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(54) **Title:** ASSAY METHOD

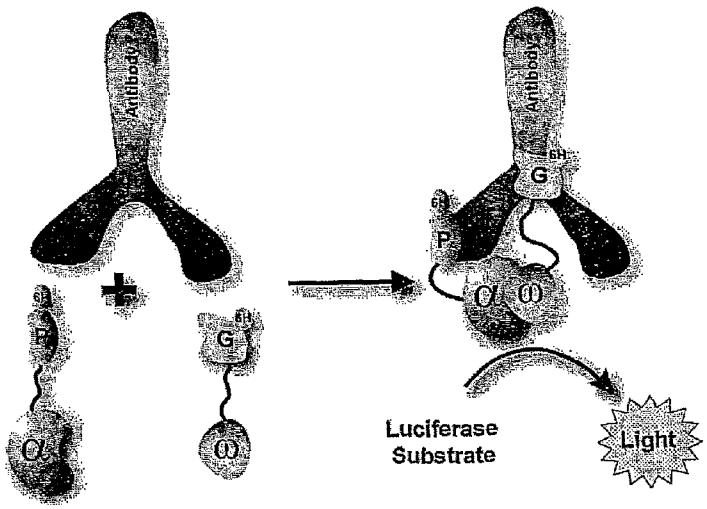

FIG. 5

(57) **Abstract:** A method is described for determining the presence of an analyte of interest in a sample which, method comprises contacting the sample with: (i) a purified first reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest; and (ii) a purified second reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest and operable in reconstituting an active enzyme upon association with the first reporter fragment pair member through the affinities of the interactor domains of the first and second reporter fragment pair members with the analyte of interest; under in vitro assay conditions which allow the first and second reporter fragment pair members to associate in the presence of analyte of interest in the sample through the affinity of the interactor domains with the analyte to produce a glow luminescent signal through the action of the reconstituted active enzyme on a substrate; and detecting the presence or absence, or extent, of a glow luminescent signal.

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ASSAY METHOD

FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology and diagnostics. More specifically, the present invention relates to in vitro forced enzyme complementation assays based on glow luminescence.

BACKGROUND OF THE INVENTION

Heterogeneous enzyme immunoassays (EIAs) such as the enzyme-linked immunosorbent assay (ELISA) for the clinical diagnosis of disease have dominated the diagnostic industry for several decades due to their high sensitivity and specificity. The increasing use of luminescent substrates; whose sensitivity is several orders of magnitude greater than conventional colorimetric and fluorometric substrates; in combination with heterogeneous EIAs; has further improved limits of detection. However, the principle drawback associated with heterogeneous EIAs is their lengthy processing time and requirement of multiple wash and incubation steps, necessitating specialized instrumentation for the purpose of automation.

Increasingly, the requirement for greater sensitivity and ease of operability has generated substantial activity in the generation of alternative luminescent platform technologies. Luminescence is broadly used herein to describe chemiluminescence and/or bioluminescence. Luminescent detection methodologies typically provide sensitivities that are up to 100-1000 fold higher than may be possible with fluorescent or colorimetric enzyme substrates, respectively (Olesen et al., 2000, Methods Enzymol. 326: 175-202). As a result, luminescence provides a greater dynamic range (as illustrated in FIG. 1) with broad linearity and low inherent background (Fan and Wood, 2007, Assay Drug Dev. Technol. 5(1): 127-136). In addition, homogeneous assays permit the formation and detection of an enzyme-labelled analyte complex (e.g. antibody-antigen complex) within a single solution, obviating the need for a solid support, allowing for more simple and rapid assays. Therefore, luminescent homogeneous EIAs present an alternative platform for diagnosis of disease in a single reaction vessel with limited processing requirements and exquisite sensitivities. The

elimination of solid supports and washing to remove unbound label provides significant advantages to the application of homogeneous EIAs to chemistry analysers for high throughput, automated immunoassays.

Assays utilizing split reporter proteins for the detection of protein-protein interactions *in vivo* are known as forced enzyme complementation (FEC) assays or protein fragment complementation assays (PCA). These assays are described generally in, for example: WO 01/71702; US Patents 6,270,964; 6,294,330; 6,428,951; 6,342,345, 6,828,099 and published US Patent Application 20030175836. The fundamental principle that underlies FEC is the use of a complementing protein fragment pair that may functionally reassemble into an active reporter protein producing a detectable signal. Central to the assay's functionality is the reassembly of the reporter protein fragments via binding of an independent target (analyte), to recognition moieties present within each member of the fragment pair that have been attached to the reporter protein fragments. As used herein, these interaction moieties are termed "interactors" or "interactor domains". Interactor domains are separate moieties or domains contained within each member of the fragment pair constructs, and include, but are not limited to, disease-specific antigen, antibodies or protein binding partners. Interactor domains may be joined directly to the reporter fragment domains or may be joined by a linker domain. The protein fragment pair members are typically constructed so that, in the absence of the interactor domain target, the reporter fragments do not spontaneously functionally reassemble. Thus, the interactor domains of each member of the pair assist the reconstitution of a functional reporter protein from its fragments through binding of target to the interactor domains.

Functional *in vivo* FEC assays have been constructed using several different reporter proteins. See, for example, those disclosed in WO 01/71702; US Patents 6,270,964; 6,294,330; 6,428,951; 6,342,345, 6,828,099 and published US Patent Application 20030175836. However, *in vitro* application of FEC assays present specific challenges, including the preparation of pure, soluble, stable and active enzyme fragments with highly characterized properties for manufacturing and quality assurance purposes. The *in vitro* use of FEC assays producing a luminescent signal not only needs to overcome these specific challenges but also needs to produce an output signal suitable for detection in the clinical laboratory. To provide the greatest level of

sensitivity and dynamic range possible luminescence detection can be determined in either of two modes - flash or glow. Both detection methods have benefits and limitations and the use of either is generally dominated by particular assay requirements.

In the field of *in vitro* protein-protein interaction determination, glow luminescence allows for kinetic determination of reaction rates and is intrinsically more sensitive than endpoint readings provided with flash luminescence. This preference for use of glow luminescence is evidenced by the current multi-mode microplate reader trends, which indicate that glow luminescence detection accounts for 13% of total detection modalities while flash luminescence is still in its infancy (HTStec 2007 Market Report). Flash luminescence is transient in nature and reaches maximal light intensity within seconds or milliseconds compared with glow luminescence, which is a steady-state kinetic approach to signal generation and is long lasting (minutes to hours). Other benefits of glow luminescence mostly relate to instrumentation, as flash luminescence requires reagent dispensing, mixing and measurement onboard the instrument. Flash luminescence is performed on a well-by-well (well mode) basis due to the rapidity of the light emission; therefore limiting its utility for diagnostic purposes, as fluid injectors and detection throughput compromise assay flexibility. In contrast, glow luminescence reagents can be added outside of the reader and luminescent determinations can be made on the entire plate (plate mode). Injectors are not required, there is greater flexibility in plate processing and a single reagent addition and incubation can be performed. For this reason glow luminescence has been the detection method of choice for the ELISA market as glow luminescent detection is compatible with current EIA liquid handling instruments. Glow luminescence also provides the added advantage of dual analyte detection by multiplexing response signals using multimode detection; by combining color and luminescence; fluorescence and luminescence; flash and glow luminescence into a single reaction for *in vitro* purposes.

Luminescent reporter enzymes known as luciferases produce detectable photon emission as a result of the oxidation of luciferin to oxyluciferin in a process known generally as bioluminescence (Wilson et al., 1998, Annu. Rev. Cell Dev. Biol. 14:197-230). Several luciferases have been split and successfully used to determine protein-protein interactions via PCA; including, firefly *Photinus pyralis* luciferase in

Paulmurugan et al., 2002, Proc. Natl. Acad. Sci. USA 99(24): 15608-15613 and Paulmurugan et al., 2007, Anal. Chem. 79(6): 2346-2353, sea pansy *Renilla remiformis* luciferase in Paulmurugan et al., 2003, Anal. Chem. 75(7): 1584-1589 and Kaihara et al., 2003, Anal. Chem. 75(16): 4176-4181 and marine copepod *Gaussia princeps* luciferase in Remy et al., 2006, Nat. Methods 3(12): 977-999.

The creation of broadly applicable *in vitro* homogeneous assays based upon the principle of bioluminescent FEC presents novel challenges to the design and implementation of FEC within a homogeneous assay format. These include, but are not limited to, the type of luminescent output signal, the particular assay environment created within the homogeneous assay format, the nature and source of analytes intended for detection and the manufacturing challenges associated with the production of such homogeneous assays.

Homogeneous assays are typically constituted of isolated, purified components in order to ensure specificity, reliability, manufacturing ease and robust characteristics in use. The design of appropriate protein fragment pairs (including appropriate reporter fragments, interactor domains, linking domains, and whole fusion constructs or reporter fragment pairs incorporating each) as well as robust assay conditions that also produce the necessary solubility, stability, and amenability to manufacture for *in vitro* diagnostic use, is crucial to creating a broadly applicable homogeneous assay platform. In contrast to *in vivo* PCA assay conditions, *in vitro* diagnostic assay conditions are extra-cellular and relatively harsh and may not be conducive to appropriate protein folding and protein-protein interactions that may readily occur *in vivo*.

The application and use of the bioluminescent *Renilla* luciferase reporter protein utilizing a luminescent substrate presents additional challenges, including issues associated with fragment stability, substrate autoluminescence and in particular the generation of extended luminescence signal rather than flash emission. Therefore, in addition to the challenges of implementing FEC in an *in vitro* assay generally, there are additional challenges presented by implementation of luminescent FEC in an *in vitro* homogeneous assay platform.

Further, central to the development of a luminescent-based homogeneous assay platform is the elimination of wash steps, therefore diagnostic tests may be carried out in the presence of serum, or at least some serum components. Thus, sample fractions containing analytes may be processed without washing, likely leaving homogeneous

platforms, especially those based upon luminescent FEC, susceptible to cross-reactivity, interference and inhibitory effects due to serum components or other contaminants.

SUMMARY OF THE INVENTION

The present invention is based on the successful development of a sensitive homogeneous FEC assay using a split enzyme reporter system that produces a glow luminescent signal. We have demonstrated the use of such a system in detecting analytes with high sensitivity (100%) and specificity (100%). Thus, we have demonstrated the successful application of an *in vitro* homogeneous FEC assay based on extended glow luminescence for the detection of a diagnostically relevant target. Further the timeframe required (approximately 1 hour) is suitable for uptake of this methodology into pathology laboratories. The present invention therefore provides significant advantages to the current immunodiagnostic field.

Accordingly, in a first aspect, the present invention provides a method of determining the presence of an analyte of interest in a sample which method comprises contacting the sample with:

(i) a purified first reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest; and

(ii) a purified second reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest and operable in reconstituting an active enzyme upon association with the first reporter fragment pair member through the affinities of the interactor domains of the first and second reporter fragment pair members with the analyte of interest;

under *in vitro* assay conditions which allow the first and second reporter fragment pair members to associate in the presence of analyte of interest in the sample through the affinity of the interactor domains with the analyte to produce a glow luminescent signal through the action of the reconstituted active enzyme on a substrate;

and detecting the presence or absence, or extent, of a glow luminescent signal.

In a related aspect, the present invention provides a method of assaying for the presence of an analyte in a sample, the method comprising the steps of:

(a) providing a sample to be tested for the presence of an analyte of interest;

(b) providing a purified first reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest;

(c) providing a purified second reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest and operable in reconstituting an active enzyme upon association with the first reporter fragment pair member through the affinities of the interactor domains of the first and second reporter fragment pair members with the analyte of interest; and

(d) contacting the sample with the first and second reporter fragments under *in vitro* assay conditions which allow the first and second reporter fragment pair members to associate in the presence of analyte of interest in the sample through the affinity of the interactor domains with the analyte, to produce a glow luminescent signal through the action of the reconstituted active enzyme on a substrate, wherein a glow luminescent signal indicates the presence of the analyte of interest in the sample.

In a preferred embodiment, the active enzyme is a luciferase, preferably a *Renilla* luciferase or a derivative thereof, such as a luciferase comprising a C124A mutation.

In one embodiment, the first and second reporter fragment pair members are alpha and omega fragments of a luciferase polypeptide formed by a breakpoint between amino acid residues serine 91 and tyrosine 92 or glycine 229 and lysine 230 of the *Renilla* luciferase amino acid sequence shown in SEQ ID NO:10, or the equivalent position in another luciferase sequence.

In developing a glow luminescence-based FEC assay, we have also determined a range of assay conditions that provide an improved glow luminescence signal, i.e. one or more of the following:

(i) one or more reducing agents, such as DTT, preferably at a total concentration at from about 1 to about 2% w/v;

(ii) one or more denaturants, such as thiourea, preferably at a total concentration of from about 1 to about 2% w/v;

(iii) one or more detergents, such as CHAPS, preferably at a total concentration of from about 0.1 to about 0.2% w/v;

(iv) from about 0.25 to 0.5 M salt; and/or

(v) Tris.HCl as a buffer.

We have also identified preferred luciferase substrates, namely a luciferase substrate selected from the group consisting of coelenterazine analogues f, h and n (see Figure 3).

In a particular embodiment, the sample is a biological sample, such as a blood or serum sample.

In a second aspect, the present invention provides a kit for performing a homogeneous *in vitro* forced enzyme fragment complementation assay to identify the presence of an analyte of interest in a sample, which kit comprises :

(a) a purified first reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest;

(b) a purified second reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest and operable in reconstituting active enzyme upon association with the first reporter fragment pair member through the affinities of the interactor domains of the first and second reporter fragment pair members with the analyte of interest; and

(c) reagents for providing *in vitro* assay conditions which allow the first and second reporter fragment pair members to associate in the presence of analyte of interest in the sample, through the affinity of the interactor domains with the analyte, to produce a glow luminescent signal through the action of the active enzyme on a substrate, wherein a glow luminescent signal indicates the presence of the analyte of interest in the sample.

In a related aspect, the present invention provides the use of a kit for determining the presence of an analyte of interest in a sample, for example an antibody to a pathogen such as Herpes Simplex Virus or a Hepatitis B virus.

We have also developed a storage buffer than improves the stability of the reporter polypeptides and hence the results obtained with the glow luminescence assay format. One of the problems that arises with *in vitro* assays is that the concentration of reporter polypeptides is relatively high. In particular it is known that full length *Renilla*

luciferase is susceptible to loss of activity and prone to aggregation at a concentration of greater than 0.5 mg/ml (approx 10 nM) at 4°C (Matthews et al., 1977, Biochemistry 16(1): 85-91. We have found that the addition of one or sugars, such as sucrose and trehalose, and/or one or more inert proteins, such as bovine serum albumin, reduces significantly the reduction in reporter polypeptide activity following storage.

Accordingly, in a third aspect, the present invention provides an assay reagent storage composition comprising (i) one or more reporter polypeptides at a total concentration of greater than about 10 nM and (ii) one or more sugars and/or one or more inert proteins.

In a preferred embodiment, the one or more reporter polypeptides are luciferase reporter polypeptides for an *in vitro* forced enzyme complementation assay.

Preferably the one or more sugars are selected from the group consisting of sucrose, trehalose and mixtures thereof.

Preferably the one or more inert proteins are selected from the group consisting of serum albumin, gelatine and mixtures thereof.

In a related aspect the present invention provides the use of a composition comprising one or more sugars and/or one or more inert proteins to stabilise one or reporter polypeptides in an assay reagent storage composition comprising the one or more reporter polypeptides at a total concentration of greater than about 10 nM.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1. Comparison between the colorimetri , fluorometric and luminescent analyte signal detection range.

FIG. 2. Bioluminescent reaction catalysed by *Renilla* luciferase.

FIG. 3. Native coelenterazine synthetic derivatives (**A-H**) that differ from each other in terms of luminescence intensity and response time.

FIG. 4. *Renilla* luciferase model structure illustrating the position of two functional split points, S91/Y92 (**A**) and G229/K230 (**B**).

FIG. 5. Schematic illustration of *Renilla* luciferase FEC assay and its basic components. Enzyme fragments, α and ω, are linked to analyte binding moieties such as a disease-specific antigenic peptide (P) and one domain of protein G. In the presence of analyte (disease-specific antibody), the

fragments are forced into close proximity (right), thereby initiating catalysis of a bioluminescent reaction.

FIG. 6 Schematic representation of FEC fragments (**A** and **B**) used in this study. RLuc refers to *Renilla* Luciferase alpha (α blue) and omega (ω green) fragments. Analyte binding moieties (red), ProG, refers to the protein G domain and P (P1 and P2) refers to the HSV type-specific antigenic peptides for HSV-1 and HSV-2 respectively. Histidine tags are shown in grey. (Gly_4Ser) linkers were used to join the enzyme fragments to the analyte binding moieties.

FIG. 7. Purification of α protein fragments (RLuc_α_{229}P1 and RLuc_α_{229}P2) of the invention. A) RLuc_α_{229}P1 affinity purification profile and gel electrophoresis of pure protein, MW 35,214.22 Da. B) Affinity purification profile and gel electrophoresis of pure RLuc_α_{229}P2, MW 38,603.98 Da. Chromatograms labelled "Affinity purified" illustrate embodiments of purified enzyme fragments. Similar results are obtained for other fragments of the invention.

FIG. 8. Purification of ω protein fragment (RLuc_$_{230}$ωProG) of the invention. A) RLuc_$_{230}$ωProG affinity purification profile. B) Gel filtration profile and gel electrophoresis of affinity purified RLuc_$_{230}$ωProG, MW 18,149.57 Da. Chromatograms labelled "Affinity purified" and "Gel filtration profile" both illustrate embodiments of purified enzyme fragments. Similar results are obtained for other fragments of the invention.

FIG. 9. Performance of RLuc fragments split at S91/Y92 (**A**) compared to G229/K230 (**B**).

FIG. 10. Comparison of the use of (**A**) a commercial Renilla Luciferase 5 x Promega lysis buffer (proprietary composition) with (**B**) optimised 5 x PB enhancer buffer C (150 mM HEPES pH 8, 1% CHAPS, 5% thiourea and 25% glycerol) of the invention.

FIG. 11. Luciferase FEC assay for HSV-1 specific IgG. Five sera samples from HSV negative, HSV-1 or HSV-2 positive individuals were assayed using luciferase fragments RLuc_α_{229}P1/ RLuc_$_{230}$ωProG. Cut off = 1.16.

Sensitivity = 100 %. Specificity = 100 %. The normalized enzyme activity is represented as a box and whiskers plot for each set of 5 patient samples.

BRIEF DESCRIPTION OF SEQUENCE LISTINGS

SEQ ID NO: 1. Amino acids 92 to 148 of gG1 of HSV1.

SEQ ID NO: 2. P1 trunc, HSV-1 truncated antigen nucleotide sequence.

SEQ ID NO: 3. P1 trunc, HSV-1 truncated antigen amino acid sequence.

SEQ ID NO: 4. Amino acids 551 to 641 of gG2 of HVS2.

SEQ ID NO: 5. P2 trunc, HSV-2 truncated antigen nucleotide sequence.

SEQ ID NO: 6. P2 trunc, HSV-2 truncated antigen amino acid sequence.

SEQ ID NO: 7. Nucleotide sequence of Protein G subunit.

SEQ ID NO: 8. Amino acid sequence of Protein G subunit.

SEQ ID NO: 9. Nucleotide sequence of synthetic/codon optimised *Renilla* luciferase (RLuc) reporter enzyme incorporating a C124A mutation.

SEQ ID NO: 10. Amino acid sequence of synthetic/codon optimised *Renilla* luciferase (RLuc) reporter enzyme incorporating a C124A mutation.

SEQ ID NOS: 11 to 18 – primers shown in Table 2

SEQ ID NO: 19. Rluc_α_{91}P1, *Renilla* luciferase alpha fragment (split at S91/Y92) P1 trunc construct nucleotide sequence.

SEQ ID NO: 20. Rluc_α_{91}P1, *Renilla* luciferase alpha fragment (split at S91/Y92) P1 trunc construct amino acid sequence.

SEQ ID NO: 21. RLuc_α_{229}P1, *Renilla* luciferase alpha fragment (split at G229/K230) P1 trunc construct nucleotide sequence.

SEQ ID NO: 22. RLuc_α_{229}P1, *Renilla* luciferase alpha fragment (split at G229/K230) P1 trunc construct amino acid sequence.

SEQ ID NO: 23. Rluc_α_{91}P2, *Renilla* luciferase alpha fragment (split at S91/Y92) P2 trunc construct nucleotide sequence.

SEQ ID NO: 24. Rluc_α_{91}P2, *Renilla* luciferase alpha fragment (split at S91/Y92) P2 trunc construct amino acid sequence.

SEQ ID NO: 25. RLuc_α_{229}P2, *Renilla* luciferase alpha fragment (split at G229/K230) P2 trunc construct nucleotide sequence.

SEQ ID NO: 26. RLuc_α_{229}P2, *Renilla* luciferase alpha fragment (split at G229/K230) P2 trunc construct amino acid sequence.

SEQ ID NO: 27. RLuc_$_{92}$ωProG, *Renilla* luciferase omega fragment (split at S91/Y92) Protein G subunit construct nucleotide sequence.

SEQ ID NO: 28. RLuc_$_{92}$ωProG, *Renilla* luciferase omega fragment (split at S91/Y92) Protein G subunit construct amino acid sequence.

SEQ ID NO: 29. RLuc_$_{230}$ωProG, *Renilla* luciferase omega fragment (split at G229/K230) Protein G subunit construct nucleotide sequence.

SEQ ID NO: 30. RLuc_$_{230}$ωProG, *Renilla* luciferase omega fragment (split at G229/K230) Protein G subunit construct amino acid sequence.

SEQ ID NO: 31. Hepatitis B surface antigen (HBsAg) A5 single chain (scFv) construct nucleotide sequence.

SEQ ID NO: 32. Hepatitis B surface antigen (HBsAg) A5 single chain (scFv) construct.amino acid sequence.

SEQ ID NO: 33. Hepatitis B surface antigen (HBsAg) E11 single chain (scFv) construct nucleotide sequence.

SEQ ID NO: 34. Hepatitis B surface antigen (HBsAg) E11 single chain (scFv) construct amino acid sequence.

SEQ ID NO: 35. RLuc_α_{91}A5, *Renilla* luciferase omega fragment (split at S91/Y92) Hepatitis B surface antigen (HBsAg) A5 single chain (scFv) construct nucleotide sequence.

SEQ ID NO: 36. RLuc_α_{91}A5, *Renilla* luciferase omega fragment (split at S91/Y92) Hepatitis B surface antigen (HBsAg) A5 single chain (scFv) construct amino acid sequence.

SEQ ID NO: 37. RLuc_92ωE11, *Renilla* luciferase omega fragment (split at S91/Y92) Hepatitis B surface antigen (HBsAg) E11 single chain (scFv) construct nucleotide sequence.

SEQ ID NO: 38. RLuc_92ωE11, *Renilla* luciferase omega fragment (split at S91/Y92) Hepatitis B surface antigen (HBsAg) E11 single chain (scFv) construct amino acid sequence.

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g. in cell biology, chemistry and molecular biology). Standard techniques used for molecular and biochemical methods can be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology).

The following detailed descriptions of particular embodiments and examples are offered by way of illustration and not by way of limitation. Unless contraindicated or noted otherwise, in these descriptions and throughout this specification, the terms "a" and "an" mean one or more. Similarly the term "or" means "and/or".

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range.

Reporter Components and Reporter Polypeptide Fragments

The assay methods of the present invention use reporter components that comprise or consist of polypeptides that are adapted for use as isolated and purified components of homogeneous *in vitro* FEC assays. The reporter components form part of a reporter system. As discussed above, the reporter system in FEC assays includes two or more polypeptide fragments that when they associate, form a reporter protein complex that can give rise to a detectable signal. Accordingly, each reporter component includes at least one such polypeptide fragment or subunit (herein termed a "reporter fragment"). Thus for a system based on luciferase activity, a first reporter component may comprise an alpha fragment of an enzyme, such as a luciferase and a second reporter component may comprise an omega fragment of an enzyme, such as a luciferase such that when the two reporter components associate under assay conditions, the resulting complex has luciferase activity. A combination of a first reporter component and a second reporter component which together can give rise to detectable enzyme activity when their respective reporter polypeptide fragments (also termed "subunits") are brought into association, is termed herein a "reporter component pair", the respective reporter polypeptide fragments being collectively termed a "reporter fragment pair".

Several luciferases have been split and successfully used to determine protein-protein interactions via FEC; including, firefly *Photinus pyralis* luciferase in Paulmurugan et al., 2002, Proc. Natl. Acad. Sci. USA 99(24): 15608-15613 and Paulmurugan et al., 2007, Anal. Chem. 79(6): 2346-2353, sea pansy *Renilla remiformis* luciferase in Paulmurugan et al., 2003, Anal. Chem. 75(7): 1584-1589 and Kaihara et al., 2003, Anal. Chem. 75(16): 4176-4181 and marine copepod *Gaussia princeps* luciferase in Remy et al., 2006, Nat. Methods 3(12): 977-999. In addition to the wild-type proteins, a number of luciferases have been modified to alter the wavelength of

light emitted (see for example US Patent No. 6,495,355 which describes a red-shifted luciferase) and/or to improve in vitro characterstics. The luciferase variants are within the scope of the present invention.

For the purpose of producing an *in vitro* homogeneous assay amenable to commercialisation and manufacture, considerations such as luciferase stability, size and substrate utility (Table 1) are relevant to the selection of the specific luciferase. *Renilla remiformis* luciferase (EC 1.13.12.5) which is responsible for the production of light via oxidation of coelenterazine (FIG. 2) is particularly suitable for application to the FEC format as it is small (36 kDa), monomeric and contains only three cysteines, none of which form disulphide bonds (Matthews et al., 1977, Biochemistry 16(1): 85-91). Furthermore, improved stability can be achieved by mutation of cysteine at position 124 to alanine (Liu et al., 1999, Gene 237: 153-159). Additional improvements to *Renilla* luciferase stability and light output can be achieved with the use of several supplementary mutations (Loening et al., 2006, Protein Eng. Des. Sel. 19(9): 391-400 and Loening et al., 2007, Nat. Methods 4(8): 641-643) when used in combination with analogs of coelenterazine that vary in their light intensity and response times (FIG. 3).

Table 1 – Comparison of various luciferases

	Firefly	Renilla	Gaussia*
Brightness *in vivo*	1	1	1000
Stable *in vitro*	No	Yes	Unknown
Crystal structure	Yes	Model only	No
Number of cysteine	4	3 (C124A improves stability)	11
Glycosylation	Yes	No	No
***in vivo* FEC**	Yes	Yes	Yes
Size (kDa)	61	36	18
Substrate	Luciferin	Coelenterazine	Coelenterazine
Substrate auto luminescence	No	Low	Low
Published split points	416-417 [a]	91-92 or 229-230 [b]	93-94 [c]

* Humanized form of *Gaussia princeps* luciferase
[a] Luker et al., 2004, Proc. Natl. Acad. Sci. USA 101(33): 12288-12293
[b] Kaihara et al., 2003, Anal. Chem. 75(16): 4176-4181
[c] Remy et al., 2006, Nat. Methods 3(12): 977-999

An alternative strategy to the use of split bioluminescent reporter enzymes as previously described is the use of split reporter enzymes that do not traditionally catalyse chemiluminescent reactions, by combining these with synthetically produced luminescent substrates or pro-luminescent substrates, for example, pro-luciferin (Fan and Wood, 2007, Assay Drug Dev. Technol. 5(1): 127-136) glow luminescence can be

produced. Several patents disclose the synthesis and use of luminescent β-lactamase substrates, for example, those disclosed in; US Patents 5,955,604, 7,018,802 B2 and published US Patent Application 20060292656 A1. Recently, the first bioluminogenic substrate for β-lactamase termed 'Bluco' was described in the literature in Yao et al., 2007, Angew. Chem. Int. Ed. 46: 1-5. Bluco is a pro-luciferin in which the 6-hydroxy group of D-luciferin is coupled to a cephalosporin through an ether bond. β-lactamase acts to open the β-lactam ring resulting in spontaneous fragmentation allowing firefly luciferase to subsequently act on the luciferin resulting in light emission.

Accordingly, the assay methods of the present invention are not limited to reporter polypeptides based on bioluminescent proteins such as luciferases, but also include the use of any reporter polypeptides that can generate a luminescent signal in the presence of an appropriate substrate, herein termed a luminescence reporter polypeptide. Accordingly, examples of reporter polypeptides that can be used as the basis for reporter fragments and systems include bioluminescent proteins such as luciferases (e.g. firefly and Renilla luciferases) and beta-lactamase (e.g. TEM-1 beta-lactamase: EC: 3.5.2.6).

As mentioned above, the reporter polypeptides are typically split into two fragments which when they associate can reconstitute the activity of the original full length polypeptide. For example, luciferase and beta-lactamase are typically split into two fragments, an alpha and an omega fragment. Suitable breakpoints in the amino acid sequences of these various proteins to generate the two fragments have been described previously.

Reporter polypeptides/fragments are selected so that they are suitable for *in vitro* use (for the avoidance of doubt, in the present context the term *in vitro* means that the assays take place outside of living cells). The reporter polypeptides are typically variants of wild type sequences that have amino acid changes that improve their suitability for *in vitro* use, for example to enhance stability, improve solubility and/or reduce aggregation. In a particularly preferred embodiment, the reporter polypeptides/fragments have reduced sensitivity, compared to wild type polypeptides, to inhibitors, such as enzyme inhibitors, of the activity of the polypeptide required for reporter function, e.g. beta lactamase activity. Such inhibitors include compounds found in samples, e.g. biological samples such as blood and serum samples, that it is desired to test for the presence of analytes of interest. Particular examples of such

inhibitors are inhibitors of beta-lactamase activity found in blood as a result of administering antibiotics to individuals from whom the blood samples are taken.

Under assay conditions, the association of the reporter fragments to form an active complex having reporter activity is typically mediated by the interaction between other regions of the reporter components and a target analyte. Thus, the reporter components typically comprise an interactor moiety or domain.

Interactor domains have binding specificity for a target analyte of interest. Interactor domains include, for example, peptides, glycoproteins, polysaccharides, antigens, antibodies and antigen-binding fragments of antibodies such as complementarity determining regions (CDRs). Antigens include antigens derived from pathogens, such as viral or bacterial antigens. Antibodies/CDRs include sequences that bind to antigens derived from pathogens, such as viruses or bacteria. Particular examples of interactor domains include the IgG-binding domain of Protein G, and Herpes Simple Virus antigens (particularly preferred versions of which are truncated glycoprotein G1 envelope proteins from HSV1 or truncated glycoprotein G2 envelope proteins from HSV2, such as amino acids 92 to 148 of gG1 or 551 to 641 of gG2).

Interactor domains may be joined directly to the reporter fragments or via a linker. Suitable linker domains include peptides, such as glycine rich repeat sequences (e.g. G_4S repeat sequences - *i.e.* GGGGS sequence repeats). The number of linker domains, for example, G_4S domains may be 1, 2, 3, 4, 5, 6, or more. In one embodiment, the number of glycine rich repeat sequences is preferably 2 or 3, particularly where the interactor domain is a polypeptide having fewer than 150 or 100 amino acids. Where larger interactor domains are used, it may be desirable to increase linker length.

In one embodiment, the linker domain is flexible. In another embodiment the linker domain is rigid.

The selection of an appropriate linker domain sequence and/or number of linker domain repeats for any particular interactor and reporter fragment construct and fragment pairing is within the routine level of experimentation by the skilled artisan.

Reporter fragment polypeptides, linker domains and interactor domains may be joined by covalent or non-covalent means to form a reporter component.

In one embodiment, the linker domains and interactor domains are polypeptides. Thus the reporter component may be a single polypeptide.

In another embodiment, the reporter fragments are linked to interactor domains by conjugation e.g. covalent coupling via, for example, thiol-thiol, amine-carboxyl or amine-aldehyde functional groups. Particular examples include cross-linking of polypeptides to glycoproteins via the carbohydrate groups; cross-linking via primary amines(found at the N-terminus and on lysine residues) e.g. using heterobifunctional cross linkers with an amine reactive group and a sulfhydryl reactive group; cross-linking via carboxyl groups (found at C-terminus and as side groups on glutamic acid and aspartic acid residues); cross-linking via free sulfhydryl groups; and disulphide exchange.

Non-covalent methods include avidin-biotin systems and hybridization of oligonucleotide-protein conjugates.

As used herein, a peptide/polypeptide/protein is said to be "isolated" or "purified" when it is substantially free of cellular material or free of chemical precursors or other chemicals. The peptides may be purified to homogeneity or other degrees of purity. The level of purification will be based on the intended use. The intended use in the present invention is as operable components of homogenous *in vitro* FEC assays for specific analytes.

In some uses, "substantially free of cellular material" includes preparations of the peptide having less than about 30% (by dry weight) of other proteins (*i.e.*, contaminating protein), less than about 20% other proteins, less than about 10% other proteins, or less than about 5% other proteins. When the peptide is recombinantly produced, it may also be substantially free of culture medium, *i.e.*, culture medium represents less than about 20% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of the peptide in which it is separated from chemical precursors or other chemicals that are involved in its synthesis. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of the enzyme peptide having less than about 30% (by dry weight) chemical precursors or other chemicals, less than about 20% chemical precursors or other chemicals, less than about 10% chemical precursors or other chemicals, or less than about 5% chemical precursors or other chemicals.

The isolated reporter fragment and polypeptide reporter components may be purified from cells that have been altered to express it (recombinant), or synthesized

using known protein synthesis methods. For example, a nucleic acid molecule encoding the enzyme peptide is cloned into an expression vector, the expression vector introduced into a host cell and the protein expressed in the host cell. Suitable host cells are described in more detail below. The protein may then be isolated from the cells by an appropriate purification scheme using appropriate protein purification techniques. Exemplary techniques of the invention are described in detail in the Examples set out below.

In one aspect, the reporter polypeptides are proteins that consist of the amino acid sequences provided herein. A protein consists of an amino acid sequence when the amino acid sequence is the final amino acid sequence of the protein. In an additional aspect, the present invention further provides proteins that consist essentially of the amino acid sequences provided. A protein consists essentially of an amino acid sequence when such an amino acid sequence is present with only a few additional amino acid residues that do not alter the functional characteristics of the proteins of the invention. In yet a further aspect, the reporter polypeptides are proteins that comprise the amino acid sequences provided. A protein comprises an amino acid sequence when the amino acid sequence is at least part of the final amino acid sequence of the protein. In such a fashion, the protein may be only the peptide or have additional amino acid molecules, such as amino acid residues (contiguous encoded sequence) that are associated with it or heterologous amino acid residues or peptide sequences. Such a protein may have a few additional amino acid residues or may comprise several hundred or more additional amino acids. A brief description of how various types of these proteins may be made or isolated is provided below.

The reporter polypeptides used in the present invention may be attached to heterologous sequences to form chimeric or fusion proteins. Such chimeric and fusion proteins may comprise an enzyme peptide operatively linked to a heterologous protein having an amino acid sequence not substantially homologous to the enzyme peptide. "Operatively linked" indicates that the enzyme peptide and the heterologous protein are fused such that the operability of each is not destroyed. The heterologous protein may be fused to the N-terminus or C-terminus of the enzyme peptide.

A chimeric or fusion protein may be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different protein sequences are ligated together in-frame in accordance with conventional techniques. In another

embodiment, the fusion gene may be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification or ligation of gene fragments may be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which may subsequently be annealed and re-amplified to generate a chimeric gene sequence (see Ausubel *et al.*, Current Protocols in Molecular Biology, 1998). Moreover, many expression vectors are commercially available that already encode a fusion moiety. An enzyme peptide-encoding nucleic acid may be cloned into such an expression vector such that the fusion moiety is linked in-frame to the enzyme peptide, which is one means by which the fusion protein is made without destroying the operability of each component.

Identification of homologous positions in alternative sequence numbering schemes

To determine homologous positions in comparing two amino acid or nucleotide sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps may be introduced in one or both of a first and a second amino acid or nucleotide sequence for optimal alignment and non-homologous sequences may be disregarded for comparison purposes). In a preferred implementation, at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% or more of the length of a reference sequence is aligned for comparison purposes. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position and the position is homologous in the two. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity and similarity between two sequences may be accomplished using a mathematical algorithm. (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence

Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

For example, pairwise alignments and levels of sequence identity and homology may be determined using the BestFit program in the GCG software package. The percent identity between two amino acid sequences may be determined using the algorithm of Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)), which has been incorporated into the GAP program in the GCG software package. The algorithm is typically employed using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide sequences may be determined using the GAP program (Devereux, J., *et al.*, Nucleic Acids Res. 12(1):387 (1984)) with a NSWgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6.

Thus, a substitution at a position in an amino acid sequence or peptide is indicated by the one letter designation for the amino acid, followed by the position number of the relevant non-substituted sequence or peptide, followed by one or more one letter designations for replacement amino acids. For example, substitution of Threonine for Valine at position 74 of the α fragment of TEM-1 Beta-lactamase would be designated as V74T. Similar designations will be clear from the context and further details provided herein.

Amino acid Modifications to Improve Reporter Functionality *In Vitro*

Reporter polypeptide fragments for use in the assays of the invention may comprise amino acid sequence changes or modifications that improve the suitability of the reporter polypeptide for use in such assays. In particular, in a preferred embodiment, at least one of the reporter polypeptide fragments comprises a variation in or modification to its amino acid sequence which renders a reconstituted active reporter polypeptide complex less susceptible to inhibition by substances that are inhibitors of the unmodified (e.g. wild type) amino acid sequence, especially substances that are found in samples to be tested, such as biological samples including serum.

Such variants typically contain one or more non-conservative amino acid substitutions, deletions, insertions, inversions, or truncation or a substitution, insertion, inversion, or deletion in a critical residue or critical region. Variants of altered function may also contain substitution of similar amino acids that result in no change or an

insignificant change in function. In one embodiment, variants contain 1, 2, 3, 4 or 5 amino acid changes.

Our co-pending application PCT/AU2007/000508, the contents of which are incorporated by reference, describes modified Beta-lactamase peptide sequences comprising single amino acid substitutions or multiple substitutions in combination that are especially adapted for use in homogeneous *in vitro* FEC assays as further exemplified herein. Specific examples of such substitutions are substitutions at amino acid position 69 (preferably M69L or M69I) in the alpha fragment, which reduces inhibition by beta-lactamase inhibitors and substitutions at amino acid position 276 (preferably N276D) in the omega fragment, which also reduces inhibition by beta-lactamase inhibitors. Other examples are selected from substitutions at one or more of amino acid positions 74, 182, 208, 211 and 230 (preferably one or more of V74T, M182T, I208T, M211Q and F230Y).

Further examples are mutations of the following hydrophobic amino acid residues to any residue which is considered to be less hydrophobic may give rise to an improved assay: V44, **Y46**, L49, **L51**, **F66**, **V74**, **L81**, F151, P183, V184, A187, **L190**, **L194**, L198, L199, **L207**, I208, **W210**, M211, A232, I247, A249, P257, **I260**, I261, **I262**, I263, **Y264**, I282, L286 (particularly residues that are in bold type and underlined).

The amino acid numbering given is with reference to the wild-type TEM-1 beta lactamase sequence shown in SEQ ID NOS: 1 and 3 of PCT/AU2007/000508. However, it will be appreciated that the modifications can also be applied to homologous beta lactamase sequences at the equivalent positions (see preceding section for determination of equivalent positions by sequence alignments).

As mentioned above, improved stability can be achieved for *Renilla* luciferase stability and light output by mutation of cysteine at position 124 to alanine (Liu et al., 1999, Gene 237: 153-159). Additional improvements to *Renilla* luciferase stability and light output can be achieved with the use of several supplementary mutations (Loening et al., 2006, Protein Eng. Des. Sel. 19(9): 391-400 and Loening et al., 2007, Nat. Methods 4(8): 641-643) when used in combination with analogs of coelenterazine that vary in their light intensity and response times (FIG. 3).

Modified reporter fragments having improved properties for use in *in vitro* assays, such as homogeneous assays, can be obtained using various techniques. For

example, sequence changes may be introduced by site-directed mutagenesis. The selection of suitable sites may, for example, be guided by the primary amino acid sequence and/or secondary/tertiary structural information included structural information determined by techniques such as x-ray crystallography or NMR. For example, the 3D structure of the active site of an enzyme can be used to assist in designing variants which have reduced susceptibility to inhibition (see for example, the crystal structure of TEM1 as described in Jelsch, C., F. Lenfant, *et al.*, 1992, FEBS Lett 299(2): 135-42)

Modified reporter fragments having improved properties can also be obtained by techniques such as random mutagenesis or directed molecular evolution followed by selection of variants having the desired properties (e.g. by testing variants for enzyme activity in the presence of an inhibitor of the unmodified protein).

Additional modification useful in the present invention may include sequences containing amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids. Further modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Accordingly, peptides and constructs for use in the present invention also encompass derivatives or analogs in which a substituted amino acid residue is not one encoded by the genetic code, in which a substituent group is included, in which the mature enzyme peptide is fused with another compound, such as a compound to increase the half-life of the enzyme peptide (for example, polyethylene glycol), or in which the additional amino acids are fused to the mature enzyme peptide, such as a leader or secretory sequence or a sequence for purification of the mature enzyme peptide or a pro-protein sequence.

Glow Luminescence *in vitro* FEC Assays

The present invention is directed to *in vitro* FEC assays to detect analytes of interest in samples where the presence or absence of the analyte is measured by detecting a glow luminescence signal.

The method typically involves contacting the sample with luminescence reporter components, as described above, which are capable of reconstituting an enzyme activity in the presence of an analyte of interest in a sample, which enzyme activity can act on a suitable substrate present in the reaction mixture to give rise to a luminescence signal. Thus, where the analyte of interest is present in the sample, active enzyme is produced which acts on the substrate in the reaction mixture to generate a luminescent signal. This signal is then detected.

The *in vitro* assay conditions, as described herein, are such that the signal generated includes glow luminescence, as opposed to flash luminescence. The assay conditions used need not necessarily exclude the possibility that a flash luminescence signal is generated in addition to the detectable glow luminescence signal, and in some embodiments both types of signals may be generated in the presence of the analyte of interest.

Analytes of interest include those present in environmental or biological samples. Biological samples include whole blood, serum, saliva and urine. The term '*in vitro*" is taken to mean in the present context that the assays are conducted outside of living cells, such as in cell-free assays. Analytes of interest include antibodies, such as antibodies to pathogens, antigens, such as antigens derived from pathogens, clinical markers such as hormones and host molecules e.g. prostate-specific antigen and other disease-related diagnostic marker molecules. The term "pathogen" includes viruses, bacteria and prions (e.g. Herpes Simplex Viruses and Hepatitis virus such as HBV and HCV).

Suitable luminescence reporter components are described in detail above. The substrate used will depend on the type of enzyme activity reconstituted by the association of the luminescence reporter components in the presence of an analyte of interest and is selected so that the action of the reconstituted enzyme on the substrate generates a luminescent signal. For example, in the case of biologically-derived luciferases such as *Renilla*, the substrate is typically coelenterazine or an analog thereof,

such as one of the analogs shown in Figure 3, with coelenterazine analogues f, h and n being particularly preferred. In the case of Firefly luciferase, the substrate is typically luciferin or an analog thereof.

Suitable chemiluminescent substrates can also be designed for non-bioluminescent enzymes such as beta-lactamase, for example, pro-luciferin (Fan and Wood, 2007, Assay Drug Dev. Technol. 5(1): 127-136). Several patents disclose the synthesis and use of luminescent β-lactamase substrates, for example, those disclosed in; US Patents 5,955,604, 7,018,802 and published US Patent Application 20060292656. Recently, the first bioluminogenic substrate for β-lactamase termed 'Bluco' was described in the literature in Yao et al., 2007, Angew. Chem. Int. Ed. 46: 1-5. Bluco is a pro-luciferin in which the 6-hydroxy group of D-luciferin is coupled to a cephalosporin through an ether bond. β-lactamase acts to open the β-lactam ring resulting in spontaneous fragmentation allowing firefly luciferase to subsequently act on the luciferin resulting in light emission.

The amount of reporter component required for an *in vitro* assay is generally higher than that found *in vivo*. For example, the concentration of reporter components, such as polypeptides, in the reaction mix may be at least 1 pM, such as at least 10 or 100 pM, or 1 or 10 nM.

In developing a glow luminescence-based FEC assay, we have determined a range of assay conditions that provide an improved glow luminescence signal. These assay conditions include the presence of one or more of the following, and any combination thereof, particularly (i), (ii) and (iii):

(i) Reducing agents: the inclusion of one or reducing agents was found to be important for the generation of an extended glow luminescence signal. Reducing agents as referred to herein are able to act reversibly on protein disulphide bonds and therefore include thiol reducing agents such as DTT and related compounds as well as 2-mercaptoethanol and related compounds and phosphines and related compounds. The preferred total concentration of reducing agents is from about 1 to about 2% w/v. However, the optimum concentration can be determined empirically for a given system e.g. using the procedures described in the examples.

(ii) Denaturants: one or more denaturants are preferably included to reduce the natural affinity of the reporter enzyme fragments for each other (spontaneous complementation). Denaturants include urea, thiourea and guanidine hydrochloride,

with thiourea being particularly preferred. The total concentration of denaturants may be from about 1 to about 2% w/v, such as from about 1.3 to about 1.6 w/v. However, again, the optimum concentration can be determined empirically for a given system e.g. using the procedures described in the examples.

(iii) Detergents: one or more detergents are preferably included to reduce protein-protein interactions, particularly self-association of reporter fragments. Preferred detergents are nondenaturing zwitterionic detergents such as aminosulphobetaine-14, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), [(3-[3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propane sulfonate (CHAPSO) and other sulphobetaine derivatives of cholic acid, preferably at a total concentration of from about 0.1 to about 0.2% w/v. Again, the optimum concentration can be determined empirically for a given system e.g. using the procedures described in the examples.

(iv) Salt: this is preferably included at a concentration from from about 0.25 M to 0.5 M. NaCl is a preferred example but other salts may be used. The optimum concentration can be determined empirically for a given system e.g. using the procedures described in the examples.

(v) Tris.HCl as a buffer. This was shown to give improved results compared with sodium or potassium phosphate buffers. The concentration of Tris.HCl used is typically in the range of from about 20 mM to about 200 mM, such as about 50 mM. The pH of the reaction mix is preferably from about 6.5 to about 8.0, such as from about 6.8 to about 7.5. The optimum concentration and pH can be determined empirically for a given system e.g. using the procedures described in the examples.

(vi) Metal chelators: one or more metal chelators such as EDTA and variants thereof, e.g. EGTA. This is especially relevant where samples to be tested, such as blood samples, include metal ions. Typically total concentrations of metal chelators are from about 1 mM to 10 mM, such as from 7 mM to 9 mM, e.g. about 5 mM. The optimum concentration can be determined empirically for a given system e.g. using the procedures described in the examples.

Light emission resulting from the presence of analyte of interest can be detected using methods familiar to those skilled in the art, such as a luminometer.

The various assay conditions described above are preferably selected so as to provide for an extended period of glow luminescence, in the presence of an analyte of interest, of at least 5 minutes, more preferably at least 10 minutes.

Rates of light emission are relative to the integration time, fragment concentration and substrate concentration, among other factors. However, preferred rates of light emission are greater than 1 mRLU/min for kinetic determinations, such as greater than 2, 5, 10, 20 or 50 mRLU/min; greater than 1 RLU for endpoint determinations, such as greater than 2, 5 or 10 RLU. Preferred signal-to-noise ratios are greater than 2 for both kinetic and endpoint detection modalities, such as greater than 5 or 10.

The methods of the invention may be applied to both *in vitro* homogenous assays and *in vitro* heterogeneous assays, the latter being where at least one reporter fragment or reporter component is immobilized to a solid phase.

In one embodiment, the assays are conducted in parallel in a plurality of reaction vessels, such as in different wells of a microtiter plate. For example, assays may be conducted substantially simultaneously in at least 6 wells, such as in at least 10 or 12 wells. The generation of an extended glow luminescence signal improves the ability to then read the luminescence signal in all of the wells before the signal has decayed.

In some embodiments, multiple analytes are detected in the same reaction using different reporter components which generate distinct luminescent signals, e.g. a dual glow signal, such as a glow signal produced by firefly luciferase and a glow signal produced by *Renilla* luciferase. Simultaneous detection of two independent analytes within a single sample is known as multiplexing. In an alternative embodiment, multiple analytes are detected in the same reaction using different reporter components, at least one of which can give rise to an extended glow luminescent signal and at least one of which gives rise to a non-luminescent signal, such as a colorimetric signal.

Kits

The present invention also provides reporter fragments and/or reporter components as kits together with other kit components required for the generatation of a glow luminescence signal in the present of an analyte of interest. Such kits include the reporter components described above together with reagents for providing *in vitro* assayconditions which allow the reporter components to associate in the presence of

analyte of interest in the sample, through the affinity of the interactor domains of the reporter components with the analyte, to produce a glow luminescent signal through the action of the resulting active enzyme on a substrate. The reagents include one or more of the various components described in items (i) to (vi) above. In one embodiment, the reagents are provided as a concentrated reaction buffer which is diluted prior to use, e.g. a 2X to 20X concentrate, such as a 5X to 10X concentrate.

Assay Reagent Storage Compositions

Another aspect of the invention relates to improvements in storage of proteinaceous reporter components. In particular, the present invention provides an assay reagent storage composition comprising (i) one or more reporter polypeptides at a total concentration of greater than about 10 nM and (ii) one or more sugars and/or one or more inert proteins. The sugars and/or inert proteins are used to stabilise/extend the half-life of reporter polypeptides during storage, e.g. at about 4°C, and optionally also to reduce reporter polypeptide aggregation.

Reporter polypeptides in the context of the storage compositions of the invention include both luminescent reporter polypeptides as described above and other types of reporter polypepides. In a preferred embodiment, at least one reporter polypeptide or reporter polypeptide pair is a luminescent reporter polypeptide, such as a luciferase reporter polypeptides for an *in vitro* forced enzyme complementation assay. In a particular embodiment the luciferase is a *Renilla* luciferase.

The one or more reporter polypeptides are present in the compositon at a total concentration of greater than about 10 nM, such as greater than about 20, 30, 40 or 50 nM.

The one or more sugars are preferably selected from the group consisting of sucrose, trehalose and mixtures thereof. Sucrose is particularly preferred.

In one embodiment, the total concentration of the one or more sugars is at least about 50 mM, such as at least about 100, 250 or 500 mM. The total concentration of the one or more sugars may be about 5 M or less, such as about 2.5 M or less. In one embodiment the total concentration of the one or more sugars is from 1 M to 2.5 M.

The one or more inert proteins is selected from the group consisting of serum albumin, gelatin and mixtures thereof. The term "inert protein" means that the protein does not react with the reporter polypeptides, analytes of interest or substrates. The

serum albumin is preferably bovine serum albumin. A particular example of gelatin is fish gelatin.

In one embodiment, the total concentration of the one or more inert proteins is at least about 0.2 mg/ml, such as at least about 0.4 or 0.5 mg/ml.

The present invention will now be described further with reference to the following examples which are illustrative only and non-limiting.

EXAMPLES

Introduction of a breakpoint between the junction of glycine at position 229 and lysine at position 230 (FIG. 4) of native *Renilla* luciferase (RLuc) results in the generation of optimal α and ω fragments. FEC assays derived from alternative breakpoint positions of RLuc also give rise to operable fragments, for example, breakpoint between serine at position 91 and tyrosine at position 92 (also demonstrated in the invention). In conjunction with combinations of mutations to address solubility, stability, sensitivity and/or resistance to enzyme inhibitors, operative FEC assays (FIG. 5) can be designed as functionally required. The functionality of the engineered fragments *in vitro* relies principally on the reaction conditions and composition, without which glow luminescent detection and relevant FEC signal to noise ratios cannot be obtained.

Example 1: Homogeneous *in vitro Renilla* luciferase FEC assays for antibody detection using glow luminescence.

The procedures outlined herein describe the synthesis and characterisation of RLuc fragments generated by splitting the full-length parental enzyme at amino acids 229/230. The α fragment (amino acid residues 2-229) was subsequently juxtaposed to a flexible linker $(G_4S)_3$, HSV-1 (SEQ ID NOS: 1, 2 and 3) or HSV-2 (SEQ ID NOS: 4, 5 and 6) truncated antigen and polyhistidine tag (6H) at the breakpoint termini. A single point mutation, C124A, was introduced into the α fragment to enhance enzyme stability resulting in the generation of RLuc_α_{229}P1 and RLuc_α_{229}P2, for HSV-1 and HSV-2 detection, respectively. Introduction of a polyhistidine tag (6H), flexible linker $(G_4S)_3$ and protein-G subunit (SEQ ID NOS: 7 and 8) at the breakpoint termini of the ω fragment (amino acid residues 230-311) resulted in the generation of an IgG antibody

binding reporter enzyme fragment designated RLuc$_{230}$ωProG. DNA sequence data indicating the locations of the flexible linker $(G_4S)_3$, polyhistidine tags (6H), point mutation and analyte interaction moieties are provided. Schematic representations of the RLuc constructs engineered in this invention are illustrated in FIG. 6. The corresponding custom-made oligonucleotide primers (Sigma-Genosys, Australia) used for the construction of all plasmids is listed in Table 2. These fragment pair members were isolated, purified and used to demonstrate forced enzyme complementation in the presence of an antibody analyte (anti penta-histidine monoclonal antibody binding to histidine tag) in a homogeneous *in vitro* assay format.

Renilla luciferase synthetic synthesis

A codon optimised gene encoding full-length RLuc incorporating the C124A mutation (SEQ ID NOS 9 and 10) for enhanced folding and stability was synthesised by DNA2.0 (Menlo Park, U.S.A) for optimal *E.coli* expression. Synthetic pJ36-RLuc plasmid (2 μg) was supplied on a filter disk that was purified using the QIAprep spin miniprep kit (Qiagen, Australia) by incubating the filter disk in appropriate buffer, applying the supernatant to a QIAprep spin column and completing the purification according to the manufacturer's instructions. Purified plasmid was transformed into XL1-Blue competent cells (Stratagene, c/o Integrated Sciences, Australia) according to the manufacturer's instructions. Colonies were screened to confirm the presence of the plasmid and sequence integrity was checked by DNA sequencing (using primers RLuc1 and RLuc2 – Table 2).

pET26 subcloning of full-length Renilla luciferase for protein expression

RLuc was excised from pJ36-RLuc by digestion with *Nde*I and *Xho*I restriction endonucleases (New England Biolabs, c/o Genesearch Pty Ltd, Australia) and ligated with *Nde*I and *Xho*I digested pET-26b(+) vector (Novagen, Australia), a prokaryotic expression vector that allows for inducible expression of recombinant proteins in *E. coli*. Ligated plasmids were transformed into BL21-Gold (DE3) competent cells (Stratagene) according to the manufacturer's instructions. Five to ten colonies from each transformation were screened to confirm the presence of the cloned insert sequence within the pET-26b(+) vector by restriction digestion with *Nde*I/*Xho*I and digestion

products were analysed by gel electrophoresis. Positive clones were also confirmed by DNA sequencing (using primers RLuc1 and RLuc2 – Table 2).

Generation of RLuc α and ω fragments

All amplifications were performed using Platinum *Taq* DNA polymerase (Invitrogen, Australia) according to the manufacturer's recommendations. RLuc fragments were generated by PCR amplification of appropriate regions from the full-length enzyme using specific primers (see Table 2 – Primer Sequences for a description of the primers used for PCR). Reagents for an antibody homogeneous assay to both HSV-1 and HSV-2 were constructed for both RLuc break points, S91/Y92 and G229/K230.

Construction of pET-RLuc_α_{91}P1, pET-RLuc_α_{91}P2, pET-RLuc_α_{229}P1, pET-RLuc_α_{229}P2

RLuc_α_{91} (residues 2-91) was amplified using forward primer RLuc3 and reverse primer RLuc4. RLuc_α_{229} (residues 2-229) incorporating the C124A mutation was amplified using forward primer RLuc3 and reverse primer RLuc7. Amplification products were purified directly from the reaction tube using the QIAquick PCR purification kit (Qiagen) and were subsequently digested with *NdeI/KpnI* and cloned into each *NdeI/KpnI* digested universal pET-αP1 and pET-αP2 plasmids encoding a C-terminal HSV-1 and HSV-2 specific cassette [$(G_4S)_3$ linker, truncated HSV antigen (P1 and P2), G_4S linker and histidine tag (6H)], yielding pET-RLuc_α_{91}P1 (SEQ ID NOS 19 and 20), pET-RLuc_α_{229}P1 (SEQ ID NOS 21 and 22), pET-RLuc_α_{91}P2 (SEQ ID NOS 23 and 24) and pET-RLuc_α_{229}P2 (SEQ ID NOS 25 and 26) respectively. Ligated plasmids were transformed into BL21-Gold (DE3) competent cells and selected colonies from each transformation were screened to confirm the presence of the cloned insert. Positive clones were again confirmed by DNA sequencing (using primers RLuc1 and RLuc2 – Table 2).

Construction of pET-RLuc_ω92ProG and pET-RLuc_ω230ProG

RLuc_92ω (residues 92-311) incorporating the C124A mutation was amplified using forward primer RLuc5 and reverse primer RLuc6. RLuc_230ω (residues 230-311) was amplified using forward primer RLuc8 and reverse primer RLuc6. Amplification products were digested with *NdeI/XhoI* and each were cloned into *NdeI/XhoI* digested universal pET-ωProG plasmid encoding an N-terminal protein-G subunit specific cassette [histidine tag (6H), G_4S linker, protein-G subunit (ProG) and $(G_4S)_3$ linker], yielding pET-RLuc_92ωProG (SEQ ID NOS 27 and 28) and pET-RLuc_230ωProG (SEQ ID NOS 29 and 30). Ligated plasmids were transformed into BL21-Gold (DE3) competent cells and selected colonies from each transformation were screened to confirm the presence of the cloned insert by sequence confirmation (using primers RLuc1 and RLuc2 – Table 2).

Table 2 – Primer Sequences

Oligo	Full Name	Sequence (5' - 3')	SEQ ID NO:
RLuc1	pET-T7terminator seq	GCTAGTTATTGCTCAGCGG	11
RLuc2	pET-T7promoter seq	TAATACGACTCACTATAGGGG	12
RLuc3	RLuc-alpha1-F1	TATAACTTTAATCGGCATATGACCAGCAAAGTG TATGATCCGG	13
RLuc4	RLuc-alpha1-R1	AACTTTAATCGGGGTACCGCTGCCGTTGCCGC TTTTGCC	14
RLuc5	RLuc-omega1-F1	AACTTATGATTATCCGGCTAGCTATCGTCTGCT GGATCATTATAAATATCTGACC	15
RLuc6	RLuc-omega1-R1	AACTTATGATTATCCGCTCGAGTTACTACTGTT CGTTTTTCAGCACACGTTCCAC	16
RLuc7	RLuc-alpha2-R1	AACTTTAATCGGGGTACCGCCGCCTTTCACCA GCGG	17
RLuc8	RLuc-omega2-F1	AACTTATATTATCTAGCTAGCAAACCGGATGTG GTGCAGATTGTG	18

Universal plasmids encoding cassette domains

To simplify the engineering of fusion constructs, universal αP1/2 and ωProG fusion genes were synthesized by DNA2.0 (Menlo Park, U.S.A) incorporating restriction endonuclease sites located between the enzyme fragment, linkers and binding

moieties to enable the substitution of various domain sequences. The universal αP1/2 construct, pET-αP1/P2 was designed to have *Kpn*I, *Bam*HI and *Spe*I sites between the α sequence, $(G_4S)_3$ linker and antigen encoding moieties with *Nde*I and *Xho*I sites on either end for ligation into the *NdeI/XhoI* site of pET-26b(+). Likewise, the universal ωProG construct, pET- ωProG, was designed to have *Bam*HI, *Spe*I and *Nhe*I sites inserted between the ProG, $(G_4S)_3$ linker and ω encoding domains with *Nde*I and *Xho*I sites on either end for ligation into the *NdeI/XhoI* site of pET-26b(+). The sequence encoding the C2 IgG-binding domain of *Streptococcus* strain G148 Protein G was obtained by back-translation of the published amino acid sequence (Gülich et al., 2002, Protein Eng. 15(10): 835-42) with an *E. coli* codon usage table, using Vector NTI (Invitrogen). The locations of flanking restriction endonuclease sites placed in between domains are indicated as underlined font in the attached sequences of the invention (SEQ ID NOS 19, 21, 23, 25, 27 and 29).

HSV-1 and HSV-2 antigenic peptide design

In order to detect and discriminate between antibodies against HSV-1 and HSV-2 in serum, two type-specific truncated HSV antigens were designed as analyte binding moieties. The HSV-1 specific antigen (SEQ ID NOS 1, 2 and 3) is comprised of residues 92-148 of glycoprotein G1 (gG1). This region of gG1 contains an immunodominant epitope (residues 112-127), and two key amino acids within a second epitope known to confer an HSV type-1 specific response in humans. The HSV-2 specific peptide (SEQ ID NOS 4, 5 and 6) is composed of residues 551-641 of glycoprotein G2 (gG2) and is comprised of two immunodominant epitopes (residues 561-578 and 626-640) known to confer an HSV type-2 specific response in humans.

Expression of α and ω RLuc fragments

Expression of fragments in BL21-Gold (DE3) was done as follows: 10 ml of LB broth supplemented with 50 μg/ml of kanamycin was inoculated with a single colony of interest and the liquid culture was incubated overnight (~14 hours) at 37 °C with shaking at 250 rpm. The overnight culture was diluted 1:25 into 250 ml of Overnight Express Instant TB Medium (Novagen, Australia) supplemented with 50 μg/ml of kanamycin, which was subsequently incubated at 37 °C, 250 rpm for 24 hours. Cells

were pelleted at 2,057 g for 30 min (4°C) and stored at –20 °C for no more than 6 months.

Purification of α and ω reporter fragments

All enzyme fragments (RLuc_α_{91}P1, RLuc_α_{229}P1, RLuc_α_{91}P2, RLuc_α_{229}P2, RLuc_$_{92}$ωProG and RLuc_$_{230}$ωProG) were extracted under denaturing conditions and refolded on a 1 ml HisTrap HP column (GE Healthcare, Sydney, Australia). All purified proteins were characterised and assayed in the same manner, in order to directly compare their rate of catalysis and signal to noise ratio upon forced enzyme complementation with anti penta-histidine monoclonal antibody.

Extraction of recombinant proteins under denaturing conditions

The pellet from a 250 ml overnight induction was lysed in 10 ml/g (wet pellet weight) of lysis buffer (6 M GuHCl, 100 mM sodium phosphate, 200 mM L-arginine, 20 mM imidazole, 2 mM DTT pH 8). Following a 1-hour incubation at 4 °C with shaking at 100 rpm, each suspension was sonicated in an ice bath for 5 cycles of 30 seconds on/30 seconds off (70% duty cycle) using a Branson 250 sonifier. Soluble protein was recovered by centrifugation at 18,514 g at 4 °C for 30 min. Supernatant (10 ml) was passed through a 0.2 μm filter.

Immobilised metal affinity chromatography (IMAC) and on-column refolding of RLuc_αP1, RLuc_αP2 and RLuc_ωProG

Chromatography instruments and columns used for FPLC were from GE Healthcare (Sydney, Australia). Fusion proteins were purified using a 1 ml HisTrap™ HP column under the control of an AKTA-Purifier using the Unicorn 5.1 controller software at 4 °C. The HisTrap column was equilibrated with 10 column volumes (CV) of gradient buffer (8 M urea, 100 mM sodium phosphate, 200 mM L-arginine, pH 8) at a flow rate of 1 ml/min. Cleared *E. coli* lysates were directly loaded onto the column using a 50 ml superloop to inject sample directly via the injection valve (INV-907) at 1 ml/min. Bound protein was refolded over a 20 CV gradient from 8 M urea, 100 mM sodium phosphate, 200 mM L-arginine, pH 8 to 100 mM sodium phosphate, 200 mM L-

arginine, pH 8 at 1 ml/min. Contaminating protein was washed off the column with 10 CV of 50 mM imidazole, 100 mM sodium phosphate, 300 mM NaCl, pH 7.5, followed by a second 10 CV wash of 100 mM imidazole, 100 mM sodium phosphate, 300 mM NaCl, pH 7.5. Histidine-tagged proteins were eluted with 10 CV of 500 mM imidazole, 100 mM sodium phosphate, 300 mM NaCl, pH 7.5 and collected into 1 ml fractions (FIG. 7 and 8). Fractions from each chromatography run were assessed for purity by SDS-PAGE using a NuPAGE® Novex4-12% Bis-Tris gel (Invitrogen). Fractions containing the protein peak (2.5 ml) were pooled and buffer exchanged into 50% glycerol, 50 mM sodium phosphate, pH 7.0, using a PD10 column (GE Healthcare) and stored at –20 °C. Protein was quantified using a Bradford assay (Pierce, c/o Quantum Scientific, Australia), prior to storage at –20 °C.

Gel filtration

In some cases, following affinity purification of the enzyme fragments; pooled eluates were subjected to size exclusion chromatography using a Superdex 200 GL column under the control of an AKTA-Purifier using the Unicorn 5.1 controller software. Gel filtration was used for either polishing of purified enzyme fragments (removal of contaminating proteins) or to determine the proportion of monomeric versus aggregated enzyme fragments following on-column refolding. The protocol was as follows: equilibration with 2 CV of 50 mM sodium phosphate, 150 mM NaCl, pH 7 at 0.6 ml/min; injection of 250 – 500 µl of sample at 0.6 ml/min; and isocratic flow of 1 CV of 50 mM sodium phosphate, 150 mM NaCl, pH 7 at 0.6ml/min. See FIG. 8 for exemplary results.

Mass Spectroscopy

The molecular mass of purified proteins was confirmed using the HPLC/TOF mass spectroscopy services at the Institute for Molecular Bioscience, University of Queensland, Australia. Respective experimental data was collected and analysed for comparison with the theoretical molecular weight values.

Development of glow luminescence using Full-length Renilla luciferase

The use of luminescent signal detection for diagnostic purposes requires an easy to read format compatible with high sample processivity and throughput. Luminescence from full-length RLuc is typically recorded as a flash emission that reaches maximal light intensity within seconds or milliseconds. Due to the speed of light emission, the reaction needs to be initiated and recorded in front of the light detection device where the time interval between substrate addition and detection is kept constant between samples. An alternate detection method is glow luminescence, a steady state kinetic signal. The constant signal is preferable as the reaction can be initiated outside of the detection chamber yielding more sensitive and reproducible results. We performed assays using full-length RLuc in combination with a commercially available *Renilla* Luciferase Assay System (Promega, Sydney, Australia) to adapt reaction conditions in order to produce glow luminescence. Prior disclosures, US Patents 5,618,682 and 6,171,809, demonstrate the use of dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) for conversion of a flash luminescent signal to a glow signal for *Firefly* luciferase and *Renilla* luciferase, respectively.

Assays were performed as described herein. RLuc (20 nM) was combined with sodium phosphate pH 7 in a final reaction volume of 100 µL in 96-well white Costar plates (Corning, Australia). Combinations of 5 mM DTT, 1 x Promega lysis buffer (PLB, proprietary composition), 5 mM EDTA and control serum (1/100) were added and compared with the use of Promega assay buffer (proprietary composition). Immediately following the reaction setup, 1 µl of 100 x assay substrate (Promega, proprietary composition) was added and the light emission was measured over 10 min with a 1 sec integration per well using an LmaxII^{384} luminometer (Molecular Devices, Melbourne, Australia). The rates (mRLU·min^{-1}) were determined using SoftMaxPro V5 software to determine the extent of glow luminescence.

Table 3 – Rate of light emission (mRLU·min^{-1}) generated by full-length RLuc

Full-length RLuc	-DTT	+DTT	PLB -DTT	PLB +DTT	PLB +DTT + serum	PLB +DTT +serum +EDTA
Phosphate Buffer	-6203	-170000	-2099	23972*	29884*	34794*
Promega Assay Buffer	-26495	-15196				48231*

*** positive mRLU·min^{-1} indicates glow luminescence.**

The assay results (Table 3) demonstrate the requirement of both DTT and lysis buffer to the generation of glow luminescence with EDTA and serum enhancing the effect.

Enzyme fragment complementation

Purified α and ω fragments containing the polyhistidine tag (6H) able to bind anti penta-histidine monoclonal antibody (His MAb; Merck, Australia), were used to compare the functionality of fragments generated by the two alternative split points (S91/Y92 and G229/K230). In addition, the impact of various substrates and buffer additives on the signal (His MAb forced enzyme complementation) and noise (spontaneous enzyme complementation without His MAb) were investigated to evaluate and determine the optimal reagent composition.

Comparison of His MAb directed FEC between split point S91/Y92 and G229/K230

RLuc FEC assays were performed in 96-well white Costar plates (Corning, Australia) using components from the *Renilla* Luciferase Assay Kit (Promega, Sydney, Australia) as indicated below. Variable α (RLuc_α_{91}P1 or RLuc_α_{229}P1) and ω (RLuc_ω_{92}ProG or RLuc_ω_{230}ProG) fragment concentrations (5nM → 50 nM, as indicated in FIG. 9), were added to a final reaction volume of 200 µL containing 0.5 x PLB, 0.5 x PBS pH 7.3, 0.5 M NaCl and His MAb analyte (5 nM → 25 nM, as indicated in FIG. 9). The reactions were incubated for 30 min at room temperature (RT) on a platform rocker. DTT was then added for a final concentration of 2.5 mM, followed by the addition of 1 µl of 100 x Promega assay substrate. Light emission was measured over 10 min with a 1 sec integration per well using an LmaxII^{384} luminometer (Molecular Devices, Melbourne, Australia). The rates (mRLU·min^{-1}) were calculated over the first 20 V_{max} points (using SoftMaxPro V5 software) to determine signal to noise ratios.

It was determined that the signal to noise ratio presented in FIG. 9 was comparable between 5 nM fragments split at G229/K230 and 50 nM fragments split at S91/Y92. The light emitted by 5 nM fragments split at G229/K230 was also similar to that emitted by 50 nM fragments split at S91/Y92, hence the G229/K230 split point is superior in its ability to undergo His MAb directed FEC, particularly since the S91/Y92

light emission of 20 nM fragments was below 1RLU and that of 5 nM fragments was undetectable.

Optimisation of His MAb directed in vitro FEC of RLuc fragments split at G229/K230 for improved assay sensitivity

Traditionally, *Renilla* luciferase *in vitro* assay buffer, known as 1 x Matthew's buffer (Matthews et al., 1977, Biochemistry 16(1): 85-91), has a standard composition that consists of:

100 mM potassium phosphate pH 7.6

500 mM sodium chloride

1 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)

0.02% w/v bovine serum albumin (BSA)

0.6 mM sodium azide (NaN_3)

In addition Matthew's lysis buffer (Promega technical manual Part# TM055) has a composition that consists of:

150 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulonic acid (HEPES) pH 8

0.25% octylphenol ethoxylate (Triton X-100)

1 mg/mL porcine gelatin

10% glycerol

0.05% Antifoam 289

In an attempt to improve the signal to noise ratio of RLuc_α_{229}P1 and RLuc_ω_{230}ProG His MAb forced fragment complementation, the standard *Renilla* luciferase *in vitro* assay buffer (1 x Matthew's buffer) and Matthew's lysis buffer were used as the benchmark from which to modify components. Consequently, the effect of various buffers, and buffer additives such as salts, metal chelators, solvents, detergents and protein denaturants were investigated. Assays were performed as described herein.

RLuc FEC assays were performed in 96-well white Costar plates as above using components from the *Renilla* Luciferase Assay Kit (Promega, Sydney, Australia) as indicated. Five nM α (RLuc_α_{229}P1) and ω (RLuc_ω_{230}ProG) fragments were added to a final reaction volume of 100 µL containing assay buffer (refer to Table 4), 1 x PLB,

0.5 M NaCl, 5 mM EDTA, 5mM DTT and control serum (1/100). Immediately, following the reaction setup, His MAb (5 nM) was added and the reactions were incubated for 10 min at RT without agitation. Promega 100 x assay substrate (1 µl per reaction) was added and the light emission was measured over 10 min with a 1 sec integration per well for kinetic readouts. Endpoint readings were subsequently taken (at the completion of the kinetic read) with a 5 sec integration using an LmaxII^{384} luminometer (Molecular Devices, Melbourne, Australia). The rates (mRLU·min^{-1}) were calculated over the first 50-100 V_{max} points (using SoftMaxPro V5 software) to determine signal to noise ratios. Each reaction component was individually tested and components substituted as required. Specific reaction conditions are stated below.

Buffer comparison

Initially, the base buffer and pH were assessed in order to produce optimal signal to noise ratios as indicated above. Four buffers were tested including, potassium phosphate (KPi) pH 7.4, sodium phosphate (NaPi) pH 7.4, tris hydrochloride (Tris.HCl) pH 7.4 and Promega assay buffer (unknown pH). The best performing buffer (Table 4) was then selected and a pH range of 6 - 8.5 was evaluated (Table 5).

Table 4 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using different buffers

Comparison of Buffer pH 7.4	mRLU/min	mRLU/min	Mean mRLU/min	Ratio
100 mM KPi +His MAb	28.68	32.70	30.69	16.34
100 mM KPi -His MAb	1.27	2.48	1.88	
100 mM NaPi +His MAb	23.99	23.91	23.95	13.08
100 mM NaPi -His MAb	1.56	2.10	1.83	
50 mM Tris.HCl +His MAb[#]	16.95	23.11	20.03	25.39
50 mM Tris.HCl -His MAb	0.94	0.64	0.79	
Promega buffer +His MAb	6.59	17.77	12.18	15.32
Promega buffer -His MAb	0.78	0.81	0.80	

[#] 50 mM Tris.HCl pH 7.4 buffer provides optimal signal to noise ratio

Table 5 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC in 50 mM Tris.HCl of various pH values

Comparison of Buffer pH	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
pH 6.0 +His MAb	33.83	36.61	4.33	9.40
pH 6.0 -His MAb	0.92		0.46	
pH 6.5 +His MAb	31.00	31.28	3.66	8.64
pH 6.5 -His MAb	0.99		0.42	
pH 7.0 +His MAb[#]	33.30	61.32	3.87	8.62
pH 7.0 -His MAb	0.54		0.45	

pH 7.5 +His MAb	23.64	14.94	2.96	7.07
pH 7.5 -His MAb	1.58		0.42	
pH 8.0 +His MAb	15.87	10.32	1.93	4.47
pH 8.0 -His MAb	1.54		0.43	
pH 8.5 +His MAb	10.91	5.90	1.32	3.14
pH 8.5 -His MAb	1.85		0.42	

[#] 50 mM Tris.HCl pH 7.0 buffer provides optimal signal to noise ratio

The experimental results indicate that in the presence of 1/100 diluted serum the use of certain buffers impacts on the rate of luminescence signal (produced by His MAb forced enzyme complementation) as well as influencing the rate of spontaneous/self complementation (background luminescence in the absence of analyte), resulting in variable signal to noise ratios. Tris.HCl limits background luminescence whilst maintaining excellent luminescent output resulting in the best possible signal to noise ratio, particularly at pH 7.0. All ensuing reactions were done using 50 mM Tris.HCl pH 7.0.

Metal chelators

Native RLuc is inhibited by divalent metal cations (Matthews et al., 1977, Biochemistry 16(1): 85-91), a process that is reversible by metal chelation. In addition, FEC is designed for the detection of analyte in human serum samples which contain high levels of metal cations, including for example, zinc (70-150 µg/dL), copper (70-155 µg/dL), iron (60-180 µg/dL) and manganese (0.04-1.4 µg/dL) (Fundamentals of Clinical Chemistry. N.W. Teitz, Ed. Philadelphia, W.B. Saunders Company, 1987). Therefore the use of metal chelators is obligatory for human serum analyte detection. The assay was prepared as follows: 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG) was added to a dilution series of EDTA (10 mM to 1 mM), in the presence of 50 mM Tris.HCl pH 7.0, 1 x PLB, 0.5 M NaCl, 5 mM DTT and control serum (1/100) in a final reaction volume of 100 µL. To the first set of wells, 5 nM His MAb was added and mixed, whereas the second set contained no His MAb. The plate was incubated for 10 minutes at RT following which 1 µl Promega 100 x assay substrate was added per well and the kinetics of light emission were measured over 10 min with a 1 sec integration per well. Endpoint readings were subsequently taken (at the completion of the kinetic read) with a 5 sec integration using an LmaxII^{384} luminometer (Molecular Devices, Melbourne, Australia). Signal to noise ratios were

determined by comparing the rates (mRLU·min^{-1}) of reaction calculated over the first 50-100 V_{max} points (using SoftMaxPro V5 software). It was determined for example; the effect of EDTA in final concentrations of 10 mM, 5mM and 1 mM, the signal to noise ratio was 19.54, 24.61 and 25.95 and 8.66, 10.04 and 9.70 respectively for kinetic reads or endpoint determination, respectively.

It was observed that in the presence of 1/100 diluted serum a certain range of concentration of metal chelator is required to provide optimal signal to noise ratios, which in this case was achieved with 5 mM EDTA. Therefore, as a precaution, all subsequent reactions had 5 mM EDTA included in order to limit the inhibition of reconstituted RLuc activity by metal cations.

Salts

Electrostatic effects have an important function on both substrate catalysis and protein-protein interactions, and these effects can be modulated or modified using different salt concentrations. Different sodium chloride (NaCl) concentrations were tested to determine their effect on forced enzyme complementation and on the resulting light emission (Table-5). The assay was prepared as follows: 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG) was added to a dilution series of NaCl (1 M, 0.75 M, 0.5 M, 0.25 M and 0 M), in the presence of 50 mM Tris.HCl pH 7.0, 1 x PLB, 5 mM DTT and control serum (1/100) in a final reaction volume of 100 µL. To the first set of wells, 5 nM His MAb was added and mixed, while the second set contained no His MAb. The plate was incubated for 10 minutes at RT following which 1 µl Promega 100 x assay substrate was added per well and the kinetics of light emission were measured over 10 min with a 1 sec integration per well using an LmaxII^{384} luminometer (Molecular Devices, Melbourne, Australia).

Table 6 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC in variable NaCl concentrations

Comparison of [NaCl]	Kinetic value + His MAb	Kinetic value - His MAb	Kinetic Ratio
0 M NaCl	92.85	9.65	9.63
0.25 M NaCl#	59.29	2.38	24.90
0.5 M NaCl	26.28	2.71	9.72
0.75 M NaCl	19.31	2.60	7.43
1 M NaCl	7.23	3.64	1.99

0.25 M provides an optimal signal to noise ratio

It was determined that the absence of NaCl from the reaction resulted in very high background autoluminescence/self-complementation whilst also providing a very high rate of light emission (Table 6). This leads to a poor signal to noise ratio, therefore the inclusion of 0.25 M NaCl is able to maintain high rates of reaction and low rates of background luminescence, resulting in high signal to noise ratios. Addition of NaCl above 0.5 M to the FEC assay does not provide any further benefit, however it does adversely affect the signal.

Reducing agents

The use of reducing agents was shown to be essential for the generation of extended glow luminescence rather than flash emission (refer to Table 3). It is important to empirically determine the concentration of reducing agents in order to ensure that critical disulphide bonds present in proteins such as the target antibody are not reduced thus maintaining analyte integrity, whilst ensuring that the extended glow luminescent signal is not impeded. Various DTT concentrations were tested to determine their effect on light emission and the ability of the analyte to induce forced enzyme complementation (Table 7). The final concentrations of DTT tested were 5 mM, 2 mM and 1 mM and the assay was prepared as follows: 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG) was added to 50 mM Tris.HCl pH 7.0, 1 x PLB, 0.25 M NaCl, 5 mM EDTA and control serum (1/100) in a final reaction volume of 100 µL. Five nM His MAb was added to the first set of wells, while control wells did not contain His MAb. The plate was incubated for 10 minutes at RT following which 1 µl Promega 100 x assay substrate was added to each well and the kinetics of light emission were measured over 10 min with a 1 sec integration per well. Endpoint readings were subsequently taken (at the completion of the kinetic read) with a 5 sec integration using an LmaxII^{384} luminometer.

Table 7 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using various DTT concentrations

Comparison of [DTT]	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
1 mM DTT + His MAb	9.32	15.28	1.74	5.19
1 mM DTT - His MAb	0.61		0.34	
2 mM DTT + His MAb#	13.23	19.10	2.14	5.39
2 mM DTT - His MAb	0.69		0.40	
5 mM DTT + His MAb#	19.94	17.04	3.19	6.72
5 mM DTT - His MAb	1.17		0.47	

[#] 2-5 mM DTT provides an optimal signal to noise ratio

It was observed that increasing concentrations of DTT could result in increased levels of fragment self-association (background) and increased light emission as determined with either kinetic or endpoint readings (Table 7). The optimal DTT concentration required is therefore a balance between producing the best signal to noise ratio. Furthermore, the temporal addition of DTT is important in maintaining high signal to noise ratios. The signal to noise ratio of DTT addition at the completion of the analyte incubation time is less than that of DTT added to the reaction prior to analyte incubation, 7.1:1 and 74:1 with kinetic reading or 4.3:1 and 5.8:1 with endpoint reading respectively.

Denaturants

Previous experience with the FEC technology has indicated that a degree of protein denaturation is essential for minimising the natural affinity of reporter enzyme fragments for each other (spontaneous complementation), whilst allowing for interaction and conformational change when fragments are directed into close proximity by the analyte (forced complementation). In addition, it has been previously reported that human serum albumin (HSA) is the primary cause of coelenterazine auto-oxidation (Zhao et al., 2004, Mol Imaging 3, 43-54). Given that human serum is the primary sample medium for FEC development and that the HSA reference range is 3.5-5.0 g/dL (Fundamentals of Clinical Chemistry. N.W. Teitz, Ed. Philadelphia, W.B. Saunders Company, 1987) in healthy human serum, we found that thiourea derivatives are able to both bind albumin and behave as strong denaturants (Cui et al., 2007, Anal Sci 23, 719-725). In addition, US Patent 7,118,878 describes the use of thiourea to effectively reduce analyte-independent luminescence without affecting analyte-dependent luminescence. Assays were performed in which concentrations of thiourea ranging from 6% w/v – 0% w/v were tested with 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG). Reactions were carried out in 50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT, control serum (1/100) and an in-house formulation of 1 x enhancer buffer (30 mM HEPES pH 8.0, 10% glycerol, 0.2% CHAPS) in a final reaction volume of 100 µL. Five nM His MAb was added to the first set of wells, while no His MAb was added to the second set of wells and the reaction was incubated for 10

minutes at RT following which 1 µl Promega 100 x assay substrate was added to each well and the kinetics of light emission (10 min with a 1 sec integration) and endpoint readings (5 sec integration) were recorded with an LmaxII^{384} luminometer.

Table 8 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using various thiourea concentrations

Comparison of [Thiourea]	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
0% thiourea + His MAb	47.55	1.40	23.51	1.35
0% thiourea - His MAb	34.00		17.42	
0.5% thiourea + His MAb	167.72	6.82	23.34	4.65
0.5% thiourea - His MAb	24.59		5.02	
1% thiourea + His MAb	182.14	19.78	19.93	9.25
1% thiourea - His MAb	9.21		2.15	
1.1% thiourea + His MAb	176.74	23.10	18.31	10.76
1.1% thiourea - His MAb	7.65		1.70	
1.2% thiourea + His MAb	148.59	15.38	15.34	8.93
1.2% thiourea - His MAb	9.66		1.72	
1.3% thiourea + His MAb	131.05	23.74	13.45	11.55
1.3% thiourea - His MAb	5.52		1.17	
1.4% thiourea + His MAb#	133.70	35.05	13.67	15.17
1.4% thiourea - His MAb	3.82		0.90	
1.5% thiourea + His MAb#	97.61	39.57	10.79	13.64
1.5% thiourea - His MAb	2.47		0.79	
2% thiourea + His MAb	33.99	31.94	3.92	9.46
2% thiourea - His MAb	1.06		0.41	
4% thiourea + His MAb	0.02		0.07	1.06
4% thiourea - His MAb	-0.49		0.06	
6% thiourea + His MAb	-0.27		0.05	1.09
6% thiourea - His MAb	-0.18		0.05	

1.4% w/v – 1.5% w/v thiourea provides an optimal signal to noise ratio

The results (Table 8) demonstrate that the addition of thiourea at approximately 1.4% w/v is essential in generating optimal signal to noise ratios by increasing FEC derived light emission while at the same time decreasing background. In particular the omission of thiourea from the reaction conditions results in extremely high autoluminescence/fragment self-association. It was understood that at low concentrations of thiourea (less than or equal to 2%) the enzyme fragments are partially denatured, thereby reducing their natural affinity for each other limiting spontaneous complementation, whilst still allowing for interaction and conformational change when directed into close proximity by the analyte (forced complementation). Thiourea also likely restricts coelenterazine auto-oxidation caused by HSA.

Detergents

Detergents are widely used to reduce protein-protein interactions and non-specific binding in immunoassays. The use of ionic detergents in FEC to reduce self-association of fragments in the absence of analyte is critical to further increase the signal to noise ratio. We selected the nondenaturing zwitterionic detergent 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) to limit any further denaturation of the RLuc fragments already maintained in a mildly denaturing environment with the use of thiourea. Various concentrations of CHAPS (2% w/v – 0% w/v) were tested in the presence of 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG) in 50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT, control serum (1/100) and an in-house formulation of 1 x enhancer buffer (30mM HEPES pH 8.0, 10% glycerol, 1.4% w/v thiourea) in a final reaction volume of 100 µL. Analyte (5 nM His MAb) was added and the reactions were incubated for 10 minutes at RT. Substrate was then added (1 µl Promega 100 x assay substrate) to each well and the kinetics of light emission (10 min with 1 sec integration) and endpoint readings (5 sec integration) were read using an LmaxII^{384} luminometer.

Table 9 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using various CHAPS concentrations

Comparison of [CHAPS]	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
0% CHAPS + His MAb	104.13	12.97	13.66	6.46
0% CHAPS - His MAb	8.03		2.11	
0.1% CHAPS + His MAb	115.63	70.42	12.45	15.26
0.1% CHAPS - His MAb	1.64		0.82	
0.2% CHAPS + His MAb	63.19	63.07	6.58	14.68
0.2% CHAPS - His MAb	1.00		0.45	
0.3% CHAPS + His MAb	31.11		3.28	14.06
0.3% CHAPS - His MAb	-0.24		0.23	
0.4% CHAPS + His MAb	13.25		1.48	9.34
0.4% CHAPS - His MAb	-0.36		0.16	
0.5% CHAPS + His MAb	4.33		0.78	7.14
0.5% CHAPS - His MAb	-0.40		0.11	
1% CHAPS + His MAb	-0.30		0.10	1.20
1% CHAPS - His MAb	-0.29		0.08	
2% CHAPS + His MAb	-1.20		0.08	1.00
2% CHAPS - His MAb	-0.34		0.08	

[#] 0.1% w/v – 0.2% w/v CHAPS provides an optimal signal to noise ratio

At certain concentrations of detergent, for example 0.1% w/v - 0.2% w/v CHAPS (Table 9) the increase in the signal to noise ratio was effectively produced by

decreasing the background luminescent signal to a greater extent than reducing analyte derived FEC signal.

Comparison of enhancer buffer compositions

The use of Matthew's lysis buffer is for the resuspension of mammalian cells expressing RLuc reporter enzyme. We previously determined that the generation of extended glow luminescence from full-length RLuc using the commercially available *Renilla* Luciferase Assay System (Promega) relied on the addition of DTT and EDTA, as well as the 5 x Promega lysis buffer (PLB) component (proprietary composition) of the *Renilla* Luciferase Assay System (Promega). Therefore we formulated an in-house 5 x enhancer buffer using the known Matthew's lysis buffer composition and based on observations listed in the present invention. The in-house 5 x enhancer buffers were formulated as follows:

5 x PB enhancer buffer A

150 mM HEPES pH 8
1% CHAPS
5% thiourea
50% glycerol

5 x PB enhancer buffer B

150 mM HEPES pH 8
1% CHAPS
7% thiourea
50% glycerol

5 x PB enhancer buffer C

150 mM HEPES pH 8
1% CHAPS
5% thiourea
25% glycerol

We tested each of the enhancer buffers (1 x final conc.) in the presence of 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG) in 50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT and control serum (1/100) in a final reaction volume of 100 µL. To the first set of wells, 5 nM His MAb was added and mixed, while the second set contained no His MAb. The plate was incubated at RT (10 min) and 1 µl Promega 100 x assay substrate was added. Using an LmaxII^{384} luminometer, kinetic (10 min, 1 sec integration) and endpoint (5 sec integration) readings were obtained and signal to noise ratios were determined as above.

Table 10 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using various 5 x enhancer buffer compositions

Comparison of different lysis buffer formulations	Mean Kinetic value	Ratio	Mean Endpoint value	Ratio
Promega lysis buffer + His MAb	43.71	41.71	5.09	10.80
Promega lysis buffer - His MAb	1.05		0.47	
PB enhancer buffer A + His MAb	172.23	16.42	21.29	8.88
PB enhancer buffer A - His MAb	10.49		2.40	
PB enhancer buffer B + His MAb	55.62	37.10	5.95	12.36
PB enhancer buffer B - His MAb	1.50		0.48	
PB enhancer buffer C + His MAb#	37.40	95.64	4.16	14.39
PB enhancer buffer C - His MAb	0.39		0.29	

PB enhancer buffer C provides an optimal signal to noise ratio

It was observed that PB enhancer buffer C could provide 2.3 fold and 1.3 fold increases in the signal to noise ratio with kinetic and endpoint readings respectively (Table 10), over that obtained with the Promega lysis buffer. PB enhancer buffer C (30 mM HEPES pH 8, 0.2% CHAPS, 1% thiourea, 5% glycerol – final reaction concentration) in combination with 50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, and 2.5 mM DTT in the presence of a 1/100 dilution of serum can yield an excellent signal (37 mRLU·min^{-1}) and negligible self-association/autoluminescence (0.4 mRLU·min^{-1}) resulting in a signal to noise ratio of 96:1 and 14:1 with kinetic and endpoint reading respectively (exemplary results are shown in FIG. 10).

Comparison of coelenterazine analogs

Synthetic analogs of native coelenterazine (FIG. 3) produce variable luminescent intensities and response times (Inouye et al., 1997, Biochem. Biophys. Res. Commun. 233, 349-353). RLuc FEC produces excellent results using the Promega 100 x assay substrate; however the composition and substrate concentration are unknown.

We compared 8 synthetic coelenterazine analogs to native coelenterazine and to the Promega 100 x assay substrate. RLuc_α_{229}P1 and RLuc_ω_{230}ProG (5 nM) were assayed in 50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT, 1 x PB enhancer buffer C and control serum (1/100) in a final reaction volume of 100 µL. Analyte was added (5 nM His MAb) and the plate was incubated at RT (10 min). Stock coelenterazine substrates (coelenterazine sampler kit, Sigma, Australia) were resuspended in 100% ethanol (500 µM substrate) and used at a final concentration of 5 µM. Therefore, 1 µl of coelenterazine substrate including Promega 100 x assay substrate was added to the assay at the completion of analyte incubation and read using an LmaxII^{384} luminometer. Kinetic (10 min with 1 sec integration) and endpoint (5 sec integration) readings were obtained and signal to noise ratios were determined.

Table 11 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using various coelenterazine analogs

Comparison of Coelenterazine analogs	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
5 µM coelenterazine native + His MAb	4.39		0.64	6.09#
5 µM coelenterazine native - His MAb	-0.31		0.11	
5 µM coelenterazine cp + His MAb	-0.14		0.13	2.56
5 µM coelenterazine cp - His MAb	-0.50		0.05	
5 µM coelenterazine f + His MAb	2.74		0.46	4.16#
5 µM coelenterazine f - His MAb	-0.55		0.11	
5 µM coelenterazine fcp + His MAb	0.52		0.27	2.77
5 µM coelenterazine fcp - His MAb	-0.58		0.10	
5 µM coelenterazine h + His MAb	7.23		1.41	6.57#
5 µM coelenterazine h - His MAb	-1.22		0.21	
5 µM coelenterazine hcp + His MAb	0.80		0.35	2.39
5 µM coelenterazine hcp - His MAb	-0.57		0.15	
5 µM coelenterazine i + His MAb	-0.59		0.20	1.43
5 µM coelenterazine i - His MAb	-0.60		0.14	
5 µM coelenterazine ip + His MAb	-0.65		0.08	1.63
5 µM coelenterazine ip - His MAb	-0.57		0.05	
5 µM coelenterazine n + His MAb	1.15		0.45	4.69#
5 µM coelenterazine n - His MAb	-1.28		0.10	
1 x Promega assay substrate + His MAb	43.10	74.69	6.04	12.96
1 x Promega assay substrate - His MAb	0.58		0.47	

Native coelenterazine and coelenterazine analogs f, h and n produce good signal to noise ratios

It was observed that synthetic coelenterazine h analog outperforms other substrate analogs (Table 11), however at 5 µM it produces a signal to noise ratio that is ½ (endpoint) that obtained with the Promega assay substrate. Further testing of native coelenterazine and coelenterazine analogs f, h and n with some modification to the current PB assay and PB enhancer buffer compositions will result in further

improvements to the light emission produced by RLuc fragments forced into close proximity by analyte in the presence of human serum. Of particular interest is the total absence of background luminescence (autoluminescence/fragment self-association) in the assays utilising 5 μM coelenterazine substrate analogs with either kinetic or endpoint determination.

Example 2: Homogeneous *in vitro Renilla* luciferase FEC reagent storage buffer components.

Full-length RLuc is susceptible to loss of activity and prone to aggregation upon storage at a concentration of greater than 0.5 mg/mL at 4°C (Matthews et al., 1977, Biochemistry 16(1): 85-91). It is expected that splitting the full-length RLuc parental enzyme at amino acids 229/230 followed by addition of analyte interaction moieties, may increase the propensity of the recombinant fragments to lose activity and stability. Indeed, producing fragments from a monomeric enzyme can result in the exposure of the hydrophobic core resulting in aggregation upon storage. A single point mutation, C124A, introduced into the α fragment of RLuc to enhance enzyme stability (Liu et al., 1999, Gene 237: 153-159), goes some way to addressing these concerns. However, chemical components added to the fragment storage buffer are a benign way in which to tackle protein stability during storage without affecting the FEC process. The procedures outlined herein describe the components used to extend the half-life of dilute concentrations of RLuc fragments stored at 4°C. RLuc fragments used in this example include RLuc_α_{229}P1 and RLuc_$_{230}$ωProG, their synthesis and characterisation are described in example 1 of the invention.

RLuc fragment stabilising excipients

Buffer additives known to increase the stability of dilute concentrations of proteins in solution can be composed of solvents, inert proteins and sugars. Protein degradation can be reduced upon storage by the inclusion of reducing agents, hydrophobic additives, protease inhibitors and antibacterial agents. Finally, lyophilization can also be used for long-term protein storage. In this example, we focus on increasing the stability of working dilutions (100 nM) of both the α and ω purified fragments. Sugars (trehalose and sucrose) and inert proteins (BSA and gelatin) were

tested for their ability to retain fragment integrity and activity as determined by His MAb directed FEC following extended fragment storage at 4°C. Separate α and ω fragment working dilutions (100 nM) were prepared by dissolving individual purified fragments into solutions of 1.5 M trehalose, 1.5 M sucrose, 2 mg/mL BSA (Pierce), 2 mg/mL fish gelatin (Sigma) and 50 mM sodium phosphate buffer pH 7.0. Assays were performed as described herein; 5 nM of each fragment (RLuc_α_{229}P1 and RLuc_ω_{230}ProG) from the various stabilising solutions were added to 50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT, control serum (1/100) and 1 x PLB, in a final reaction volume of 100 µL. Analyte (5 nM His MAb) was added and the reactions were incubated for 10 minutes at RT. Substrate was then added (1 µl Promega 100 x assay substrate) to each well and the kinetics of light emission (10 min with 1 sec integration) and endpoint readings (5 sec integration) were read using an LmaxII^{384} luminometer. Assays were repeated at various time intervals up to 6 days storage at 4°C.

Table 12 – Rate of light emission (mRLU·min^{-1}) generated by His MAb FEC using various fragment stabilising excipients

Comparison of fragment stabilisers	Time = 0 hrs				Time = 4 hrs			
	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
Trehalose + His MAb	27.89	5.18	4.00	2.80	38.49	13.87	5.49	4.53
Trehalose - His MAb	5.38		1.43		2.78		1.21	
Sucrose + His MAb	42.41	8.58	5.08	3.74	70.50	31.33	7.80	7.13
Sucrose - His MAb	4.95		1.36		2.25		1.09	
BSA + His MAb	39.90	21.04	4.60	5.21	56.47	150.99	6.60	8.43
BSA - His MAb	1.90		0.88		0.37		0.78	
Gelatin + His MAb	38.96	19.47	4.39	5.55	60.15	76.04	6.62	10.23
Gelatin - His MAb	2.00		0.79		0.79		0.65	
NaPi pH7 + His MAb	38.49	40.56	4.32	7.62	43.00	31.69	4.62	9.84
NaPi pH7 - His MAb	0.95		0.57		1.36		0.47	
Comparison of fragment stabilisers	**Time = 24 hrs**				**Time = 6 days**			
	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio	Kinetic value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
Trehalose + His MAb	51.44	7.91	6.87	4.45	33.74	9.32	5.23	4.58
Trehalose - His MAb	6.50		1.54		3.62		1.14	
Sucrose + His MAb	60.91	8.65	7.29	5.42	47.31	19.52	5.89	6.93
Sucrose - His MAb	7.04		1.35		2.42		0.85	
BSA + His MAb	61.23	24.51	7.18	7.52	27.88	14.36	3.64	6.12
BSA - His MAb	2.50		0.95		1.94		0.60	
Gelatin + His MAb	39.24	20.87	4.53	8.43	10.19	105.06	1.34	5.14
Gelatin - His MAb	1.88		0.54		0.10		0.26	
NaPi pH7 + His MAb	29.06	14.82	3.39	7.84	7.63	15.32	1.03	4.52
NaPi pH7 - His MAb	1.96		0.43		0.50		0.23	

Fragment dissolution in sucrose, BSA and fish gelatin produced excellent stabilisation of low concentration fragments stored for up to 6 days at 4°C, resulting in high signal to noise ratios (Table 12). Fragments stored in 50 mM sodium phosphate buffer pH 7.0 lost significant enzymatic activity resulting in a decrease of 80% and 76% in light emission for kinetic and endpoint readings, respectively. Stabilising solutions composed of half strength solutions mixed in combinations as follows; 0.75 M sucrose, 1 mg/mL BSA (diluent #1) and 0.75 M sucrose, 1 mg/mL fish gelatin (diluent #2) were also tested as above following storage at 4°C for up to 18 days (Table 13).

Table 13 – Rate of light emission ($mRLU \cdot min^{-1}$) generated by His MAb FEC using various fragment stabilising diluents

Comparison of fragment stabilizers	Time = 0 days				Time = 6 days			
	Kinetic Value	Kinetic Ratio	Endpoint Value	Endpoint Ratio	Kinetic Value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
Diluent #1 + His MAb	65.04	20.74	8.49	10.19	47.89	10.61	1.23	5.80
Diluent #1 - His MAb	3.14		0.83		4.51		0.21	
Diluent #2 + His MAb	35.54	58.93	5.12	15.73	22.95	16.29	0.56	5.40
Diluent #2 - His MAb	0.60		0.33		1.41		0.10	
NaPi + His MAb	136.69	38.88	17.76	25.99	0.02		0.04	1.41
NaPi - His MAb	3.52		0.68		-0.89		0.03	
37.5 mM Sucrose + His MAb	23.23		4.27	9.33	34.97	17.16	0.86	7.32
37.5 mM Sucrose - His MAb	-0.88		0.46		2.04		0.12	
0.5 mg/mL BSA + His MAb	16.30		2.86	13.41	41.81		0.96	9.66
0.5 mg/mL BSA - His MAb	-0.85		0.21		-0.51		0.10	
0.5 mg/mL Fish Gelatin + His MAb	31.98		5.64	11.26	-0.12		0.03	1.00
0.5 mg/mL Fish Gelatin - His MAb	-0.15		0.50		-0.57		0.03	
Comparison of fragment stabilizers	**Time = 12 days**				**Time = 18 days**			
	Kinetic Value	Kinetic Ratio	Endpoint Value	Endpoint Ratio	Kinetic Value	Kinetic Ratio	Endpoint Value	Endpoint Ratio
Diluent #1 + His MAb	91.55	25.61	1.93	11.83	25.56	26.27	0.70	5.96
Diluent #1 - His MAb	3.57		0.16		0.97		0.12	
Diluent #2 + His MAb	0.04		0.05	1.69				
Diluent #2 - His MAb	-1.14		0.03					
NaPi + His MAb	0.08		0.03	1.04				
NaPi - His MAb	-1.15		0.02					
37.5 mM Sucrose + His MAb	39.86	44.39	0.90	8.20	11.66		0.35	6.96
37.5 mM Sucrose - His MAb	0.90		0.11		-0.96		0.05	
0.5 mg/mL BSA + His MAb	31.15	24.94	0.69	8.16	8.70		0.25	7.26
0.5 mg/mL BSA - His MAb	1.25		0.08		-1.28		0.03	
0.5 mg/mL Fish Gelatin + His MAb	-0.13		0.03	1.29				
0.5 mg/mL Fish Gelatin - His MAb	-0.64		0.02					

It was observed that combinations of certain concentrations of sugars and inert proteins are able to maintain excellent light emission, negligible background (autoluminescence/fragment self-association) and diagnostically relevant signal to noise ratios. Extended RLuc fragment storage (18 days) at 4°C resulted in increased stability

and decreased aggregation of the final product, both of which provide significant advantages for commercialisation of homogeneous *in vitro* FEC assays based on RLuc.

Example 3: Homogeneous *in vitro Renilla* luciferase FEC assays for polyclonal HSV-1 and HSV-2 IgG specific patient antibody detection using glow luminescence.

Examples 1 and 2 describe the synthesis, purification and characterisation of RLuc fragments generated by splitting the full-length parental enzyme (RLuc) at amino acids 229/230. The α fragments incorporating a single point mutation (C124A) were linked to a flexible linker [$(G_4S)_3$], HSV-1 or HSV-2 truncated antigen and polyhistidine tag at the breakpoint termini resulting in the generation of RLuc_α_{229}P1 and RLuc_α_{229}P2, for HSV-1 and HSV-2 detection, respectively. Introduction of a polyhistidine tag, flexible linker and protein-G subunit at the breakpoint termini of the ω fragment resulted in the generation of an IgG antibody binding reporter enzyme fragment designated RLuc_$_{230}$ωProG. These fragment pair members were used to demonstrate forced enzyme complementation in the presence of an antibody analyte (anti penta-histidine monoclonal antibody binding to histidine tag) in an optimised homogeneous *in vitro* assay format utilising PB assay buffer (50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT) and 5 x PB enhancer buffer C (150 mM HEPES pH 8, 1% CHAPS, 5% thiourea and 25% glycerol) of the invention.

In this example, the enzyme fragments were used to detect disease specific IgG antibodies in patient serum samples (HSV-1 and HSV-2 IgG specific patient polyclonal antibody binding to P1 and P2 truncated antigens respectively) by forced enzyme complementation in a homogeneous *in vitro* assay format.

Renilla luciferase based FEC assay for HSV-1 specific IgG detection

RLuc FEC assays were performed in 96-well white Costar plates (Corning, Australia) using the *Renilla* Luciferase Assay Kit (Promega, Australia). Sera from five normal individuals (HSV-1/HSV-2 negative) and five individuals infected with HSV-1 positive/HSV-2 negative and HSV-2 positive/HSV-1 negative (Brisbane, Australia) were confirmed using an existing commercial assay, HerpeSelect1 and 2 ELISA IgG

(Focus Diagnostics, USA). FEC homogeneous assays were carried out in a 200 µL reaction mix containing 5 nM RLuc_α_{229}P1 and RLuc_$_{230}\omega$ProG, 0.5 x Promega lysis buffer (PLB), 0.5 x PBS pH 7.3, 0.5 M NaCl. Sera were added to a final concentration of 1/50 and reactions were incubated for 30 min at RT on a platform rocker. DTT was then added for a final concentration of 2.5 mM, followed by the addition of 0.5 µl of 100 x Promega assay substrate. Light emission was measured with 1.5 sec integration per well and rates ($mRLU \cdot min^{-1}$) were calculated over the first 20 minutes (using an $Lmax\Pi^{384}$ luminometer). All readings were obtained in triplicate and the mean and standard errors were determined using GraphPad Prism 4.0 (GraphPad Software, Inc., CA, USA). Results were normalised against a sample of pooled (n=5) serum testing negative for both HSV-1 and HSV-2 (dividing data by the mean of the HSV negative population) resulting in arbitrary units defined as the HA Index.

Exemplary results are demonstrated in FIG. 11 of the invention and illustrate that *Renilla* luciferase based FEC can successfully detect type specific HSV-1 antibodies with high sensitivity (100%) and specificity (100%). Furthermore, use of RLuc FEC components of the invention, including PB assay buffer (50 mM Tris.HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 2.5 mM DTT), 5 x PB enhancer buffer C (150 mM HEPES pH 8, 1% CHAPS, 5% thiourea, 25% glycerol) and fragment stabilisation/storage buffer (0.75 M sucrose, 1 mg/mL BSA) will further improve the limit of detection and dynamic range of these results. This has been demonstrated by way of increased light emission, reduced autoluminescence derived from substrate auto-oxidation in the presence of human serum and from fragment self-association in the absence of analyte. We demonstrate the successful application of an *in vitro* homogeneous FEC assay for the detection of a diagnostically relevant target and within a suitable timeframe (approximately 1 hour) for uptake into pathology laboratories. The present invention provides significant advantages to the current immunodiagnostic field and provides the impetus for further advancement of homogeneous *in vitro* FEC assays based on RLuc for the detection of antigens.

Example 4: Homogeneous *in vitro Renilla* luciferase FEC assays for Hepatitis B surface antigen detection using glow luminescence.

Example 3 describes the use RLuc enzyme fragments for the detection of disease specific IgG antibodies in patient serum samples (HSV-1 IgG specific patient polyclonal antibody binding to P1 truncated antigen) by forced enzyme complementation in a homogeneous *in vitro* assay format. The limit of detection (LOD) required for antibody-based analytes in human serum is in the high picomolar (pM, 10^{-12} M) range, whilst the detection of antigens, for example, Hepatitis B surface antigen (HBsAg), is in the low femtomolar (fM, 10^{-15} M) to attomolar (aM, 10^{-18} M) range (Anderson et al., 2002, Mol. Cell Proteomics 1(11): 845-867), as illustrated in FIG. 1. In addition to the need for greater LODs, antigen detection requires the use of antigen binding proteins such as antibodies on both the α and ω reporter enzyme fragments. In this example, the design and synthesis of fragments capable of HBsAg detection is described. Synthetic full-length parental enzyme (RLuc) incorporating a single point mutation (C124A) for enhanced stability was split at amino acid position S91/Y92. The α fragment was linked to a flexible linker, HBsAg single chain antibody (scFv) and polyhistidine tag at the breakpoint termini resulting in the generation of RLuc_α_{91}A5. Bose et al. (2003) cloned and expressed an scFv of a mouse monoclonal antibody (5S) against the 'a' epitope of the HBsAg S domain. Likewise, the ω fragment was linked to a polyhistidine tag, flexible linker and second HBsAg scFv at the breakpoint termini of the ω fragment generating RLuc_$_{92}$ωE11. The E11 designated scFv was originally cloned and expressed from a mouse monoclonal antibody (125E11) against the PreS1 (21-47) fragment of the large HBsAg (Yang et al., 2005, Protein Expr. Purif. 41:341-348). Together these two fragments should be capable of *in vitro* homogeneous FEC detection of HBsAg in human serum samples.

HBsAg A5 and E11 scFv synthetic synthesis and cloning into universal plasmids encoding cassette domains

Codon optimised A5-scFv (SEQ ID NOS 31 and 32) and E11-scFv (SEQ ID NOS 33 and 34) genes were synthesised by DNA2.0 (Menlo Park, U.S.A) for *E.coli* expression. Synthetic pJ36-A5 and pJ36-E11 plasmid (2 μg) was supplied on a filter disk that was purified using the QIAprep spin miniprep kit (Qiagen, Australia) by

incubating the filter disk in appropriate buffer, applying the supernatant to a QIAprep spin column and completing the purification according to the manufacturer's instructions. Purified plasmid was digested with *BamHI/Spe*I and the A5-scFV *BamHI/Spe*I insert was cloned into the *BamHI/Spe*I digested universal pET-α plasmid encoding a C-terminal antigen specific cassette [$(G_4S)_3$ linker, antigen and G_4S linker and histidine tag (6H)], yielding pET- αA5. Similarly, the E11-scFV *BamHI/Spe*I insert was cloned into the *BamHI/Spe*I digested universal pET-ω plasmid encoding a N-terminal antigen specific cassette [histidine tag (6H), G_4S linker, antigen and $(G_4S)_3$ linker], yielding pET- ωE11.

Construction of pET-Rluc_α_{91}A5

RLuc_α_{91} (residues 2-91) was amplified using forward primer RLuc3 and reverse primer RLuc4 as previously described in example 1. Amplification products were purified directly from the reaction tube using the QIAquick PCR purification kit (Qiagen) and were subsequently digested with *NdeI/KpnI* and cloned into *NdeI/KpnI* digested universal pET-αA5 destination plasmid encoding a C-terminal HBsAg scFv cassette [$(G_4S)_3$ linker, HBsAg-A5, G_4S linker and histidine tag], yielding pET-Rluc_α_{91}A5 (SEQ ID NOS 35 and 36).

Construction of pET-Rluc_ω_{92}E11

RLuc_$_{92}\omega$ (residues 92-311) incorporating the C124A mutation was amplified using forward primer RLuc5 and reverse primer RLuc6 as previously described in example 1. Amplification products were purified directly from the reaction tube using the QIAquick PCR purification kit (Qiagen) and were subsequently digested with *NdeI/KpnI* and cloned into *NdeI/KpnI* digested universal pET-ωE11 destination plasmid encoding a N-terminal HBsAg scFv cassette [histidine tag (6H), G_4S linker, HBsAg-E11 and $(G_4S)_3$ linker], yielding pET- Rluc_ω_{92}E11 (SEQ ID NOS 37 and 38).

Homogeneous in vitro FEC antigen assay

The detection of HBsAg in patient samples using Rluc_α_{91}A5 and Rluc_ω_{92}E11 is now possible given that appropriate HBsAg scFv antibodies have been identified and incorporated into RLuc fragments suitable for FEC. The description in the present invention of suitable reaction composition and conditions suggests that extended glow luminescence as a result of HBsAg directed FEC will likely produce a commercially viable HBsAg diagnostic.

Example 5: Application of glow luminescent homogeneous FEC assays for multiple analyte detection.

Example 3 describes the use of RLuc enzyme fragments for the detection of disease specific IgG antibodies in patient serum samples (HSV-1 IgG specific patient polyclonal antibody binding to P1 truncated antigen) by forced enzyme complementation in a homogeneous *in vitro* assay format. Simultaneous detection of two independent analytes within a single sample is known as multiplexing and in this instance can be applied to the detection of both HSV-1 and HSV-2. Dual analyte detection can be performed with the use of glow luminescent RLuc FEC to detect HSV-1 in combination with colorimetric β-lactamase FEC to detect HSV-2. The description and application of β-lactamase *in vitro* FEC for detection of HSV-1 and 2 in a homogeneous assay format has been described in PCT/AU2007/000508. In this example the reaction mix contains all the necessary components for generation of a colorimetric output signal from β-lactamase HSV-2 and for luminescent detection from RLuc HSV-1. The HSV-2 positive sample will be detected by an increase in relative light units (RLU) while the HSV-2 positive sample will be detected by an increase in absorbance at OD_{492nm} accompanying a colorimetric change; both signals are typically proportionate to the amount of analyte present in the sample. This design will combine two independent homogeneous assays into one single well resulting in the differentiation of HSV-1 and HSV-2 disease from a single patient sample in one single homogeneous assay reaction. Further potential to detect more than two disease markers is anticipated, as one can simply change between different wavelengths on the light filter used in the photomultiplier tube (PMT) of a luminometer and on the spectrophotometer for different enzyme substrates used.

Table 14. Expected result for multiplexing signals

	HSV-1 pos HSV-2 pos	HSV-1 pos HSV-2 neg	HSV-1 neg HSV-2 pos	HSV-1 neg HSV-2 neg
Renilla Luciferase based HSV-1 HA bioluminescent detection (RLU)	+	+	-	-
β-lactamase based HSV-2 HA colorimetric detection (OD_{492nm})	+	-	+	-

Example 6: Homogeneous *in vitro* β-lactamase FEC assays for glow luminescent detection.

An alternative strategy to the use of split bioluminescent reporter enzymes as previously described in Example 1, 3 and 4, is the use of split reporter enzymes that do not traditionally catalyse chemiluminescent reactions, by combining these with synthetically produced luminescent substrates that generate glow luminescence. In this example, the recently described bioluminogenic substrate for β-lactamase termed 'Bluco' is used in combination with split β-lactamase FEC in a homogeneous format to detect HSV-2 using glow luminescence *in vitro*. β-lactamase acts to open the β-lactam ring of the pro-luciferin substrate resulting in spontaneous fragmentation; *Firefly* luciferase subsequently acts on the luciferin producing light emission. *In vitro* β-lactamase FEC homogeneous assays for the detection of HSV-2 are described in patent PCT/AU2007/000508, which uses β-lactamase fragments fused to HSV-1 or 2 antigens for forced enzyme complementation (FEC) detection of patient HSV specific IgG. Here the same format is used however the chromogenic substrate nitrocefin is substituted with the β-lactamase substrate Bluco and firefly luciferase, ATP and $Mg2^{+}$.

β-lactamase based FEC assay for HSV-2 specific IgG detection using glow luminescence

β-lactamase FEC assays were performed in 96-well white Costar plates (Corning, Australia) using Bluco (Stanford University) and *Firefly* luciferase (Promega, Australia). Sera from normal individuals (HSV-1/HSV-2 negative) and individuals infected with HSV-2 positive/HSV-1 negative (Brisbane, Australia) were confirmed using an existing commercial assay, HerpeSelect1 and 2 ELISA IgG (Focus

Diagnostics, USA). FEC homogeneous assays were carried out in a 200 µL reaction mix containing 2 nM BLαM182T-HSV-2 and 4 nM BLωN276D-ProG in 50 mM sodium phosphate buffer, 150 mM NaCl, 0.05% CHAPS, 5 mM o-phenanthroline (OP), 1 mM EDTA, pH7.6 containing 100 µM Bluco, 1.5 mM ATP and 1.5 mM MgCl. Sera were added to a final concentration of 1/100 and reactions were incubated for 60 min at RT followed by the addition of 100 nM *Firefly* luciferase. Light emission was measured with 2 sec integration per well and rates ($mRLU \cdot min^{-1}$) were calculated over the first 30 minutes (using an $LmaxII^{384}$ luminometer). Exemplary results are demonstrated in Table 15 of the invention and illustrate that glow luminescent detection of β-lactamase based FEC employing a pro-luciferin substrate termed 'Bluco' in combination with *Firefly* luciferase can successfully detect type specific HSV-2 antibodies in a quantifiable manner. The differentiation between high, medium and low positive samples demonstrates the enhanced capacity of glow luminescence to provide a greater dynamic range thus improving the limit of detection of *in vitro* homogeneous FEC based assays. This has been demonstrated by way of increased light emission, reduced autoluminescence derived from substrate auto-oxidation in the presence of human serum and from reduced fragment self-association in the absence of analyte. We demonstrate the successful application of an *in vitro* homogeneous FEC assay for the detection of a diagnostically relevant target and within a suitable timeframe (approximately 1 hour) for uptake into pathology laboratories. The present invention provides significant advantages to the current immunodiagnostic field and provides the impetus for further advancement of homogeneous *in vitro* FEC assays based on glow luminescence for the detection of more complex analytes at sub femptomolar concentrations.

Table 15 - Result for β-lactamase glow luminescent HSV-2 detection using Bluco

	High Positive	Medium Positive	Low Positive	Negative
Kinetic Reading (mRLU/min)	1326	642	323	167
End point (RLU/2sec integration)	775	627	563	537

****

The various features and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, *mutatis mutandis*.

Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The references specified in the above text are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

CLAIMS

1. A method of determining the presence of an analyte of interest in a sample which method comprises contacting the sample with:

(i) a purified first reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest; and

(ii) a purified second reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest and operable in reconstituting an active enzyme upon association with the first reporter fragment pair member through the affinities of the interactor domains of the first and second reporter fragment pair members with the analyte of interest;

under *in vitro* assay conditions which allow the first and second reporter fragment pair members to associate in the presence of analyte of interest in the sample through the affinity of the interactor domains with the analyte to produce a glow luminescent signal through the action of the reconstituted active enzyme on a substrate;

and detecting the presence or absence, or extent, of a glow luminescent signal.

2. A method according to claim 1 wherein the first and second reporter fragment pair members are alpha and omega fragments of a luciferase polypeptide formed by breakpoint between amino acid residues serine 91 and tyrosine 92 or glycine 229 and lysine 230 of the *Renilla* luciferase amino acid sequence shown in SEQ ID NO:10, or the equivalent position in another luciferase sequence.

3. A method according to claim 1 wherein the active enzyme is a *Renilla* luciferase or a derivative thereof.

4. A method according to claim 3 wherein the luciferase comprises a C124A mutation.

5. A method according to any one of the preceding claims wherein the assay conditions comprise the presence of one or more reducing agents.

6. A method according to claim 5 wherein the total concentration of reducing agent is from about 1 to about 2% w/v.

7. A method according to any one of the preceding claims wherein the assay conditions comprise the presence of a denaturant.

8. A method according to claim 7 wherein the total concentration of denaturant is from about 1 to about 2% w/v.

9. A method according to any one of the preceding claims wherein the assay conditions comprise the presence of a detergent.

10. A method according to claim 9 wherein the detergent is CHAPS.

11. A method according to claim 9 or claim 10 wherein the total concentration of detergent is from about 0.1 to about 0.2% w/v.

12. A method according to any one of the preceding claims wherein the assay conditions comprise the presence of from about 0.25 to 0.5 M salt.

13. A method according to any one of the preceding claims wherein the assay conditions comprise the presence of Tris.HCl as a buffer.

14. A method according to any one of the preceding claims wherein the assay conditions comprise the presence of a luciferase substrate selected from the group consisting of coelenterazine analogues f, h and n.

15. A method according to any one of claims 1 to 14 wherein the interactor domain of at least one of the first and second reporter fragment pair members comprises one or more epitopes.

16. A method according to any one of claims 1 to 14 wherein the interactor domains of at least one of the first and second reporter fragment pair members comprises one or more complementarity determining regions.

17. A method according to any one of the preceding claims wherein the sample is a biological sample, such as a blood or serum sample.

18. A kit for performing a homogeneous *in vitro* forced enzyme fragment complementation assay to identify the presence of an analyte of interest in a sample, which kit comprises:

(a) a purified first reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest;

(b) a purified second reporter fragment pair member comprising an interactor domain with affinity for the analyte of interest and operable in reconstituting enzyme activity upon association with the first reporter fragment pair member through the affinities of the interactor domains of the first and second reporter fragment pair members with the analyte of interest; and

(c) reagents for providing *in vitro* assay conditions which allow the first and second reporter fragment pair members to associate in the presence of analyte of interest in the sample, through the affinity of the interactor domains with the analyte, to produce a glow luminescent signal through the action of the active enzyme on a substrate, wherein a glow luminescent signal indicates the presence of the analyte of interest in the sample.

19. Use of a kit according to claim 18 for determining the presence of an analyte of interest in a sample.

20. Use according to claim 19 wherein the analyte of interest is an antibody.

21. Use according to claim 19 wherein the analyte of interest is an antigen.

22. An assay reagent storage composition comprising (i) one or more reporter polypeptides at a total concentration of greater than about 10 nM and (ii) one or more sugars and/or one or more inert proteins.

23. A composition according to claim 22 wherein the one or more reporter polypeptides are luciferase reporter polypeptides for an *in vitro* forced enzyme complementation assay.

24. A composition according to claim 22 or claim 23 wherein the one or more reporter polypeptides are present at a total concentration of greater than about 50 nM.

25. A composition according to any one of claims 22 to 24 where the one or more sugars are selected from the group consisting of sucrose, trehalose and mixtures thereof.

26. A composition according to any one of claims 22 to 25 wherein the total concentration of the one or more sugars is at least about 50 mM.

27. A composition according to any one of claims 22 to 26 where the one or more inert proteins is selected from the group consisting of serum albumin, gelatine and mixtures thereof.

28. A composition according to any one of claims 22 to 27 wherein the total concentration of the one or more inert proteins is at least about 1 mg/ml.

29. Use of a composition comprising one or more sugars and/or one or more inert proteins to stabilise one or reporter polypeptides in an assay reagent storage composition comprising the one or more reporter polypeptides at a total concentration of greater than about 10 nM.

30. Use according to claim 29 wherein the one or reporter polypeptides are luciferase reporter polypeptides suitable for an *in vitro* forced enzyme complementation assay.

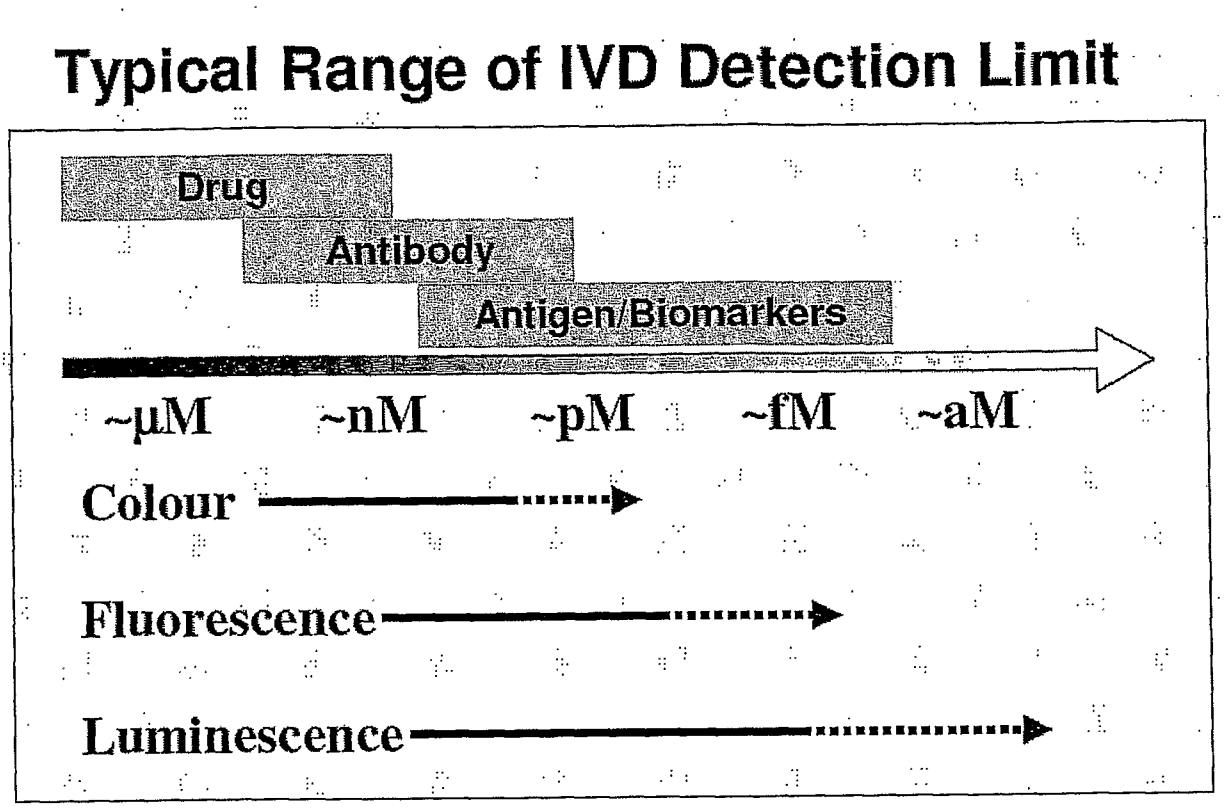
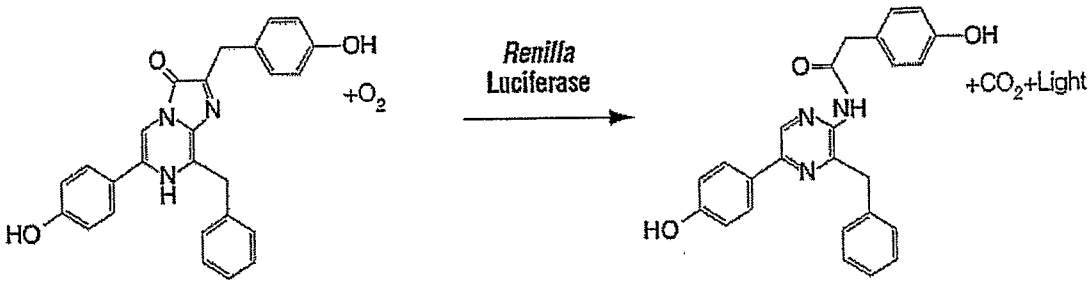

Typical Range of IVD Detection Limit
Drug
Antibody
Antigen/Biomarkers
~µM
~nM
~pM
~fM
~aM
Colour
Fluorescence
Luminescence

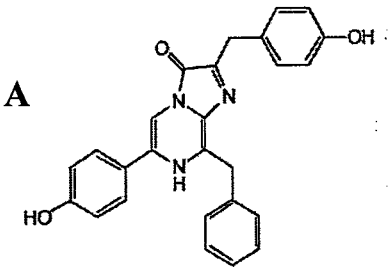
FIG. 1



Coelenterazine Coelenteramide

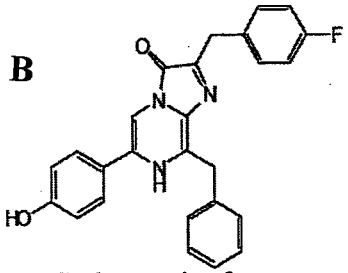
FIG.2

A



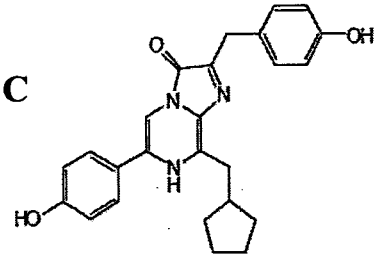
Coelenterazine, native

B



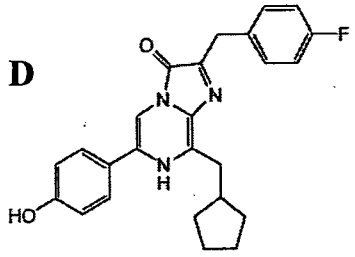
Coelenterazine f
20X higher luminescence intensity

C



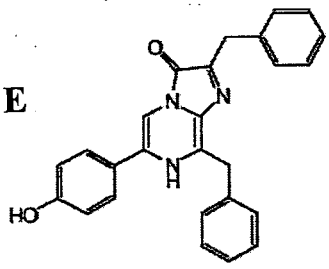
Coelenterazine cp
15X higher luminescence intensity

D



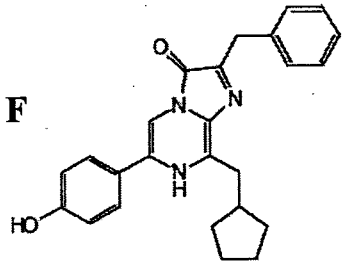
Coelenterazine fcp
135X higher luminescence intensity

E



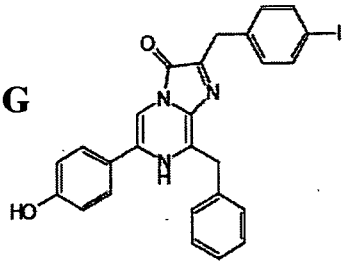
Coelenterazine h
10X higher luminescence intensity

F



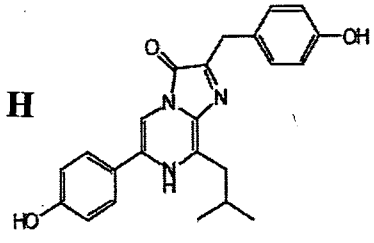
Coelenterazine hcp
190X higher luminescence intensity

G



Coelenterazine i
3% luminescence intensity of native coelenterazine

H



Coelenterazine ip
50X higher luminescence intensity

FIG. 3

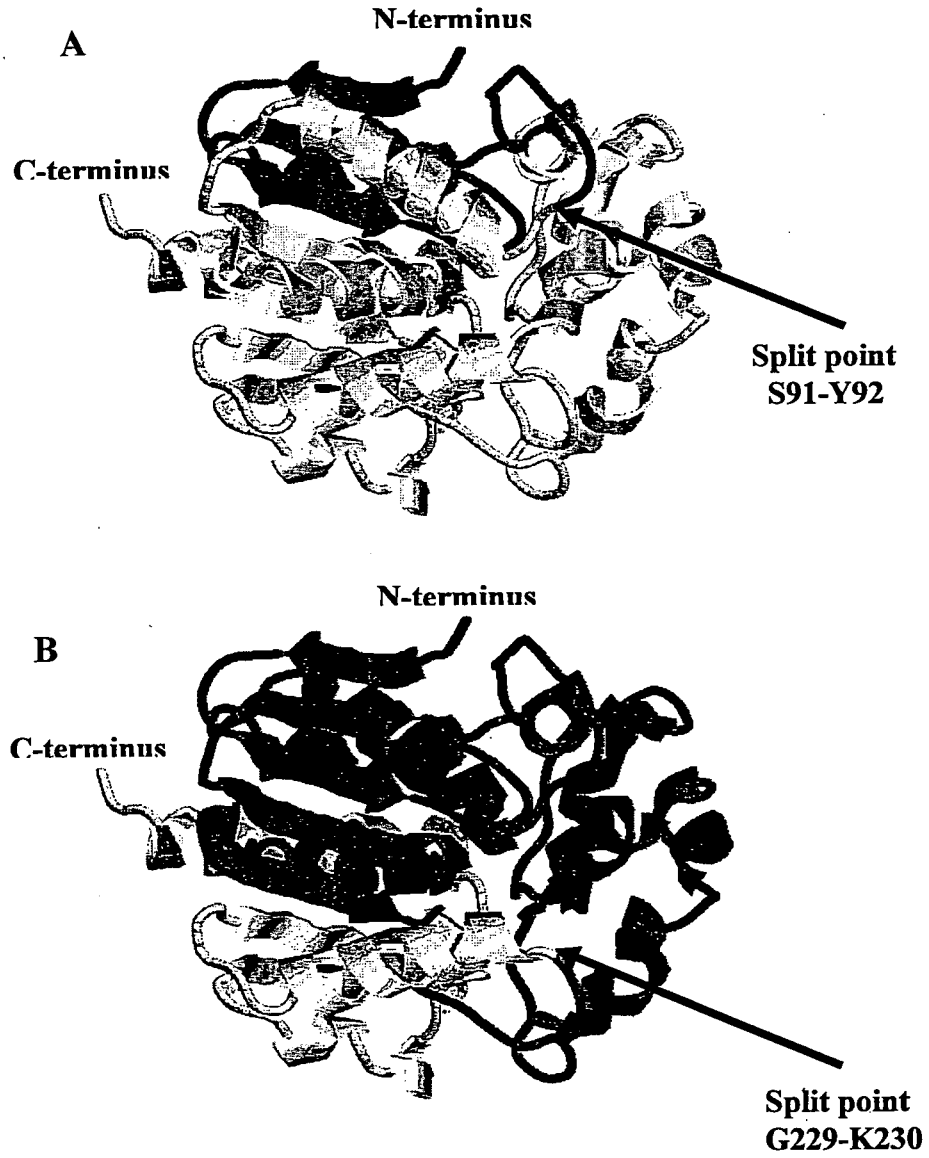

A
N-terminus
C-terminus
Split point
S91-Y92
B
N-terminus
C-terminus
Split point
G229-K230

FIG. 4

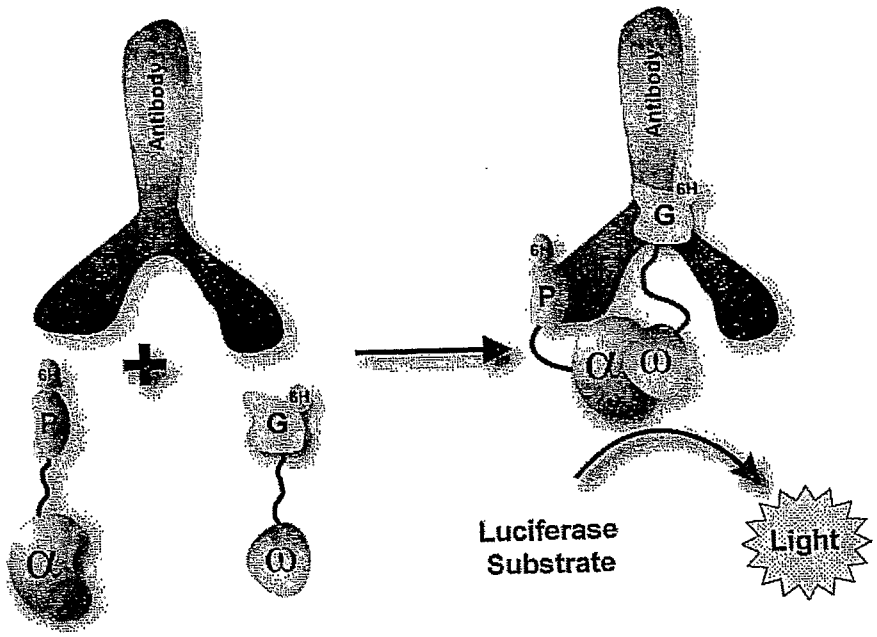

Antibody
6H
P
Antibody
6H
G
6H
P
G
Luciferase
Substrate
Light

FIG. 5

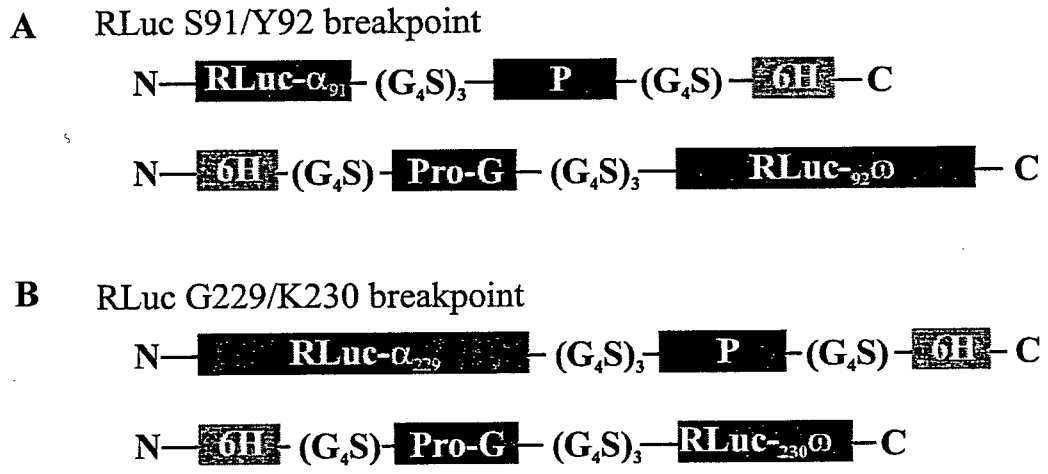
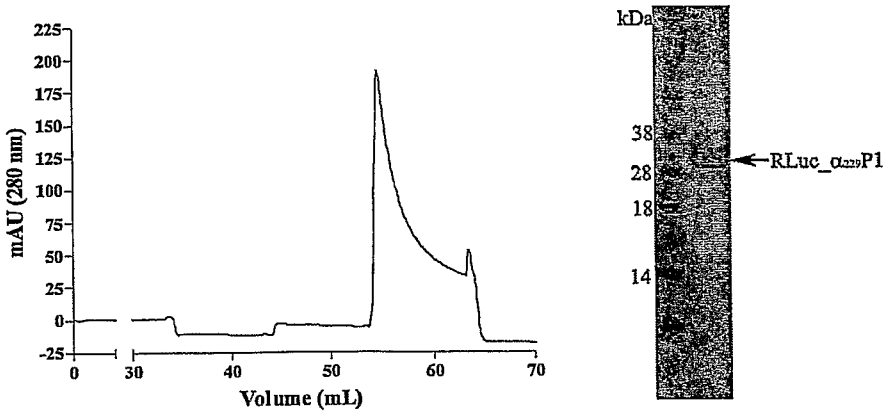

A
RLuc S91/Y92 breakpoint
N
RLuc-α91
(G4S)3
P
(G4S)
6H
C
N
6H
(G4S)
Pro-G
(G4S)3
RLuc-92ω
C
B
RLuc G229/K230 breakpoint
N
RLuc-α229
(G4S)3
P
(G4S)
6H
C
N
6H
(G4S)
Pro-G
(G4S)3
RLuc-230ω
C

FIG. 6

A) Affinity purified RLuc_α_{229}P1



B) Affinity purified RLuc_α_{229}P2

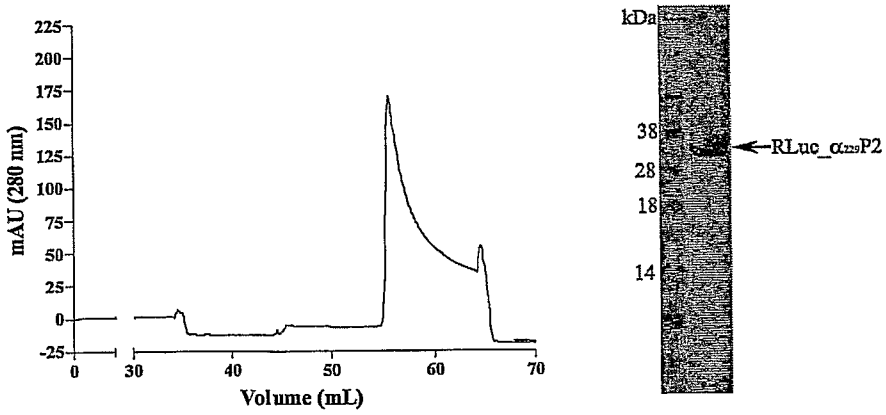


FIG. 7

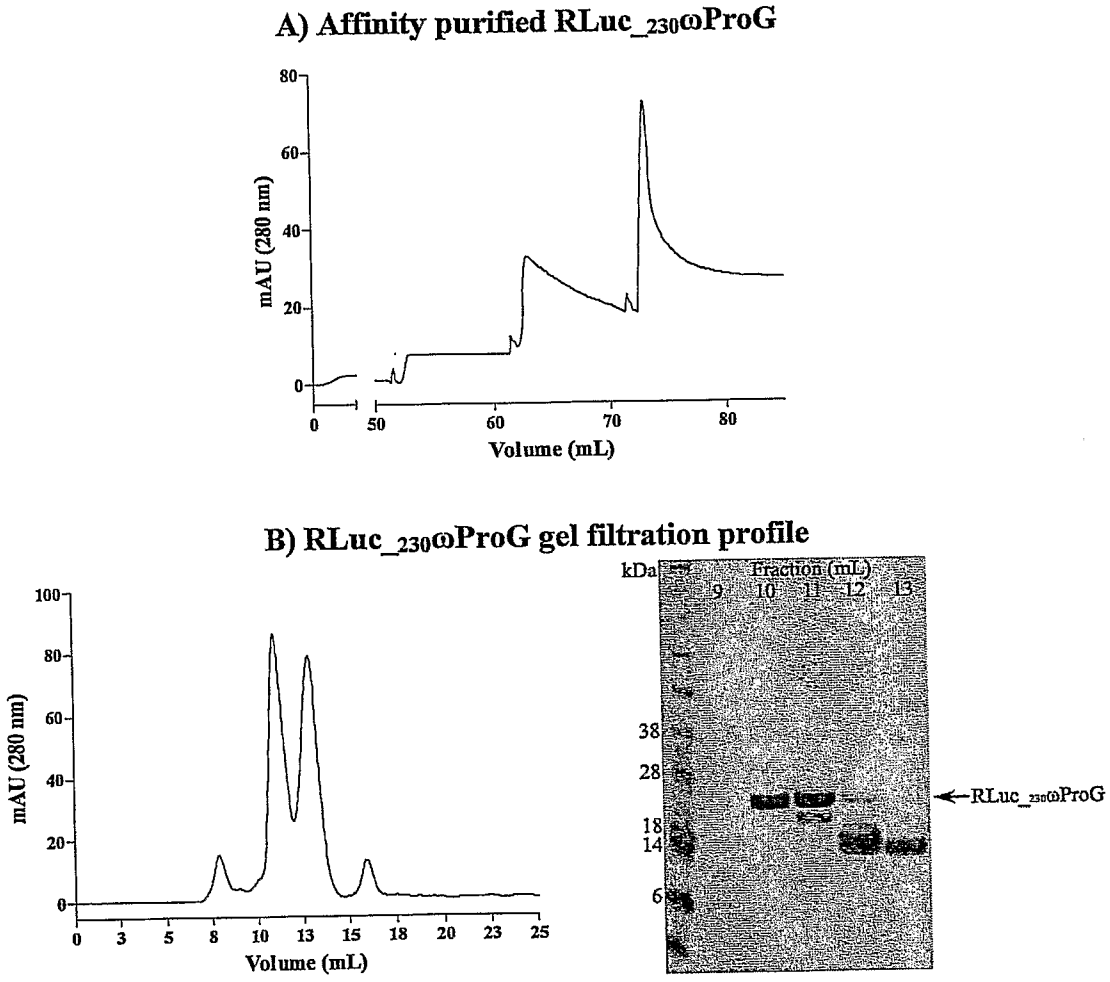
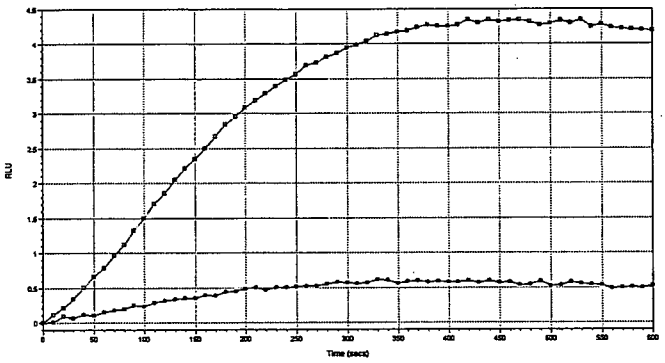

A) Affinity purified RLuc_230ωProG
mAU (280 nm)
Volume (mL)
B) RLuc_230ωProG gel filtration profile
mAU (280 nm)
Volume (mL)
kDa
Fraction (mL)
9
10
11
12
13
38
28
18
14
6
←RLuc_230ωProG

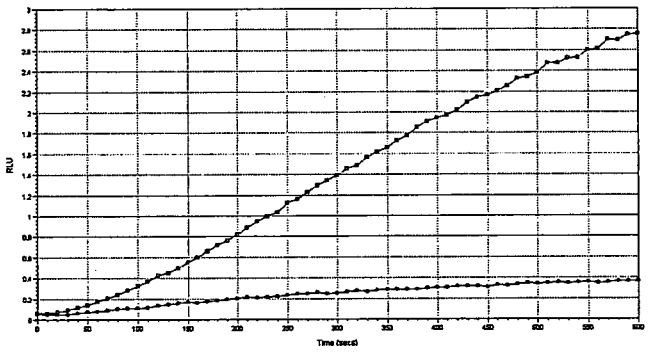
FIG. 8

A



50 nM RLuc split at S91/Y92
+ His = 985 mRLU·min^{-1}
- His = 143 mRLU·min^{-1}
Signal to noise ratio = 6.9:1

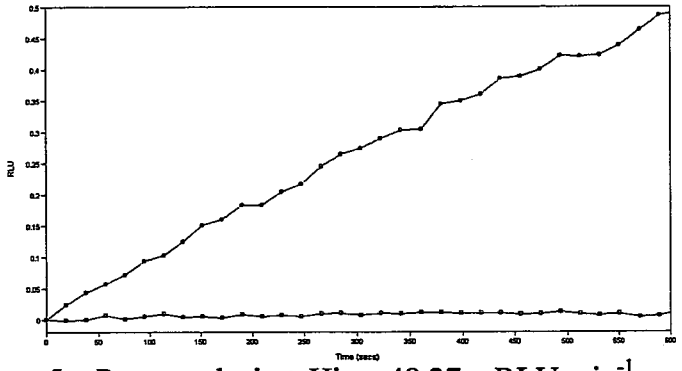
B



5 nM RLuc split at G229/K230
+ His = 339 mRLU·min^{-1}
- His = 53 mRLU·min^{-1}
Signal to noise ratio = 6.4:1

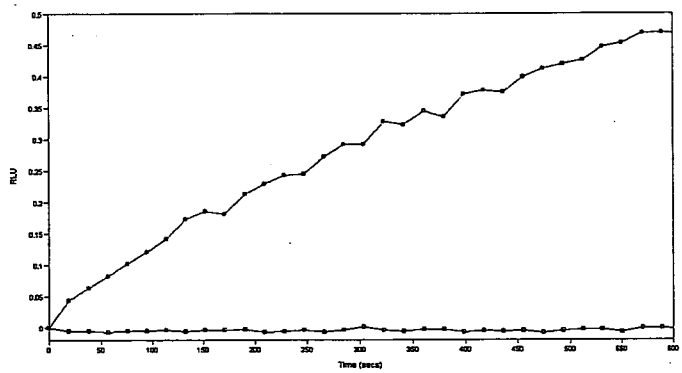
FIG. 9

A



5 x Promega lysis + His = 48.27 mRLU·min^{-1}
5 x Promega lysis - His = 0.75 mRLU·min^{-1}
Signal to noise ratio = 64:1

B



5 x PB enhancer buffer C + His = 45.53 mRLU·min^{-1}
5 x PB enhancer buffer C - His = 0.05 mRLU·min^{-1}
Signal to noise ratio = 911:1

FIG. 10

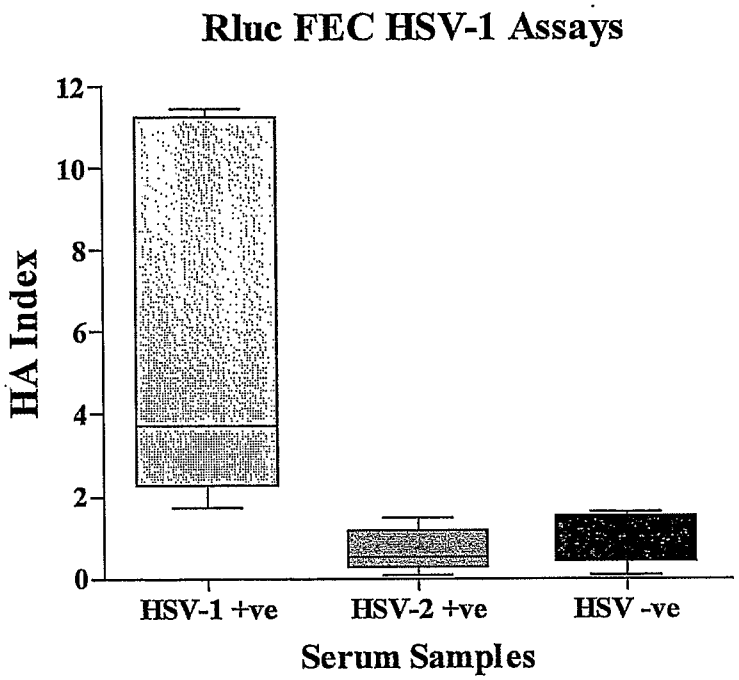

Rluc FEC HSV-1 Assays
HA Index
12
10
8
6
4
2
0
HSV-1 +ve
HSV-2 +ve
HSV -ve
Serum Samples

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001919

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/66 (2006.01) *G01N 33/53* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, HCA, BIOSIS, BIOTECHABS, WPI, EPODOC (forced enzyme complementation assay, forced protein complementation assay, FEC, enzyme fragment complementation assay, protein fragment complementation assay, forced enzyme binding complementation assay, forced protein binding complementation assay, luminescence, luciferase, rluc, reporter, bioluminescence, analyte, antibody, immunoglobulin, antigen, fragment, complement, split, interact, bind, storage, stabilise, sugar, sucrose, trehalose, inert, serum albumin, gelatin, BSA)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/0073537 A1 (CAIRNS et al) 6 April 2006 See whole document especially Abstract; Page 2, [0020]; Page 8, [0110]; Table 7; Claims 14 and 15	22, 24-29
X	WANG C.-Y. et al, "Purification and Preservation of Firefly Luciferase", Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects, Proceedings of the International Symposium on Bioluminescence and Chemiluminescence, 8th, Cambridge, UK, Sept 5-8, 1994, pages 423-426 See whole document especially Introduction; Materials and Methods	22, 24-26, 29

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 February 2009	19 FEB 2009

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001919

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ERIKSSON J. et al, "Method enabling firefly luciferase-based bioluminometric assays at elevated temperatures", Analytical Biochemistry, 2003, Vol. 314, No. 1, pages 158-161 See whole document especially Page 158, right hand column first paragraph; Page 159, left hand column, first paragraph; Page 160, right hand column, last paragraph; Figure 1	22, 24-26, 29
X	HALL M. S. and Leach F. R., "Stability of Firefly Luciferase in Tricine Buffer and in a Commercial Enzyme Stabilizer", Journal of Bioluminescence and Chemiluminescence, 1988, Vol. 2, No. 1, pages 41-44 See whole document especially Abstract; Page 42, Methods; Page 43, Discussion	22, 24, 27, 29
X	BEKHOR I. et al, "Purification and Properties of Luciferase From Firefly Lanterns", Bi-Annu. ATP Methodol. Symp., 2nd, 1977, pages 27-57 See whole document especially Abstract; Page 29, first paragraph; Figure 3	22, 24, 27-29
X	WO 2002/048393 A2 (CARDIOGENICS INC.) 20 June 2002 See whole document especially Abstract; Page 9, lines 11-18; Page 14, lines 12-17; Claims 9 and 10	1, 3, 16, 17
X	KAIHARA A. et al, "Locating a Protein-Protein Interaction in Living Cells via Split *Renilla* Luciferase Complementation", Anal. Chem., 2003, Vol. 75, pages 4176-4181 See whole document especially Abstract; Page 4176, right hand column, 2nd full paragraph; Page 4177, Experimental Section, Plasmid Construction; Figure 1	1-4, 18-21
P, X	WO 2008/095222 A1 (PANBIO LIMITED) 14 August 2008 See whole document especially Abstract; Page 15, lines 24-26; Page 33, lines 17-18; Page 34, lines 15-24; Claims 6, 18, 21, 22 and 39	1, 3, 7, 8, 15-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001919

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(*See Supplemental Box*)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001919

Supplemental Box
(To be used when the space in any of Boxes I to IV is not sufficient)

Continuation of Box No: III

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, I have given consideration to those features which can be considered to potentially distinguish the claimed combination of features from the prior art. Where different claims have different distinguishing features they define different inventions.

This International Searching Authority has found that there are 2 different inventions as follows:

- Invention 1 (Claims 1-21). It is considered that methods and kits for determining the presence of an analyte in a sample, comprising two reporter fragments each comprising separate interactor domains both having affinity for the analyte of interest, where under in vitro assay conditions and in the presence of the analyte both reporter fragments associate to produce a glow luminescent signal, comprises a first distinguishing feature.

- Invention 2 (Claims 22-30). It is considered that a composition comprising one or more reporter polypeptides and one or more sugars and/or one or more inert proteins, comprises a second distinguishing feature.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

Each of the abovementioned groups of claims has a different distinguishing feature and they do not share any feature which could satisfy the requirement for being a special technical feature. Because there is no common special technical feature it follows that there is no technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention *a priori.*

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2008/001919

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	2006/0073537	NONE					
WO	2002/048393	AU	15765/02	CA	2328684	CA	2431785
		US	2004063165				
WO	2008/095222	NONE					

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

专利名称(译)	分析方法		
公开(公告)号	EP2231868A4	公开(公告)日	2011-02-16
申请号	EP2008866208	申请日	2008-12-24
[标]申请(专利权)人(译)	瑞士了Alere		
申请(专利权)人(译)	瑞士了Alere GMBH		
当前申请(专利权)人(译)	瑞士了Alere GMBH		
[标]发明人	HUANG CHANG YI DE LAS HERAS RACHEL FRY SCOTT ROBERT LI JUN		
发明人	HUANG, CHANG-YI DE LAS HERAS, RACHEL FRY, SCOTT, ROBERT LI, JUN		
IPC分类号	C12Q1/66 G01N33/53		
CPC分类号	C12Q1/66 G01N33/581		
优先权	2008900006 2008-01-02 AU		
其他公开文献	EP2231868A1		
外部链接	Espacenet		

摘要(译)

描述了一种用于确定样品中感兴趣分析物的存在的方法，该方法包括使样品与以下物质接触：（i）纯化的第一报告片段对成员，其包含对目标分析物具有亲和力的相互作用结构域;（ii）纯化的第二报道片段对成员，其包含对目标分析物具有亲和力的相互作用结构域，并且可通过第一和第二的相互作用结构域的亲和力在与第一报告片段对成员结合时重构活性酶。报告片段成员与感兴趣的分析物成员;在体外测定条件下，允许第一和第二报告片段对成员通过相互作用结构域与分析物的亲和力在样品中感兴趣的分析物存在下缔合，通过重构活性物质的作用产生发光发光信号酶在基质上;并检测辉光发光信号的存在或不存在或程度。