CHAPTER TWELVE

A Highly Sensitive Biosensor for ATP Using a Chimeric Firefly Luciferase

Bruce R. Branchini[1], **Tara L. Southworth**
Connecticut College, New London, CT, United States
[1]Corresponding author: e-mail address: brbra@conncoll.edu

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Abstract

Firefly luciferases, which emit visible light in a highly specific ATP-dependent process, have been adapted for a variety of applications based on the detection of the enzymes or using the proteins to measure ATP levels. Based on studies of chimeric luciferases, we engineered a novel luciferase called PLG2 that has enhanced specific activity, and thermal and pH stability compared to the commonly used *Photinus pyralis* luciferase. We present here protocols for preparing a single assay mixture containing PLG2 that can be used to readily detect femtomole levels of ATP. Our methodology can be used with a variety of samples, including human and bacterial cells, where measurements of ATP can be used as a biosensor for the detection of viable cells.

1. INTRODUCTION

Bioluminescence, the emission of visible light by living organisms, is broadly distributed in the marine and terrestrial environments. Typically,

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light is produced by enzymes (luciferases) and substrates (luciferins and O_2) in biochemical reactions that often require additional cofactors. Methods for analyte detection based on a variety of bioluminescence materials are important research tools in cell biology, molecular biology, and analytical chemistry (Roda, 2011; Widder & Falls, 2014). The general advantages of bioluminescence methods include: relative ease of performance, high sensitivity due to low background, wide dynamic range, and low cost of photomultiplier tube (PMT) and charge-coupled device detectors. Numerous applications of beetle luciferases, especially those from fireflies, include whole-cell-based biosensors (Binkowski et al., 2011; Bottcher, Pollet, Delius, & Niehrs, 2004; Etzov, Cohen, & Marks, 2015; Hakkila, Maksimow, Karp, & Virta, 2002; Roda et al., 2011; Shi et al., 2014; Urban et al., 2007), gene expression assays (Gould & Subramani, 1988; Leclerc, Boockfor, Faught, & Frawley, 2000; Paddison, Caudy, & Hannon, 2002; Vannini et al., 2012), and imaging disease states in live mammals (Cevenini et al., 2014; Kuo, Auriau, Pierre-Eugene, & Issad, 2014; Lewis et al., 2014; Taylor & Kelly, 2014). Many of these applications can be applied in high-throughput screening format in support of drug development (Afshari, Uhde-Stone, & Lu, 2015; Che, Cui, Kutsch, Cui, & Li, 2012; Hattori & Ozawa, 2015; Roda, Guardigli, Michelini, & Mirasoli, 2009; Thorne et al., 2012; Yoon, Namkung, & Lee, 2013). In addition to the general advantages of bioluminescence methods, beetle bioluminescence methods take advantage of the stringent requirement for ATP, the universal biological energy source. Beetle luciferase (abbreviated here as Luc) assays for the production, consumption, or presence of ATP are based on the role of the metabolite in the activation of beetle luciferin (LH_2), the first step (Eq. 1) in the production of light (Eqs. 1–3).

$$\mathrm{Luc} + \mathrm{LH_2} + \mathrm{ATP} \overset{\mathrm{Mg^{2+}}}{\leftrightarrows} \mathrm{Luc \cdot LH_2 - AMP} + \mathrm{PP}_i \quad (1)$$

$$\mathrm{Luc \cdot LH_2 - AMP} + \mathrm{O_2} \rightarrow \mathrm{Luc \cdot AMP \cdot Oxyluciferin^*} + \mathrm{CO_2} \quad (2)$$

$$\mathrm{Luc \cdot AMP \cdot Oxyluciferin^*} \rightarrow \mathrm{Luc} + \mathrm{Oxyluciferin} + \mathrm{AMP} + h\nu \quad (3)$$

The *Photinus pyralis* Luc bioluminescence system is highly efficient with a quantum yield of $41.0\% \pm 7.4\%$ (Ando et al., 2008). It has been estimated that attomole levels of Luc can be quantified (Lundin, 1993), and unlike with fluorescence systems that require an external light source,

Table 1 Properties of Luciferases

Enzyme	Relative Integrated Specific Activity[a]	K_m (μ*M*)		Bioluminescence λ_{max}[b] (nm)		Thermal Inactivation[c] (h) at 37°C
		LH_2	Mg-ATP	pH 7.8	pH 6.5	
PpyWT[d]	100 ± 2	15 ± 2	86 ± 7	560 (70)	609, 560 sh (92)	0.3 ± 0.03
PLG2	425 ± 2	52 ± 6	79 ± 5	559 (67)	561 (71)	>24 ± 5

[a]Specific activities are based on total light emission in 15 min, were obtained at pH 7.8 with LH_2 (400 μ*M*) and Mg-ATP (2 m*M*), and are expressed relative to the PpyWT value, which is defined as 100.
[b]Bioluminescence emission maxima were obtained from two trials and the standard deviation is ±1 nm. The bandwidth at half-maximal intensity is shown in parentheses and sh indicates a shoulder.
[c]Time for the maximum initial activity to decay to 50% at 37°C. All activity values were obtained from at least three trials and are reported as means ± standard deviation.
[d]Recombinant *P. pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer (Branchini et al., 2014).

there is virtually no nonspecific light production induced in the cellular environment.

We have developed improved biosensor methods for the detection of ATP by taking advantage of the highly specific and sensitive detection of ATP generally afforded by beetle Lucs. The key component is a highly engineered version of *P. pyralis* Luc called PLG2 (Table 1) (Branchini et al., 2015). The design of this enzyme was inspired by earlier studies of the enhanced specific activity of a chimeric Luc (Branchini et al., 2014), which contains the N-domain of *P. pyralis* Luc joined to the C-domain of the *Luciola italica* enzyme (Branchini, Southworth, DeAngelis, Roda, & Michelini, 2006). We present procedures using PLG2 in in vitro assays designed to detect femtomole levels of ATP from biological samples including living cells.

2. ATP DETECTION PROTOCOL

2.1 Sample Preparation

The assay protocol described here can be used to determine levels of cells and microorganisms in a variety of products including foodstuffs, water, and surfaces based on their ATP content. Methods of sample preparation, including extraction procedures when necessary, will vary according to the specific materials being analyzed. An excellent guide to sample preparation can be found in the technical bulletin of the Promega ENLITEN® ATP Assay

System (ENLITEN ATP Assay System Bioluminescence Detection Kit for ATP Measurement, https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/enliten-atp-assay-system-bioluminescence-detection-kit-for-atp-protocl.pdf).

2.2 Light Measurement

1. Luminometer. Microwell plate reading and single tube luminometers are available from several manufacturers and can be purchased from Promega, Thermo Scientific, Berthold Technologies, BioTek, and others. Although not essential, it is preferable to use an instrument with a single automatic injector. Assays can be performed at room temperature, but standard curves should be made at the temperature where unknown assays will be performed. The detection limit of ATP assays will ultimately depend on the "dark background," the signal produced in the absence of ATP. For the protocols described here, we used a Thermo Luminoskan Ascent instrument equipped with a PMT operated at 900 V and a single automatic injector. The reagent lines and injector were cleaned prior to and after use with 70% ethanol. If more stringent cleaning procedures are required, consult the technical bulletin of the Promega ENLITEN® ATP Assay System (ENLIGHTEN ATP Assay System Bioluminescence Detection Kit for ATP Measurement).
2. Measurement of relative light units (RLUs). Luciferase activity is commonly expressed in RLUs that are defined for the equipment being employed. The RLUs in the protocols presented here have been related to the definition described in the Sigma-Aldrich catalog for recombinant *P. pyralis* luciferase. We prepared a stock solution of Sigma product SRE0045 by dissolving a 1 mg vial of reagent in 1 mL of 1 *M* Tris buffer, pH 7.6 with brief gentle mixing. In triplicate, aliquots of a 1:10 dilution of the stock solution (2 μL, 2×10^{-4} mg enzyme) were pipetted into wells of a 96-well white plate (Greiner Bio-One North America, Inc., Monroe, NC). The luminometer injector was loaded with a solution comprising Promega LH_2 potassium salt (150 μ*M*) and Sigma Mg-ATP (0.4 μ*M*) in 50 m*M* Tricine buffer, pH 7.6, and 0.1 mL of the solution was injected into the wells containing enzyme. Light emission from the wells was monitored until the signals were steady and not decaying and then the RLUs were recorded, for example, at 10 s. The Luminoskan mean value reading of 1340 (after blank subtraction) was set to 4×10^6 RLU $[(2 \times 10^{-4}\ \text{mg}) \times (2 \times 10^{10}\ \text{RLU/mg})]$; therefore, 1 Luminoskan

unit = 2985 RLU. Using our standard methods (Branchini et al., 2014) for measuring specific activity, we obtained values for our recombinant *P. pyralis* luciferase essentially equivalent to the Promega Luc2 enzyme.

2.3 Preparation of PLG2

1. The PLG2 luciferase required for PLG2 Assay Mix preparation can be obtained from the procedures below by transforming *E. coli* with the pGEX-6P-2 PLG2 plasmid and inducing expression of the GST-luciferase fusion protein. Please contact the authors to obtain the plasmid or a sample of the PLG2 Assay Mix (see Section 2.4 later).
2. Preparation of protein expression reagents. LB/AMP media is prepared by dissolving 25 g Luria Bertani media (Fisher Scientific) in 1 L of distilled deionized H_2O (ddH_2O), autoclaving the media, and adding 1 mL of Ampicillin solution (100 mg/mL in ddH_2O, sterile filtered) once the media has cooled. Store at 5°C. IPTG solution (24 mg/mL) is made up by dissolving isopropyl-α-D-thiogalactoside in ddH_2O. Solutions should be sterile filtered, divided into aliquots and stored at −20°C.
3. Preparation of bacteria cell pellet. For a 0.25-L culture whose expression typically yields ~6 mg of purified enzyme, prepare a starter culture by using a sterile inoculating loop to scrape the surface of a glycerol stock of pGEX-6P-2 PLG2 plasmid (previously transformed into *E. coli* strain BL21(DE3)pLysS cells), inoculating 5 mL of LB/AMP, and growing it overnight at 37°C with vigorous shaking (325 rpm). Add 3 mL of starter culture to 250 mL of LB/AMP in a 1-L flask and grow at 37°C with shaking while monitoring cell growth of 1 mL aliquots by absorbance at 600 nm. Within 1.5–2 h following inoculation, the A_{600} nm should reach 0.4–0.6, and at this point, the incubator temperature is adjusted to 22°C. After 15 min, add 0.25 mL of IPTG solution and continue shaking at 22°C for 19 h. Harvest cells by centrifugation at 5000 × *g* for 10 min at 4°C and discard the supernatant. Freeze the cell pellet at −80°C for 15 min, thaw, and continue the preparation or store (stable for months) at this temperature.
4. Preparation of protein purification reagents. Store all solutions at 4°C and keep on ice during protein purification. Prepare 1 L of 10 × PBS buffer: 400 m*M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, and 18 m*M* KH_2PO_4 in ddH_2O, pH 7.3, and 1 L of 1 × PBS by dilution of 10 × PBS in ddH_2O; buffers are sterilized by filtration. Resuspension

buffer is prepared immediately prior to use by adding 50 μL of 25 mg/mL phenylmethylsulfonyl fluoride (PMSF, Sigma) solution (made by dissolving 2.5 mg of PMSF in 0.1 mL of 95% ethanol with vigorous mixing) to 50 mL of 1 × PBS containing 3.8 mg of DTT. Lysozyme (Sigma, from egg white) solution is prepared by dissolving 10 mg/mL lysozyme in 1 × PBS. RNaseA solution (Sigma, from bovine pancreas) in 10 mg/mL, RNaseA in 10 m*M* Tris buffer, pH 7.5, containing 15 m*M* NaCl. DNaseI solution is 5 mg/mL DNaseI (Sigma, from bovine pancreas) in 10 m*M* Tris buffer, pH 7.5 containing glycerol (1:1, v/v), 50 m*M* NaCl, 10 m*M* $MgCl_2$, and 1 m*M* DTT. Protease cleavage buffer (PCB) comprises 50 m*M* Tris, pH 7.0, containing 150 m*M* NaCl, 1 m*M* EDTA, and 1 m*M* DTT.

5. Preparation of PLG2 activity assay solutions. LH_2 solution is 0.52 m*M* LH_2 (Promega, K salt) in 50 m*M* Tricine buffer, pH 7.8. Dissolve ~2 mg LH_2 in 10 mL of buffer and adjust concentration by dilution based on UV using a molar absorptivity coefficient of 7600 *M*/cm at 266 nm. ATP solution is prepared by dissolving 9 m*M* ATP (Sigma, Mg salt) in 50 m*M* Tricine buffer, pH 7.8 as follows: Dissolve ~60 mg Mg-ATP in 10 mL buffer, readjust pH to ~7.8 with 1 N NaOH, and adjust concentration using a molar absorptivity coefficient of 15,400 *M*/cm at 259 nm.
6. Equilibration of Glutathione Sepharose® 4B media. In a cold room (5°C), pipet 1.5 mL of slurry of Glutathione Sepharose® 4B (GE Healthcare Life Sciences) into a 50 mL plastic (polypropylene or polycarbonate plastics, but not polystyrene, are compatible with luciferases) centrifuge tube, add 25 mL of cold 1 × PBS, and centrifuge at 500 × *g* for 5 min. Carefully pipet off ~15 mL of supernatant making sure not to remove resin. Wash resin three times with 25 mL portions of cold 1 × PBS and after the third wash, pipet off supernatant to bring down the volume to 3 mL, creating a 50% resin slurry. Store on ice.
7. Isolation of crude enzyme. Samples should be kept on ice during all procedures. Thaw bacteria pellet (Section 2.3 step 3) for 30 min (on ice) and add 25 mL of resuspension buffer. Gently resuspend the pellet by withdrawing and expelling the mixture with a pipet, making sure to avoid foaming and bubbling. Add 2.5 mL of lysozyme solution, let stand for 20 min, and sonicate. We use a Virsonic Model 475 instrument with a 0.5-in. tip diameter at setting 3 (95 W) and apply 3 bursts at 10 s each. It is important to avoid frothing during the sonication.

Add 25 μL RNaseA solution and 25 μL DNaseI solution and incubate mixture for 15 min. Add 1.25 mL 20% Triton X-100 (aq., v/v), mix, transfer the suspension into a 50 mL centrifuge tube, and centrifuge at $20{,}000 \times g$ for 45 min at 4°C, carefully decant the supernatant (avoid taking any suspended pellet) into a centrifuge tube and record the volume of the crude protein solution (should be ~25 mL).

8. Perform enzyme activity measurement on crude supernatant. It is a good idea to check the total activity of the supernatant prior to continuing to the affinity chromatography step. However, it is necessary to do this quickly to avoid loss of activity. Assay the supernatant by pipetting 3 μL aliquots into wells of a 96-well white plate (Greiner Bio-One North America, Inc., Monroe, NC) containing 0.2 mL of 0.52 m*M* LH_2 solution (prepared as described in Section 2.3 step 5). Load the luminometer injector with the solution of 9 m*M* Mg-ATP (Section 2.3 step 5) and inject 60 μL into the wells containing enzyme and LH_2. Monitor the RLUs for ~30 s and record the maximum RLU value ($\sim 1.3 \times 10^{10}$ RLU).
9. Glutathione Sepharose® 4B affinity chromatography. Additional general information is available in the GST Gene Fusion System Handbook 18-1157-58 (GE Healthcare http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/GST_gene_fusion_system_handbook.pdf). All procedures are performed in a cold room (5°C). Add 3 mL of resin slurry (Section 2.3 step 6) to the crude supernatant (Section 2.3 step 7) in a centrifuge tube with gentle agitation (we use a VWR rocking platform at setting 3.5) for 2 h. Centrifuge at $500 \times g$ at 4°C for 5 min, carefully remove the supernatant (avoid removing resin), and record the volume of the supernatant (typically ~20 mL). Determine the percentage of RLU remaining in the supernatant (Section 2.3 step 8), typically ~25–45%. Wash resin (three times) using 30 mL cold PBS with brief gentle mixing, centrifuge ($500 \times g$ at 4°C for 2 min), and remove supernatant by pipet. Next wash resin with 30 mL of PCB, centrifuge, remove supernatant, and transfer to a 5 mL polypropylene tube keeping ~3 mL of the ~50% slurry. Add 100 units of PreScission (GE Healthcare Life Sciences) to remove the GST tag and gently mix overnight (~16 h) (we use a VWR Tube Rotator). Transfer the resin slurry to a gravity flow column, collect, and save the flow through (~1.5 mL) and elute with 3 mL PCB

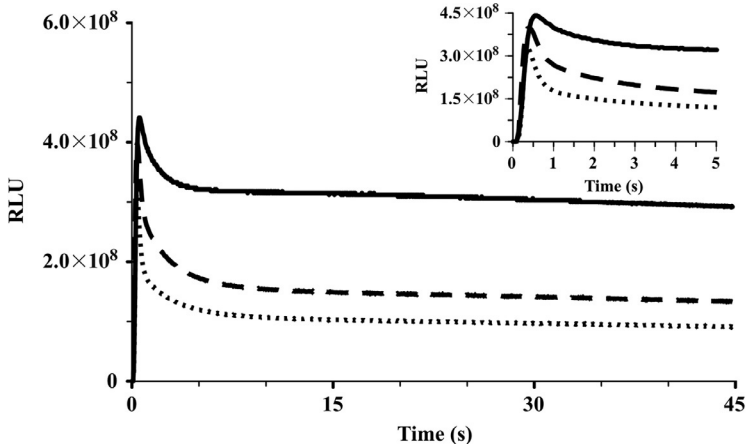

Fig. 1 Bioluminescence time course of reactions of PLG2 (–), recombinant *P. pyralis* luciferase containing the N-terminal peptide GlyProLeuGlySer (- -) and Sigma recombinant *P. pyralis* luciferase (····). Aliquots of purified enzymes (2 μL of 0.01 mg/mL solution) in PCB were added to the wells of a 96-well white plate. The reaction was initiated by the injection of a solution containing 0.40 m*M* LH_2 and 2 m*M* Mg-ATP in 50 m*M* Tricine, pH 8.

(collect 1 mL fractions). Determine the protein concentration of the flow through and column fractions and combine those that are at least ~1 mg/mL. We recommend the Bio-Rad Protein Assay System using bovine serum albumin as the standard.

10. Determine the specific activity of purified PLG2. Measure the activity of the pooled protein and calculate the specific activity, which should be $\sim 2.2 \times 10^{13}$ RLU/mg. By comparison, the specific activity of the Sigma luciferase is 1.6×10^{13} RLU/mg (Fig. 1).
11. Enzyme storage. PLG2 solutions (~2 mg/mL in PCB) can be stored for 1–2 months at 5°C. For long-term storage, flash freeze 0.1–0.3 mL aliquots in thin-walled PCR tubes by submerging in liquid nitrogen for 60 s and store at −80°C. Enzyme solutions can be thawed by gently swirling tubes in a 37°C water bath until only a small amount (size of a grain of rice) remains and immediately place on ice. Thawed aliquots can be used to make PLG2 Assay Mix.

2.4 PLG2 Assay Mix for ATP Determination

Approximately 100 ATP assays can be performed with 5 mL of the PLG2 Assay Mix (3.3 μ*M* PLG2, 0.8 m*M* LH_2 and 10 m*M* $MgSO_4$ in 50 m*M* Tricine buffer, pH 8).

1. Autoclave 0.5 L of 50 m*M* Tricine buffer, pH 8, microcentrifuge tubes, 5–10 mL plastic tubes and caps, and pipet tips.
2. Prepare 100 m*M* $MgSO_4$ stock solution in 50 m*M* Tricine buffer, pH 8.0, and store at 5°C.
3. Prepare a 5 m*M* LH_2 stock solution by dissolving ~2 mg of LH_2 potassium salt in 1 mL of 50 m*M* Tricine buffer, pH 8.0, and adjust the concentration by dilution based on UV using a molar absorptivity coefficient of 7600 at 266 nm.
4. For 5 mL of PLG2 Assay Mix, add 0.8 mL LH_2 stock solution, 0.5 $MgSO_4$ stock solution, 1 mL of 1.0 mg/mL PLG2 in PCB, and 50 m*M* Tricine buffer, pH 8 to volume. For highest sensitivity (ATP levels below $\sim 1 \times 10^{-14}$mol), background emission can be reduced by storing the PLG2 Assay Mix overnight at 5°C. This is necessary to detect ATP levels below $\sim 1 \times 10^{-14}$ mol. PLG2 Assay Mix can be stored at 5°C for 48 h without measurable loss of activity, while after 2 weeks, the solution retained 85% activity. For convenience, the assay solution can be lyophilized, stored at −20°C for months, and reconstituted with ATP-free ddH_2O.

2.5 ATP Standard Curve and Analysis

1. Prepare a standard 2 m*M* solution of ATP (Sigma, disodium salt hydrate) in 50 m*M* Tricine, pH 8. Dissolve ~6 mg of ATP in 5 mL buffer, readjust pH to 8.0 with 1 N NaOH and adjust the concentration using a molar absorptivity coefficient of 15,400 at 259 nm. Serially dilute the 2 m*M* ATP standard solution into the Tricine buffer to make a set of concentrations ranging from 0.2 μ*M* to 0.2 p*M*. The stock ATP solution can be stored at 5°C for at least a week and the dilutions can be made, stored at 5°C, and used the next day.
2. Assay 0.05 mL aliquots of the ATP dilutions (corresponding to 1×10^{-11} to 1×10^{-17} mol of ATP) in triplicate in white 96-well plates (Greiner Bio-One North America, Inc., Monroe, NC) and a blank (Tricine buffer, no ATP) by automatic injection of 0.05 mL of the PLG2 Assay Mix. Record light emission for 1 s after a 1 s delay, record the mean test values after subtraction of the instrument blank. A representative standard curve is shown in Fig. 2. The data were analyzed using the regression function of the Analysis ToolPack of Microsoft Excel version 14.0. To detect statistical correlations, Pearson's correlation coefficient (*r*) was calculated between the light emission and moles of ATP. The associated

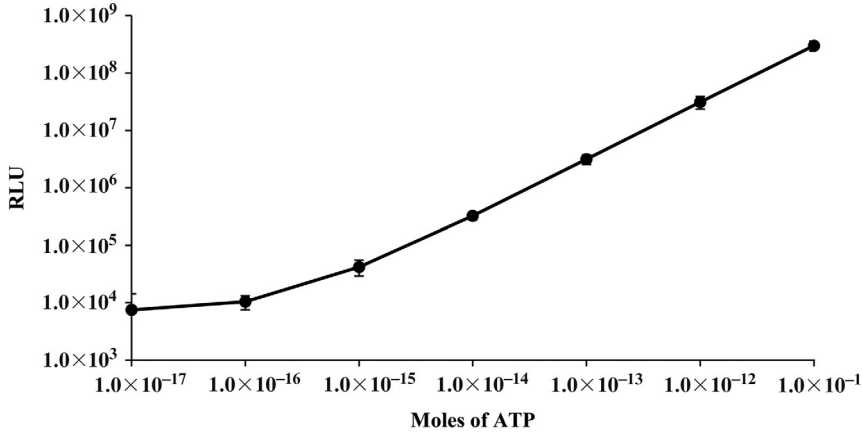

Fig. 2 ATP standard curve generated from the bioluminescent signals with PLG2 Assay Mix (Section 2.5). The mean relative light units (RLUs) for 1 s integrals from three independent experiments are plotted as a function of ATP levels and the standard deviations are indicated by error bars. The correlation coefficient (*r*) for the portion of the curve from 1×10^{-16} to 1×10^{-11} mol of ATP is 0.999 and the associated significance is $P < 0.001$. Source*: Branchini, B. R., Southworth, T. L., Fontaine, D. M., Kohrt, D., Talukder, M., Michelini, E.,…Grossel, M. J. (2015). An enhanced chimeric firefly luciferase-inspired enzyme for ATP detection and bioluminescence reporter and imaging applications.* Analytical Biochemistry, 484*, 148–153. doi:10.1016/j.ab.2015.05.020.*

probability (*P*) was determined using the Student *t*-distribution function of Excel.

3. User samples should be assayed on the same day that the standard curve is constructed.

2.6 Viable Cell Quantitation Protocol

1. Generate an ATP standard curve (Section 2.5) by preparing the ATP solutions in 50 m*M* Tricine buffer, pH 8 containing 10% PBS.
2. We validated this application by demonstrating that ATP levels correlated with cell number using HEK293T cells (Fig. 3). Aliquots of cell suspensions were washed twice with PBS and counted using a TC10 automated cell counter (Bio-Rad). Triplicate cell suspensions (200,000 cells in 0.1 mL of PBS) were serially diluted in PBS and lysed by five cycles of freezing at −80°C and thawing at 30°C. The lysed cell suspensions were diluted 10-fold in 50 m*M* Tricine buffer, pH 8, and aliquots (0.05 mL) were assayed in triplicate as described in Section 2.5 step 2.

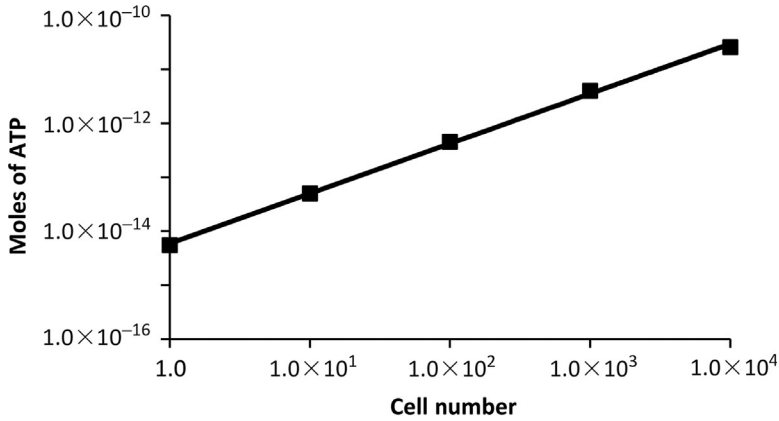

Fig. 3 Correlation between HEK293T cell numbers and ATP levels. Using the protocol described in Section 2.6, a standard ATP curve was made and used to measure actual ATP content in the mammalian cells. Bioluminescence was measured with the PLG2 Assay Mix in experiments that were performed three times with individual assays performed in triplicate. Symbols represent mean values, and the line represents the linear data fit. Error bars indicate the standard deviations among replicates. Pearson's correlation coefficient (*r*) and the associated significance (*P*) value were: $r = 0.999$, $P < 0.001$. Source*: Branchini, B. R., Southworth, T. L., Fontaine, D. M., Kohrt, D., Talukder, M., Michelini, E., … Grossel, M. J. (2015). An enhanced chimeric firefly luciferase-inspired enzyme for ATP detection and bioluminescence reporter and imaging applications.* Analytical Biochemistry, 484*, 148–153. doi:10.1016/j.ab.2015.05.020.*

3. CONCLUSIONS

3.1 PLG2-Based ATP Analysis

A rapid and simple method for ATP assays using PLG2 has been described that, based on the standard curve provided in Fig. 2, provides linearity over six orders of magnitude with a detection limit of $\sim 1 \times 10^{-16}$mol of ATP. The method provides better sensitivity than the femtomole detection capabilities of the Promega ENLITEN® kit and represents detection of ATP at a level limited only by instrument noise. The sensitivity improvement that is possible with PLG2 enables using less enzyme to reach femtomole detection. Moreover, the enhanced stability of PLG2 should provide performance advantages compared to currently available standard luciferases. While results in individual labs may vary, especially depending on the type of light measuring device that is used, the detection of ATP is straightforward and feasible with a stable, inexpensive single assay reagent, and a basic detection device. Additionally, the methodology can be used to detect viable

mammalian and bacterial cells (Branchini et al., 2015). Using mammalian cells, light intensity and cell number were proportional over four orders of magnitude, from 1 to 10,000 mammalian cells (Fig. 3, $r = 0.999$, $P < 0.001$). Using a standard curve similar to the one in Fig. 2, we determined that there were 5.5 ± 0.4 fmol of ATP in a single HEK293T cell, in very good agreement with the reported value of 2–6 fmol ATP per viable mammalian cell (Sonderhoff, Kilburn, & Piret, 1992), thus validating our protocol as we had previously done with bacterial cells (Branchini et al., 2015).

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