LUMIGEN
A Beckman Coulter Company

EVALUATION OF LUMIGEN HYPERBLU™ TECHNOLOGY FOR HIGH-THROUGHPUT DETECTION OF ENZYMATICALLY PRODUCED HYDROGEN PEROXIDE

Abstract

The enzymatic production of hydrogen peroxide in cells has been linked to oxidative stress and attending cellular damage[1]. Hydrogen peroxide is normally detected in vitro assays using colorimetric and fluorescent methodologies; the latter includes the use of horseradish peroxidase and resorufin-based fluorescence in a coupled-enzyme system. HyPerBlu is a dioxetane-boronic acid which reacts directly with hydrogen peroxide to produce a sustained chemiluminescent signal. HyPerBlu reagent's wide tolerance for common assay additives (e.g., DMSO), the lack of the need for coupling enzymes, chemical specificity, one component structure, and excellent stability lead to assay simplification with excellent sensitivity over a wide range of hydrogen peroxide concentrations. We demonstrate that HyPerBlu can be used as a highly sensitive assay to measure xanthine oxidase activity in 1536-well format, and compare the results of compound profiling with the traditional horseradish peroxidase fluorescence assay technology.

Key Words

Hydrogen peroxide, high-throughput screening, HyPerBlu, chemiluminescence, oxidase

INTRODUCTION

Hydrogen peroxide is a common enzymatic product in numerous biological pathways[1], and exerts untoward physiological effects when allowed to accumulate within cells[2]. The evolution of hydrogen peroxide in cells and tissues has been implicated in the pathology of both Alzheimer's Disease[3] and chronic pulmonary obstructive disease[4] Research continues to demonstrate additional clinical relevance to the production of hydrogen peroxide and the pathologies of aging[5]. These factors are driving a demand for efficient and reliable detection technologies relevant to high-throughput screening.

Assay methodologies used in high throughput screening (HTS) present a variety of unique challenges particularly, the need for homogeneous assay formats which are amenable to robotic liquid handling and the ability to add multiple assay components into solution volumes of 10μL or less. Naturally-fluorescent compounds in a screening library and the use of coupling enzymes necessitate de-replication of hits which may inhibit the coupling enzymes and can compromise the value of these assay methodologies. The opportunity to detect hydrogen peroxide by the chemiluminescent methodology afforded by the use of the direct and specific reaction of a dioxetaneboronic acid with hydrogen peroxide eschews these complications. The resulting production of light is based on the half-life of the intermediate compound (Figure 1). In a basic environment dioxetaneboronic acid reacts with hydrogen peroxide producing an unstable intermediate, which collapses to produce adamantan-2-one, 3-hydroxy-methyl benzoate and light.

Xanthine oxidase is a molybdopterin-containing flavoprotein which exists as a homodimer of ~290kDa subunits, composed of an N-terminal 20kDa domain with two

iron sulfur centers, a central 40kDa FAD-containing domain, and a C-terminal 85-kDa molybdopterin-binding domain with four linearly aligned redox centers. Physiologically, xanthine oxidase is the last enzyme in the purine degradation pathway which catalyzes the successive oxidations of hypoxanthine to xanthine and then to uric acid utilizing two moles of O_2 producing two moles of hydrogen peroxide (Figure 2). The oxidation of hypoxanthine and xanthine takes place at the molybdopterin center[6].

We chose to evaluate the HyPerBlu for the detection of hydrogen peroxide for use in assays for high throughput compound screening, using the commercially-available bovine xanthine oxidase as a model system because the enzyme is well characterized and many inhibitors with varying potencies have been previously reported with this system. This evaluation included the full development of a kinetic assay including the characterization of inhibitors, miniaturization to a high-throughput 1536-well format, assay performance evaluation on a compound library, and comparison of compound profiling results with the conventional fluorescence increase assay utilizing horse radish peroxidase and Amplex Red™ (N-acetyl-3,7-dihydroxyphenoxazine). In the current comparison, we chose to evaluate the fluorogenic substrate Amplex Red with HRP because it has been demonstrated in a variety of enzymatic assays to be 5-10x more sensitive than other fluorometric and spectrophotometric assays[7-9] and is highly amenable to high-throughput screening formats[10,11].

MATERIALS & METHODS

Materials

Urea peroxide, hypoxanthine, bovine xanthine oxidase, dibasic potassium phosphate, sodium chloride, bovine serum albumin (BSA), 3-[3-(cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), ethylenediaminetetraacetic acid (EDTA), horse radish peroxidase (HRP), catalase beads, allopurinol, luteolin, and oxypurinol were purchased from Sigma (St. Louis, MO). Diphenylene iodium (DPI) was purchased from Enzo Life Sciences (Farmingdale, NY). Nuclease-free water was purchased from Ambion (Austin, TX). Dimethyl sulfoxide (DMSO) 99.97% pure, chromatography grade, was purchased from EMD Biosciences (Darmstadt, Germany). Amplex Red was purchased from Invitrogen (Carlsbad, CA).

HyPerBlu Reagent Sensitivity Determination

A serial dilution of urea peroxide ranging from 0.001-500μM was made in nuclease-free water. These peroxide solutions (50μL) were added to the wells of an assay plate (96-well, white Thermo Fisher) in triplicate. 50μL aliquots of HyPerBlu solution were added to the wells and the plate was incubated at room temperature for 35 minutes. Chemiluminescence was measured on a Luminoskan™ luminometer (Labystems, Finland) with an integration time of 1 second per well. The triplicates were averaged and plotted on a log-log scale. A standard linear fit was used to produce the R^2 value.

Steady-State Enzyme Kinetic Characterization

A serial dilution of hypoxanthine ranging from 8-1000μM was made in buffer A (50mM K_2HPO_4, pH 8.0, 50mM NaCl, 0.005% (v/v) BSA, and 1mM CHAPS). Hypoxanthine solutions (2.5μL) were added to the assay plate (384-well, white low

volume Greiner). Reactions were initiated by addition of 2.5µL of 2nM enzyme in buffer A and incubated at room temperature for 21 minutes. Reactions were quenched by addition of 1µL of 1mM DPI in neat DMSO and then 5µL HyPerBlu reagent was added, followed by brief centrifugation at 500rpm. After the plates were incubated for 20min at room temperature, wells were detected using a Viewlux™ plate imager (PerkinElmer, Waltham, MA) with a clear filter and 5 second exposure.

Rates of reaction for each substrate concentration were calculated by linear regression. These rates were then plotted versus hypoxanthine concentration and fitted to the traditional Michaelis-Menten model, equation 1 below, to yield Km values for the substrate in Grafit 5.0 (Erithacus Software Ltd). Equation 1 is defined as:

$$v = Vmax * A / (A + K_m) \qquad (1)$$

where v is the initial rate of reaction, Vmax is the maximal velocity, A is the substrate concentration, and Km is the Michaelis constant.

Compound Inhibition

The reference compounds that were tested include allopurinol, oxypurinol, luteolin, and the mechanism-based inactivator DPI. Serial dilutions of these compounds ranging from 300nM to 10mM were prepared and 50nL of each were added to a 384-well, white low volume Greiner plate using a Hummingbird™ dispenser (Genomic Solutions, Ann Arbor, MI). Then, 2.5µL of 2nM enzyme solution in buffer A was added to the plate wells, followed by the addition of 2.5µL of 100µM hypoxanthine in buffer A. Reactions were centrifuged briefly at 500rpm and then incubated for 10min at room temperature. Reactions were quenched with 1µL of 1mM DPI in neat DMSO and then

5μL of HyPerBlu reagent was added, followed by brief centrifugation at 500rpm. After the plates were incubated for 20min at room temperature, luminescence was detected using a Viewlux plate imager with a clear filter and 5 second exposure. IC50 values were determined by fitting % inhibition to equation 2 in Grafit™ 5.0 (Erithacus Software, UK):

$$\% \text{ Inhibition} = 100 / (1 + [I]/IC50)^s \qquad (2)$$

% Inhibition is calculated relative to DMSO only controls, [I] is the concentration of inhibitor, and s is the slope of the dose response curve.

Tolerance of Assay to Common Additives

The presence of 0-10% v/v DMSO, 0-10mM $MgCl_2$, 0-10mM EDTA, 0-0.005% (v/v) BSA, and 0-1mM CHAPS were individually assessed as additives in the assay. For these experiments, twice the final concentration of the desired additive was added to a 100μM hypoxanthine solution in buffer A, and 2.5μL of this solution was added to wells in a 384-well white low volume Greiner plate. Then 2.5μL of 2nM enzyme in buffer A was added to initiate the reactions. Reactions were quenched after 10min, incubated at room temperature with 1μL 1mM DPI in buffer A, and then 5μL of HyPerBlu reagent was added, followed by brief centrifugation at 500rpm. After the plates were incubated for 20min at room temperature, wells were detected using a Viewlux plate imager with a clear filter and 5 second exposure.

HyPerBlu Luminescence Assay

50nL of 1mM library compounds were added to wells in a 1536-well, white Greiner plate using a Hummingbird dispenser. 2μL of 2nM xanthine oxidase enzyme solution in buffer A was added to each assay plate well using a Combi™ microplate dispenser (Thermo Scientific, Waltham, MA) for this and subsequent dispensing. For signal free samples ("low controls"), 2μL of enzyme-free solution (buffer A only) was added to columns 35 and 36 of the plate, for calculation of a Z', signal to background ratio (S/B) and to set the 100% inhibition threshold. The reaction was initiated by the addition of 2μL of 100μM hypoxanthine in buffer A and the plate then incubated at room temperature for 10min. Reactions were quenched by addition of 0.5μL of 1mM DPI in neat DMSO. 4μL of HyPerBlu solution was dispensed to each well of the. Plates were centrifuged briefly at 500rpm immediately after the HyPerBlu reagent addition and incubated for 20min at room temperature. Plates were then read on a Viewlux imager with a clear filter and 5 second exposure.

Horseradish Peroxidase (HRP) Fluorescence Assay

50nL of 1mM library compounds were added to wells in a 384-well, black Greiner plate using a Hummingbird dispenser. 2.5μL of 2nM xanthine oxidase enzyme solution containing 4U/mL HRP in buffer B (50mM K_2HPO_4, pH 7.5 buffer, 0.2mM CHAPS) was added to each assay plate well using a CyBi™ microplate dispenser (CyBio, Woburn, MA). For low control, 0.5μL of 1mM DPI solution was added to column 18 of the plate using a Combi™ microplate dispenser, and for calculation of a Z', S/B, and to set the 100% inhibition threshold. The reaction was initiated by the addition of 2.5μL of 100μM hypoxanthine containing 10 μM Amplex Red in buffer B using a

Cybi microplate dispenser in a dark environment, and the plate was incubated at room temperature for 15min. The 100 μM hypoxanthine solution containing 10μM Amplex Red was pre-treated with 50U/mL Catalase beads for 30min at room temperature in a dark environment on a tilt table, and the solution was subsequently filtered. Treatment using catalase beads served to reduce the hydrogen peroxide background, for improvement of the overall signal window. Reactions were quenched by addition of 0.5μL of 1mM DPI in neat DMSO. Plates were centrifuged briefly at 500rpm immediately. Plates were then read on a Viewlux imager with a 525nm excitation filter with 20nm bandwidth, a 598nm emission filter with a 25nm bandwidth, and 1 second exposure.

RESULTS

HyPerBlu Reagent Sensitivity Determination

Use of the HyPerBlu reagent demonstrates detection of hydrogen peroxide as low as 5nM, which was linear over a 50,000-fold range of concentrations 25 nmol to 500 fmol (250 μM to 5nM) (Figure 3). The lower level of detection (LLOD) was the lowest peroxide standard which yielded a signal greater than three standard deviations from the background signal. In comparison to the HRP/Amplex Red assay, the lower limit of detection of hydrogen peroxide was found to be 5 pmol (50nM) with a linear range up to 20 μM which is the upper detection limit[8]. HyPerBlu has greater dynamic range with improved sensitivity at lower concentrations and higher tolerance for larger concentrations.

Development of a HyPerBlu Xanthine Oxidase HTS Assay

In order to evaluate the HyPerBlu detection reagent, a robust assay for the peroxide producing enzyme, xanthine oxidase, was developed. A time course of the quenched reaction showed a linear response up to at least 20min using 50μM hypoxanthine and 1nM enzyme (Figure 4). Next, the Km of Hypoxanthine was determined to be 15 ± 2.0μM (Figure 5). The true Km value for hypoxanthine has proved difficult to determine experimentally, because the product of the first reaction is a substrate for the second (see Figure 2). Because determination of a true Km value requires that product does not effectively bind to the enzyme, which is not the case for xanthine oxidase, only an apparent Km value ($K_{m,app}$) can be measured. The $K_{m,app}$ determined in the HRP/Amplex Red assay was 3.3 ± 0.6μM (data not shown). Overall,

an experimental value of 15μM is reasonable, given the estimated Km values of 2μM in literature and the complexity of determining a reliable Km value[12].

After a robust assay was developed, several different classes of inhibitors were evaluated for their potency to confirm pharmacologic relevance of this assay (Figure 6). Allopurinol and oxypurinol are substrate mimetic inhibitors of xanthine oxidase[6]. Both allopurinol and oxypurinol displayed IC50 values of 490 ± 13nM and 5600 ± 490nM, respectively. Luteolin, a known antioxidant, is also a competitive inhibitor of bovine xanthine oxidase, with reported Ki = 1900nM.[13] An IC50 value of Luteolin = 1900 ± 160nM was obtained using HyPerBlu reagent, which is in good agreement with the literature value. Finally, an irreversible flavin inactivator[14], diphenylene iodium (DPI) had IC50 39 ± 1.4nM. Overall, many different classes of inhibitors ranging from substrate mimetics to irreversible flavin modifiers were detected in this assay.

Next, we tested the effects of DMSO, BSA, CHAPS, Mg^{2+}, and EDTA individually in the assay. These additives were selected for the following reasons: (1) Many compound libraries are stored as solutions in neat DMSO, requiring tolerability to this organic solvent. (2) Detergents, like CHAPS, are often added to HTS assays to aid in liquid handling, primarily to reduce well to well variability in signal. (3) Metal ions, such as Mg^{2+}, are often co-factors for enzymatic reactions. (4) EDTA is often used to quench metal dependent catalytic reactions. (5) Carrier proteins such as BSA are commonly added to HTS assays to improve hit quality by increasing enzyme activity which may otherwise be lost due to adhesion to plastic or apparent inhibition due to compound aggregation.

This assay's performance, in terms of S/B and Z', was improved by the addition of CHAPS and BSA up to 1mM and 0.005%, respectively. Given the low enzyme concentration used in the assay (1nM), it is likely both additives improve enzyme solubility or reduce enzyme adhesion to well surfaces. The addition of up to 10mM Mg^{2+}, 10mM EDTA, and 10% v/v DMSO had no effect on assay performance.

The Z' factor is a universal parameter for determining assay robustness and is an indication of the separation between positive and negative control values[15]. The Z' value ranges from 0 to 1, where higher numbers indicate a greater statistical separation between positive and negative controls. The ability to distinguish "hits" from "noise" is imperative when screening at single doses of compounds, and usually requires a $Z' > 0.4$. The Z' of HyPerBlu was > 0.6 and demonstrates that this assay is suitable for screening compounds at single doses.

HyPerBlu Assay Robustness Evaluation

In order to create an assay amenable to high-throughput screening, the assay must perform well in a 1536-well format (requiring volume miniaturization) and use of a plate dispenser for all reagent additions. Both requirements were easily achieved; dispensing on a Multidrop Combi™ and reduction of total assay volume from 10 µL to 8µL were immediately successful.

The assay was validated for HTS purposes by assaying in duplicate a set of 1408 compounds at 12.5µM concentrations (Figure 7A). Besides measuring sample Z', this step evaluates the data reproducibility and tests the performance of liquid handling under industrialized high throughput conditions. This step provides assay quality measures that

can be predictive of the HTS performance allowing intervention when issues become apparent before committing time and monetary resources to a full HTS campaign. Key performance indicators include false-positive hit rate identification, interclass correlation coefficient (ICC), mean, standard deviation (SD), and the statistical robust cutoff (defined herein as 3-times the sample population standard deviation + sample population mean) with resulting hit rate. No edge related artifacts were observed, nor was the signal dependant on position on the plate. The xanthine oxidase HyPerBlu assay had no negative performance indicators identified, with average Z' = 0.85, signal to background ratio of 5-6-fold, ICC = 0.94 indicating excellent data reproducibility, 5.2% hit rate at 18.4% inhibition statistical robust cut-off, and a 0% false positive hit rate (Table 1).

Horseradish Peroxidase Assay Robustness Evaluation

Identical to the HyPerBlu compound evaluation above, the HRP/Amplex Red assay was assessed on the set of 1408 compounds in duplicate at 10μM (Figure 7B). The HRP/Amplex Red assay had similar statistical characteristics to the HyPerBlu assay, with key performance indicators: average Z' = 0.72, signal to background ratio of 2.5-3.5-fold, ICC = 0.94 indicating excellent data reproducibility, and a 0% false positive hit rate (Table 1). The only major difference from the HyPerBlu assay was a 5.0% hit rate at 51.4% inhibition robust cut-off.

DISCUSSION

We have characterized HyPerBlu, a novel dioxetaneboronic acid compound, which generates a chemiluminescent species upon reaction with hydrogen peroxide.

HyPerBlu is 10-fold more sensitive than the conventional HRP/Amplex Red assay. The high sensitivity of HyPerBlu arises because it is totally non-luminescent until reacted with hydrogen peroxide, giving a greatly reduced background compared to many colorogenic and fluorogenic compounds. Additionally, it can be used at high concentrations (up to 250 μM hydrogen peroxide), which is approximately 10-fold higher than Amplex Red which suffers from instrument detection limitations in fluorescence-detection mode[8].

HyPerBlu's broad hydrogen peroxide concentration detection range provides many advantages. First, it can accommodate assay development for a range of hydrogen peroxide producing targets whose K_m value for substrates greatly differ. Secondly, the higher sensitivity allows for development of assays using lower enzyme concentrations (reducing cost per well) or for enzymes with slow catalytic rates. Likewise, the improved upper limit of detection facilitates substrate competitiveness studies, a key experiment for mechanism of action studies. HyPerBlu stability (Figure 8) indicates that one lot of material could be kept at room temperature and used for an entire screen.

Herein, we demonstrated the HyPerBlu technology can be easily adapted into a biochemical assay quantifying hydrogen peroxide produced by the oxidation of hypoxanthine by xanthine oxidase. Linear reaction time courses (Figure 4) were easily achieved, and the $K_{m,app}$ value for the substrate was accurately determined as ~15 μM. Additionally, the sensitivity of HyPerBlu to a broad range of inhibitor potencies was demonstrated. Figure 6 shows inhibitors whose potency differs by greater than 2-log units with IC50 39nM for the most potent compound.

High throughput screening (HTS) assays present a variety of unique challenges for assay technologies. Naturally fluorescent or fluorescence quenching compounds and inhibition of coupling enzymes instead of the true biological target can decrease the utility of an assay and increase the complexities of hit evaluation following high throughput compound screening. Lumigen HyPerBlu technology avoids these interferences by utilizing a technology based on the specific reaction of a dioxetaneboronic acid with hydrogen peroxide to produce a luminescence de novo, rather than the assessment of changes in sample fluorescence for other assay methodologies. As demonstrated from the testing of compounds (n = 1408 at 10μM in duplicate), most of the traditional performance indicators for assay robustness were similar between the HyPerBlu and HRP/Amplex Red assays, ie. Z', S/B, hit rate, ICC, and false positive rate. Although the hit rates were identical, the robust statistical cut-off value for HRP/Amplex Red was 2.8-fold higher than the 18.4% value obtained for the HyPerBlu assay. The higher cut-off value hinders the ability to identify true "hits" that are of lower potency because the "noise" in the assay is set at a higher % inhibition threshold. In this respect, the HyPerBlu assay is superior to the HRP/Amplex Red assay because the lower robust cut-off value achieved allows a broader range of "hit" potencies to be sampled when screening at single doses of compound.

SUMMARY

We developed a low-volume, high throughput luminescence assay for measuring hydrogen peroxide production from xanthine oxidase oxidation of hypoxanthine. The direct and accurate measurement of hydrogen peroxide makes it a fit-for-purpose assay

for HTS, profiling, as well as mode of action studies. The direct detection, rather than traditional fluorescence assays which require coupling enzymes like HRP, allows for more robust screening assays due to lower incidence of compound interference via inhibition of coupling enzymes or through fluorescence artifacts.

*All trademarks including HyPerBlu, Labsystems, ViewLux, Hummingbird, Grafit, CyBi, Combi and Amplex Red are property of their respective owners.

REFERENCES

[1] Schafer, FQ, and Buettner, GR. (2001). Redox Environment of the cell as viewed through the Redox State of the Glutathione Disulfide/Glutathione couple, Free Radical Biology & Medicine, Vol. 30, No.11: 1191-1212.

[2] Halliwell, B & Gutteridge, J. M. (1984). Oxygen Toxicity, Oxygen Radicals, Transition Metals & Disease, Biochemical J;219: 1-14.

[3] Behl, C, Davis, JB, Lesley, R, and Schubert, D. (1994), Hydrogen Peroxide Mediates Amyloid β Toxicity, Cell;77: 817-827.

[4] Dekhuijzen, PN R, Aben, KKKH, Dekker, I, Aarts, LPHJ, Wielders, PLML, van Herwaarden, CLA, Bast, A. (1996). Increased Exhalation of Hydrogen Peroxide in Patients with Stable and Unstable Chronic Obstructive Pulmonary Disease, Am J Resp Crit Care;154: 813-816.

[5] Gems, D, and Partridge, L. Stress-Response Hormesis and Aging: That which Does Not Kill Us Makes Us Stronger, Cell Metabolism 2008;7: 200-203.

[6] Pacher, P, Nivorozhkin, A, and Szabo, C. Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol, Pharmacol Rev 2006;58: 87-114.

[7] Palamakumbura, AH, and Trakman, PC. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. Anal Biochem 2002;300: 245-251.

[8] Zhou, M, Diwi, Z, Panchuk-Voloshina, N, and Haugland, RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem 1997;253: 162-168.

[9] Zhou, M, and Panchuk-Voloshina, N. A one-step fluorometric method for the continuous measurement of monoamine oxidase activity. Anal Biochem 1997;253: 169-174.

[10] Brandish, PE, Chi-Sung, C, Schneeweis, J, Brandon, NJ, Leech, CL, Kornienko, O, Scolnick, EM, Strulovici, B, and Zheng, W. A Cell-Based Ultra-High-Throughput Screening Assay for Identifying Inhibitiors of D-Amino Acid Oxidase. J Biomol Screen 2006;11: 481-487.

[11] Keller, PM, Rust, T, Murphy, DJ, Matico, R, Trill, JJ, Krawiec, JA, Jurewicz, A, Jaye, M, Harpel, M, Thrall, S, and Schwartz, B. A High-Throughput Screen for Endothelial Lipase Using HDL as Substrate. J Biomol Screen 2008;13: 468-475.

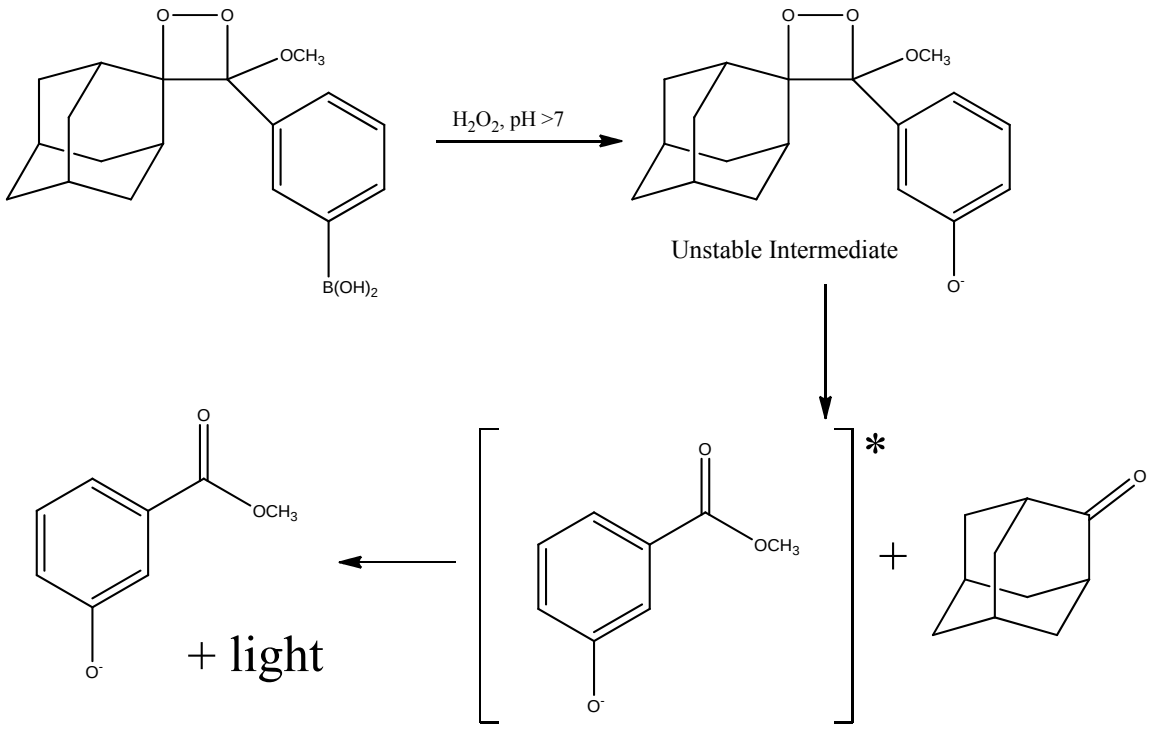
[12] Escribano, J, Garcia-Canovas, F, and Garcia-Carmona, F. A Kinetic Study of Hypoxanthine Oxidation by Milk Xanthine Oxidase, Biochem J 1988;254: 829-833.

[13] Pauff, JM and Hille, R. Inhibition studies of Bovine Xanthine Oxidase by Luteolin, Silibinin, Quercetin, and Curcumin, J Nat Prod 2009;72: 725-731.

[14] O'Donnell, VB, Tew, DG, Jones, OTG, and England, PJ. Studies on the Inhibitory Mechanism of Iodonium Compounds with Special Reference to Neutrophil NADPH Oxidase, Biochem J 1993;290: 41-49.

[15] Zhang, JH, Chung, TD, Oldenburg, KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 1999;4: 67-73.

Figure 1 HyPerBlu Reaction Scheme:



Caption: In a basic environment dioxetaneboronic acid reacts with hydrogen peroxide producing an unstable intermediate. This intermediate breaks down and produces light.

Figure 2 Mechanism of Hydrogen Peroxide Production by Xantine Oxidase:

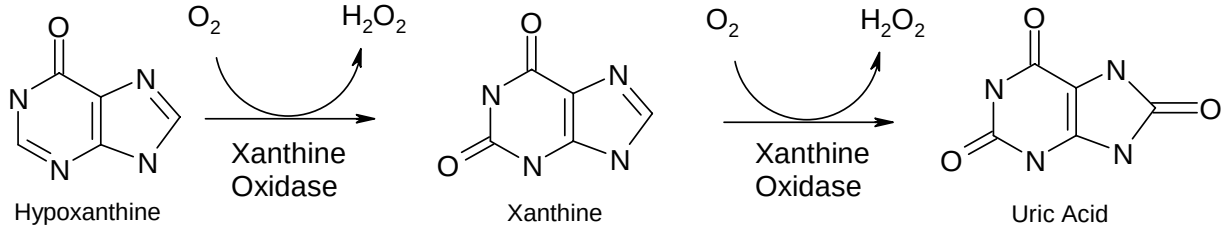
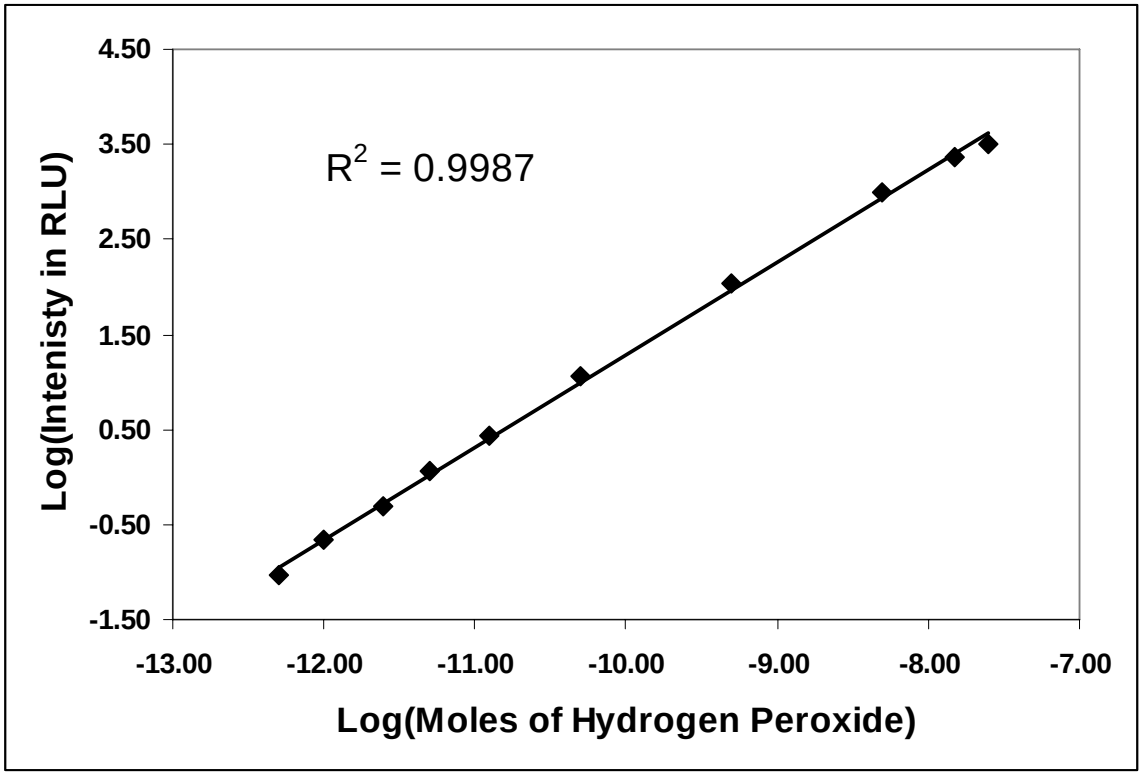
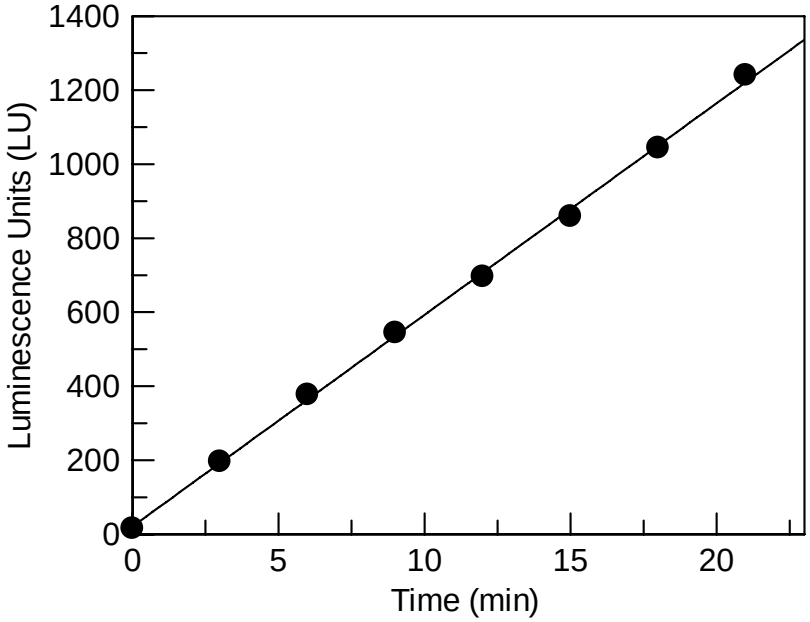


Figure 3 HyPerBlu Reagent Peroxide Sensitivity Determination:



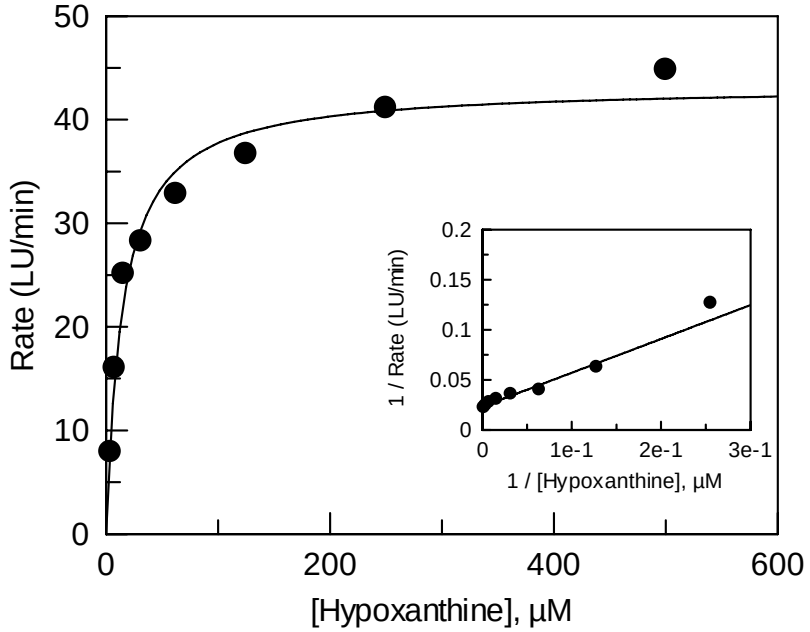
Caption: HyPerBlu reagent demonstrates a linear detection range from 2.5 x 10^{-8} to 5 x 10^{-13} moles hydrogen peroxide.

Figure 4
Reaction Time Course:



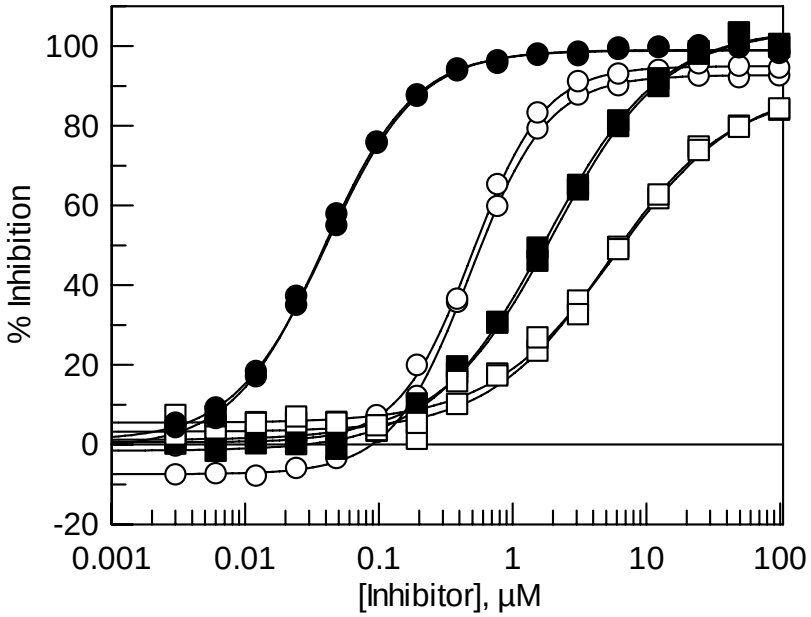
Caption: Example time course in bovine xanthine oxidase-hypoxanthine assay. Hypoxanthine turnover (50μM) was measured in the luminescent test format with with HyPerBlu reagent and 1nM bovine xanthine oxidase present.

Figure 5
Hypoxanthine Km Determination:



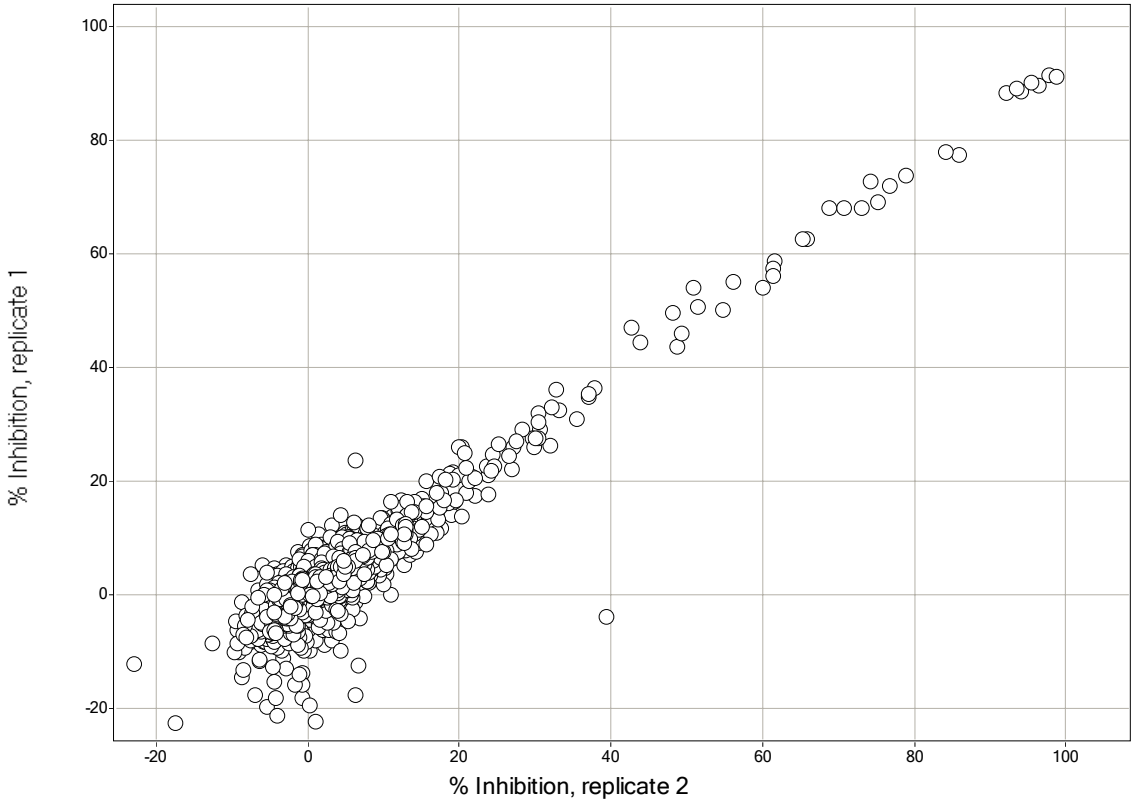
Caption: Hypoxanthine Km determination for bovine xanthine oxidase. Fitting the rate (Relative Luminescence Units per min) verses the concentration of Hypoxanthine to the standard Michaelis-Menten equation (Rate = Vmax * [Sub] / ([Sub] + Km)) yielded Km = 15 ± 2.0µM. Inset is Lineweaver-Burk double reciprocal representation of data.

Figure 6
Inhibitors:



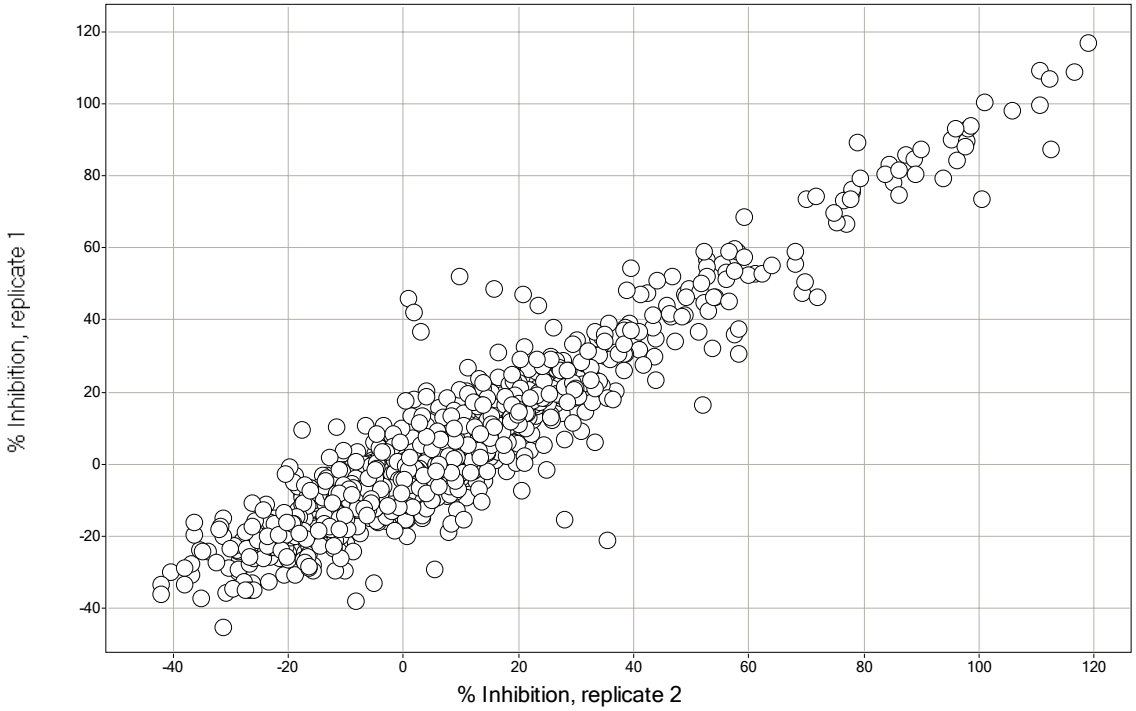
Caption: Data showing the IC50 determination for 4 inhibitors of bovine xanthine oxidase in the presence of 50µM Hypoxanthine substrate. Closed circles (●) represent DPI, open circles (○) represent Allopurinol, closed squares (■) represent Luteolin, and open squares (□) represent Oxypurinol. Curve fit shown was obtained by fitting % inhibition to the 4-parameter IC50 fit; IC50s were as follows: DPI = 39 ± 1.4nM, Allopurinol = 490 ± 13nM, Luteolin = 1900 ± 160nM, Oxypurinol = 5600 ± 490nM.

Figure 7A 1536 Single Shot Compound Screening, Duplicate:



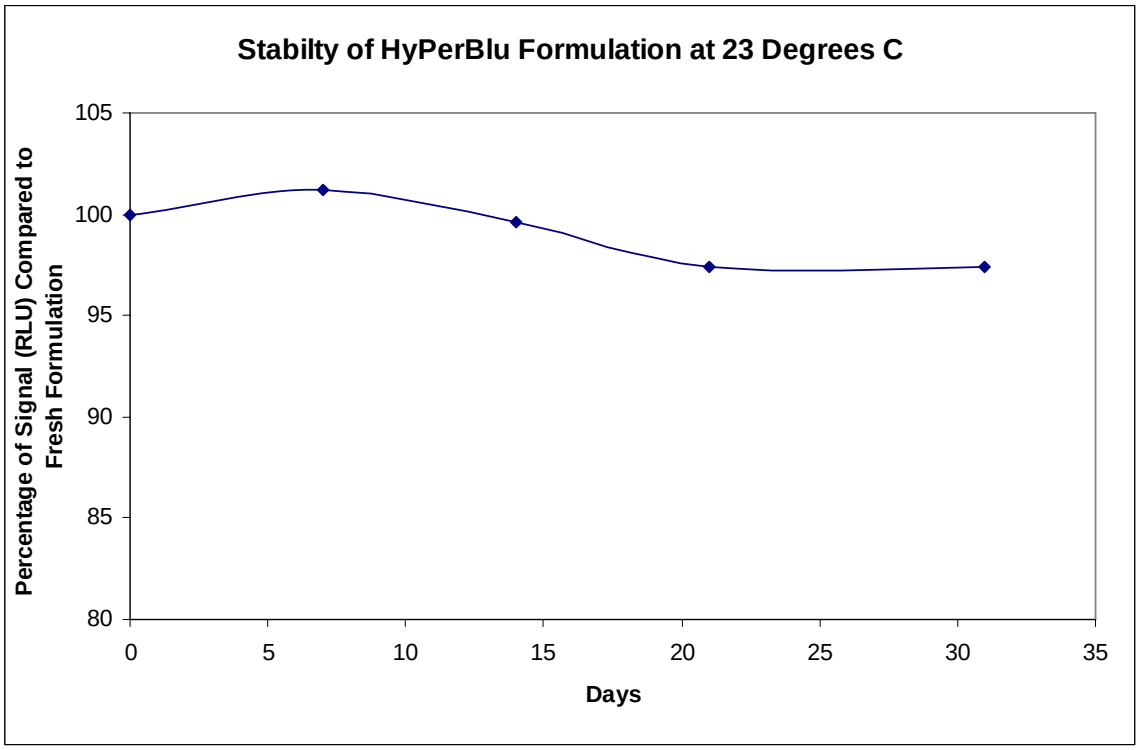
Caption: Percentage inhibition values for 1408 compounds, screened at 12.5µM in duplicate in the HyPerBlu assay. Replicate 1 is on the y-axis, replicate 2 is on the x-axis.

Figure 7B



Caption: Percentage inhibition values for 1408 compounds, screened at 10μM in duplicate in the HRP/Amplex Red assay. Replicate 1 is on the y-axis, replicate 2 is on the x-axis.

Figure 8 HyPerBlu Formulation Stability



Caption: A formulation of Lumigen HyPerBlu was allowed to remain on a bench top at 23 degrees. Samples were periodically measured against a freshly prepared formulation. After 31 days the formulation yielded over 95% of the the light output of the fresh formulation.

Table 1.

Statistical Measure	HyPerBlu Luminescence Assay	HRP/Amplex Red Fluorescence Assay
Hit Rate	5-6%	5-6%
Robust Cut-off	18%	50-55%
False Positives	0%	0%
Interclass Correlation Coefficient	0.94	0.94