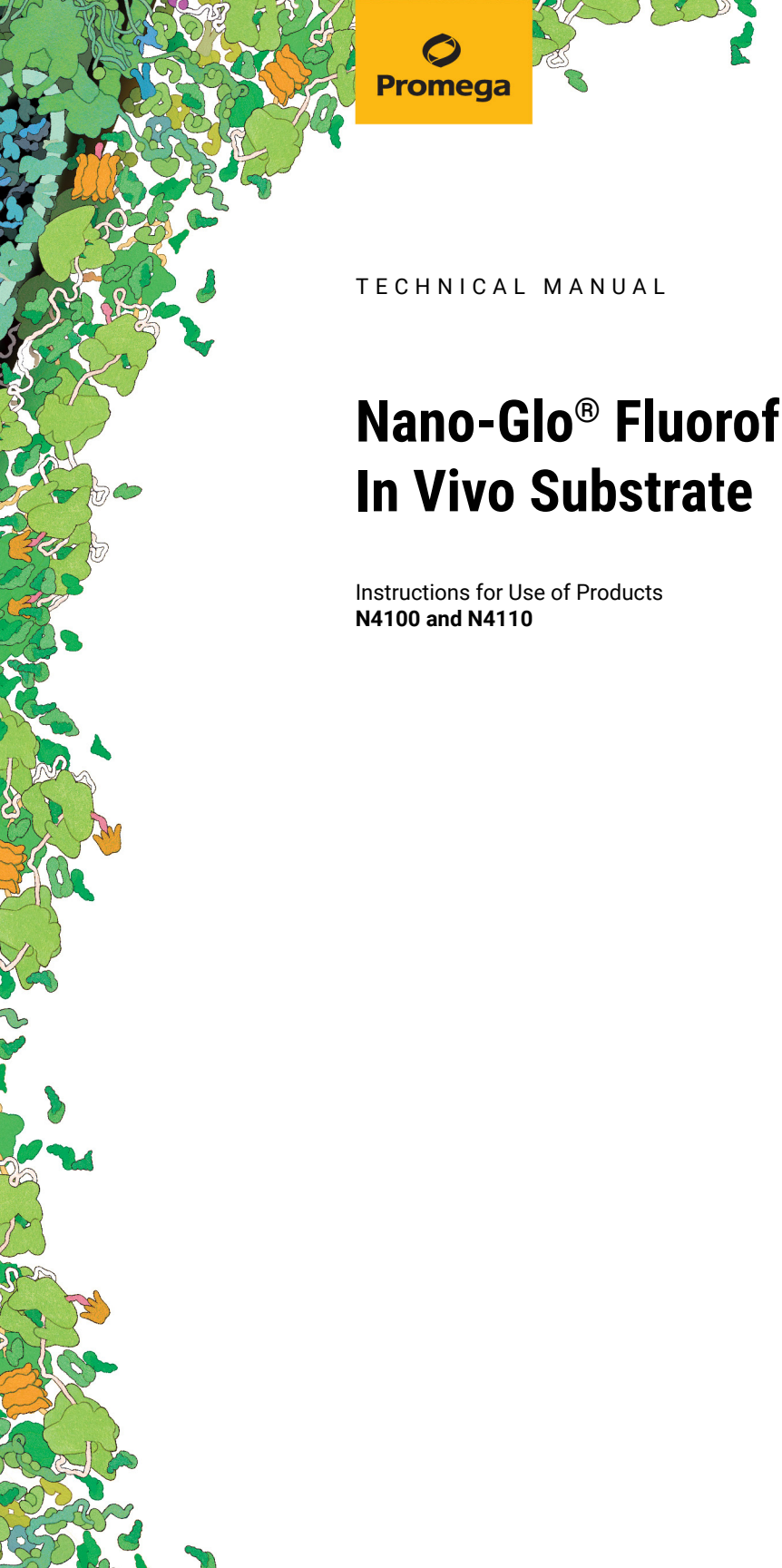

TECHNICAL MANUAL

Nano-Glo® Fluorofurimazine In Vivo Substrate

Instructions for Use of Products
N4100 and N4110

11/23
TM722

Nano-Glo® Fluorofurimazine In Vivo Substrate

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Nano-Glo® Fluorofurimazine In Vivo Substrate[a,b] is an optimized formulation for bioluminescence imaging of NanoLuc® luciferase, NanoLuc® fusion proteins or reconstituted NanoBiT® luciferase in animal models. The formulation contains fluorofurimazine (FFz), a furimazine derivative with increased aqueous solubility, and poloxamer-407 (P-407). This formulation provides increased substrate bioavailability in vivo, leading to signals that are substantially brighter than formulations containing furimazine (1,2). The structure of fluorofurimazine is shown in Figure 1. With NanoLuc® luciferase, the emission maximum is 459nm, similar to furimazine (Figure 5).

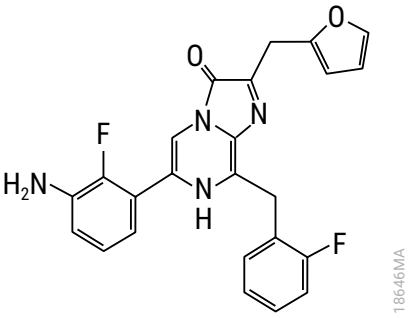

Figure 1. Fluorofurimazine chemical structure. Fluorofurimazine is a substrate for NanoLuc® and NanoBiT® luciferases. NanoBiT® luciferase is formed by complementation of Large BiT (LgBiT) with either Small BiT (SmBiT) or High BiT (HiBiT) peptides. Molecular weight = 432.43g/mol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Nano-Glo® Fluorofurimazine In Vivo Substrate	**1 vial**	**N4100**
	5 vials	**N4110**

Storage Conditions: Store lyophilized substrate at less than –65°C and protect from light. We recommend reconstituting a new vial for each in vivo imaging experiment.

3. Protocol

3.A. Overview

The Nano-Glo® Fluorofurimazine In Vivo Substrate was developed to improve bioluminescence imaging of NanoLuc® luciferase, NanoLuc® fusion proteins or NanoBiT® luciferase in animal models.The formulation contains fluorofurimazine, a furimazine derivative with increased aqueous solubility (Figure 1), that increases substrate bioavailability in vivo. Bioavailability is further enhanced by including a hydrophilic, nonionic surfactant, poloxamer 407 (P-407), in the lyophilized formulation.

Endotoxin levels are measured for each lot of the Nano-Glo® Fluorofurimazine In Vivo Substrate, and are ≤0.1EU/mg based on the lumulus amebocyte lysate (LAL) method.

3.B. Reconstituting the Nano-Glo® Fluorofurimazine In Vivo Substrate

Each vial contains a lyophilized mixture of fluorofurimazine substrate (4.6µmoles) and P-407. Add 525µl of sterile phosphate buffered saline (PBS) or sterile Dulbecco's PBS (DPBS), and gently swirl by hand to hydrate the lyophilized solid. **Do not** vortex. The reconstituted solution will appear orange. The substrate solution can be stored at room temperature for up to 8 hours with <15% decrease in fluorofurimazine concentration or at 4°C for up to 8 hours with <5% decrease in fluorofurimazine concentration. We recommend reconstituting a new vial for each in vivo imaging experiment.

If desired, the lyophilized material can be resuspended in up to 1.5ml of sterile PBS or DPBS to deliver lower doses of the fluorofurimazine substrate per injection. This will conserve substrate for experimental systems with ample brightness.

3.C. Injecting the Nano-Glo® Fluorofurimazine In Vivo Substrate

Follow standard protocols for injecting your animal model. Fully comply with your institution's guidelines for the humane treatment of animals.

Users should determine the optimal dose per injection based on their animal model and sensitivity requirements. For optimizing both intravenous (i.v.) and interperitoneal (i.p.) injection of mice, the recommended starting dose is 0.44µmoles of fluorofurimazine substrate per 24-hour period. Following injection, measure bioluminescence at several time points to generate a kinetic curve (Figure 3) and use the kinetic curve to identify an optimal time point for subsequent measurements.

For mouse i.p. injection, the maximum recommended dose is 1.5µmoles of fluorofurimazine per 24-hour period (unpublished data). Monitor for increased toxicity when exceeding the recommended dose or with more frequent injections.

For mouse i.v. injection, monitor for increased toxicity when using more than 0.44µmoles per 24-hour period or with more frequent injections.

3.D. In Vivo Imaging

Image mice following established protocols and fully comply with your institution's requirements for the humane treatment of animals. Cover the injection site if the autoluminescent signal contribution is problematic.

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4. Representative Data

Orthotopic implantation of mouse 4T1 cells into a mammary fat pad of female BALB/c mice is a syngeneic model system for breast cancer research (3). Primary tumors appear within 2–4 weeks, and both primary tumors and lung metastases can be visualized using bioluminescence (3,4).

A 4T1 cell line was created stably expressing both NanoLuc® and firefly luciferases. When tested in vitro using the Nano-Glo® Dual Luciferase® Reporter Assay System (Cat.# N1620), the NanoLuc® signal was approximately 70-fold brighter than the firefly signal, a result consistent with the known relative brightness of these reporters.

Following orthotopic implantation of the 4T1/Nluc-P2A-Fluc cell line into the #3 mammary fat pad of female BALB/c mice, tumors were allowed to grow for 22 days before binning into cohorts with tumors of similar size. Mice were i.p. or i.v. injected with the Nano-Glo® Fluorofurimazine In Vivo Substrate to measure NanoLuc® luminescence (Figure 2). Likewise, mice were i.p. injected with Vivo-Glo® Luciferin, In Vivo Grade, to measure firefly luminescence (Figure 2). In all cases, luminescence was measured every 5 minutes for 75 minutes to compare signal intensity vs. time for the different experimental conditions (Figures 2 and 3).

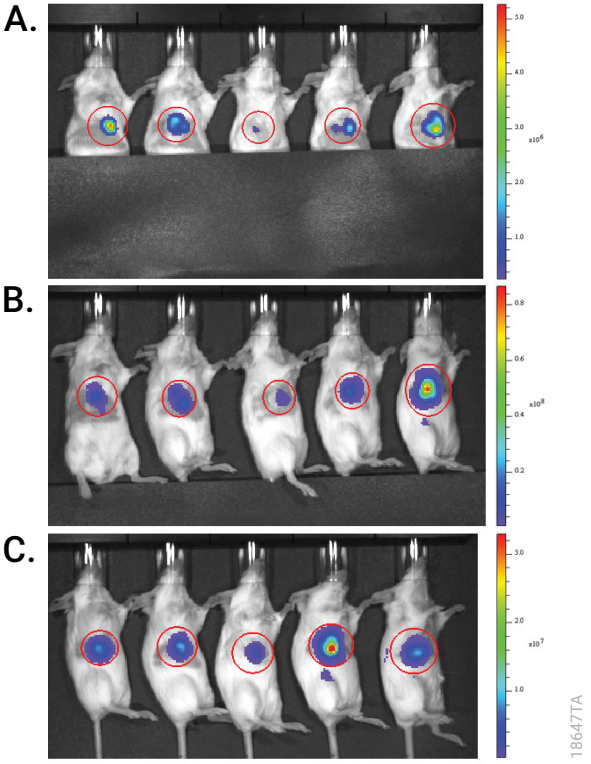

Figure 2. Bioluminescence images of 4T1 primary tumors in BALB/c mice. Ten thousand 4T1 cells expressing both NanoLuc® and firefly luciferases were implanted orthotopically into the #3 mammary fat pad of female BALB/c mice. Primary tumors were allowed to grow for 22 days prior to binning into three cohorts based on tumor size. One day after binning, bioluminescence was measured every 5 minutes for 75 minutes following injection of respective substrates. The hair in the vicinity of primary tumors was removed by shaving. **Panel A.** Cohort #1 (n = 5) was imaged 20 minutes following contralateral i.p injection of 0.44µmoles of Nano-Glo® Fluorofurimazine In Vivo Substrate (0.19mg in 50µl total volume). The color table ranges were 2.63×10^5–5.26×10^6 photons/second/cm^2/ steradian (sr). Autoluminescence from the i.p. injection site was blocked using a black, nonfluorescent plastic sheet. **Panel B.** Cohort #2 (n = 5) was imaged 10 minutes following i.v. injection of 0.44µmoles of Nano-Glo® Fluorofurimazine In Vivo Substrate (0.19mg in 50µl total volume). Autoluminescence from the tail was blocked using a black, nonfluorescent plastic sheet. The color table ranges were 6.08×10^5–8.67×10^7 photons/second/cm^2/sr. **Panel C.** Cohort #3 (n = 5) was imaged 20 minutes following contralateral i.p. injection of 9.4µmoles of Vivo-Glo® Luciferin, In Vivo Grade (3mg in 100µl). The color table ranges were 2.93×10^5–3.29×10^7 photons/second/cm^2/sr. Prior to binning, primary tumor sizes were similar for all three cohorts, with an average tumor volume of 135 ± 50mm^3. Images were acquired using an IVIS Spectrum In Vivo Imaging System (Perkin Elmer). IVIS settings: Emission filter, open; FOV, 22.4 x 22.4cm; f stop, 1; binning, M(8); autoexposure times, 15, 1 and 3 seconds for Panels A, B and C, respectively. Bioluminescence imaging was done at the University of Wisconsin Small Animal Imaging and Radiotherapy Facility, Madison, WI. Images were analyzed using Living Image software (version 4.7.4). Regions of interest for calculating total photon flux (see Figure 3) are indicated in each image.

4. Representative Data (continued)

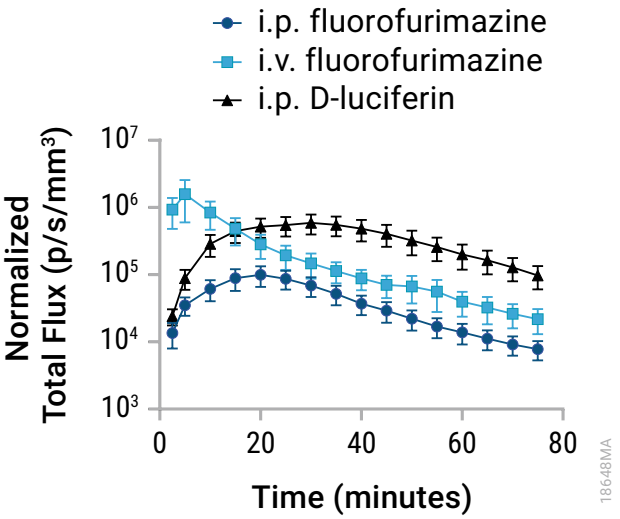

Figure 3. Kinetic curves for i.p. and i.v. injection of Nano-Glo® Fluorofurimazine In Vivo Substrate and Vivo-Glo® Luciferin, In Vivo Grade. For the experiment in Figure 2, the average value of the normalized total flux (photons/sec/mm^3) for each cohort is plotted at the indicated time points. Error is represented as standard error of the mean (SEM). Regions of interest for the calculation of total photon flux are indicated in Figure 2.

5. Appendix

5.A. Autoluminescence Following Intraperitoneal Injection

Like furimazine, fluorofurimazine will undergo enzyme-independent oxidation with concomitant emission from an excited state to produce autoluminescence. The intensity of the autoluminescence signal can be variable (Figure 4), and signal intensity will vary as a function of time post-injection (Figure 4). Physically block the injection site (e.g., with a black, nonfluorescent plastic sheet) or pick an alternative injection route to reduce or eliminate interfering autoluminescence.

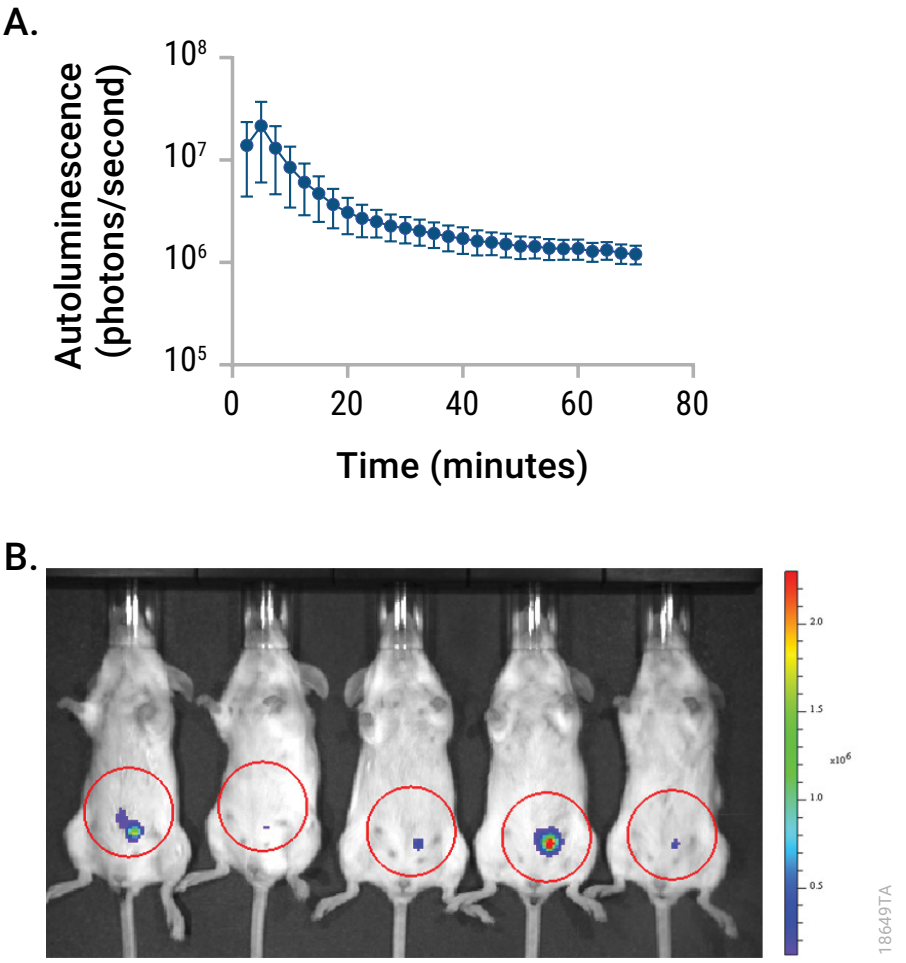

Figure 4. Autoluminescence following intraperitoneal injection of the Nano-Glo® Fluorofurimazine In Vivo Substrate. Unshaven female BALB/c mice (n = 5) received an intraperitoneal injection of 0.44µmoles of the Nano-Glo® Fluorofurimazine In Vivo Substrate. Following injection, luminescence was measured every 2.5 minutes for 70 minutes using an IVIS Spectrum In Vivo Imaging System (Perkin Elmer). **Panel A.** Average total photon flux (photons/second) plotted vs. time post-injection. Error is represented as standard error of the mean (SEM). **Panel B.** Mice were imaged 20 minutes post i.p. injection using a 20-second exposure. The color table ranges are 1.2×10^5–2.3×10^6 photons/second/cm^2/ steradian (sr). Regions of interest for the calculation of total photon flux are indicated for each mouse. IVIS settings: emission filter, open; FOV, 22.4 × 22.4cm; f stop, 1; binning, M(8); autoexposure times, 3–60 seconds. Bioluminescence imaging was done at the University of Wisconsin Small Animal Imaging and Radiotherapy Facility, Madison, WI. Images were analyzed using Living Image software (version 4.7.4).

5.B. Emission Spectra of Fluorofurimazine and Furimazine

NanoLuc®-mediated turnover of fluorofurimazine and furimazine produce similar emission spectra (Figure 5). As both substrates are blue-light emitters, signal attenuation will occur at deeper tissue depths due to hemoglobin absorbance and light scattering.

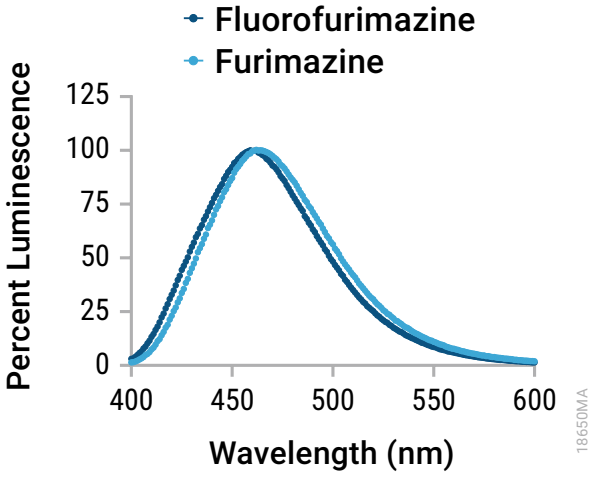

Figure 5. Normalized emission spectra of fluorofurimazine and furimazine in vitro. Purified NanoLuc®-HaloTag® fusion protein was diluted in TBS + 0.01% BSA to a final concentration of 12nM. Fluorofurimazine or furimazine were diluted in TBS + 0.01% BSA to a final concentration of 40µM. Equal volumes of enzyme and substate were mixed in a white, 96-well plate and then immediately scanned over the emission wavelength (Infinite® M1000 PRO multimode reader, Tecan). Final concentrations of enzyme and substrate were 6nM and 20µM, respectively. The emission maximum for fluorofurimazine was 459nm. The emission maximum for furimazine was 462nm.

5.C. Frequently Asked Questions

Question: I have leftover reconstituted material after completing my experiment. Can I store this material for subsequent use?

Answer: We recommend reconstituting a new vial of Nano-Glo® Fluorofurimazine In Vivo Substrate for each in vivo imaging experiment. If you do choose to store reconstituted material, we recommend only short-term storage at 4°C, where you can expect <5% decrease in fluorofurimazine concentration over 8 hours. We cannot guarantee acceptable performance for your in vivo imaging application if stored long term at colder temperatures or following freeze/thaw cycles.

Question: Can firefly and NanoLuc® luciferases be multiplexed in vivo?

Answer: Yes. The optimal dosing regimen must be determined for each experimental system. For example, dose respective substrates on consecutive days (1).

Question: Can the Nano-Glo® Fluorofurimazine In Vivo Substrate be used to image NanoLuc®, NanoLuc® fusion proteins or NanoBiT® luciferase at deeper tissue depths?

Answer: Yes. Although signal attenuation will occur due to hemoglobin absorbance and light scattering (Section 5.B), NanoLuc® luciferase can be used to image deep tissue (2). Higher doses of FFz may be required. Alternatively, a NanoLuc®-based construct that relies on resonance energy transfer and red-shifted emission can be used instead (1,5).

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Autoluminescence at the i.p. injection site that obscures the signal	• Try a different injection route (e.g., i.v.). • Use a contralateral i.p. injection. • Inject less substrate. • Cover the i.p. injection site during imaging (e.g., with a black, nonfluorescent plastic sheet).
Signal is dim or undetectable	Factors such as tissue depth and substrate availability will affect signal strength. If using i.p. injection, increase the amount of substrate delivered to 1.5µmoles, the maximum recommended dose. Alternatively, switch to i.v. injection of ≥0.44µmoles of fluorofurimazine. If attempting to measure luminescence in deep tissues, switch to a NanoLuc®-based construct that relies on resonance energy transfer and red-shifted emission to mitigate signal attenuation (5).

7. References

1. Su, Y. *et al*. (2020) Novel NanoLuc substrates enable bright two-population bioluminescence imaging in animals. *Nat. Methods* **17**, 852–60.
2. Gasper, N. *et al*. (2021) Evaluation of NanoLuc substrates for bioluminescence imaging of transferred cells in mice. *J. Photochem. Photobiol. B*. **216**, 112118.
3. Pulaski, B.A. and Ostrand-Rosenberg, S. (2001) Mouse 4T1 breast tumor model. In: *Current Protocols in Immunology* Chapter 20, Unit 20.2.
4. Paschall, A.V. and Liu, K. (2016) An orthotopic mouse model of spontaneous breast cancer metastasis. *J. Vis. Exp.* **114**, 54040.
5. Chu, J. *et al*. (2016) A bright cyan-excitable orange fluorescent protein facilitates dual-emission microscopy and enhances bioluminescence imaging in vivo. *Nat. Biotechnol*. **34**, 760–7.

8. Related Products

Product	Size	Cat.#
VivoGlo™ Luciferin, In Vivo Grade	50mg	P1041
	250mg	P1042
	1g	P1043

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(a) use NanoBRET™-branded energy acceptors (e.g., BRET-optimized HaloTag® ligands) for all determinations of energy transfer activity by this product; or

(b) contact Promega to obtain a license for use of the product for energy transfer assays to energy acceptors not manufactured by Promega.

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[b]U.S. Pat. No. 11,691,976 and other patents pending.

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