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An enhanced Gaussia luciferase blood assay for monitoring of in vivo biological processes

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Abstract

Secreted *Gaussia* Luciferase (Gluc) has been shown to be a useful tool for *ex vivo* monitoring of *in vivo* biological processes. The Gluc level in the blood was used to detect tumor growth, metastasis and response to therapy, gene transfer, circulating cells viability, as well as transcription factors activation, complementing *in vivo* bioluminescence imaging. The sensitivity of the Gluc blood assay is limited due to the absorption of blue light by pigmented molecules such as hemoglobin resulting in quenching of the signal and therefore lower sensitivity. To overcome this problem, we designed an alternative microtiter well-based binding assay in which Gluc is captured first from blood using a specific antibody followed by the addition of coelenterazine and signal acquisition using a luminometer. This assay showed to be over one order of magnitude more sensitive in detecting Gluc in the blood as compared to the direct Gluc blood assay enhancing *ex vivo* monitoring of biological processes.

INTRODUCTION

Secreted blood reporters are valuable tools for sensitive and fast detection, quantification and non invasive monitoring of in vivo biological processes[1–5]. The level of these secreted reporters can be measured over time to generate multiple data sets without the need to sacrifice the animal, since only a small amount of blood is required. *Gaussia* Luciferase (Gluc) has been recently shown to be a promising blood reporter for *ex vivo* monitoring of *in vivo* biological processes[4–6]. This small luciferase (19.9 kDa) emits a blue flash light (480 nm) upon catalyzing the oxidation of its substrate coelenterazine[7]. Gluc cDNA possesses a signal sequence and therefore is naturally secreted in an active form upon expression in mammalian cells[7]. Further, the level of secreted Gluc in blood is linear with respect to cell number, growth and proliferation, and therefore can be used as a marker for monitoring of biological processes including tumor growth, metastasis and response to therapy[6,8,9], gene transfer[6,9–11], viral infection[12], circulating cells viability[6], as well as transcription factors activation[13,14], complementing *in vivo* bioluminescence imaging. Compared to other widely used secreted blood reporters such as the secreted alkaline phosphatase or SEAP, Gluc has several advantages including a much shorter assay time, an increased sensitivity and linear range as well as shorter half life in circulation allowing multiple measurements within a short period of time without accumulation of signal[4,5].

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One of the major disadvantages of using Gluc as a blood reporter is the absorption of its blue light by pigmented molecules such as hemoglobin resulting in quenching of the signal and therefore lower sensitivity. To overcome this problem, we designed an alternative microtiter well-based assay in which Gluc is captured first from blood using a specific antibody followed by the addition of coelenterazine and signal acquisition using a luminometer. This optimized microtiter well-based assay showed to be over one order of magnitude more sensitive than the typical direct Gluc blood assay. This optimized assay is useful for the detection of subtle luciferase levels *in vivo* facilitating non-invasive *ex vivo* monitoring of various biological processes.

EXPERIMENTAL SECTION

Animal studies and blood collection

All animal studies were approved by the Massachusetts General Hospital Review Board. U87 human glioma cells (ATCC) were transduced by a lentivirus vector to stably express Gluc as we previously described[6]. To generate tumors, 1 million of these cells (in 50 µl) were mixed with equal volume of Matrigel and injected subcutaneously in the flanks of athymic nude mice. Blood samples were collected from these mice as well as mice with no tumors by making a small incision in the tail and directly adding it to an eppendorf tube containing EDTA as an anti-coagulant (10 mM final concentration).

Microtiter well-based Gluc assay

High binding microtiter 96 well plates (Thermo Fisher Scientific, Rochester, NY) were coated overnight with different amounts of polyclonal rabbit anti-Gluc antibody (Nanolight, Pinetop, AZ) or monoclonal mouse anti-Gluc (generated through the Massachusetts General Hospital antibody production facility[15]) diluted in 100 mM carbonate buffer pH 9.6 [or phosphate-buffer saline (PBS) or 50 mM Tris-HCl, pH 7.8 in the presence of 0.5 g/l NaN_3 for optimization studies] in a total volume of 50 µl (unless otherwise stated). Eighteen to twenty four hours later, the plates were washed 2× with 200 mM Tris-HCl pH 7.8 (or PBS or 100 mM carbonate pH 9.6 for optimization studies). Blood samples were then mixed in 200 mM Tris pH 7.8 to a final volume of 50 µl, centrifuged at 200× g, and the supernatants were added to the coated wells, incubated for one hour (unless otherwise stated) at room temperature with shaking. The plates were then washed once with 200 mM Tris-HCl, pH 7.8 (PBS or 100 mM carbonate buffer, pH 9.6) and analyzed by injecting 50 µl 50 µg/ml (unless otherwise stated) coelenterazine diluted in PBS (or 200 mM Tris-HCl pH 7.8 in the presence or absence of 5 mM NaCl) and acquiring photon counts for 10 sec using a microplate luminometer (Dynex, Chantilly, Va).

Direct Gluc blood assay

Five µl (unless otherwise stated) of Gluc-containing blood or Gluc negative blood were added directly to a 96-well white microtiter plate and the Gluc activity was detected by injecting 50 µl 50 µg/ml (unless otherwise stated) coelenterazine and acquiring photon counts for 10 sec using a microplate luminometer as described[4].

Statistical Analysis

All experiments were repeated atleast 3 times to achieve statistical significance. Data are presented as the mean relative light units (RLU) ± standard deviation (SD) from 5 different replicates in each experiment. P values were calculated using Student's t-test. Reproducibility was assessed by calculting the % coefficient of variation (%CV) at 3 different Gluc concentrations.

RESULTS AND DISCUSSION

We performed several optimization steps for the microtiter well-based Gluc binding assay. Two Gluc specific antibodies are available, a polyclonal rabbit anti-Gluc antibody and a monoclonal mouse anti-Gluc antibody. We first compared these two antibodies by coating the microtiter plates with 50 µl (diluted 1:100 in 50 mM Tris-HCl pH 7.8) with either of these antibodies. Twenty-four hours later, plates were washed with Tris-HCl buffer and 5 µl blood (Gluc positive and negative) were mixed with 45 µl Tris-HCl, centrifuged for 5 min at 200× g and the supernatant was added to each well (in triplicates) and incubated for 1 hour at room temperature with shaking. Wells were then washed with Tris-HCl buffer and analyzed with 50 µl 50 µg/ml (diluted in PBS) coelenterazine using a luminometer. The polyclonal antibody showed to have higher binding capacity of Gluc from the blood and therefore was used for all subsequent studies (Fig. 1A).

We next checked the optimum amount of polycolonal antibody for coating the microtiter plates. Polyclonal Gluc antibody was serially diluted in 50 mM Tris-HCl, pH 7.8 and added to all wells. Twenty-four hours later, the Gluc assay was performed as above. A 1:500 dilution of the antibody yielded the highest Gluc binding (Fig. 1B) without any effect on the noise defined as the signal obtained from the Gluc negative blood samples (data not shown). Several buffers were then tested for coating the microtiter wells with the Gluc polyclonal antibody including PBS, 50 mM Tris-HCl pH 7.8 and 100 mM carbonate pH 9.6. The carbonate buffer showed the highest signal and the lowest background yielding around 2-fold higher signal-to-noise ratio (calculated by dividing the signals obtained from Gluc-positive blood to Gluc-negative blood) as compared to all other buffers tested (Fig. 1C). Therefore, the polyclonal antibody diluted 1:500 in carbonate buffer was used for all subsequent studies.

We also optimized the assay time for binding Gluc on antibody-coated plates. The optimum coating protocol above was used to capture 5 µl Gluc-containing blood in Tris-HCl buffer for different time points ranging from 5 min to 24 hours. One hour incubation time gave high binding of Gluc which even increased further by 2-fold over 24 hours (Fig. 1D). For simplicity purposes, one hour binding time can be used. We also compared the assay volume, in which all assay steps including coating, binding and detection were performed, in 50 or 100 µl. No statistical significance differences were observed between the two volumes tested and therefore the lowest 50 µl volume was chosen for further studies (Fig. 1E).

After optimizing the assay conditions, we focused on improving the bioluminescent-detection step. Different volumes and concentrations of coelenterazine, the Gluc substrate, were tested using the optimized protocol above. A concentration of 50 µg/ml in a total volume of 50 µl was found to give the best signal-to-noise ratio (Fig. 2A and B). We then compared different buffers to dilute the coelenterazine for the detection of Gluc. We used PBS or 50 mM Tris-HCl in the presence or absence of NaCl, since this salt is known to enhance the Gluc reaction. We found that PBS resulted in the highest signal-to-noise ratio and therefore was chosen for final analysis (Fig. 2C).

Previously, we showed that 5 µl of blood gives the highest Gluc signal which is substantially decreased when using higher volumes due to quenching of the bioluminescence signal by hemoglobin and other molecules[8]. Further, detecting Gluc in serum did not show an increased sensitivity due to auto-oxidation of coelenterazine yielding higher noise[6,8]. We confirmed these data using the direct Gluc blood assay and compared them to our optimized microtiter plate-based Gluc blood assay. Different volume of Gluc-containing blood (or Gluc negative blood) were analyzed with both assays. As expected, using the direct assay, a linear decrease of Gluc signal with increasing amount of blood was observed showing

quenching of light by hemoglobin. On the other hand, a linear increase in signal reaching a plateau at around 40 μl was observed with the microtiter plate-based binding assay showing that this assay can be used to detect higher blood volume (Fig. 2D). The background noise for the binding assay did not change with increasing blood volume (average 0.186 RLU). On the other hand, the direct assay showed a decrease in background RLUs ranging from 0.136 RLU for 5 μl blood to 0.08 RLU for 80 μl blood. More importantly, higher signal-to-noise ratio was obtained with all different blood volumes tested yielding around 20-fold higher ratio when assaying 40 μl blood using the microtiter plate binding assay as compared to the typical 5 μl direct detection of Gluc (Fig. 2D).

Finally, using the optimized protocol above, we compared the sensitivity of the microtiter plate-based binding assay to the typical direct assay for detecting Gluc in the blood. Blood was collected from nude mice bearing tumors expressing Gluc, serially diluted with Gluc-negative blood and analyzed with both assays. The microtiter well-based binding assay showed to be over 16-fold more sensitive in detecting Gluc in the blood as compared to the direct standard approach showing that our optimized protocol could be used for detection of subtle luciferase levels *in vivo* facilitating non-invasive monitoring of various biological processes (Fig. 2E). To assess reproducibility, we analyzed 3 different dilutions (1:1, 1:10, 1:100) of Gluc-containing blood (n=9) using both the binding and direct assays. We found that the %CV to be ≤ 9% for the binding assay and ≤ 15% for the direct assay.

In conclusion, we developed a microtiter plate-based binding assay and showed it to be more sensitive in detecting Gluc in the blood as compared to the typical direct measurement. This assay is suited for the detection of Gluc in any sample including conditioned medium from mammalian cell cultures as well as urine from small animals. The optimized assay is very useful for *ex vivo* monitoring of *in vivo* biological processes where high sensitivity is required such as detecting few circulating cells, early tumor metastasis and apoptosis[6,8,16].

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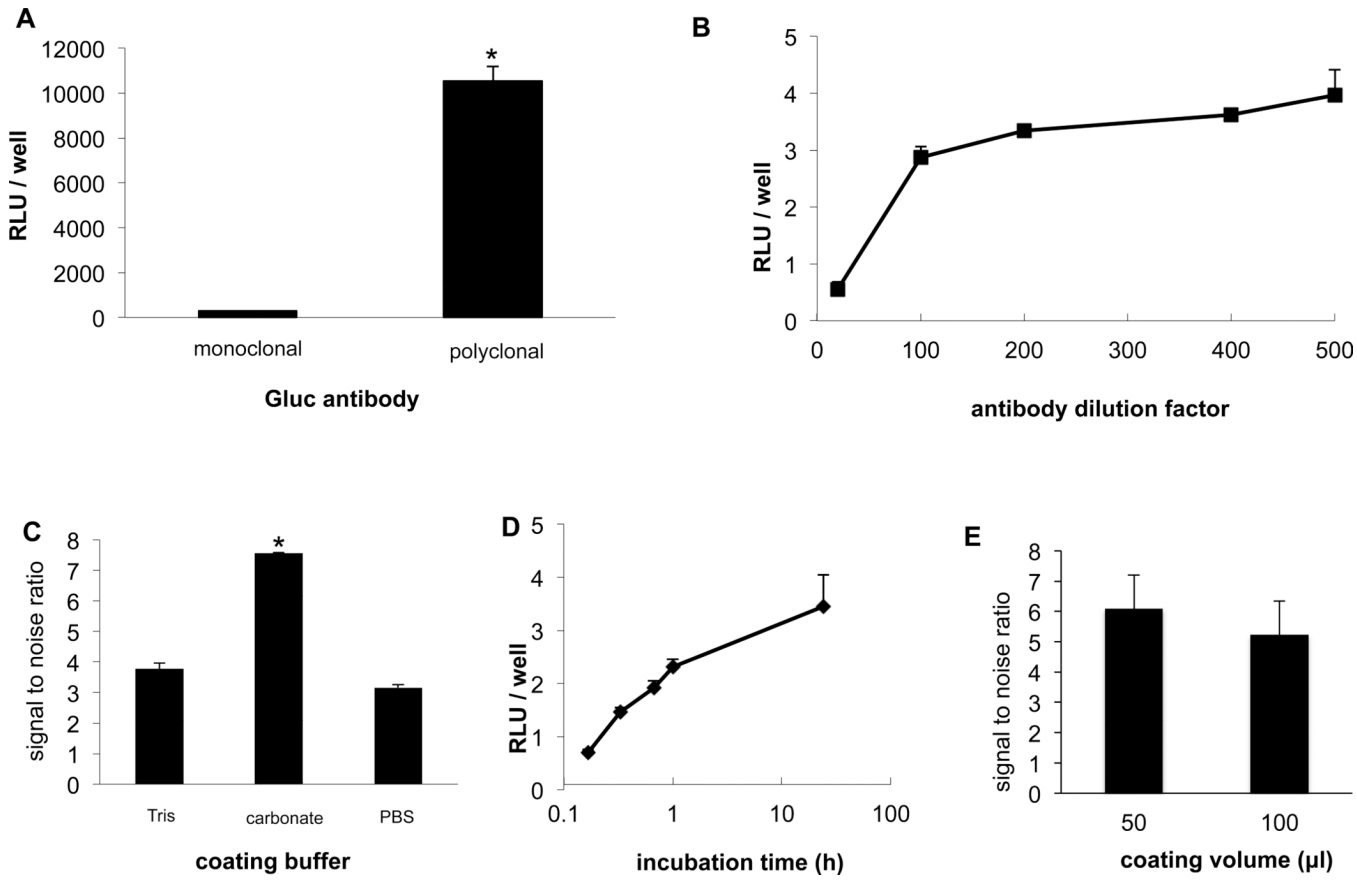

Figure 1. Optimization of the Gluc blood binding assay

(**A**) Polycolonal or monoclonal anti-Gluc antibody diluted 1:100 in 50 mM Tris-HCl, pH 7.8 were used to coat the microtiter plates. Twenty-four hours later, plates were washed and 5 µl blood containing Gluc or negative blood (mixed with 45 µl 200 mM Tris pH 7.8) were added to each well in triplicates, incubated for 1 hour, and analyzed after the addition of 50 µl 50 µg/ml coelenterazine using a luminometer. (**B–C**) Different dilutions of the polyclonal anti-Gluc antibody diluted in 50 mM Tris-HCl buffer (B) or the same antibody diluted 1:500 in different buffers (C) were used to detect 5 µl of blood-containing Gluc as in (A). (**D**) 5 µl blood (mixed with 45 µl Tris-HCl buffer) were added to the microtiter wells pre-coated with anti-Gluc antibody and incubated at different time points. Plates were then washed and analyzed as in (A). (**E**) Different volume of the polyclonoal anti-Gluc antibody (diluted 1:500 in carbonate buffer) was used to coat the microtiter wells and was used to detect 5 µl of blood as in (A). Data presented as average ± SD from 3 independent experiments with 5 replicates per experiment. *p<0.001.

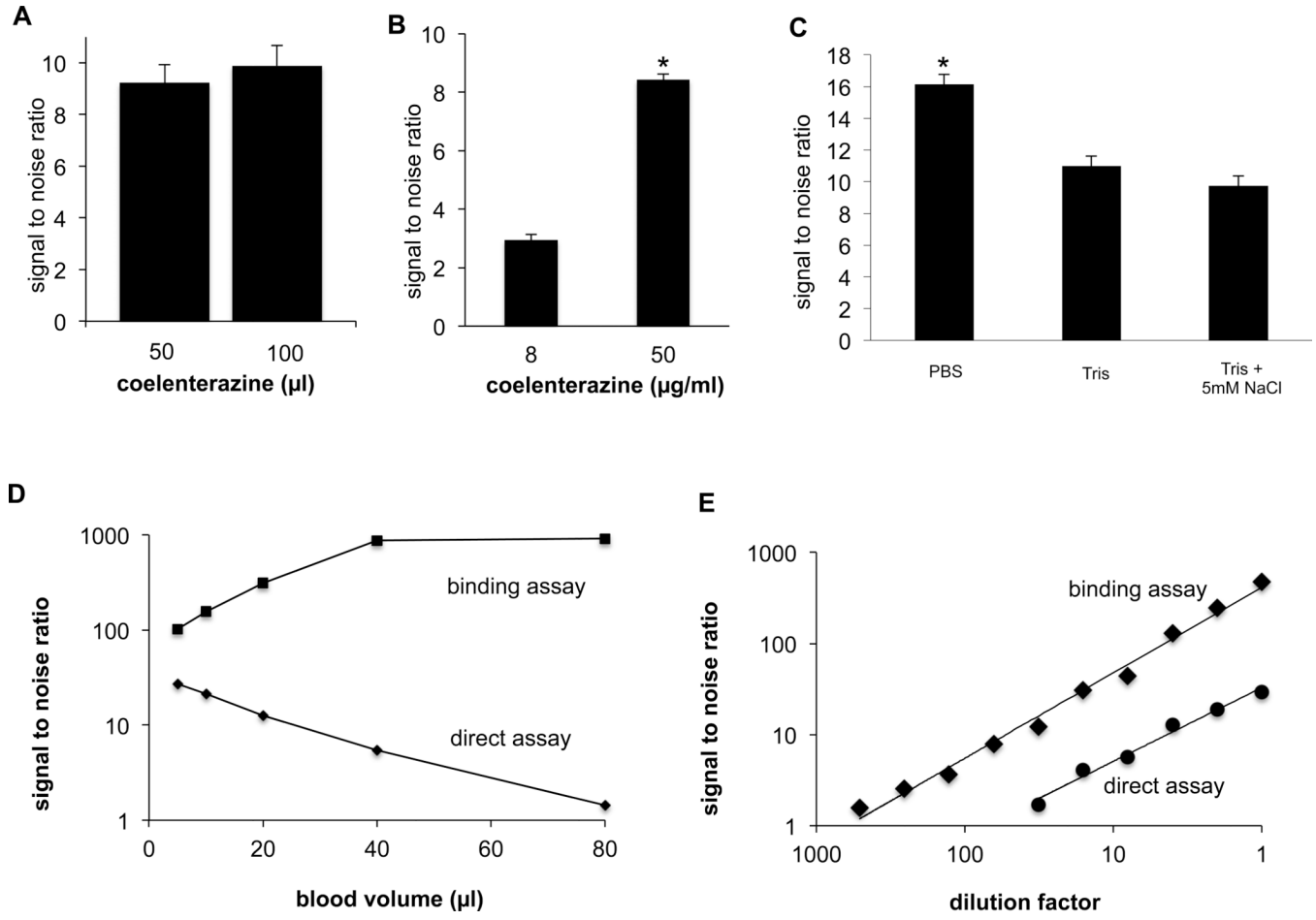

Figure 2. Microtiter plate-based binding assay for the detection of Gluc in blood

Microtiter wells were coated with 50 µl of the polyclonal rabbit anti-Gluc antibody diluted 1:500 in carbonate buffer. (**A–C**) Five µl blood-containing Gluc or negative blood (mixed with 45 µl 200 mM Tris pH 7.8) were added to each well in triplicates, incubated for 1 hour and analyzed with different volumes of 50 µg/ml (a) or amounts (b) of coelenterazine diluted in PBS or in 50 mM Tris-HCl pH 7.8 in the presence or absence of 5 mM NaCl (c). (**D**) Different amounts of blood-containing Gluc or negative blood (background noise) were mixed with 200 mM Tris pH 7.8 and added to each well in triplicates. One hour later, plates were washed and analyzed with 50 µl 50 µg/ml coelenterazine (diluted in PBS) using a luminometer. The same samples were also analyzed using the standard direct Gluc blood assay. The signal (RLUs from Gluc-positive cells) over noise (RLUs from Gluc-negative cells) ratio with respect to blod volume are showing. (**E**) Blood-containing Gluc was serially diluted with Gluc-free blood and analyzed by both the microtiter plate-based binding assay as well as the direct assay. Data presented as average ± SD from 3 independent experiments with 5 replicates per experiment. *p<0.001.