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(71) Applicant: **PROMEGA CORPORATION** [US/US]; 2800 Woods Hollow Road, Madison, Wisconsin 53711 (US).

(72) Inventors: **SHI, Ce**; 2800 Woods Hollow Road, Madison, Wisconsin 53711 (US). **KIRKLAND, Thomas**; 2800 Woods Hollow Road, Madison, Wisconsin 53711 (US). **MEISENHEIMER, Poncho**; 2800 Woods Hollow Road, Madison, Wisconsin 53711 (US). **ENCELL, Lance P.**; 2800 Woods Hollow Road, Madison, Wisconsin 53711 (US). **HALL, Mary**; 2800 Woods Hollow Road, Madison, Wisconsin 53711 (US).

(74) Agent: **STAPLE, David W.**; 2275 Deming Way, Suite 310, Middleton, Wisconsin 53562 (US).

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(54) Title: THERMAL STABLE LUCIFERASE WITH IMPROVED RESISTANCE TO INHIBITION BY LUCIFERIN BREAK-DOWN PRODUCTS

(57) Abstract: Provided herein are inhibitor-resistant luciferase mutants, and methods of use thereof. In particular, luciferase mutants are provided that are thermal stable and exhibit improved stability in the presence of luciferin break-down products such, such as dehydroluciferin.

THERMAL STABLE LUCIFERASE WITH IMPROVED RESISTANCE TO INHIBITION BY LUCIFERIN BREAK-DOWN PRODUCTS

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. Provisional Patent Application Serial No. 62/407,815 filed October 13, 2016, which is hereby incorporated by reference in its entirety.

FIELD

Provided herein are inhibitor-resistant luciferase mutants and methods of use thereof. In particular, luciferase mutants are provided that are thermal stable and exhibit improved resistance to inhibition by luciferin break-down products such as dehydroluciferin.

BACKGROUND

Quantitative detection of ATP is commonly performed using a luciferase enzyme (e.g., firefly luciferase) and its substrate (e.g., D-luciferin).

SUMMARY

Provided herein are inhibitor-resistant luciferase mutants and methods of use thereof. In particular, luciferase mutants are provided that are thermal stable and exhibit improved resistance to inhibition by luciferin break-down products such as dehydroluciferin.

The stability and reliability of ATP-detection assays and reagents is compromised in the presence of luciferin break-down products which inhibit luciferase and result in decreased light output. These breakdown products are particularly problematic when luciferin and luciferase are stored together as single liquid reagent, resulting in reduced shelf-life. Dehydroluciferin has been identified as the major inhibitory breakdown product of luciferin.

In some embodiments, provided herein are inhibitor-resistant luciferases comprising enhanced resistance to inhibition by dehydroluciferin compared to a luciferase of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase exhibits a smaller relative reduction in activity when exposed to dehydroluciferin than a luciferase of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 100%) sequence identity with SEQ ID NO: 3 and comprises at least one substitution relative to SEQ ID NO: 3 at a position selected from 240, 244, 254, 300, 344, and/or 396 of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase

comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from I240L, H244R, Y254S, C300G, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions. In some embodiments, the inhibitor-resistant luciferase comprises a substitution relative to SEQ ID NO: 3 at position 396 of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase comprises an I396K substitution, or a conservative or semi-conservative variation thereof, relative to SEQ ID NO: 3.

In some embodiments, provided herein are inhibitor-resistant luciferases comprising enhanced resistance inhibition by to dehydroluciferin compared to a luciferase of SEQ ID NO: 1. In some embodiments, the inhibitor-resistant luciferase exhibits a smaller relative reduction in activity when exposed to dehydroluciferin than a luciferase of SEQ ID NO: 1. In some embodiments, the inhibitor-resistant luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 100%) sequence identity with SEQ ID NO: 1 and comprises at least one substitution relative to SEQ ID NO: 1 at a position selected from 240, 244, 254, 300, 344, and/or 396 of SEQ ID NO: 1. In some embodiments, the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 1, the at least one substitution selected from I240L, H244R, Y254S, C300G, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions. In some embodiments, the inhibitor-resistant luciferase comprises a substitution relative to SEQ ID NO: 1 at position 396 of SEQ ID NO: 1. In some embodiments, the inhibitor-resistant luciferase comprises an I396K substitution, or a conservative or semi-conservative variation thereof, relative to SEQ ID NO: 1.

In some embodiments, the inhibitor-resistant luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 100%) sequence identity with SEQ ID NO: 5 and comprises at least one substitution relative to SEQ ID NO: 3, said substitution at a position selected from 244, 300, and/or 396 of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from H244R, C300G, I396K, and/or conservative or semi-conservative variations of such substitutions.

In some embodiments, the inhibitor-resistant luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 100%) sequence identity with SEQ ID NO: 7 and comprises at least one substitution relative to SEQ ID NO: 3, said substitution at a position selected from 240, 254, 344, and/or 396 of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from I240L, Y254S, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions.

In some embodiments, the inhibitor-resistant luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 100%) sequence identity with SEQ ID NO: 9 and comprises at least one substitution relative to SEQ ID NO: 3, said substitution at a position selected from 244, 344 and/or 396 of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from H244R, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions.

In some embodiments, the inhibitor-resistant luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 100%) sequence identity with SEQ ID NO: 11 and comprises at least one substitution relative to SEQ ID NO: 3, said substitution at a position selected from 244, 300, 344 and/or 396 of SEQ ID NO: 3. In some embodiments, the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from H244R, C300G, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions.

In some embodiments, inhibitor-resistant luciferases comprise at least 10% (e.g., 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more, or ranges therebetween) of the bioluminescent activity of a luciferase of SEQ ID NO: 3, when neither inhibitor-resistant luciferase nor the luciferase of SEQ ID NO: 3 have been exposed to dehydroluciferin.

In some embodiments, provided herein are reagent compositions comprising (i) an inhibitor-resistant luciferase described herein and (ii) luciferin. In some embodiments, a reagent composition further comprises a contaminant comprising a degradation product of luciferin. In some embodiments, the contaminant is dehydroluciferin. In some embodiments, a reagent composition further comprises magnesium. In some embodiments, a reagent composition further comprises one or more additional components selected from the group consisting of: a buffer, a defoamer, an ATPase inhibitor, an enzyme stabilizer, a detergent, an inhibitor of ATP-generating enzymes, a cell lysing agent, an ATP-extraction agent, co-enzyme A, a thiol reagent, a metal ion chelator, a protease inhibitor, and a salt. In some embodiments, the reagent composition is a single liquid reagent. In some embodiments, the reagent comprises racemic luciferin. In some embodiments, the reagent comprises dehydroluciferin. In some embodiments, racemic luciferin and/or dehydroluciferin are exogenously added to a reagent composition, for example, to improve the apparent stability of the luciferase, the overall stability of the reagent, and/or the duration of the luminescence signal produced by the reagent in the presence of ATP.

In some embodiments, provided herein are reagent compositions comprising an inhibitor-resistant luciferase described herein and a cationic detergent. In some embodiments, the cationic detergent is DTAB (dodecyltrimethylammonium bromide), CTAB (cetyltmethylammonium) Benzalkonium Chloride, or BDDABr (benzyldimethyldodecylammonium bromide).

In some embodiments, provided herein are kits comprising the reagent compositions described herein. In some embodiments, a kit further comprises one or more additional components selected from the group consisting of: a buffer, a defoamer, an ATPase inhibitor, an enzyme stabilizer, a detergent, an inhibitor of ATP-generating enzymes, a cell lysing agent, an ATP-extraction agent, co-enzyme A, a thiol reagent, a metal ion chelator, a protease inhibitor, and a salt. In some embodiments, a kit further comprises instructions for performing an ATP detection or quantification assay.

In some embodiments, provided herein are assay systems for detecting or quantifying ATP in a sample, comprising: (a) a reagent composition described herein; and (b) a sample comprising or suspected of comprising ATP. In some embodiments, assay systems further comprise a device for the detection and/or measurement of luminescence (e.g., luminometer although other light detection device or instrument may be used). In some embodiments, the sample is a cell lysate.

In some embodiments, provided herein are methods of detecting ATP in a sample comprising: (a) adding to the sample a reagent composition reagent composition described herein; and (b) detecting luminescence. In some embodiments, the sample comprises cells, and the method further comprises lysing the cells to generate a cell lysate.

In some embodiments, provided herein are methods of quantifying the amount or concentration of ATP in a sample comprising: (a) adding to the sample a reagent composition reagent composition described herein; (b) quantifying luminescence from the sample; and (c) comparing the luminescence to a control value to determine the amount or concentration of ATP in the sample. In some embodiments, the control value is determined from a separate quantification of luminescence produced by a control sample comprising a known concentration of ATP. In some embodiments, methods further comprise the step of adding a known concentration of ATP to the sample. In some embodiments, luminescence is quantified at multiple time-points. In some embodiments, luminescence is quantified in real time.

In some embodiments, provided herein is the use of a reagent composition described herein for the detection and/or quantification of ATP in a sample.

In some embodiments, provided herein are methods of enhancing the apparent signal stability of an inhibitor-resistant luciferase described herein in an assay, comprising performing the assay with one or more inhibitors of the luciferase present. In some embodiments, the inhibitor is selected from one or more of a dehydroluciferin, an oxoluciferin, and L-luciferin. In some embodiments, the inhibitor-resistant luciferase exhibits an increase in enhancement of apparent signal stability compared to a native, wild-type version the luciferase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a graph depicting the luminescence as measure by relative light unit (RLU) of variant luciferases and ULTRAGLO luciferase (Promega Corp., Madison, WI).

Figure 2 shows a graph depicting calculated IC50 values for dehydroluciferin of variant luciferases and ULTRAGLO luciferase (Promega Corp., Madison, WI).

Figure 3 shows a graph depicting the apparent stability of variant luciferases and ULTRAGLO luciferase (Promega Corp., Madison, WI).

Figure 4 shows RLU and IC50 values for exemplary enzymes.

Figure 5 shows the resistance and stability of exemplary mutants in the presence of complete reagent breakdown products.

DEFINITIONS

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments described herein, some preferred methods, compositions, devices, and materials are described herein. However, before the present materials and methods are described, it is to be understood that this invention is not limited to the particular molecules, compositions, methodologies or protocols herein described, as these may vary in accordance with routine experimentation and optimization. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the embodiments described herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. However, in case of conflict, the present specification, including definitions, will control. Accordingly, in the context of the embodiments described herein, the following definitions apply.

As used herein and in the appended claims, the singular forms “a”, “an” and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a luciferase" is a reference to one or more luciferases and equivalents thereof known to those skilled in the art, and so forth.

As used herein, the term “enhanced” refers to an improvement in a particular property relative to that of a reference. For example, when used to describe a property of a luciferase variant (e.g., luminescence, signal stability, biocompatibility, protein stability (e.g., enzyme stability), or protein expression), “enhanced” refers to an improvement (e.g., 1%, 2%, 5%, 10%, 20%, 25%, 50%, 75%, 2-fold, 3-fold, 4 fold, 5-fold, 10 fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, 1000-fold, or more, or ranges therebetween) in that property compared to a reference luciferase, such as a native/wild-type version of that luciferase (e.g., SEQ ID NO: 1). A variant luciferase may exhibit one or more of “enhanced luminescence,” “enhanced signal stability,” “enhanced enzyme stability,” “enhanced protein expression,” etc.

As used herein, the term “signal duration” refers to the persistence of a signal, for example, as measured by the half-life of decay of the signal in a time-course or the length of time a signal remains constant (e.g., before detectable decay). The term “signal stability” refers to the characteristic signal duration of an enzyme (e.g., a luciferase).

“Enhanced signal stability” refers to an increase in signal duration (e.g. the persistence of a signal intensity (e.g., luminescent signal) from an enzyme (e.g., a luciferase combined with luciferin and ATP) compared to a reference enzyme (e.g., compared to a wild-type version of the luciferase (e.g., SEQ ID NO: 1) combined with luciferin and ATP)).

As used herein, the term “apparent signal stability” refers to the persistence of a signal (e.g., luminescence) from an enzyme (e.g., luciferase combined with luciferin and ATP) under a particular set of conditions (e.g., in the presence of an inhibitor). “Enhanced apparent signal stability” refers to an increase in the persistence of a signal (e.g., luminescent signal) from an enzyme (e.g., a luciferase) combined with luciferin and ATP under a particular set of conditions compared to the persistence of that same enzyme combined with luciferin and ATP under reference conditions. For example, a variant luciferase combined with luciferin and ATP may exhibit enhanced apparent signal stability in the presence of an inhibitor (e.g., by lowering the initial signal from the luciferase, the loss of signal over a time course is reduced). In some embodiments, a variant enzyme combined with luciferin and ATP exhibits “an increase in enhancement of apparent signal stability” when the variant luciferase combined with luciferin and ATP exhibits a greater enhancement of apparent signal stability

compared to a reference luciferase (e.g., compared a wild-type version of the luciferase (e.g., SEQ ID NO: 1)) combined with luciferin and ATP.

As used herein, the term "storage stability" refers to the consistency of the signal (e.g., luminescence) from an enzyme (e.g., luciferase with luciferin and ATP) when measured at various points in time (e.g., end-point measurements). For example, luminescence of aliquots of a stored luciferase (e.g., stored in an aqueous solution along with substrate) are measured (e.g., in the presence of luciferin and ATP) at various time-points relevant to the storage of the luciferase (e.g., days, weeks, etc.), and an enzyme or set of conditions that result in more consistency (e.g., less loss of signal, longer duration before decay, etc.) over time exhibits "enhanced storage stability." A variant enzyme (e.g., an inhibitor resistant variant) may exhibit enhanced storage stability relative to a wild-type enzyme, and/or a particular set of conditions (e.g., in the presence of inhibitor) may result in enhanced storage stability relative to reference conditions. Some luciferases exhibit limited storage stability when stored in the presence of luciferin, due to the formation of luciferin breakdown products that are inhibitory to the luciferase. In some embodiments, inhibitor-resistant luciferases exhibit increased storage stability. In some embodiments, storage conditions comprising both luciferin substrate and inhibitor (e.g., dehydroluciferin, fluorodehydroluciferin, aminodehydroluciferin, L-luciferin, etc.) result in enhanced storage stability, for example, by decreasing early time-point signal to more closely reflect late time-point signals.

As used herein, the terms "inhibitor resistant" and "enhanced resistance to inhibitor" refers to an enzyme that retains more activity in the presence of an inhibitor or enzyme activity than a reference version of the enzyme (e.g., a native wild-type version of the enzyme). An inhibitor-resistant enzyme is not necessarily 100% resistant to inhibitor. For example, an "inhibitor-resistant luciferase" is a polypeptide that retains more (e.g., e.g., exhibits a smaller percentage loss) luciferase activity (e.g., conversion of luciferin to oxyluciferin, RLU output, etc.) in the presence of ATP and a luciferase inhibitor (e.g., dehydroluciferin, fluorodehydroluciferin, aminodehydroluciferin, L-luciferin, etc.) when compared to a wild-type version of the luciferase (e.g., SEQ ID NO: 1).

The term "amino acid" refers to natural amino acids, unnatural amino acids, and amino acid analogs, all in their D and L stereoisomers, unless otherwise indicated, if their structures allow such stereoisomeric forms.

Natural amino acids include alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), Lysine

(Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y) and valine (Val or V).

Unnatural amino acids include, but are not limited to, azetidinecarboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, naphthylalanine ("naph"), aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisbutyric acid, 2-aminopimelic acid, tertiary-butylglycine ("tBuG"), 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, homoproline ("hPro" or "homoP"), hydroxylysine, allo-hydroxylysine, 3-hydroxyproline ("3Hyp"), 4-hydroxyproline ("4Hyp"), isodesmosine, allo-isoleucine, N-methylalanine ("MeAla" or "Nime"), N-alkylglycine ("NAG") including N-methylglycine, N-methylisoleucine, N-alkylpentylglycine ("NAPG") including N-methylpentylglycine. N-methylvaline, naphthylalanine, norvaline ("Norval"), norleucine ("Norleu"), octylglycine ("OctG"), ornithine ("Orn"), pentylglycine ("pG" or "PGly"), pipecolic acid, thioproline ("ThioP" or "tPro"), homoLysine ("hLys"), and homoArginine ("hArg").

The term "amino acid analog" refers to a natural or unnatural amino acid where one or more of the C-terminal carboxy group, the N-terminal amino group and side-chain bioactive group has been chemically blocked, reversibly or irreversibly, or otherwise modified to another bioactive group. For example, aspartic acid-(beta-methyl ester) is an amino acid analog of aspartic acid; N-ethylglycine is an amino acid analog of glycine; or alanine carboxamide is an amino acid analog of alanine. Other amino acid analogs include methionine sulfoxide, methionine sulfone, S-(carboxymethyl)-cysteine, S-(carboxymethyl)-cysteine sulfoxide and S-(carboxymethyl)-cysteine sulfone.

As used herein, the term "peptide" refers an oligomer to short polymer of amino acids linked together by peptide bonds. In contrast to other amino acid polymers (e.g., proteins, polypeptides, etc.), peptides are of about 50 amino acids or less in length. A peptide may comprise natural amino acids, non-natural amino acids, amino acid analogs, and/or modified amino acids. A peptide may be a subsequence of naturally occurring protein or a non-natural (artificial) sequence.

As used herein, the term "polypeptide" refers to a polymer of amino acids linked together by peptide bonds that is greater than about 50 amino acids in length. Polypeptides may comprise natural amino acids, non-natural amino acids, amino acid analogs and/or modified amino acids, and may be a naturally occurring sequence, or a non-natural (artificial)

sequence, or a subsequence of naturally occurring protein or a non-natural (artificial) sequence.

As used herein, the term "artificial" refers to compositions and systems that are designed or prepared by man, and are not naturally occurring. For example, an artificial sequence refers to a amino acid or nucleotide sequence that does not occur in nature (e.g., a polypeptide without 100% identity with a naturally-occurring protein or a fragment thereof).

As used herein, a "conservative" amino acid substitution refers to the substitution of an amino acid in a peptide or polypeptide with another amino acid having similar chemical properties, such as size or charge. For purposes of the present disclosure, each of the following eight groups contains amino acids that are conservative substitutions for one another:

1) Alanine (A) and Glycine (G);

2) Aspartic acid (D) and Glutamic acid (E);

3) Asparagine (N) and Glutamine (Q);

4) Arginine (R) and Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), and Valine (V);

6) Phenylalanine (F), Tyrosine (Y), and Tryptophan (W);

7) Serine (S) and Threonine (T); and

8) Cysteine (C) and Methionine (M).

Naturally occurring residues may be divided into classes based on common side chain properties, for example: polar positive (or basic) (histidine (H), lysine (K), and arginine (R)); polar negative (or acidic) (aspartic acid (D), glutamic acid (E)); polar neutral (serine (S), threonine (T), asparagine (N), glutamine (Q)); non-polar aliphatic (alanine (A), valine (V), leucine (L), isoleucine (I), methionine (M)); non-polar aromatic (phenylalanine (F), tyrosine (Y), tryptophan (W)); proline and glycine; and cysteine. As used herein, a "semi-conservative" amino acid substitution refers to the substitution of an amino acid in a peptide or polypeptide with another amino acid within the same class.

In some embodiments, unless otherwise specified, a conservative or semi-conservative amino acid substitution may also encompass non-naturally occurring amino acid residues that have similar chemical properties to the natural residue. These non-natural residues are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include, but are not limited to, peptidomimetics and other reversed or inverted forms of amino acid moieties. Embodiments herein may, in some embodiments, be limited to natural amino acids, non-natural amino acids, and/or amino acid analogs.

Non-conservative substitutions may involve the exchange of a member of one class for a member from another class.

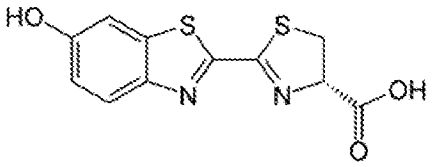
As used herein, the term "sequence identity" refers to the degree of which two polymer sequences (e.g., peptide, polypeptide, nucleic acid, etc.) have the same sequential composition of monomer subunits. The term "sequence similarity" refers to the degree with which two polymer sequences (e.g., peptide, polypeptide, nucleic acid, etc.) differ only by conservative and/or semi-conservative amino acid substitutions. The "percent sequence identity" (or "percent sequence similarity") is calculated by: (1) comparing two optimally aligned sequences over a window of comparison (e.g., the length of the longer sequence, the length of the shorter sequence, a specified window, etc.), (2) determining the number of positions containing identical (or similar) monomers (e.g., same amino acids occurs in both sequences, similar amino acid occurs in both sequences) to yield the number of matched positions, (3) dividing the number of matched positions by the total number of positions in the comparison window (e.g., the length of the longer sequence, the length of the shorter sequence, a specified window), and (4) multiplying the result by 100 to yield the percent sequence identity or percent sequence similarity. For example, if peptides A and B are both 20 amino acids in length and have identical amino acids at all but 1 position, then peptide A and peptide B have 95% sequence identity. If the amino acids at the non-identical position shared the same biophysical characteristics (e.g., both were acidic), then peptide A and peptide B would have 100% sequence similarity. As another example, if peptide C is 20 amino acids in length and peptide D is 15 amino acids in length, and 14 out of 15 amino acids in peptide D are identical to those of a portion of peptide C, then peptides C and D have 70% sequence identity, but peptide D has 93.3% sequence identity to an optimal comparison window of peptide C. For the purpose of calculating "percent sequence identity" (or "percent sequence similarity") herein, any gaps in aligned sequences are treated as mismatches at that position.

Any polypeptides described herein as having a particular percent sequence identity or similarity (e.g., at least 70%) with a reference sequence ID number, may also be expressed as having a maximum number of substitutions (or terminal deletions) with respect to that reference sequence. For example, a sequence "having at least Y% sequence identity with SEQ ID NO:Z" may have up to X substitutions relative to SEQ ID NO:Z, and may therefore also be expressed as "having X or fewer substitutions relative to SEQ ID NO:Z."

As used herein, the term "enzyme stability" refers to the capacity of an enzyme to remain active following exposure to a particular set of conditions (e.g., temperature, pH, ionic

concentration, inhibitory agents, etc.). For example, an enzyme that exhibits enhanced stability relative to a control enzyme exhibits a smaller loss of activity upon exposure to a set of conditions than the control enzyme.

As used herein, the term "luciferin" refers to a compound, having the structure:



. Luciferin may be present as "L-luciferin" or "D-luciferin" or a racemic mixture of L- and D-form luciferin. Unless specified otherwise (e.g., "L-luciferin," "a racemic luciferin mixture," etc.), the term "luciferin: refers to the D-form. The term "luciferins" refers more broadly to a class of bioluminescent compounds (or chiral sisters thereof) that serve as substrates for firefly luciferases, and include natural artificial derivatives of luciferin, such as aminoluciferin, fluoroluciferin, etc.

As used herein, the term "luciferin derivative" refers to a class of compounds that are structurally related to luciferin, having similar ring structure, and similar, but not necessarily identical, substitutes. Luciferin derivatives typically differ from luciferin by the presence or absence of double bonds in the ring structure, and or the presence or different substituents (e.g., halogen group, oxo group, amino group, OH group, CH_3 group, CN, etc.). Some luciferin derivatives are substrates of firefly luciferase, others are inhibitors of firefly luciferase.

As used herein, the term "luciferin reaction product" refers to luciferin derivatives that are not substrates for a firefly luciferase, the production of which from a luciferin is catalyzed by a luciferase (e.g., a firefly luciferase) in a light-producing reaction. Examples of luciferin reaction products include oxyluciferin, aminooxyluciferin, fluorooxyluciferin, etc.

As used herein, the term "luciferin degradation product" refers to luciferin derivatives that are not substrates for a firefly luciferase, the production of which occurs by chemical degradation of a luciferin, in a reaction that is typically not catalyzed by a luciferase and does not result in significant light production. Examples of luciferin reaction products include deydroluciferin, aminodehydroluciferin, fluorodehydroluciferin, etc.

The term "sample" is used herein in its broadest sense. It is meant to include: a specimen, culture, lysate, etc. It includes a prepared solution or mixture, and both biological and environmental samples. Biological samples may take the form of a fluid or solid, and may be obtained from any suitable biological source (e.g., animal, including human, microbiological, etc.). Environmental samples include environmental material such as

surface matter, soil, plants, and water. These examples are not to be construed as limiting the sample types applicable to the present invention.

DETAILED DESCRIPTION

Provided herein are inhibitor-resistant luciferases and methods of use thereof. In particular, luciferases are provided that are thermal stable and exhibit improved resistance to inhibition by luciferin break-down products such as dehydroluciferin.

In some embodiments, provided herein are luciferases that are thermally stable (e.g., stable at storage temperatures above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween)). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored at temperatures above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage at temperatures above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, provided herein are luciferases that are thermally stable (e.g., stable at incubation temperatures above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween)). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5%

or less)) loss of activity following incubation temperatures above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following incubation temperatures above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween).

In some embodiments, luciferases herein are stable when stored in the presence of luciferin (e.g., D-luciferin, racemic luciferin mix) and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored in the presence of luciferin and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced stability in the presence of luciferin and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.) relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage in the presence of luciferin and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, luciferases herein are stable in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or the breakdown products of luciferin derivatives

(e.g., fluorodehydroluciferin, aminodehydroluciferin, etc.). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or the breakdown products of luciferin derivatives (e.g., fluorodehydroluciferin, aminodehydroluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced resistance to inhibition by luciferin break-down products (e.g., dehydroluciferin) and/or the breakdown products of luciferin derivatives (e.g., fluorodehydroluciferin, aminodehydroluciferin, etc.) relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or the breakdown products of luciferin derivatives (e.g., fluorodehydroluciferin, aminodehydroluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, luciferases herein are stable in the presence of L-luciferin and/or a racemic luciferin mix. In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored in the presence of L-luciferin and/or a racemic luciferin mix for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced resistance to inhibition by L-luciferin and/or a racemic luciferin mix relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage in the presence of L-luciferin and/or a racemic luciferin mix for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, luciferases herein are thermally stable when stored in the presence of luciferin (e.g., D-luciferin, racemic luciferin mix) and/or luciferin derivatives

(e.g., fluoroluciferin, aminoluciferin, racemic luciferin, etc.). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) in the presence of luciferin (e.g., D-luciferin, racemic luciferin mix) and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability in the presence of luciferin (e.g., D-luciferin, racemic luciferin mix) and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.) relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) in the presence of luciferin (e.g., D-luciferin, racemic luciferin mix) and/or luciferin derivatives (e.g., fluoroluciferin, aminoluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, luciferases herein are thermally stable in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150

days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.) relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.) for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, luciferases herein are thermally stable in the presence of L-luciferin and/or a racemic luciferin mix. In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when stored above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) in the presence of L-luciferin and/or a racemic luciferin mix for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability in the presence of L-luciferin and/or a racemic luciferin mix relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following storage above 0°C (e.g., 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, or ranges therebetween) in the presence of L-luciferin and/or a racemic luciferin mix for 1 to 365 days (e.g., 1 day, 2 days, 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 365 days, or ranges therebetween).

In some embodiments, luciferases herein are thermally stable when incubated in the presence of luciferin. In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when incubated above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) in the presence of luciferin for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability when incubated in the presence of luciferin relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following incubation above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) in the presence of luciferin for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween).

In some embodiments, luciferases herein are thermally stable when incubated in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.). In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when incubated above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.) for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85

minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability when incubated in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.) relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following incubation above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) in the presence of luciferin break-down products (e.g., dehydroluciferin) and/or break-down products of luciferin derivatives (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.) for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween).

In some embodiments, luciferases herein are thermally stable when incubated in the presence of L-luciferin and/or a racemic luciferin mix. In some embodiments, a luciferase herein exhibits less than 20% (e.g., 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less, or ranges therebetween (e.g., 5% or less)) loss of activity when incubated above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) in the presence of L-luciferin and/or a racemic luciferin mix for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween). In some embodiments, a luciferase herein exhibits enhanced thermal stability when incubated in the presence of L-luciferin and/or a racemic luciferin mix relative to a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3. In some embodiments, a luciferase of SEQ ID NO: 1 and/or SEQ ID NO: 3 exhibits reduced activity relative to a luciferase herein following incubation above room temperature (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C or ranges therebetween) in the presence of L-luciferin and/or a racemic luciferin mix for 1 to 100 minutes (e.g., 1 minute, 2 minutes, 5 minutes, 10 minutes 15 minutes, 20 minutes, 25

minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, or ranges therebetween).

In some embodiments, luciferases described herein are resistant to inhibition by one or more (e.g., 2, 3, 4, 5,6, 7, 8, 9, 10, or more, or ranges therebetween) luciferin break-down products, luciferin reaction products, and other non-substrate (e.g., not a luciferase (e.g., firefly luciferase) substrate) luciferin derivatives, such as, dehydroluciferin, aminodehydroluciferin, fluorodehydroluciferin, L-luciferin, oxoluciferin, fluorooxoluciferin, aminooxoluciferin, etc.. Experiments conducted during development of embodiments herein to engineer (e.g., evolve) dehydroluciferin that have improved resistance to inhibition by luciferin break-down products (e.g., compounds that result from storage and/or incubation of luciferin).

In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises 1 or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or more, or ranges therebetween) substitutions relative to a wild-type firefly luciferase (SEQ ID NO: 1).

In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises 1 or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or more, or ranges therebetween) substitutions relative to a luciferase of SEQ ID NO: 3.

In some embodiments, a luciferase described herein exhibits enhanced signal stability (e.g., as measured by the half-life of decay of the signal in a time-course (e.g., 10% increase, 20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 2-fold increase, 3-fold increase, 4-fold increase, 5-fold increase, 6-fold increase, 7-fold increase, 8-fold increase, 9-fold increase, 10-fold increase, 20-fold increase, 50-fold increase, 100-fold increase, 1000-fold increase, or more, or ranges therebetween)) when compared to a reference luciferase (e.g., a native, wild-type luciferase (e.g., SEQ ID NO: 1), an enhanced synthetic luciferase (e.g., SEQ ID NO: 3), etc.).

In some embodiments, a luciferase described herein exhibits enhanced apparent signal stability (e.g., as measured by the half-life of decay of the signal in a time-course (e.g., 10% increase, 20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 2-fold increase, 3-fold increase, 4-fold increase, 5-fold increase, 6-fold increase, 7-fold increase, 8-fold increase, 9-fold increase, 10-fold increase, 20-fold increase, 50-fold increase, 100-fold increase, 1000-fold increase, or more, or ranges

therebetween)) in the presence of an inhibitor when compared to its stability in the absence of the inhibitor. In some embodiments, the inhibitor is one or more of a dehydroluciferin (e.g., dehydroluciferin, dehydrooxoluciferin, dehydroaminoluciferin, dehydroaminooxoluciferin, and L-luciferin

In some embodiments, a luciferase described herein exhibits an increase in enhancement of apparent signal stability (e.g., as measured by the half-life of decay of the signal in a time-course (e.g., 10% increase, 20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 2-fold increase, 3-fold increase, 4-fold increase, 5-fold increase, 6-fold increase, 7-fold increase, 8-fold increase, 9-fold increase, 10-fold increase, 20-fold increase, 50-fold increase, 100-fold increase, 1000-fold increase, or more, or ranges therebetween)) in the presence of an inhibitor when compared to its stability in the absence of the inhibitor, when compared to the signal stability enhancement in the presence of the inhibitor of a reference luciferase (e.g., a native, wild-type luciferase (e.g., SEQ ID NO: 1), an enhanced synthetic luciferase (e.g., SEQ ID NO: 3), etc.).

In some embodiments, a luciferase comprises a substitution at amino acid position 244, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a H244R substitution relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a substitution at amino acid position 300, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a C300G substitution relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a substitution at amino acid position 396, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a I396K substitution relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a substitution at nucleotide position 222, relative to SEQ ID NO: 4. In some embodiments, a luciferase comprises a C to T substitution at nucleotide position 222 relative to SEQ ID NO: 4.

In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence identity with SEQ ID NO: 5. In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence similarity (e.g., conservative or semi-conservative) with SEQ ID NO: 5. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) substitutions relative to SEQ ID NO: 5. In some

embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) non-conservative substitutions relative to SEQ ID NO: 5. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) semi-conservative substitutions relative to SEQ ID NO: 5. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) conservative substitutions relative to SEQ ID NO: 5.

In some embodiments, a luciferase comprises a substitution at amino acid position 240, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a I240L substitution relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a substitution at amino acid position 254, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a Y254S substitution relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a substitution at amino acid position 344, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a T344A substitution relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a substitution at amino acid position 396, relative to SEQ ID NO: 3. In some embodiments, a luciferase comprises a I396K substitution relative to SEQ ID NO: 3.

In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence identity with SEQ ID NO: 7. In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence similarity (e.g., conservative or semi-conservative) with SEQ ID NO: 7. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) substitutions relative to SEQ ID NO: 7. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) non-conservative substitutions relative to SEQ ID NO: 7. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) semi-conservative substitutions relative to SEQ ID NO: 7. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20,

25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) conservative substitutions relative to SEQ ID NO: 7.

In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence identity with SEQ ID NO: 9. In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence similarity (e.g., conservative or semi-conservative) with SEQ ID NO: 9. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) substitutions relative to SEQ ID NO: 9. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) non-conservative substitutions relative to SEQ ID NO: 9. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) semi-conservative substitutions relative to SEQ ID NO: 9. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) conservative substitutions relative to SEQ ID NO: 9.

In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence identity with SEQ ID NO: 11. In some embodiments, a thermally-stable and/or inhibitor-resistant (e.g., dehydroluciferin-resistant) luciferase comprises at least 70% (e.g., 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, or ranges therebetween) sequence similarity (e.g., conservative or semi-conservative) with SEQ ID NO: 11. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) substitutions relative to SEQ ID NO: 11. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) non-conservative substitutions relative to SEQ ID NO: 11. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) semi-conservative substitutions relative to SEQ ID NO: 11. In some embodiments, a luciferase comprises 100 or fewer (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20,

25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or ranges therebetween) conservative substitutions relative to SEQ ID NO: 11.

In some embodiments, a luciferase herein comprises any combination of amino acid substitutions relative to SEQ ID NO: 3, such as those selected from positions 240, 244, 254, 300, 344, and 396. In some embodiments, a luciferase herein comprises any combination of amino acid substitutions relative to SEQ ID NO: 3, such as I240L, H244R, Y254S, C300G, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions.

In some embodiments, provided herein is a luciferase comprising at least 70% sequence identity to SEQ ID NO: 3, but having at least one substitution at a position corresponding to 240, 244, 254, 300, 344, and/or 396 of SEQ ID NO: 3. In some embodiments, provided herein is a luciferase comprising at least 70% sequence identity to SEQ ID NO: 3, but having at least one substitution corresponding to I240L, H244R, Y254S, C300G, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions. In some embodiments, a luciferase comprises a substitution at position 396 (e.g., an I396K substitution).

In some embodiments, provided herein are nucleic acids (e.g., DNA, RNA, etc.) encoding the polypeptides described herein. In some embodiments, provided herein are vectors comprising nucleic acids (e.g., DNA, RNA, etc.) encoding the polypeptides described herein. In some embodiments, provided herein are cells expression the polypeptides described herein. In some embodiments, provided herein are fusion proteins comprising the polypeptides described herein.

In some embodiments, to measure luminescence and thereby determine the activity of a particular luciferase (or reagent composition comprising a luciferase), the relative light unit (RLU) value generated by the luciferase reaction at a timepoint of interest after the reagent composition is combined with a sample may be measured. In some embodiments, the relative light output may be compared to a control value (e.g., to determine the stability of the activity of the luciferase).

In some embodiments, the luciferases described herein exhibit enhanced stability and/or activity in the presence of break-down products of luciferin (e.g., compounds that inhibit ATP-dependent luciferases that utilize luciferin as a substrate (e.g., dehydroluciferin, etc.), etc.). In some embodiments, this enhanced stability allows the luciferases herein to be in contact with (e.g., stored with) luciferin under conditions (e.g., >0°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, or more) that result in the production of luciferin degradation products over time (e.g., 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8

days, 9 days, 10 days, 12 days, 14 days, 16 days, 18, days, 20 days, 25 days, 30 days, 40 days, 50 days, 75 days, 100 days, 150 days, 200 days, 250 days, 300 days, 365 days, or more). As such, in some embodiments, provided herein are stable reagent compositions comprising a luciferase described herein and luciferin. In some embodiments, reagent compositions are provided comprising a luciferase described herein, luciferin, and one or more luciferin degradation products (e.g., dehydroluciferin, etc.).

In some embodiments, reagent compositions described herein (e.g., comprising a luciferase and luciferin) are useful in a variety of assays, including for the detection of ATP in a sample. Because the bioluminescence of the luciferases described herein is dependent upon the presence of luciferin and ATP, contacting a sample comprising ATP with a reagent composition herein (e.g., comprising luciferase and luciferin) results in detectable bioluminescence. In some embodiments, because the luciferases described herein are resistant to inhibition by luciferin degradation products, such reagent compositions maintain activity over time and are useful in the quantification of ATP in sample over time (e.g., the activity of the reagent composition does not decrease (e.g., loss of activity is reduced compared to other luciferases)).

In some embodiments, in addition to a luciferase described herein and luciferin (and potentially luciferin degradation products), reagent compositions further comprise additional components for storage (e.g., for enzyme stability), handling (e.g., to facilitate dispensing of the reagent composition), and/or assay performance. In some embodiments, a reagent composition additional comprises salts or metal ions (e.g., Mg^{2+}), detergents, buffers, etc. In some embodiments, additional components are part of the reagent composition, and stored in the same container at the luciferase and luciferin. In some embodiments, a reagent composition (e.g., comprising luciferase and luciferin) is provided as part of a kit, the kit comprising additional components/reagents that are stored in a separate contained from the reagent composition.

In some embodiments, a kit or reagent composition comprises a luciferase described herein and aminothiothymidine (ATT). In some embodiments, a kit or reagent composition comprises a luciferase described herein and dehydroluciferin. In some embodiments, a kit or reagent composition comprises a luciferase described herein, ATT, and dehydroluciferin. In some embodiments, a kit or reagent composition comprises a luciferase described herein, D-luciferin, and ATT. In some embodiments, a kit or reagent composition comprises a luciferase described herein, D-luciferin, and dehydroluciferin. In some embodiments, a kit or reagent composition comprises a luciferase described herein, D-luciferin, ATT, and

dehydroluciferin. In some embodiments, a kit or reagent composition comprises a luciferase described herein and a racemic luciferin mixture. In some embodiments, a kit or reagent composition comprises a luciferase described herein and a racemic luciferin mixture. In some embodiments, a kit or reagent composition comprises a luciferase described herein, dehydroluciferin, and a racemic luciferin mixture. In some embodiments, a kit or reagent composition comprises a luciferase described herein, ATT, and a racemic luciferin mixture. In some embodiments, a kit or reagent composition comprises a luciferase described herein, ATT, dehydroluciferin, and a racemic luciferin mixture. In any of the aforementioned embodiments, luciferin may be replaced by other luciferin substrates (e.g., aminoluciferin, fluoroluciferin, etc.) and/or dehydroluciferin may be replaced by other luciferin degradation products (e.g., aminodehydroluciferin, fluorodehydroluciferin, etc.

In some embodiments, a kit or reagent composition comprises a luciferase (e.g., inhibitor resistant luciferase), luciferin (e.g., D-luciferin), and one or more inhibitors of the luciferase (e.g., dehydroluciferin, aminodehydroluciferin, fluorodehydroluciferin, L-luciferin, etc.). In some embodiments, inclusion of one or more inhibitors of the luciferase in the kit or reagent composition provides enhancement of one or more of signal duration, apparent enzyme stability, and/or storage stability. In some embodiments, the enhanced resistance of the luciferase to inhibitor provides a reagent composition or kit with one or more of enhanced signal duration, enhanced apparent enzyme stability, and/or enhanced storage stability, while providing sufficient signal (e.g., due to the inhibitor resistance) to be useful in assays and other applications.

In some embodiments, a kit or reagent composition comprises between 10:1 and 1:10 ratio (e.g., 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 18, 1:9, 1:10, or ranges therebetween) of D-luciferin (and/or D-luciferin-related substrate (e.g., aminoluciferin, fluoroluciferin, etc.) to inhibitor (e.g., dehydroluciferin, aminodehydroluciferin, fluorodehydroluciferin, L-luciferin, etc.). In some embodiments, a luciferin:inhibitor ratio is selected to minimize the net gain of inhibitor (e.g., dehyro breakdown products) over a relevant timescale (e.g., over the course of an assay (e.g., 1 minute, 5 minutes, 10 minutes, 20 minutes, 40 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, or more, or ranges therebetween), over the course of storage (e.g., 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 15 days, 20 days, 30 days, 40 days, 60 days, 80 days, 100 days, 150 days, 200 days, 300 days, or more, ore ranges therebetween), etc.). In some embodiments, conditions affecting the optimal ratio of luciferin:inhibitor include the kinetics of the luciferase and the starting or storage concentration of the luciferin.

In some embodiments, a kit or reagent composition comprises one or more suitable buffers. Any buffers that maintain suitable pH for the working solution and do not significantly interfere with the luciferase-luciferin reaction are contemplated. The preferred pH range is between about pH 4.5 and about pH 9.0 (e.g., about pH 6.0 and about pH 8.0 (e.g., 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, and ranges therebetween)). Suitable buffers include MES, citrate buffers, phosphate buffered saline (PBS), Tris-N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), borate, and any other buffer known to those of skill in the art may be suitable. Selection of appropriate buffers depends on pH buffering capacity and interaction with the luciferase-luciferin reaction.

In some embodiments, a kit or reagent composition comprises one or more defoamers. Defoaming agents are desirable to prevent foam from interfering with the detection of bioluminescence, especially in applications that quantify luminescence. Defoming agents, such as MAZU, may be organic or silicone based. Selection of defoamers depends on their ability to eliminate foam without interfering with the luciferase-luciferin reaction.

In some embodiments, a kit or reagent composition comprises magnesium. The luciferase-luciferin reaction is dependent not only on ATP, but also on magnesium ions. In some embodiments, to ensure luciferase activity, magnesium is exogenously supplied. In addition to magnesium sulfate, other salts of magnesium are contemplated, such as magnesium chloride, magnesium gluconate, magnesium acetate, magnesium bromide, magnesium carbonate, or any magnesium complex that dissociates in the reagent composition or in the sample to yield Mg^{2+} ions available to the luciferase and does not interfere with the luciferase-luciferin reaction. In some embodiments, other cations are provided in addition to or in place of magnesium, such as calcium and manganese. In some embodiments, the endogenous magnesium of the sample is sufficient to allow the luciferase-luciferin bioluminescence in the presence of ATP; in such embodiments, magnesium may not be included in a kit or reagent composition.

In some embodiments, a kit or reagent composition comprises a component containing one or more ATPase inhibitors within a solution optionally containing other functional components, such as buffers, defoamers, enzyme stabilizers, and the like. This component may be supplied as a working solution or as a concentrate. In some embodiments, an ATPase inhibitor is a detergent with a charged group (e.g., cationic detergent (e.g., DTAB (dodecyltrimethylammonium bromide), Benzalkonium Chloride, CTAB (cetyltmethylammonium), BDDABr (benzyldimethyldodecylammonium bromide),

etc.), anionic detergent (e.g., deoxycholate or SDS) or zwitterionic detergent (e.g., sulfobetaine 3-10), etc.). Such inhibitors prevent ATPases in a sample from processing ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP), for example, before the luciferase is able to utilize the ATP in the luciferase-luciferin reaction. ATPase inhibitors may inactivate ATPases directly or indirectly. They may bind to ATPases, either in the active sites, thus preventing substrate binding, or denature ATPases, such as by denaturing detergents, or they may selectively sequester ATPases from their substrates.

In some embodiments, a kit or reagent composition comprises a component containing one or more inhibitors of ATP-generating enzymes. In some samples, enzymes such as kinases may be active, allowing for continued production of ATP. Because the ATP concentration is determined at a specific time, such enzymatic activity may result in an overestimation of the ATP concentration. In some embodiments, to counter such ATP-generating activity, reagent compositions and/or kits herein comprise inhibitors of ATP production. Examples of useful compounds include NaF, which is useful at concentrations of at least 1 mM, 2 mM, 5 mM, 10 mM 20 mM 50 mM, 100 mM or more, or ranges therebetween. Any such inhibitor may be used, however, if it does not adversely affect luciferase so as to take it outside the utility of embodiments herein. Other inhibitors of ATP-generating enzymes include, but are not limited to, vanadate, paranitrophenylphosphate and dichloroacetic acid.

In some embodiments, a kit or reagent composition comprises a cell lysing agent and/or ATP-extraction agent. In embodiments in which a sample comprises cells, and intracellular ATP is desired for detection/quantification, reagents may be provided to lyse cells and/or liberate ATP from cells. In some embodiments, to free ATP sequestered within a cell and/or to lyse cells in a sample, cell lysing agents, such as non-ionic detergents, are included. Any cell lysing agent is contemplated including other non-ionic detergents, (e.g., Triton series detergents) cationic, anionic and zwitterionic detergents, bile salts, chaotropes, and any other agent that disrupts cellular membranes, including bacterial toxins such as oxylysins. Alternatively, any agent that allows for ATP extraction from a cell is contemplated (e.g., CTAB). Agents that allow for ATP extraction from a cell include detergents present at a concentration that permeablizes the cell membrane, allowing for ATP within the cell to leach into the surrounding media, but not present at such a concentration that produces a cell lysate.

In some embodiments, a kit or reagent composition comprises one or more stabilizing agents. In some embodiments, the stabilizing agent can be any compound that stabilizes the

luciferase from degradation. Suitable stabilizing agents include proteins (e.g., bovine serum albumin, gelatin, etc.), detergents (e.g., non-ionic detergents, such as THESIT), etc.

In some embodiments, other agents may be included a kit or reagent composition herein. For example, a kit or reagent composition may include substances that are known to enhance the duration of luminescence resulting from a luciferase reaction, such as co-enzyme A (CoA), thiol reagents such as dithiothreitol, β mercaptoethanol, and metal ion chelators, such as EDTA, to prolong the signal, protease inhibitors, or salts (e.g., NaCl, KCl, Na_2SO_4, $NAHCO_3$, NaH_2PO_4, etc.).

In some embodiments, a reagent composition and/or other components of a kit are contained in one or more containers or vessels. In some embodiments, the components of a reagent composition are contained within a single container or vessel. In some embodiments, a kit may comprise multiple containers or vessels containing the reagent composition. In some embodiments, a kit comprises one or more containers or vessels containing reagents other than the reagent composition (see above). In some embodiments, components and reagents included in a kit are supplied in containers of any sort such that the life of the different components are preserved, and are not adsorbed or altered by the materials of the container. Containers or vessels may comprise or consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, etc., ceramic, metal or any other material typically employed to hold reagents. Examples of suitable containers include ampules, bottles, envelopes, test tubes, vials, flasks, bottles, syringes, or the like.

In some embodiments, kits comprise, and/or reagent compositions are provided with, appropriate instruction materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audio tape, etc. Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail. In some embodiments, the instructions instruct the user to combine reagent composition (e.g., comprising luciferase and luciferin) with a sample to detect or quantify ATP.

In some embodiments, a reagent composition is provided as a liquid reagent. In some embodiments, by providing a stable luciferase and luciferin in a single premixed liquid reagent, variability introduced by rehydrating a lyophilized reagent is eliminated. In other embodiments, a reagent composition is provided in lyophilized form. In some embodiments, other components of a kit herein may be provided as one or multiple liquid or dried compositions.

Although the luciferases and reagent compositions (e.g., comprising luciferase and luciferin) described herein are not limited to use in any particular method or application, due to their stability and activity in the presence of degradation products of luciferin, the luciferases and reagent compositions described herein are particularly useful for the detection of AT in a sample.

Because the luciferase-luciferin reaction is ATP-dependent, the luciferases and reagent compositions described herein find use in assays for to detect and/or quantify ATP. The luciferase-luciferin reaction allows ATP to be detected in a sample containing as little as 10^{-16} moles of ATP or less.

In some embodiments, provided herein are methods, compositions and kits that are used to effectively and accurately detect and quantify cellular ATP levels. In some embodiments, the luciferases and reagent compositions find use in the detection of ATP on surfaces, in non-cellular samples (e.g., water), for hygiene monitoring, etc.

In some embodiments, methods comprise the addition of a single reagent composition that comprises a luciferase and luciferin (and possibly dehydroluciferin) to a sample (e.g., a sample comprising or suspected of possibly comprising ATP) and detecting luminescence. In some embodiments, additional components and /or reagents (see above) are included with the reagent composition or added separately (e.g., a kinase inhibitor, a compound that prevents accumulation of ATP, a cell-lysing agent (e.g., a polyoxyethylene such as THESIT), an ATP extracting agent, magnesium, a buffer, salts, etc.). In some embodiments, the inclusion of the luciferase and luciferin in a single reagent speeds ATP detection, simplifies assays and handling, and increases reproducibility.

As addressed throughout, the methods, compositions and kits herein are particularly useful for the qualitative or quantitative detection of ATP (or ATP an analogue which can function as a luciferase substrate) in a sample. In some embodiments, a simple qualitative experiment in which luminescence is generated in a sample using a reagent composition (e.g., comprising luciferase and luciferin) indicates the presence of ATP. In some embodiments an assay is provided in which the amount of ATP in a sample is quantitated. ATP may be detected (e.g., qualitatively) and/or quantitated as a single time-point, at multiple time-points, or in real time using the luciferases, reagent compositions, and/or kits herein.

In some embodiments, a sample is anything that contains or is suspected of containing ATP or a suitable ATP analogue, such as cell lysates, intact cells, biopsies, foods, beverages, water, swabs wiped on surfaces such as those of animals, plants, or inanimate objects, and the like. Other examples of samples include compositions of a known ATP concentration. Cells

or cell lysates may be from any organism, prokaryotic or eukaryotic. Eukaryotic cells may be from plants, animals, fungi, insects, etc. or cultured cells from such organisms. These examples are furnished only as examples and are not meant to be limiting.

A cell lysate comprises cellular components that are no longer organized into a recognizable intact cellular architecture. Cell lysates may have soluble and insoluble components, either of which may be removed before using the lysate. Lysates may be prepared by any means, including physical disruption using sonication, a dounce, mortar and pestle, freeze-thaw cycling, or any other device or process that destroys the physical integrity of cells; or lysis by detergents, such as those in which luciferase activity is maintained, such as zwitterionic and nonionic detergents, or cationic detergents DTAB or CTAB. Preferably, the cell lysate is produced in such a way that the integrity of the ATP concentration is preserved at the time the cells are harvested.

In some embodiments, to accurately detect ATP in a sample, enzymes that would degrade cellular ATP or those that would generate ATP are preferably inhibited or removed. Inhibitors of ATP-generating enzymes, those enzymes that have as a product or by-product ATP, such as the activity of kinases, may be incorporated into the reagent composition (e.g., comprising luciferase and luciferin) or into a kit comprising a reagent composition.

The luciferases, reagent compositions, methods, and kits herein permit a user to quantify the amount of ATP in a sample by quantifying the amount of luminescence. In some embodiments, the luciferase and luciferin (in a single composition) are applied to a test sample of interest. In some embodiments, the luciferase and luciferin (in a single composition) are also applied to a sample containing known amounts of ATP (control). The magnitude of the signal generated from the test sample correlates to the concentration of ATP in the sample. In some embodiments, the magnitude of the luminescent signal from the sample of unknown ATP concentration is correlated to signal generated either by internal controls (the addition of a known amount of ATP to a sample and measuring the subsequent luminescence) or external standard curves generated by measuring the luminescence of several samples of known ATP concentrations and plotting them graphically.

EXPERIMENTAL

Example 1

A library of random mutants was created using Diversify® PCR Random Mutagenesis Kit (Clonetech) and DNA from a thermal stable luciferase as a template. Mutant colonies were picked into 96-well plates and then grown for ~17 hours in LB media. The

cultures were then diluted into LB induction media containing 0.02% rhamnose and 0.05% glucose. The induced cultures were then lysed and assayed with two assay reagents: Luciferin+ATP+detergent and Luciferin+ATP+detergent+dehydroluciferin. A ratio of the RLU values for each mutant was calculated (RLU+dehydroluciferin/RLU no dehydroluciferin), and samples that had the highest ratio were selected (Figure 1). DNA from each of the mutants was isolated, pooled, and recombination mutagenesis was performed.

Select mutants in E. coli from the recombination mutagenesis were grown for secondary screening (same procedure as listed above), and then each mutant was combined with full detection reageant (Luciferin+detergent+ATP) containing varied amounts of dehydroluciferin. Samples were then measured using GloMax®-Multi+ luminometer (Promega) IC50 values were calculated using GraphPad Prism log(inhibitor) vs. normalized response regression (Figure 2).

Detection reagent (Luciferin in detergent) was prepared and then aliquots were incubated at 37°C. Aliquots were removed from 37°C at various time-points and stored at 4°C. ATP was added to an aliquot of each of the detection reagent series, and then the detection reagent was combined with each of the lysed mutant luciferases. Luminescence was measured on a GloMax®-Multi+ luminometer (Promega). An apparent stability was calculated using GraphPad Prism One phase decay regression (Figure 3).

Example 2

To determine RLU and IC50 values for the mutants described herein, two-4mL solutions of E6, B7, and UltraGlo enzymes were prepared in Detection Reagent (1% Thesit, 0.1% DTAB, 0.08% CTAB, 0.2% Chlorohexidine digluconate, 100mM MES pH 6.0, 5mM MgCl2, 10mM EGTA, 4mM NaCitrate) containing 0.1% Prionex, 0.25mM of either racemic F-LH2 or H-LH2 were added to the enzyme solutions. A 3x dilution series of F-dehydroluciferin and H-dehydroluciferin were prepared starting with 0.5mM using the earlier prepared racemic luciferin mixtures as a diluent. 50uL of each dehydroluciferin tiration series was then combined with 50uL of 100nM ATP. Samples were incubated for 1 minute, luminescence (RLU) was then measured using GloMax®-Multi+ luminometer (Promega), and IC50 values for each enzyme (Figure 4).

Example 3

Mutants E6 and B7 identified in the above recombination mutagenesis, as well as UltraGlo enzyme, were screened for their stability in the presence of breakdown products. Detection reagent + 0.1% Prionex containing either racemic 0.25mM H-LH2 or 0.25mM F-LH2 was prepared. Eight, 2mL aliquots were prepared for each substrate, and the samples incubated at 37◦C. At various time points, the aliquots were transferred to 4◦C.

After all samples had been incubated and transferred, 500uL of each sample was aliquoted in triplicate into wells of a 96-deep well plate. 0.01mg/ml of each enzyme (UltraGlo, E6, or B7) was then added to each buffer aliquot. 50uL of each substrate/enzyme solution was then combined with 50uL 0.1mM ATP. Samples were incubated for 3 minutes, and luminescence then measured using GloMax®-Multi+ luminometer (Promega) (Figure 5).

Example 4

To explore the inhibition of F-luciferin luminescence by dehydro-F-luciferin by combination mutants, the mutant Triple + 300 (H244W, T344A, I396K, C300G) was screened for its resistance to dehydroluciferin.

Purified UltraGlo and Triple +300 luciferase enzymes were serially diluted from stocks in 1xTBS+1.0% Prionex to a concentration of 0.01 mg/ml. Racemic F-luciferin was serially diluted from stocks in water to 22mM. Racemic dehydro-F-luciferin was serially diluted from stocks in water to 10mM, and then 8 consecutive 4-fold serial dilutions were carried out for each concentration tested. ATP was diluted to 1mM in BrightGlo Buffer or Detection Reagent buffer (depending on final reaction destination) to 1mM concentration.

The following components were then combined into wells of a flat bottom, white, 96-well plate: 50 ul diluted ATP in buffer (BrightGlo or Detection Reagent; 0.5 mM final concentration), 50 ul diluted luciferase enzyme (0.05 mg/ml final concentration), 5 ul diluted F-luciferin (1mM final concentration), and 4 ul diluted dehydro-F-luciferin (0.006-366.97mM final concentration)

Reactions were incubated for 3 minutes at room temperature, and luminescence was measured using a GloMax Multi+ plate reader. Data were analyzed in GraphPad Prism 7 using nonlinear regression by fitting the data to a Michaelis-Menten competitive inhibition enzyme kinetic model.

Table 1

	Ki (nM)	
	BrightGlo	Detection Reagent
UltraGlo	39.9	419.7
triple+300	71.8	562.5

SEQUENCES

SEQ ID NO: 1 – Wild-type firefly luciferase (protein)

MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVNITYAEYF
EMSVRLAEAMKRYGLNTNHRIVVCSENSLQFFMPVLGALFIGVAVAPANDIYNEREL
LNSMNISQPTVVFVSKKGLQKILNVQKKLPIIQKIIIMDSKTDYQGFQSMYTFVTSHLP
PGFNEYDFVPESFDRDKTIALIMNSSGSTGLPKGVALPHRTACVRFSHARDPIFGNQII
PDTAILSVVPFHHGFGMFTTLGYLICGFRVVLMYRFEEELFLRSLQDYKIQSALLVPT
LFSFFAKSTLIDKYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPGIRQGYGLTETTSAI
LITPEGDDKPGAVGKVVPFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNP
EATNALIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLIKYKGYQVAPAELESILLQHP
NIFDAGVAGLPDDDAGELPAAVVVLEHGKTMTEKEIVDYVASQVTTAKKLRGGVVF
VDEVPKGLTGKLDARKIREILIKAKKGGKSKL

SEQ ID NO: 2 – Wild-type firefly luciferase (nucleotide)

CTGCAGAAATAACTAGGTACTAAGCCCGTTTGTGAAAAGTGGCCAAACCCATAA
ATTTGGCAATTACAATAAAGAAGCTAAAATTGTGGTCAAACTCACAAACATTTTT
ATTATATACATTTTAGTAGCTGATGCTTATAAAAGCAATATTTAAATCGTAAACA
ACAAATAAAATAAAATTTAAACGATGTGATTAAGAGCCAAAGGTCCTCTAGAAA
AAGGTATTTAAGCAACGGAATTCCTTTGTGTTACATTCTTGAATGTCGCTCGCAG
TGACATTAGCATTCCGGTACTGTTGGTAAAATGGAAGACGCCAAAAACATAAAG
AAAGGCCCGGCGCCATTCTATCCTCTAGAGGATGGAACCGCTGGAGAGCAACTG
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GCACATATCGAGGTGAACATCACGTACGCGGAATACTTCGAAATGTCCGTTCGGT
TGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTAT
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TGCACTGATAATGAATTCCTCTGGATCTACTGGGTTACCTAAGGGTGTGGCCCTT
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ATGACGCCGGTGAACTTCCCGCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGAT
GACGGAAAAAGAGATCGTGGATTACGTCGCCAGTAAATGAATTCGTTTTACGTTA
CTCGTACTACAATTCTTTTCATAGGTCAAGTAACAACCGCGAAAAAGTTGCGCGG
AGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAAAACTCGACGC
AAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTCCAAAT
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ATGCAAATTGATGAATGGTAATTTTGTAATTGTGGGTCACTGTACTATTTTAACG
AATAATAAAATCAGGTATAGGTAACTAAAAA

SEQ ID NO: 3 – ULTRA GLO luciferase (protein)

MADKNILYGPEPFYPLEDGTAGEQMFDALSRYAAIPGCIALTNAHTKENVLYEEFLK
LSCRLAESFKKYGLKQNDTIAVCSENSLQFFLPVIASLYLGIIVAPVNDKYIERELIHSL
GIVKPRIVFCSKNTFQKVLNVKSKLKSIETIIILDLNEDLGGYQCLNNFISQNSDSNLDV
KKFKPYSFNRDDQVASIMFSSGTTGLPKGVMLTHKNIVARFSIAKDPTFGNAINPTSA
ILTVIPFHHGFGMMTTLGYFTCGFRVVLMHTFEEKLFLQSLQDYKVESTLLVPTLMA
FLAKSALCEKYDLSHLKEIASGGAPLSKEIGEMVKKRFKLNFVRQGYGLTETTSAVLI
TPKGDAKPGSTGKIVPLHAVKVVDPTTGKILGPNEPGELYFKGPMIMKGYYNNEEAT
KAIIDNDGWLRSGDIAYYDNDGHFYIVDRLKSLIKYKGYQVAPAEIEGILLQHPYIVD
AGVTGIPDEAAGELPAAGVVVQTGKYLNEQIVQDYVASQVSTAKWLRGGVKFLDEI
PKGSTGKIDRKVLRQMLEKHTNGHHHHHHHH*

SEQ ID NO: 4 – ULTRA GLO luciferase (nucleic acid)

ATGGCTGACAAAAACATCCTGTATGGTCCGGAACCGTTCTACCCACTGGAAGATG
GTACCGCTGGTGAACAGATGTTTGACGCATTATCTCGTTATGCAGCTATTCCGGG
CTGCATAGCATTGACAAATGCTCATACAAAAGAAAATGTTTTATATGAAGAGTTT
CTGAAACTGTCGTGTCGTTTAGCGGAAAGTTTTAAAAAGTATGGATTAAAACAAA
ACGACACAATAGCGGTGTGTAGCGAAAATAGTCTGCAATTTTTCCTTCCTGTAAT
TGCATCATTGTATCTTGGAATAATTGTGGCACCTGTTAACGATAAATACATTGAA
CGTGAATTAATACACAGTCTTGGTATTGTAAAACCACGCATAGTTTTTTGCTCCA
AGAATACTTTTCAAAAAGTACTGAATGTAAAATCTAAATTAAAATCTATTGAAAC
TATTATTATATTAGACTTAAATGAAGACTTAGGAGGTTATCAATGCCTCAACAAC
TTTATTTCTCAAAATTCCGATAGTAATCTGGACGTAAAAAAATTTAAACCCTATT
CTTTTAATCGAGACGATCAGGTTGCGTCGATTATGTTTTCTTCTGGTACAACTGGT
CTGCCGAAGGGAGTCATGCTAACTCACAAGAATATTGTTGCACGATTTTCTATTG
CAAAAGATCCTACTTTTGGTAACGCAATTAATCCCACGTCAGCAATTTTAACGGT
AATACCTTTCCACCATGGTTTTGGTATGATGACCACATTAGGATACTTTACTTGTG
GATTCCGAGTTGTTCTAATGCACACGTTTGAAGAAAAACTATTTCTACAATCATT
ACAAGATTATAAAGTGGAAAGTACTTTACTTGTACCAACATTAATGGCATTTCTT
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TACACGCTGTTAAAGTTGTCGATCCTACAACAGGAAAAATTTTGGGGCCAAATGA
ACCTGGAGAATTGTATTTTAAAGGCCCGATGATAATGAAGGGTTATTATAATAAT
GAAGAAGCTACTAAAGCAATTATTGATAATGACGGATGGTTGCGCTCTGGTGAT
ATTGCTTATTATGACAATGATGGCCATTTTTATATTGTGGACAGGCTGAAGTCAC
TGATTAAATATAAAGGTTATCAGGTTGCACCTGCTGAAATTGAGGGAATACTCTT
ACAACATCCGTATATTGTTGATGCCGGCGTTACTGGTATACCGGATGAAGCCGCG
GGCGAGCTTCCAGCTGCAGGTGTTGTAGTACAGACTGGAAAATATCTAAACGAA
CAAATCGTACAAGATTATGTTGCCAGTCAAGTTTCAACAGCCAAATGGCTACGTG
GTGGGGTGAAATTTTTGGATGAAATTCCCAAAGGATCAACTGGAAAAATTGACA
GAAAAGTGTTAAGACAAATGTTAGAAAAACACACCAATGGGCATCACCATCACC
ACCATCATCACTAA

SEQ ID NO: 5 – E6 luciferase mutant (protein)

MADKNILYGPEPFYPLEDGTAGEQMFDALSRYAAIPGCIALTNAHTKENVLYEEFLK
LSCRLAESFKKYGLKQNDTIAVCSENSLQFFLPVIASLYLGIIVAPVNDKYIERELIHSL
GIVKPRIVFCSKNTFQKVLNVKSKLKSIETIIILDLNEDLGGYQCLNNFISQNSDSNLDV
KKFKPYSFNRDDQVASIMFSSGTTGLPKGVMLTHKNIVARFSIAKDPTFGNAINPTSA
ILTVIPFHRGFGMMTTLGYFTCGFRVVLMHTFEEKLFLQSLQDYKVESTLLVPTLMA
FLAKSALGEKYDLSHLKEIASGGAPLSKEIGEMVKKRFKLNFVRQGYGLTETTSAVLI
TPKGDAKPGSTGKIVPLHAVKVVDPTTGKILGPNEPGELYFKGPMKMKGYYNNEEA
TKAIIDNDGWLRSGDIAYYDNDGHFYIVDRLKSLIKYKGYQVAPAEIEGILLQHPYIV

DAGVTGIPDEAAGELPAAGVVVQTGKYLNEQIVQDYVASQVSTAKWLRGGVKFLD
EIPKGSTGKIDRKVLRQMLEKHTNGHHHHHHHH*

SEQ ID NO: 6 – E6 luciferase mutant (nucleic acid)

ATGGCTGACAAAAACATCCTGTATGGTCCGGAACCGTTCTACCCACTGGAAGATG
GTACCGCTGGTGAACAGATGTTTGACGCATTATCTCGTTATGCAGCTATTCCGGG
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CTGAAACTGTCGTGTCGTTTAGCGGAAAGTTTTAAAAAGTATGGATTAAAACAAA
ATGACACAATAGCGGTGTGTAGCGAAAATAGTCTGCAATTTTTCCTTCCTGTAAT
TGCATCATTGTATCTTGGAATAATTGTGGCACCTGTTAACGATAAATACATTGAA
CGTGAATTAATACACAGTCTTGGTATTGTAAAACCACGCATAGTTTTTTGCTCCA
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ACCTGGAGAATTGTATTTTAAAGGCCCGATGAAAATGAAGGGTTATTATAATAAT
GAAGAAGCTACTAAAGCAATTATTGATAATGACGGATGGTTGCGCTCTGGTGAT
ATTGCTTATTATGACAATGATGGCCATTTTTATATTGTGGACAGGCTGAAGTCAC
TGATTAAATATAAAGGTTATCAGGTTGCACCTGCTGAAATTGAGGGAATACTCTT
ACAACATCCGTATATTGTTGATGCCGGCGTTACTGGTATACCGGATGAAGCCGCG
GGCGAGCTTCCAGCTGCAGGTGTTGTAGTACAGACTGGAAAATATCTAAACGAA
CAAATCGTACAAGATTATGTTGCCAGTCAAGTTTCAACAGCCAAATGGCTACGTG
GTGGGGTGAAATTTTTGGATGAAATTCCCAAAGGATCAACTGGAAAAATTGACA
GAAAAGTGTTAAGACAAATGTTAGAAAAACACACCAATGGGCATCACCATCACC
ACCATCATCACTAAT

SEQ ID NO: 7 – B7 luciferase mutant (protein)

MADKNILYGPEPFYPLEDGTAGEQMFDALSRYAAIPGCIALTNAHTKENVLYEEFLK
LSCRLAESFKKYGLKQNDTIAVCSENSLQFFLPVIASLYLGIIVAPVNDKYIERELIHSL
GIVKPRIVFCSKNTFQKVLNVKSKLKSIETIIILDLNEDLGGYQCLNNFISQNSDSNLDV
KKFKPYSFNRDDQVASIMFSSGTTGLPKGVMLTHKNIVARFSIAKDPTFGNAINPTSA
ILTVLPFHHGFGMMTTLGSFTCGFRVVLMHTFEEKLFLQSLQDYKVESTLLVPTLMA

FLAKSALVEKYDLSHLKEIASGGAPLSKEIGEMVKKRFKLNFVRQGYGLTEATSAVLI
TPKGDAKPGSTGKIVPLHAVKVVDPTTGKILGPNEPGELYFKGPMKMKGYYNNEEA
TKAIIDNDGWLRSGDIAYYDNDGHFYIVDRLKSLIKYKGYQVAPAEIEGILLQHPYIV
DAGVTGIPDEAAGELPAAGVVVQTGKYLNEQIVQDYVASQVSTAKWLRGGVKFLD
EIPKGSTGKIDRKVLRQMLEKHTNGHHHHHHHH*

SEQ ID NO: 8 – B7 luciferase mutant (nucleic acid)

ATGGCTGACAAAAACATCCTGTATGGTCCGGAACCGTTCTACCCACTGGAAGATG
GTACCGCTGGTGAACAGATGTTTGACGCATTATCTCGTTATGCAGCTATTCCGGG
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CTGAAACTGTCGTGTCGTTTAGCGGAAAGTTTTAAAAAGTATGGATTAAAACAAA
ACGACACAATAGCGGTGTGTAGCGAAAATAGTCTGCAATTTTTCCTTCCTGTAAT
TGCATCATTGTATCTTGGAATAATTGTGGCACCTGTTAACGATAAATACATTGAA
CGTGAATTAATACACAGTCTTGGTATTGTAAAACCACGCATAGTTTTTTGCTCCA
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CAAAAGATCCTACTTTTGGTAACGCAATTAATCCCACGTCAGCAATTTTAACGGT
ATTACCTTTCCACCATGGTTTTGGTATGATGACCACATTAGGATCCTTTACTTGTG
GATTCCGAGTTGTTCTAATGCACACGTTTGAAGAAAAACTATTTCTACAATCATT
ACAAGATTATAAAGTGGAAAGTACTTTACTTGTACCAACATTAATGGCATTTCTT
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ACAACATCCGTATATTGTTGATGCCGGCGTTACTGGTATACCGGATGAAGCCGCG
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GTGGGGTGAAATTTTTGGATGAAATTCCCAAAGGATCAACTGGAAAAATTGACA
GAAAAGTGTTAAGACAAATGTTAGAAAAACACACCAATGGGCATCACCATCACC
ACCATCATCACTAA

SEQ ID NO: 9 –UltraGlo Triple mutant (H244W+T344A+I396K) (amino acid)
MADKNILYGPEPFYPLEDGTAGEQMFDALSRYAAIPGCIALTNAHTKENVLYEEFLK
LSCRLAESFKKYGLKQNDTIAVCSENSLQFFLPVIASLYLGIIVAPVNDKYIERELIHSL
GIVKPRIVFCSKNTFQKVLNVKSKLKSIETIIILDLNEDLGGYQCLNNFISQNSDSNLDV

KKFKPYSFNRDDQVASIMFSSGTTGLPKGVMLTHKNIVARFSIAKDPTFGNAINPTSA
ILTVIPFHWGFGMMTTLGYFTCGFRVVLMHTFEEKLFLQSLQDYKVESTLLVPTLMA
FLAKSALVEKYDLSHLKEIASGGAPLSKEIGEMVKKRFKLNFVRQGYGLTEATSAVLI
TPKGDAKPGSTGKIVPLHAVKVVDPTTGKILGPNEPGELYFKGPMKMKGYYNNEEA
TKAIIDNDGWLRSGDIAYYDNDGHFYIVDRLKSLIKYKGYQVAPAEIEGILLQHPYIV
DAGVTGIPDEAAGELPAAGVVVQTGKYLNEQIVQDYVASQVSTAKWLRGGVKFLD
EIPKGSTGKIDRKVLRQMLEKHTNGHHHHHHHH

SEQ ID NO: 10 –UltraGlo Triple mutant (H244W+T344A+I396K) (nucleic acid)

ATGGCTGACAAAAACATCCTGTATGGTCCGGAACCGTTCTACCCACTGGAAGATG
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ACCTGGAGAATTGTATTTTAAAGGCCCGATGAAAATGAAGGGTTATTATAATAAT
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GGCGAGCTTCCAGCTGCAGGTGTTGTAGTACAGACTGGAAAATATCTAAACGAA
CAAATCGTACAAGATTATGTTGCCAGTCAAGTTTCAACAGCCAAATGGCTACGTG
GTGGGGTGAAATTTTTGGATGAAATTCCCAAAGGATCAACTGGAAAAATTGACA
GAAAAGTGTTAAGACAAATGTTAGAAAAACACACCAATGGGCATCACCATCACC
ACCATCATCACTAA

SEQ ID NO: 11 – UltraGlo Triple + 300 (H244W+T344A+I396K+C300G) (amino acid)

MADKNILYGPEPFYPLEDGTAGEQMFDALSRYAAIPGCIALTNAHTKENVLYEEFLK
LSCRLAESFKKYGLKQNDTIAVCSENSLQFFLPVIASLYLGIIVAPVNDKYIERELIHSL

GIVKPRIVFCSKNTFQKVLNVKSKLKSIETIIILDLNEDLGGYQCLNNFISQNSDSNLDV
KKFKPYSFNRDDQVASIMFSSGTTGLPKGVMLTHKNIVARFSIAKDPTFGNAINPTSA
ILTVIPFHWGFGMMTTLGYFTCGFRVVLMHTFEEKLFLQSLQDYKVESTLLVPTLMA
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TPKGDAKPGSTGKIVPLHAVKVVDPTTGKILGPNEPGELYFKGPMKMKGYYNNEEA
TKAIIDNDGWLRSGDIAYYDNDGHFYIVDRLKSLIKYKGYQVAPAEIEGILLQHPYIV
DAGVTGIPDEAAGELPAAGVVVQTGKYLNEQIVQDYVASQVSTAKWLRGGVKFLD
EIPKGSTGKIDRKVLRQMLEKHTNGHHHHHHHH

SEQ ID NO: 12 – UltraGlo Triple + 300 (H244W+T344A+I396K+C300G) (nucleic acid)

ATGGCTGACAAAAACATCCTGTATGGTCCGGAACCGTTCTACCCACTGGAAGATG
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ACCATCATCACTAA

CLAIMS

1. An inhibitor-resistant luciferase comprising enhanced resistance to inhibition by dehydroluciferin and derivatives thereof compared to a luciferase of SEQ ID NO: 3.

2. The inhibitor-resistant luciferase of claim 1, wherein the inhibitor-resistant luciferase exhibits a smaller relative reduction in activity when exposed to dehydroluciferin than a luciferase of SEQ ID NO: 3.

3. The inhibitor-resistant luciferase of claim 1, wherein the inhibitor-resistant luciferase comprises at least 70% sequence identity with SEQ ID NO: 3, and comprises at least one substitution relative to SEQ ID NO: 3 at a position selected from 240, 244, 254, 300, 344, and/or 396 of SEQ ID NO: 3.

3. The inhibitor-resistant luciferase of claim 3, wherein the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from I240L, H244R, Y254S, C300G, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions.

4. The inhibitor-resistant luciferase of claim 3, wherein the inhibitor-resistant luciferase comprises a substitution relative to SEQ ID NO: 3 at position 396 of SEQ ID NO: 3.

5. The inhibitor-resistant luciferase of claim 4, wherein the inhibitor-resistant luciferase comprises an I396K substitution, or a conservative or semi-conservative variation thereof, relative to SEQ ID NO: 3.

6. The inhibitor-resistant luciferase of claim 3, wherein the inhibitor-resistant luciferase comprises at least 70% sequence identity with SEQ ID NO: 5, and comprises at least one substitution relative to SEQ ID NO: 3, said substitution at a position selected from 244, 300, and/or 396 of SEQ ID NO: 3.

7. The inhibitor-resistant luciferase of claim 6, wherein the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one

substitution selected from H244R, C300G, I396K, and/or conservative or semi-conservative variations of such substitutions.

8. The inhibitor-resistant luciferase of claim 3, wherein the inhibitor-resistant luciferase comprises at least 70% sequence identity with SEQ ID NO: 7, and comprises at least one substitution relative to SEQ ID NO: 3, said substitution at a position selected from 240, 254, 344, and/or 396 of SEQ ID NO: 3.

9. The inhibitor-resistant luciferase of claim 8, wherein the inhibitor-resistant luciferase comprises at least one substitution relative to SEQ ID NO: 3, the at least one substitution selected from I240L, Y254S, T344A, I396K, and/or conservative or semi-conservative variations of such substitutions.

10. The inhibitor-resistant luciferase of claim 1, wherein the inhibitor-resistant luciferase comprises at least 10% of the bioluminescent activity of a luciferase of SEQ ID NO: 3, when neither inhibitor-resistant luciferase nor the luciferase of SEQ ID NO: 3 have been exposed to dehydroluciferin.

11. A reagent composition comprising the inhibitor-resistant luciferase of one of claims 1-10 and a luciferin substrate.

12. The reagent composition of claim 11, further comprising a contaminant comprising a degradation product of luciferin.

13. The reagent composition of claim 12, wherein the contaminant is dehydroluciferin.

14. The reagent composition of one of claims 11-13, further comprising magnesium.

15. The reagent composition of one of claims 11-14, further comprising one or more additional components selected from the group consisting of: a buffer, a defoamer, an ATPase inhibitor, L-luciferin, aminothiothymidine, an enzyme stabilizer, a detergent, an

inhibitor of ATP-generating enzymes, a cell lysing agent, an ATP-extraction agent, co-enzyme A, a thiol reagent, a metal ion chelator, a protease inhibitor, and a salt.

16. The reagent composition of one of claims 11-15, wherein the reagent composition is a single liquid reagent.

17. A reagent composition comprising the inhibitor-resistant luciferase of one of claims 1-10 and a cationic detergent.

18. The reagent composition wherein the cationic detergent is DTAB (dodecyltrimethylammonium bromide), CTAB (cetyltmethylammonium), Benzalkonium Chloride, or BDDABr (benzyldimethyldodecylammonium bromide).

19. A kit comprising a reagent composition of one of claims 11-16 or 17-18.

20. The kit of claim 19, further comprising one or more additional components selected from the group consisting of: a buffer, a defoamer, an ATPase inhibitor, an enzyme stabilizer, a detergent, an inhibitor of ATP-generating enzymes, a cell lysing agent, an ATP-extraction agent, co-enzyme A, a thiol reagent, a metal ion chelator, a protease inhibitor, and a salt.

21. The kit of claim 19 or 20, further comprising instructions for performing an ATP detection or quantification assay.

22. An assay system for detecting or quantifying ATP in a sample, comprising:
(a) a reagent composition of one of claims 11-16 or 17-18; and
(b) a sample comprising or suspected of comprising ATP.

23. The assay system of claim 22, further comprising a device for the detection and or measurement of luminescence.

24. The assay system of claim 22, wherein the sample is a cell lysate.

25. A method of detecting ATP in a sample comprising: (a) adding to the sample a reagent composition reagent composition of one of claims 11-16 or 17-18; and (b) detecting luminescence.

26. The method of claim 25, wherein the sample comprises cells and the method further comprises lysing the cells to generate a cell lysate.

27. A method of quantifying the amount or concentration of ATP in a sample comprising: (a) adding to the sample a reagent composition reagent composition of one of claims 11-16 or 17-18; (b) quantifying luminescence from the sample; and (c) comparing the luminescence to a control value to determine the amount or concentration of ATP in the sample.

28. The method of claim 27, wherein the control value is determined from a separate quantification of luminescence produced by a control sample comprising a known concentration of ATP.

29. The method of claim 27, further comprising the step of adding a known concentration of ATP to the sample.

30. The method of claim 27, wherein luminescence is quantified at multiple time-points.

31. The method of claim 27, wherein luminescence is quantified in real time.

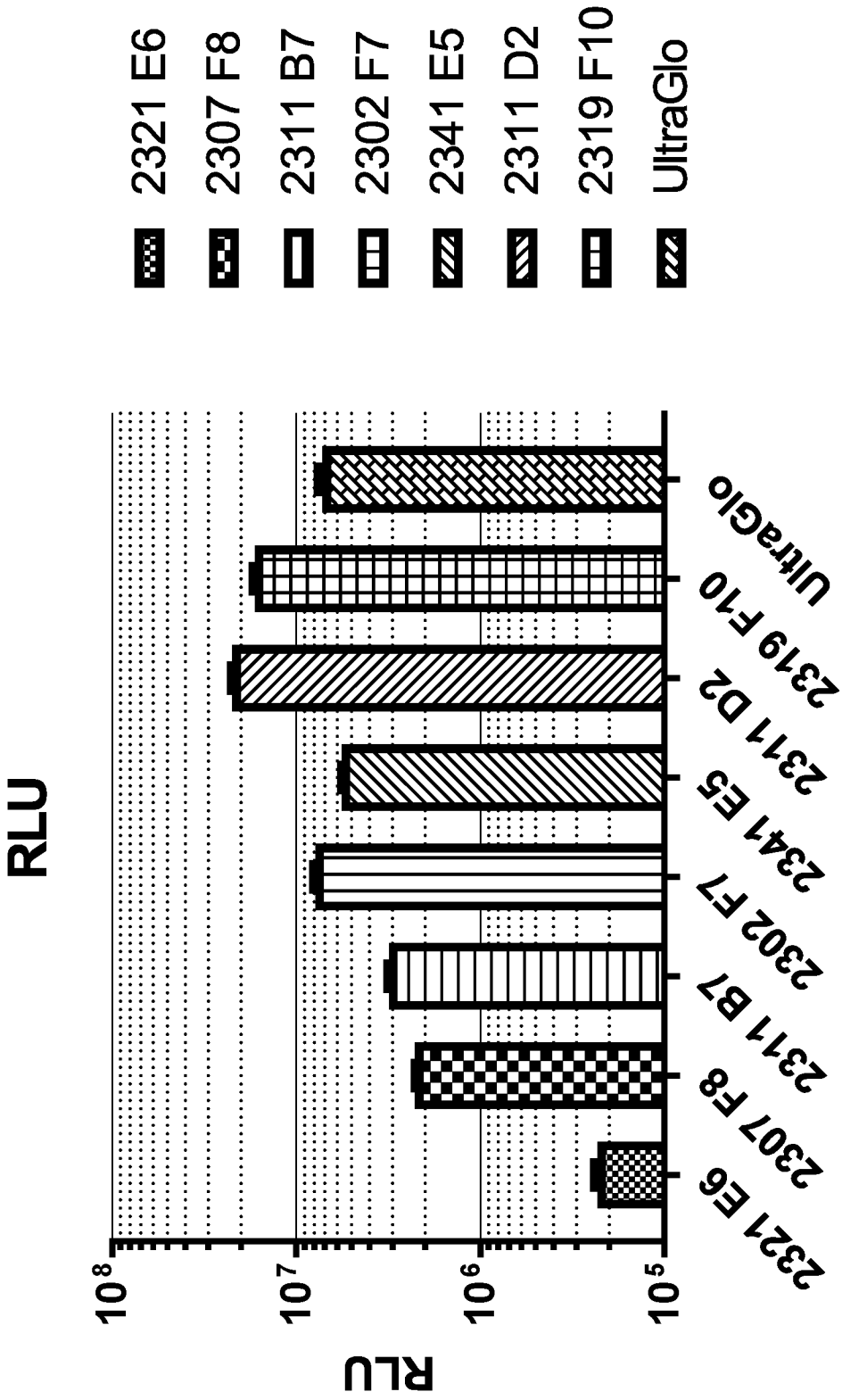
32. Use of a reagent composition of one of claims 11-16 or 17-18 for the detection and/or quantification of ATP in a sample.

33. A method of enhancing the apparent signal stability of an inhibitor-resistant luciferase of one of claims 1-10 in an assay, comprising performing the assay with one or more inhibitors of the luciferase present.

34. The method of claim 33, wherein the inhibitor is selected from one or more of a dehydroluciferin, an oxyluciferin, and L-luciferin.

35. The method of claim 33, wherein the inhibitor-resistant luciferase exhibits an increase in enhancement of apparent signal stability compared to a native, wild-type version the luciferase.

FIG. 1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10^7
10^6
10^5
RLU
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2307 F8
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2341 E5
2311 D2
2319 F10
UltraGlo
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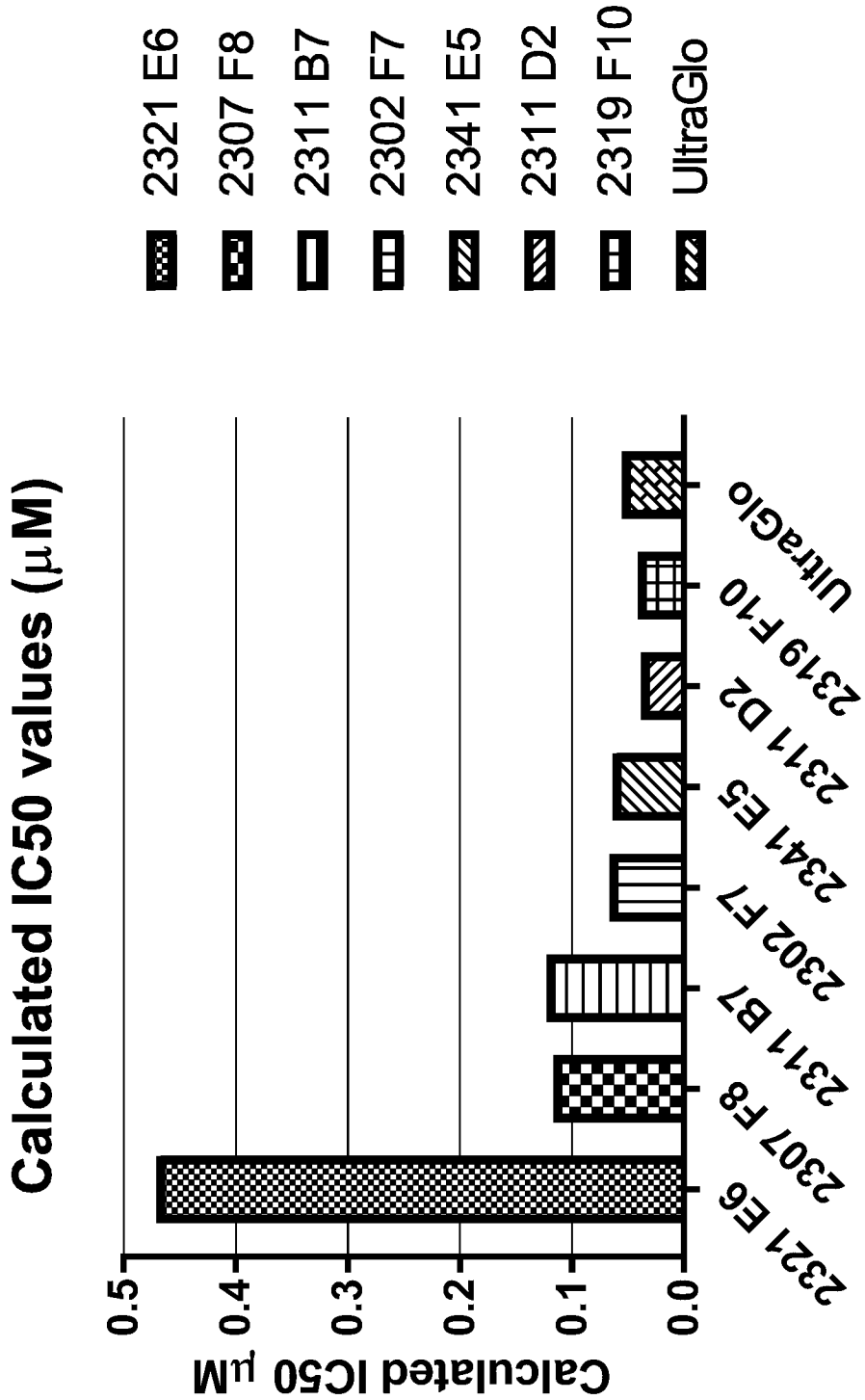
2302 F7
2341 E5
2311 D2
2319 F10
UltraGlo

FIG. 2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Calculated IC50 μM
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0.2
0.3
0.4
0.5
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2311 D2
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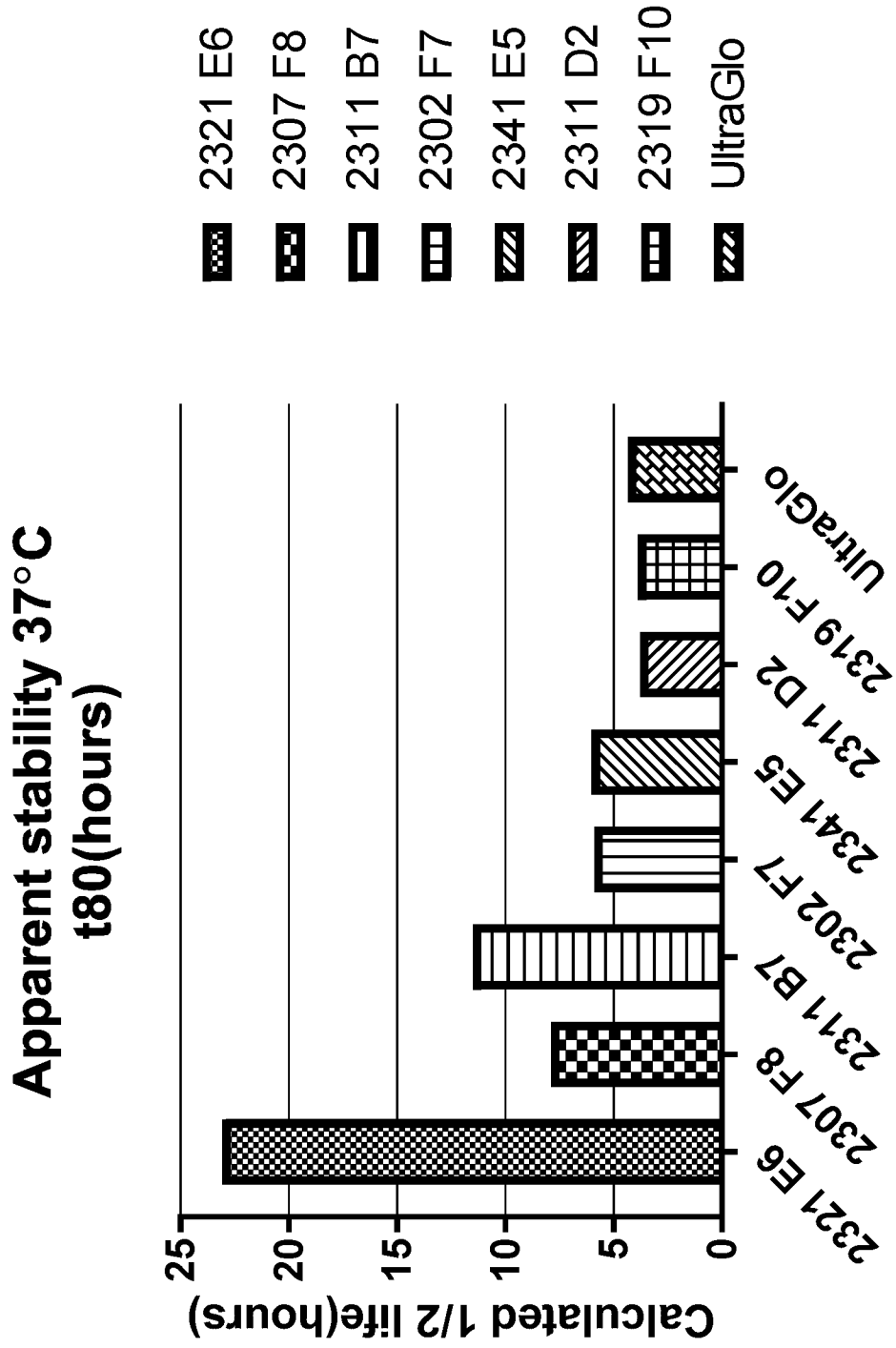
UltraGlo

FIG. 3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t80(hours)
Calculated 1/2 life(hours)
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10
15
20
25
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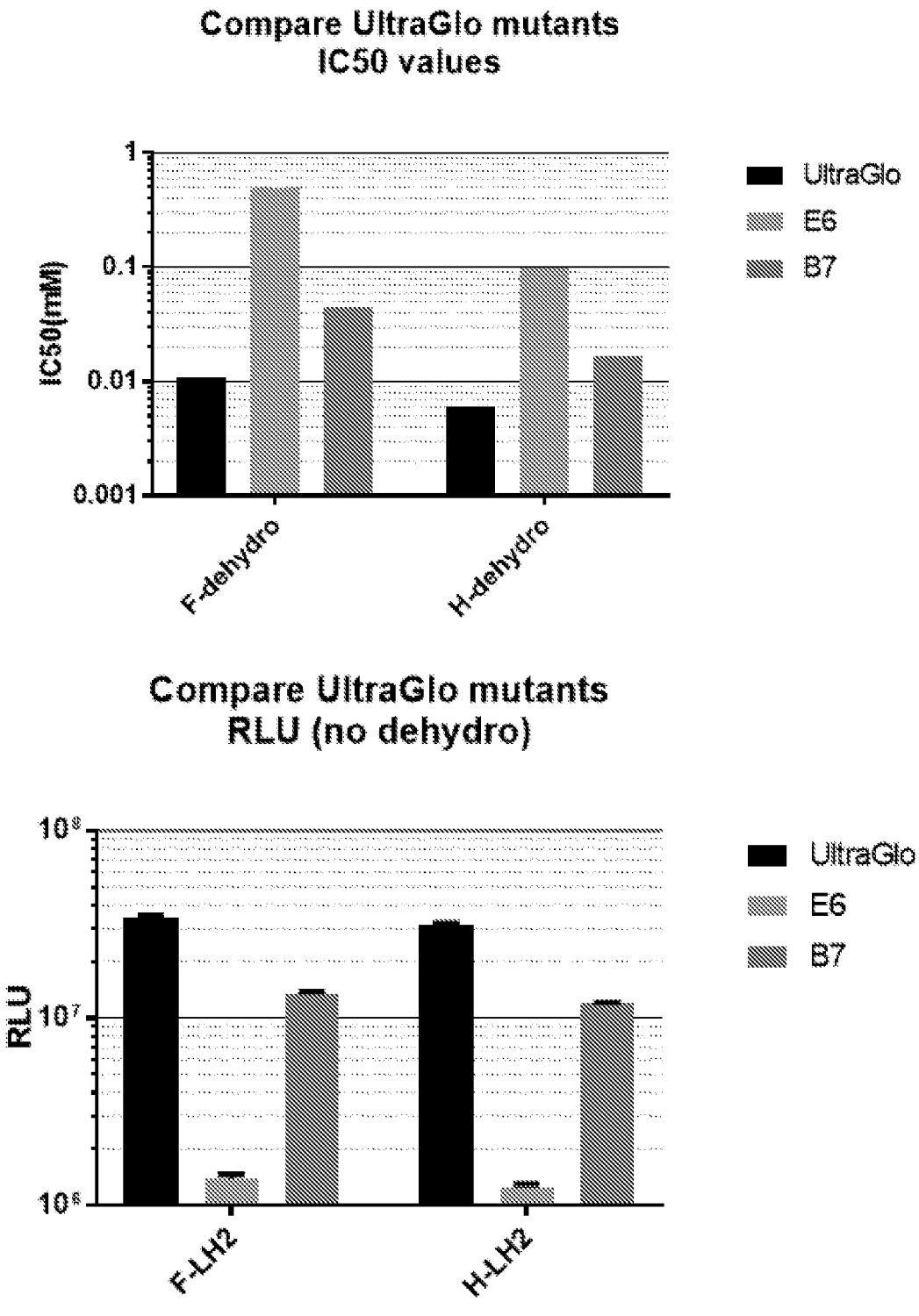
2319 F10
UltraGlo

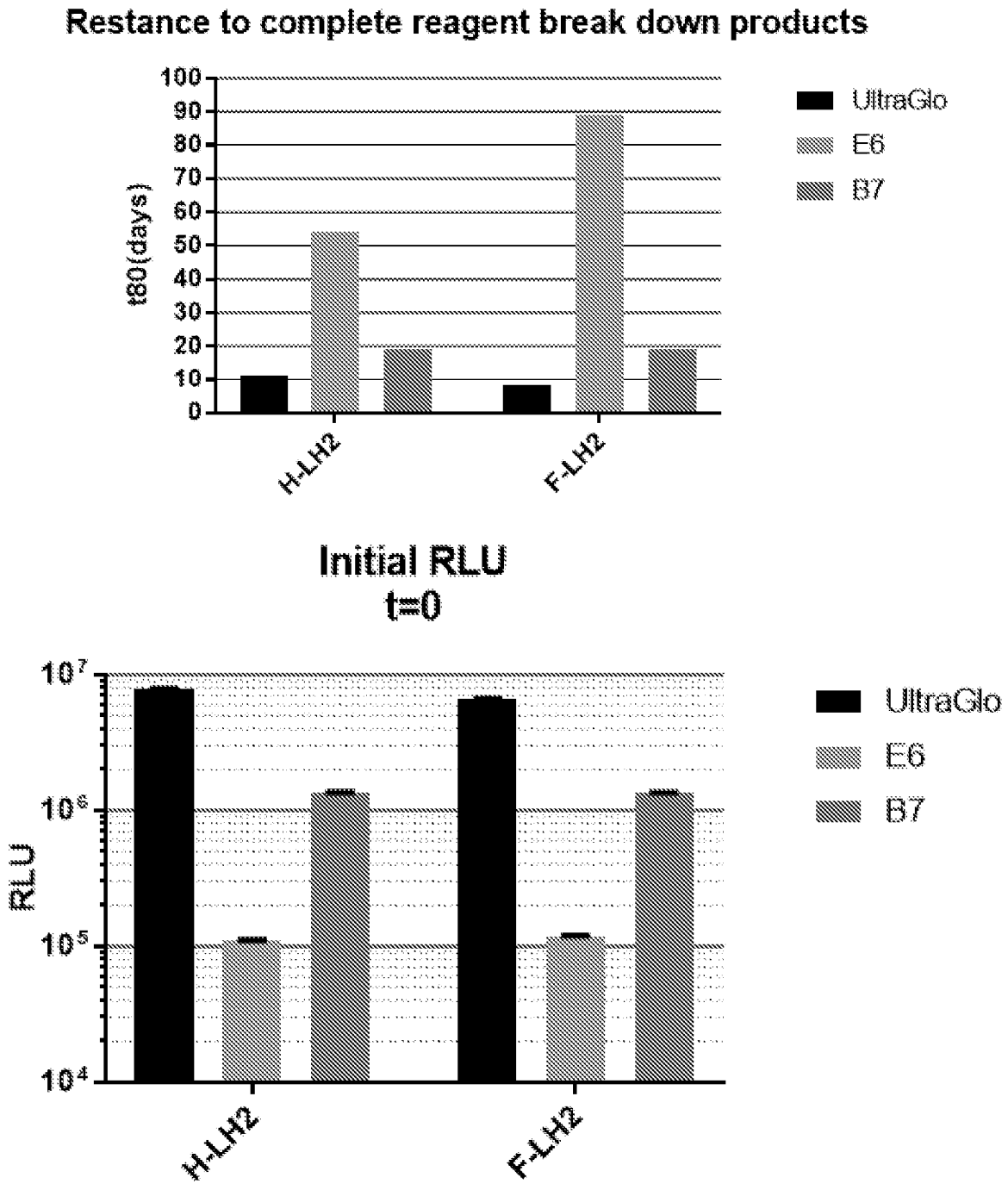
FIG. 4



E6=H244R+C300G+I396K

B7=I240L+YY254S+C300G+T344A+I396K

FIG. 5



E6=H244R+C300G+I396K

B7=I240L+YY254S+C300G+T344A+I396K