazine and a detergent, Triton X-100, catalyzes a glow-type emission kinetics suited for high-throughput functional screening applications.

EXPERIMENTAL SECTION

Construction of Gluc library

A library comprised of a pool of Gluc mutant proteins was created by shuffling of DNA fragments generated by error-prone PCR. First, the human codon-optimized cDNA sequence encoding *Gaussia* luciferase (Nanolight, Pinetop, AZ)[17] was PCR-amplified using Taq polymerase (5 PRIME, Fisher Scientific, Pittsburgh, PA) and flanking primers which included EcoRI (upstream primer) and XhoI (downstream primer) restriction sites using the following conditions: 1 cycle of 94 °C-2 min; 35 cycles of: 94 °C-30 s, 58 °C-30 s and 72 °C-30 s; 1 cycle of 72 °C -7 min. The PCR product was then digested using 0.3 Units of DNaseI (New England Biolabs, Ipswich, MA) for 10 min at room temperature followed by heat inactivated at 75°C for 15 min after the addition of EDTA. The digested DNA was separated on a 2% agarose gel electrophoresis. DNA fragments from ~50–150 base pairs were carefully excised using a sterile scalpel. The gel slice was placed in 3,500 MWCO dialysis tubing (Fisher Scientific, Pittsburgh, PA) and the DNA was eluted into TBE by electrophoresis for 15 min at 120V. The DNA was then ethanol precipitated and resuspended in nuclease-free water. The PCR fragments were reassembled into the full-sized product using Extensor Hi-Fidelity PCR enzyme mix (Thermo Scientific, Portsmouth, NH) without primers using the following conditions: 1 cycle, 94 °C-2 min; 40 cycles of: 94 °C-30 s, 45 °C-30 s and 68 °C-30 s. One μl from this reaction served as template for a second PCR using similar primers as above and the following conditions: 1 cycle, 94 °C-2 min; 25 cycles of: 94 °C-30 s, 58 °C-30 s and 68 ° C-30 s; 1 cycle, 68 °C-7 min. The PCR product was gel extracted after electropheris, digested with EcoRI and XhoI and ligated into a similarly digested pHGCx expression vector.17

Screening procedure

The vector containing the mutant Gluc sequences created above was transformed into DH10B bacterial cells and plated on five LB ampicillin Agar plates (15 cm). Approximately 2000 colonies (representing individual Gluc clones) were obtained on each plate. To ensure that the DNA shuffling procedure was successful, plasmid DNA from 10 clones was isolated and subjected to DNA sequencing. On average, each clone displayed 1–2 amino acids changes in the protein sequence when compared to the native Gluc sequence. In order to screen for colonies which gives stable light output, a 20 μM solution of coelenterazine (Nanolight, Pinetop, AZ) diluted in PBS was misted onto the surface of each plate (one plate at a time). Light emission was immediately (t = 0) measured using a cryogenically cooled, high efficiency CCD camera system (Roper Scientific; Trenton, NJ). The level of luciferase activity was measured by recording total photon counts in the CCD camera (10 s exposure) with no illumination. The plate was imaged again 5 min post-spray. An image of the plate with illumination was taken at the end of the experiment to visualize the orientation of colonies on each plate. In order to determine clones with promising light stability, the photon counts of each clone at t = 0 was compared to that of the same clone at t = 5 min. Ten clones which displayed the greatest degree

of stability in light emission were picked and 5 ml LB cultures were grown overnight. Cells were pelleted and resuspended in 300 μl lysis buffer consisting of 30 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% Triton X-100. After three freeze/thaw cycles, cell debris was pelleted with centrifugation for 5 min at 13,000 rpm. Forty μl of lysate was transferred to a 96 medium binding flat well plate (Greiner bio-one, Monroe, NC) and was mixed with an equal volume of 20 μM coelenterazine. A 5 min kinetic assay was performed (11 s read intervals) using the Spectra Max Gemini XS plate reader (Molecular Devices, Sunnyvale, CA). The Gluc encoding cDNA from the most promising clone (M43I) was then subjected to DNA sequencing. Both wild type (wt) and GlucM43I (with and without the Gluc native signal sequence) were cloned in a in pET26b(+) expression vector (Novagen, Gibbstown, NJ) in-frame between an N-terminal *pelB* periplasmic signal sequence and a C-terminal 6-Histidine tag. Proteins were expressed and purified from bacteria using a nickel charged resin column as described (Supplementary Methods).

RESULTS AND DISCUSSION

We screened a novel pool of *Gaussia* luciferase variants created by error-prone PCR and directed molecular evolution techniques to isolate a clone which yield a stable light output in the presence of its substrate coelenterazine.[20] Bacterial cells were transformed with the Gluc mutant library and colonies were misted with coelenterazine solution and imaged using a CCD camera at different time points. Colonies which retained high signal over a 5 min period were isolated. Initially, 10 different clones were observed to retain stable luminescence activity as compared to the control. Upon confirmation analysis, one clone retained higher luminescence signal over 10 min as compared to the other clones and wt Gluc (data not shown). DNA sequencing of this mutant Gluc clone revealed a single nucleotide change resulting in a change of methionine 43 to isoleucine (Fig. 1). We called this variant GlucM43I.

We next compared the activity of wt Gluc with GlucM43I mutant in bacterial cell lysates. Cells were lysed using 30 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% Triton X-100. A 5 min bioluminescence kinetic assay revealed a clear difference in light emission between the wt and the mutant enzyme (Fig. 2A). Over the first 2 min, wt Gluc retained only 30% of its starting activity while the mutant GlucM43I retained over 87% of its original light emission. Spectral analysis of GlucM43I yielded a similar peak in signal when compared to wt Gluc with no shifts in emission spectra (Supplementary Fig. 1).

Next, we cloned the wt and GlucM43I (with and without the signal sequence) into a pET-based bacterial expression plasmid in which both cDNAs were cloned in frame between an N-terminal *pelB* periplasmic signal sequence and a C-terminal 6-Histidine tag. In theory, upon expression, the *pelB* signal sequence will be cleaved and therefore it is not part of the protein. We purified both wt Gluc as well as GlucM43I from bacterial cell extracts using nickel charged resin column (Supplementary Methods). We observed that the yield and/or activity of the wt and GlucM43I with the signal sequence was very low compared to the same enzymes without the signal sequence (data not shown). These results confirm a recently published study which showed that the presence of the Gluc signal sequence severely affects protein yield and/or activity.[21] Based on these data, we decided to use the purified wt and GlucM43I without the signal sequence for all subsequent experiments. The yield of these proteins without the signal sequence was around 250 μg from a 200 ml bacterial culture. SDS-PAGE under reducing conditions showed 2 bands, a faint band around 20 KDa which corresponds to the size of Gluc and a much stronger band at 25 kDa which corresponds to the size of Gluc including the *pelB* sequence (Fig. 2B). These results suggest that the cleavage of the *pelB* sequence was not very efficient during protein expression.

In order to compare the light emission kinetics from the purified wt and GlucM43I, 10 ng of each luciferase was diluted in 30 mM Tris, pH 8.0, mixed with coelenterazine, and subjected to a 5 min kinetic analysis. Surprisingly, virtually no difference in the light decay kinetics was observed between the wt and mutant Gluc (Supplementary Fig. 2A). Similar results were observed when these enzymes were diluted in PBS (Supplementary Fig. 2B). Since Triton X-100 was included in the lysis buffer which was used in the experiments with crude bacterial extracts (Fig. 2A), we checked whether the presence of this detergent has an effect on the Gluc reaction kinetics. We repeated the 5 min kinetic analysis in the presence of 0.1% Triton X-100. Interestingly, the addition of this detergent changed the kinetics of light emission catalyzed by both wt and GlucM43I as quantified using the luminometer and visualized using the CCD camera (Fig. 2C & D; Supplementary Fig. 2C). Over the 5 min period, there was a 56.6% decrease in the wt Gluc luminescence as compared to only 21.3% for GlucM43I mutant. A 20 min kinetic assay using 10 ng of each luciferase showed a 3.1-fold increase in the half-life of light emission catalyzed by GlucM43I (930 s) compared to wt Gluc (330 s) (data not shown). These results suggest that the addition of this detergent plays a key role in stabilizing the GlucM43I light emission. Thus, all subsequent experiments (unless stated otherwise) were carried out in the presence of 0.1% Triton X-100.

We next calculated the specific activity of both the wt and mutant Gluc. No significant difference was found between the specific activity of wt and GlucM43I in the absence of Triton X-100. However in the presence of this detergent, a statistically significant increase (3.7-fold, $P<0.05$) was observed in the specific activity of wt as compared to GlucM43I (Fig. 2E). Further, the addition of Triton X-100 significantly ($P<0.05$) enhanced the specific activity of both wt Gluc (6.3-fold) and GlucM43I (1.88-fold) compared to the respective enzyme in the absence of this detergent (Fig. 2E).

To determine the effect of substrate concentration on light emission kinetics, 10 ng of either wt or GlucM43I was mixed with different concentrations of coelenterazine (diluted in 30 mM Tris-HCl pH 8.0, 0.1% Triton X-100) and a 5 min kinetic assay was performed. For wt Gluc, higher light decay occurred with higher substrate concentration (Supplementary Fig. 3A). On the other hand, the light emission kinetics catalyzed by the GlucM43I variant were not affected (Supplementary Fig. 3B). Further, light decay kinetics (5 min assay) for both wt Gluc and GlucM43I were relatively unaffected over an 8-fold range of enzyme concentration (Supplementary Fig. 4).

The data described above used Gluc purified from bacteria. To demonstrate the expanded applicability of the GlucM43I variant, we analyzed its ability to catalyze enhanced light stability when expressed in and secreted from mammalian cells (Supplementary Methods). 293T human kidney fibroblast cells were transiently transfected with a mammalian expression vector encoding either wt or GlucM43I. Forty-eight hrs later, 5 μl aliquots of the conditioned medium were transferred to a 96 well plate and subsequently 95 μl 20 μM coelenterazine (diluted in 30 mM Tris, 5 mM NaCl, pH 8.0 containing 0.1% Triton X-100) was added to each well. Kinetic analysis revealed that GlucM43I catalyzed approximately 7-fold more stable light emission as compared to wt Gluc (Fig. 2F). Bioluminescence reaction catalyzed by GlucM43I retained 81% of the original light emission compared to 12.6% for wt Gluc over a 10 min period ($P<0.0001$).

As the wt Gluc displays flash-type light emission kinetics, it is necessary to use a luminometer with a built-in injector to assay its activity. Generally, when using an injector, more substrate is used (to purge the instrument before each assay) and higher reading time/well is required (generally 10 s reading time with signal integrating over 2 s). This high reading time limits the use of Gluc for high-throughput applications. To determine whether the stability of light emission catalyzed by GlucM43I in the presence of Triton X-100 would make the Gluc assay

suited for high-throughput screening, we performed a 1 s read/well of 288 wells (three 96 well plates), each containing 10 ng of wt or GlucM43I (Supplementary Methods). Coelenterazine was diluted in 30 mM Tris-HCl pH 8.0, 0.1% Triton X-100 and was added to all wells simultaneously using a multi-channel pipette. We analyzed the data based on the way the luminometer reads the wells (column by column on the plate) and presented them in column format where signals from the left represent the earliest reads and those on the right represent later reads. For wt Gluc, a > 62% decrease in RLU between the first and last well was observed (Fig. 3A). For GlucM43I, the RLU was much more consistent between first and last well (~12% decrease; Fig. 3B). The coefficient of variation was calculated to be 28.7% for wt Gluc and only 8.53% for GlucM43I (Fig. 3C). Further, an F test for unequal variance revealed a significant difference between the two groups ($P<0.0001$).

Finally, we compared the activity of wt and mutant Gluc using the only commercially available buffer system for stabilizing light emission catalyzed by this luciferase (New England Biolabs; LumiFlex™ Gluc Assay Kit; Supplementary Methods). This kit consists of a proprietary assay buffer and “stabilizer” solution, which are mixed together with coelenterazine solution and Gluc. The drawback of using this system is that the stabilizer dramatically affects the Gluc sensitivity by up to one order of magnitude (manufacturer’s data). We tested the effect of this assay system on the stability and light output of wt Gluc versus GlucM43I by mixing 40 ng of either enzyme with coelenterazine diluted in the assay buffer containing different amounts (1–8 μl) of stabilizer. The light-emission kinetics was monitored over a 30 min period (Supplementary Fig. 5 and Supplementary Table I). GlucM43I outperformed wt Gluc under all conditions with a 2.3 to 6.1-fold increase in the half-life of light emission. Importantly, under conditions of nearly equivalent light stability, the initial starting RLU value was 7 to 10-fold higher for the GlucM43I variant as compared to the wild type (wt Gluc with 8μl stabilizer compared to GlucM43I with 1μl stabilizer or wt Gluc with 4μl stabilizer compared to GlucM43I with no stabilizer). The half-life of light emission of GlucM43I under the highest amount of stabilizer (8 μl) was 32.8 min compared to 12.95 min for wt Gluc. We also obtained a good combination of stability and signal output for GlucM43I as compared to wt under our assay conditions using 40 ng of either enzyme with 30 mM Tris pH 8.0 and 0.1% Triton X-100 (Supplementary Table I).

In summary, we isolated a variant (GlucM43I) of *Gaussia* luciferase from a screen of a mutant library created by DNA shuffling and error-prone PCR that catalyzes a stable light emission output as compared to the wt Gluc in the presence of a detergent, Triton X-100. This Gluc variant proved to be a very useful reporter for high-throughput screening applications where sensitivity and stable light emission are desired. Since *Gaussia* luciferase is naturally secreted, it allows functional screening and kinetic analysis from a single well by measuring an aliquot of conditioned medium at different time points, leaving the cells intact for conformational analysis. To our knowledge, Gluc is the only reporter available for high-throughput applications which allows kinetics analysis. Further, Gluc has the advantage over other fluorescent and bioluminescent reporters in that its level in the blood correlates to the level of its activity in a given biological system, thereby allowing semi-throughput screening/validation of novel therapeutics *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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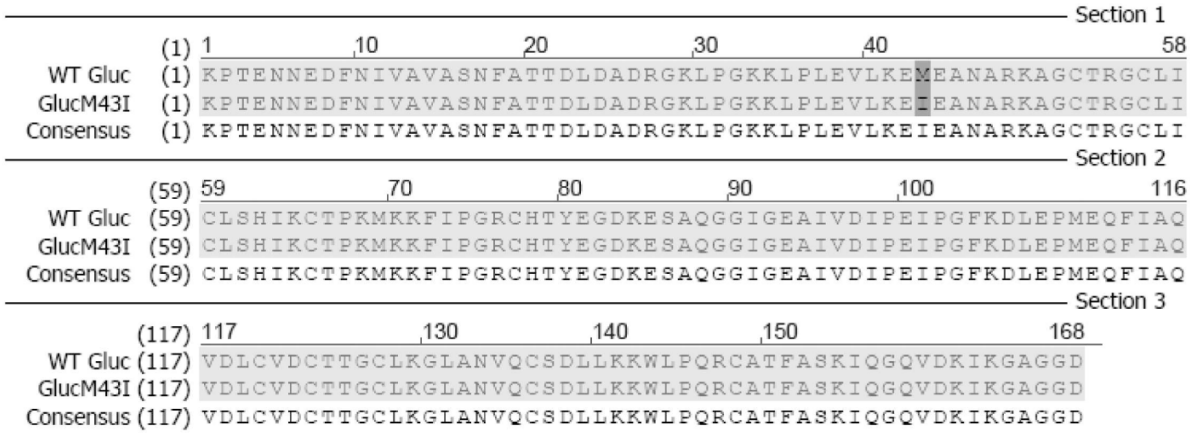

Figure 1. Amino acid composition of GlucM43I

Alignment of wt Gluc with GlucM43I using Vector NTI AlignX. The methionine to isoleucine change is highlighted. Signal peptide of Gluc is not shown.

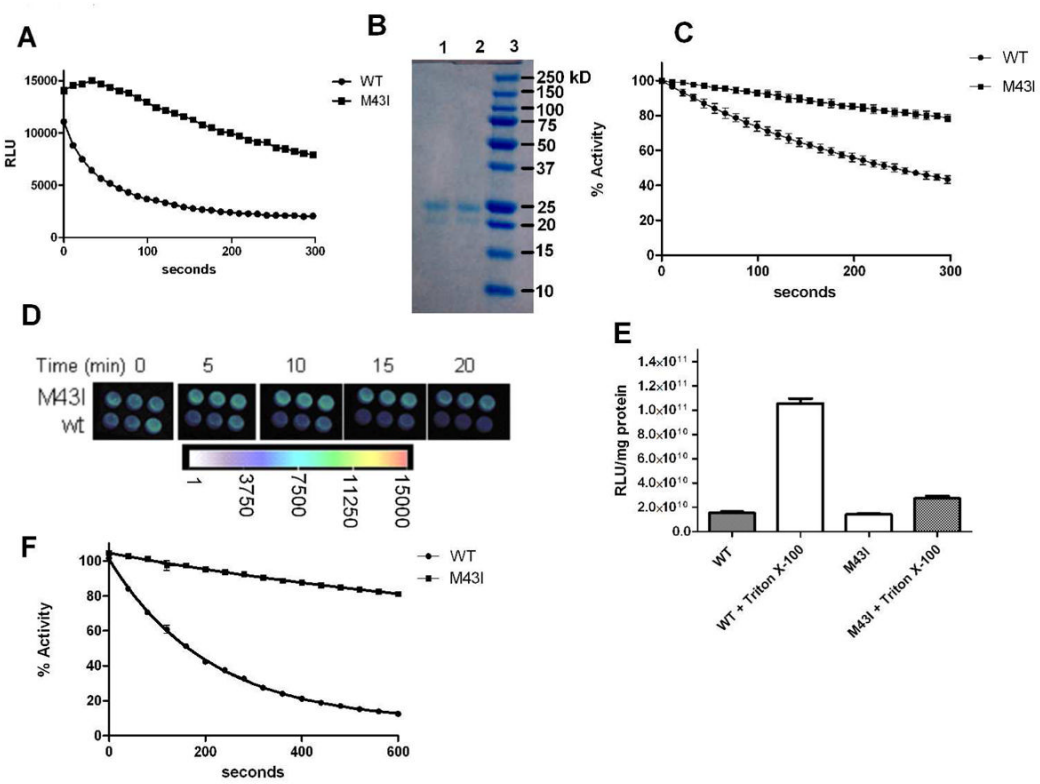

Figure 2. GlucM43I variant catalyzes a glow-type luminescence reaction in the presence of Triton X-100

(A) Small-scale bacterial cultures transformed with plasmids encoding wt Gluc or GlucM43I variant were lysed using 30 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% Triton X-100. Lysates were mixed with coelenterazine and a 5 min light emission kinetics was performed. **(B)** Purified Gluc enzymes. wt and GlucM43I were expressed as 6His-tagged proteins and purified on a Ni^{2+} column. SDS-PAGE was performed using 2 μg of each luciferase and protein was visualized by coomassie blue staining. Lane 1, wt Gluc; lane 2, GlucM43I; lane 3, protein ladder. **(C–D)** Kinetic analysis of purified wt and GlucM43I in the presence of 0.1% Triton X-100 as quantified using the luminometer (C) and visualized using a CCD camera (D). **(E)** Specific activity of wt and GlucM43I variant analyzed after addition of 50 μl coelenterazine diluted in 30 mM Tris-HCl pH 8.0 in the presence or absence of Triton X-100. (**F**) Kinetic analysis of wt and GlucM43I variant expressed in mammalian cells. 293T cells were transfected with a mammalian expression vector encoding either wt or GlucM43I variant. Forty-eight hrs post-transfection, Gluc luminescence kinetics (over 10 min) were analyzed in 5 μl aliquots of the conditioned medium after addition of 95 μl 40 μM coelenterazine (diluted in 30 mM Tris pH 8.0 with 5 mM NaCl and 0.1% Triton X-100) using a luminometer. Results presented as % in which the RLU from the first reading is set to 100%. Data presented as average ± SD.

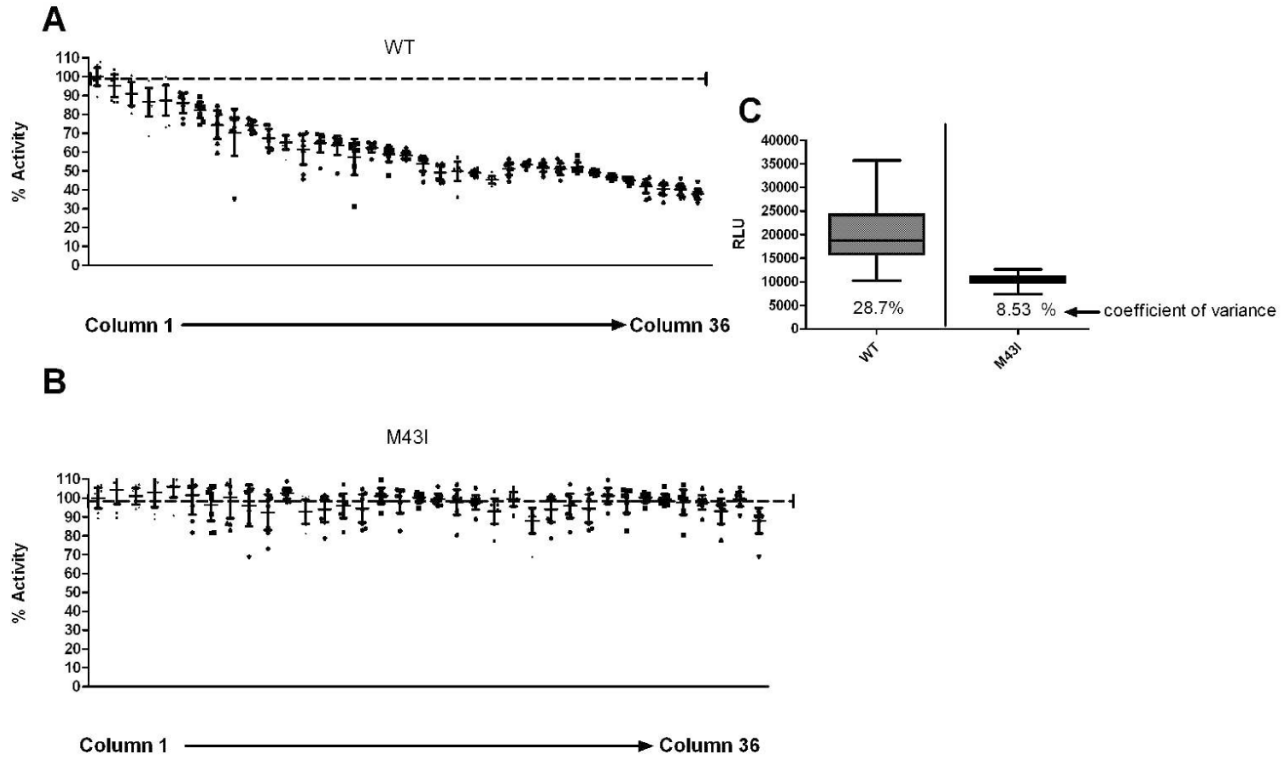

Figure 3. GlucM43I variant as a reporter for high-throughput applications

Ten ng of either wt Gluc or GlucM43I variant in 30 mM Tris, pH 8.0, 0.1% Triton X-100 was pipetted into three 96 well plates. An equal volume of 20 μM coelenterazine in PBS was then rapidly added to all wells using a multi-channel pipette and each plate was then sequentially read using a microplate luminometer. **(A–B)** Scatter plot of signals obtained from either wt Gluc (A) or GlucM43I (B) presented in a column by column basis. In both cases, the plates were read from left to right. Data presented as % Gluc activity in which the RLUs from the first well are set to 100%. Error bars represent standard deviation. **(C)** Box plot representation of the spread of total RLUs in all wells for both wt and GlucM43I. Data are representative of three independent experiments.