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(54) **ASSAY WITH REDUCED BACKGROUND**

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(57) **ABSTRACT**

In an assay, an analyte in a sample is contacted with a thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, wherein a complex is formed. ADP is added, and then formation of ATP is monitored. Prior to the addition of ADP, endogenous kinase and uncomplexed thermostable reporter adenylate kinase is substantially removed by washing and residual endogenous kinase is inactivated by heating. Prior to contacting the analyte with the thermostable reporter adenylate kinase, the sample has a background activity of at least 300,000 Relative Light Units per mg protein per ml sample when measured in the presence of luciferin/luciferase by a luminometer.

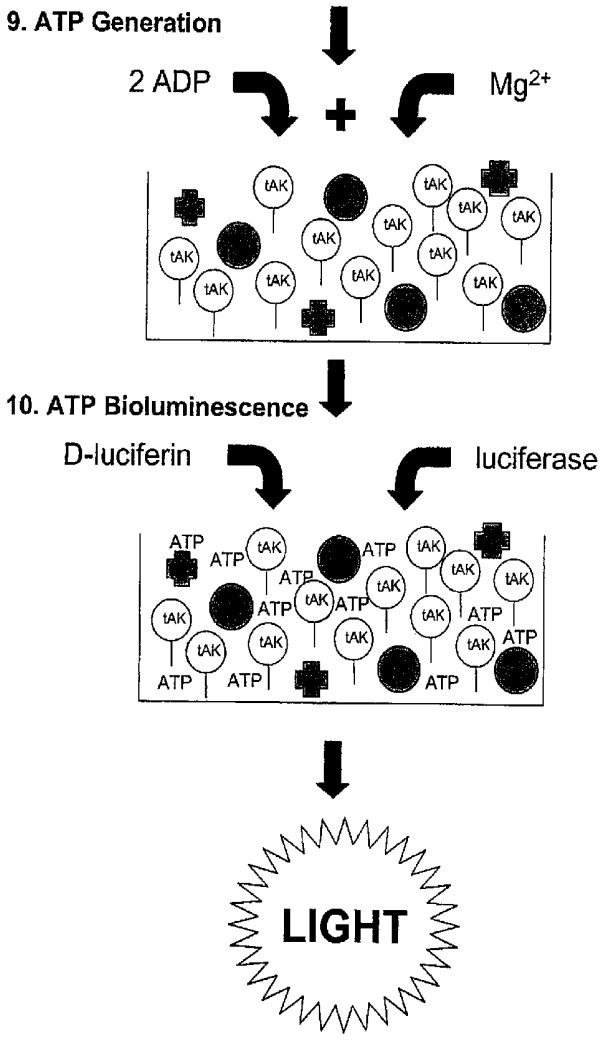


Fig. 1

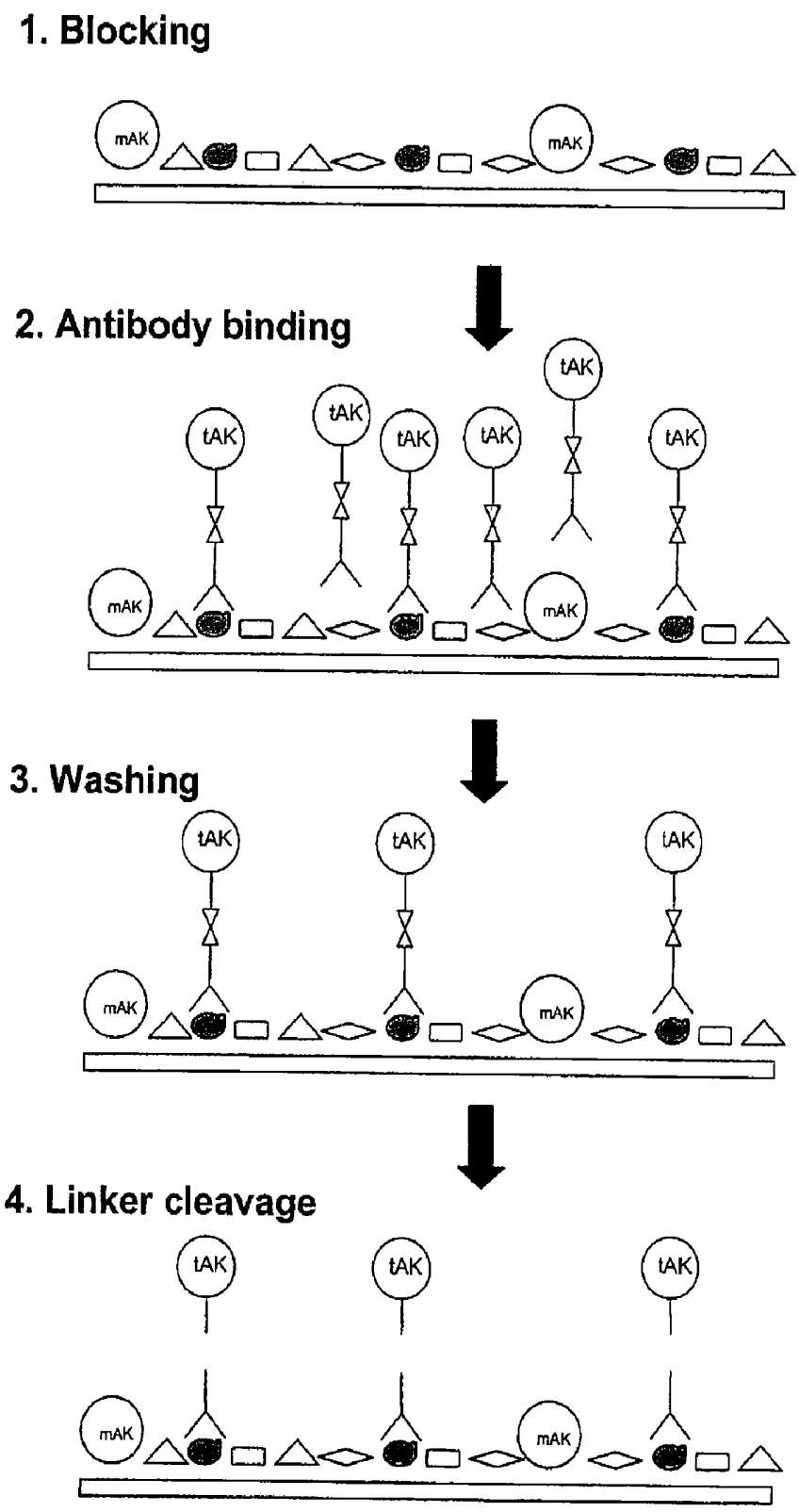

1. Blocking
mAK
mAK
2. Antibody binding
tAK
tAK
tAK
tAK
tAK
tAK
mAK
mAK
3. Washing
tAK
tAK
tAK
mAK
mAK
4. Linker cleavage
tAK
tAK
tAK
mAK
mAK

Fig. 2

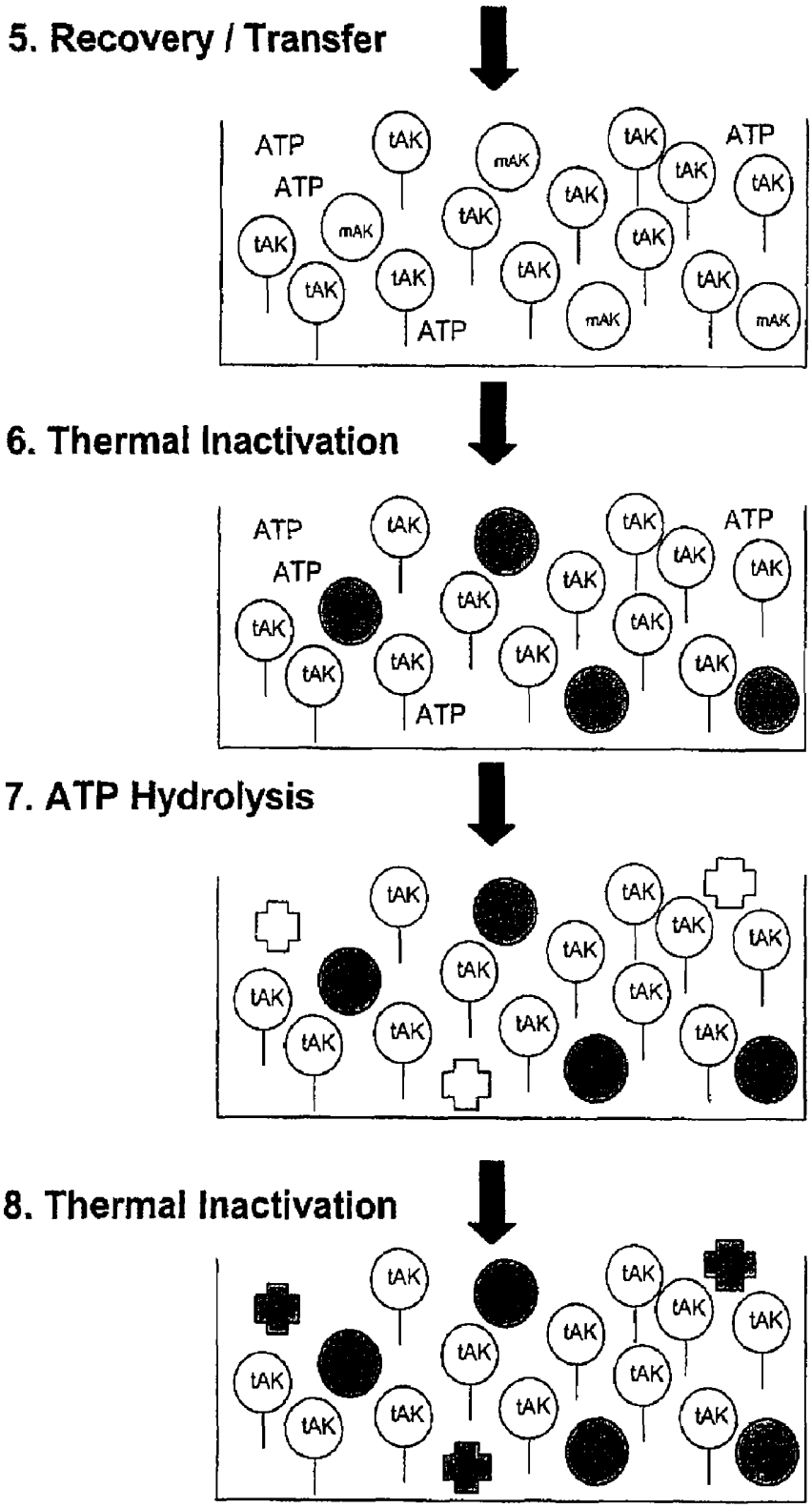

5. Recovery / Transfer
ATP
ATP
ATP
ATP
tAK
mAK
6. Thermal Inactivation
7. ATP Hydrolysis
8. Thermal Inactivation

Fig. 3

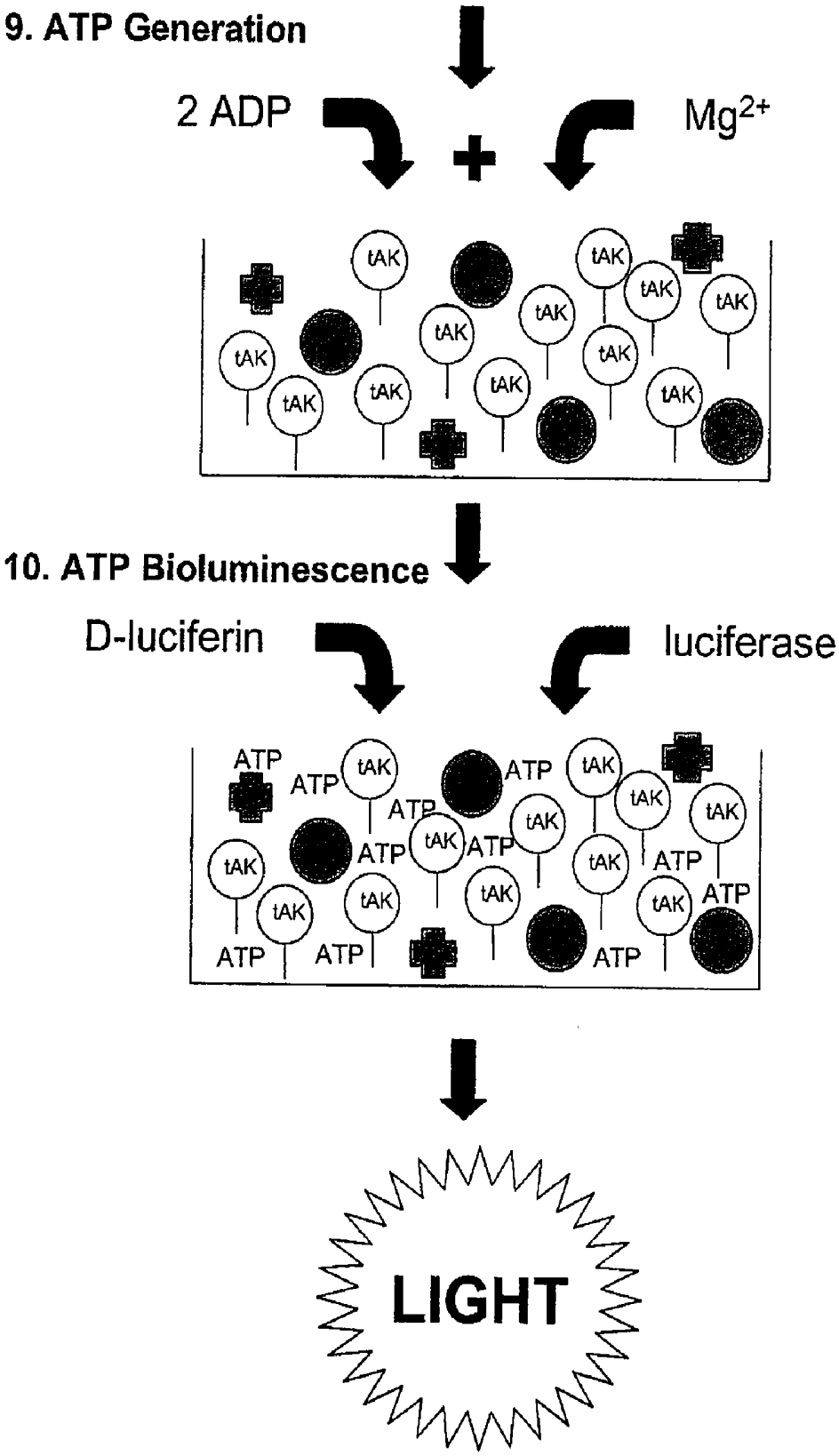

9. ATP Generation
2 ADP
Mg2+
tAK
10. ATP Bioluminescence
D-luciferin
luciferase
ATP
LIGHT

Fig. 4

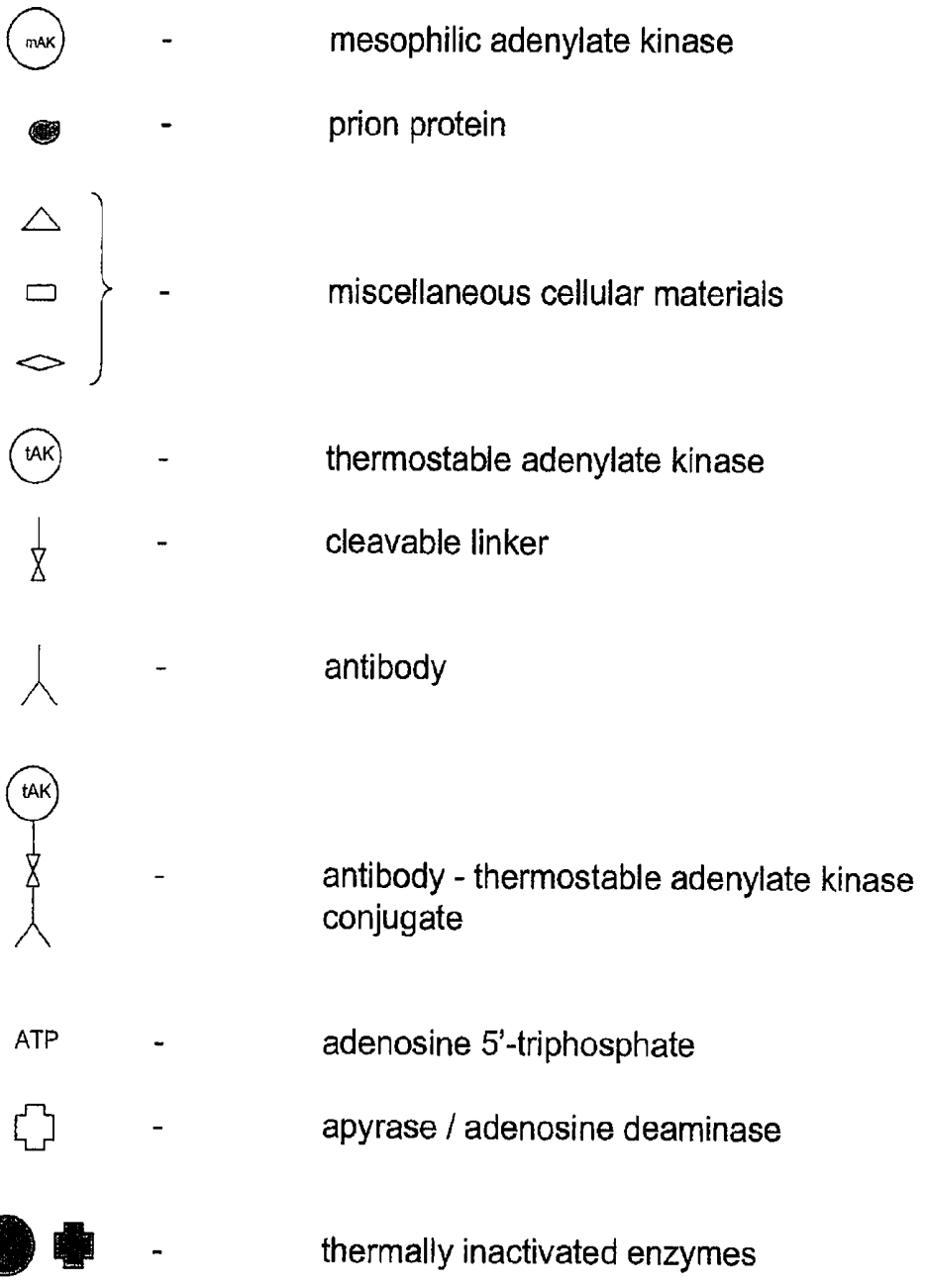

mAK
-
mesophilic adenylate kinase
-
prion protein
-
miscellaneous cellular materials
tAK
-
thermostable adenylate kinase
-
cleavable linker
-
antibody
tAK
-
antibody - thermostable adenylate kinase conjugate
ATP
-
adenosine 5'-triphosphate
-
apyrase / adenosine deaminase
-
thermally inactivated enzymes

Fig. 5A

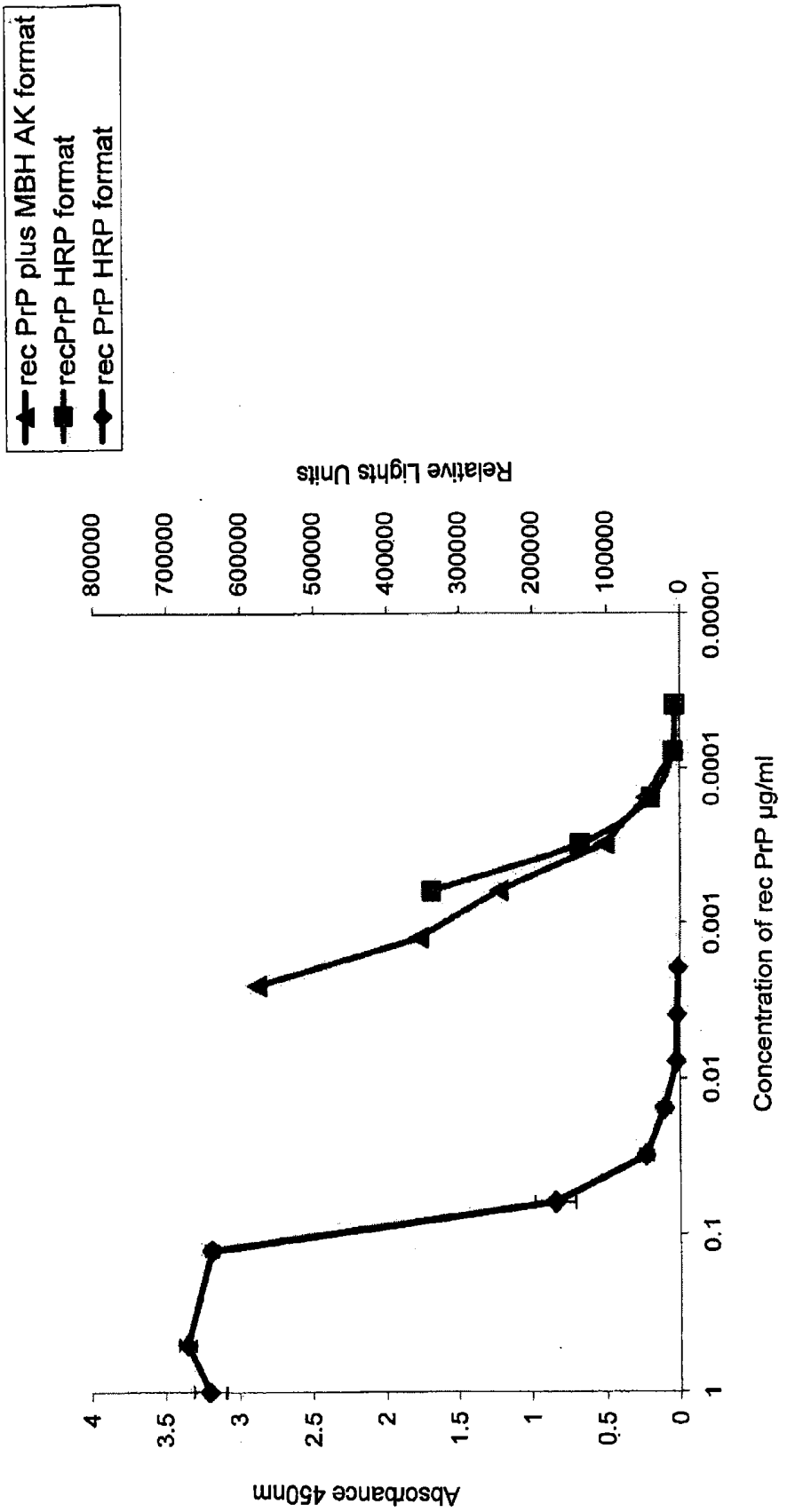

rec PrP plus MBH AK format
recPrP HRP format
rec PrP HRP format
Relative Lights Units
800000
700000
600000
500000
400000
300000
200000
100000
0
0.00001
0.0001
0.001
0.01
0.1
1
Concentration of rec PrP µg/ml
Absorbance 450nm
4
3.5
3
2.5
2
1.5
1
0.5
0

Fig. 5B

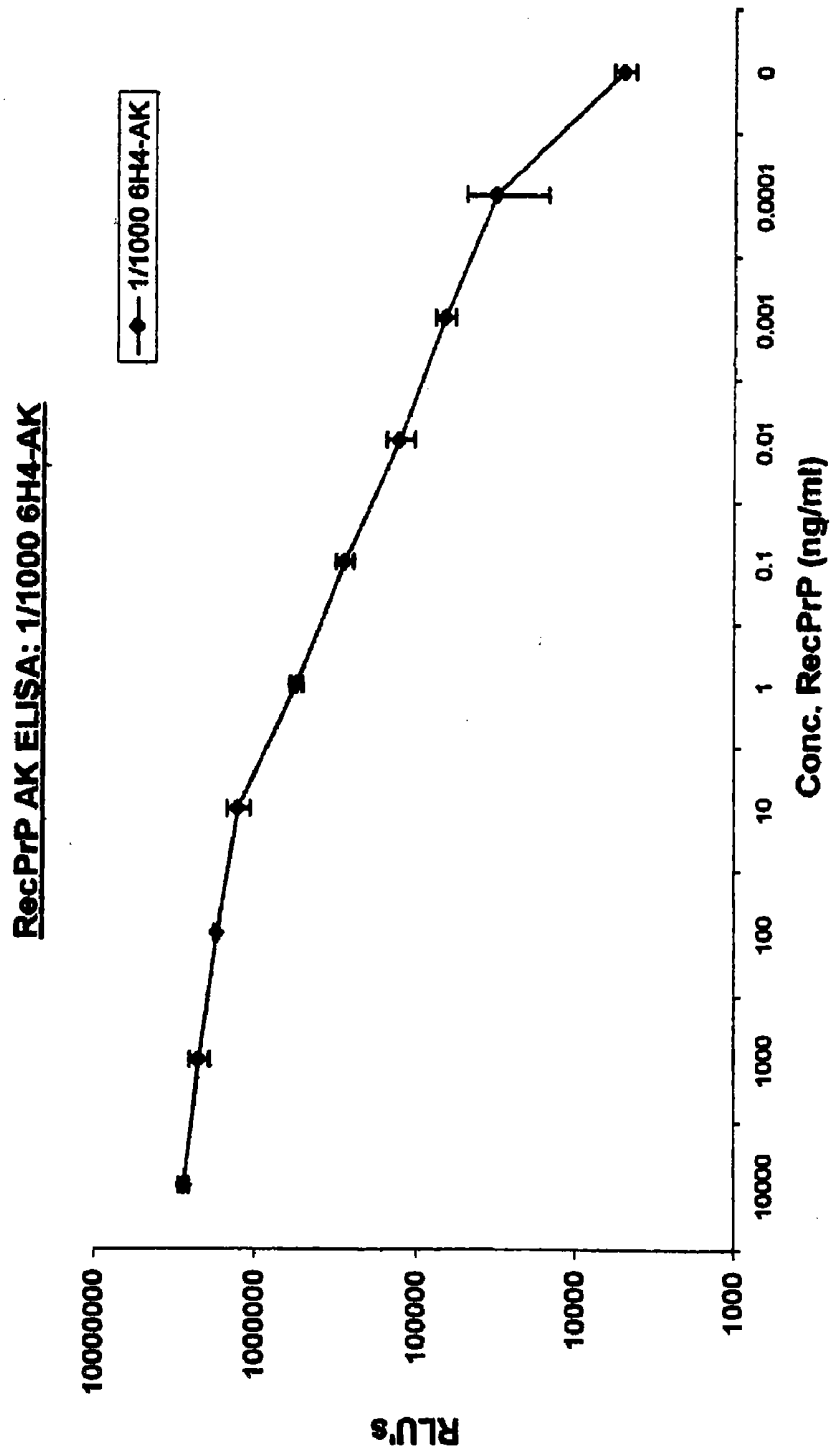

RecPrP AK ELISA: 1/1000 6H4-AK
1/1000 6H4-AK
RLU's
10000000
1000000
100000
10000
1000
Conc. RecPrP (ng/ml)
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
0

Fig. 6A

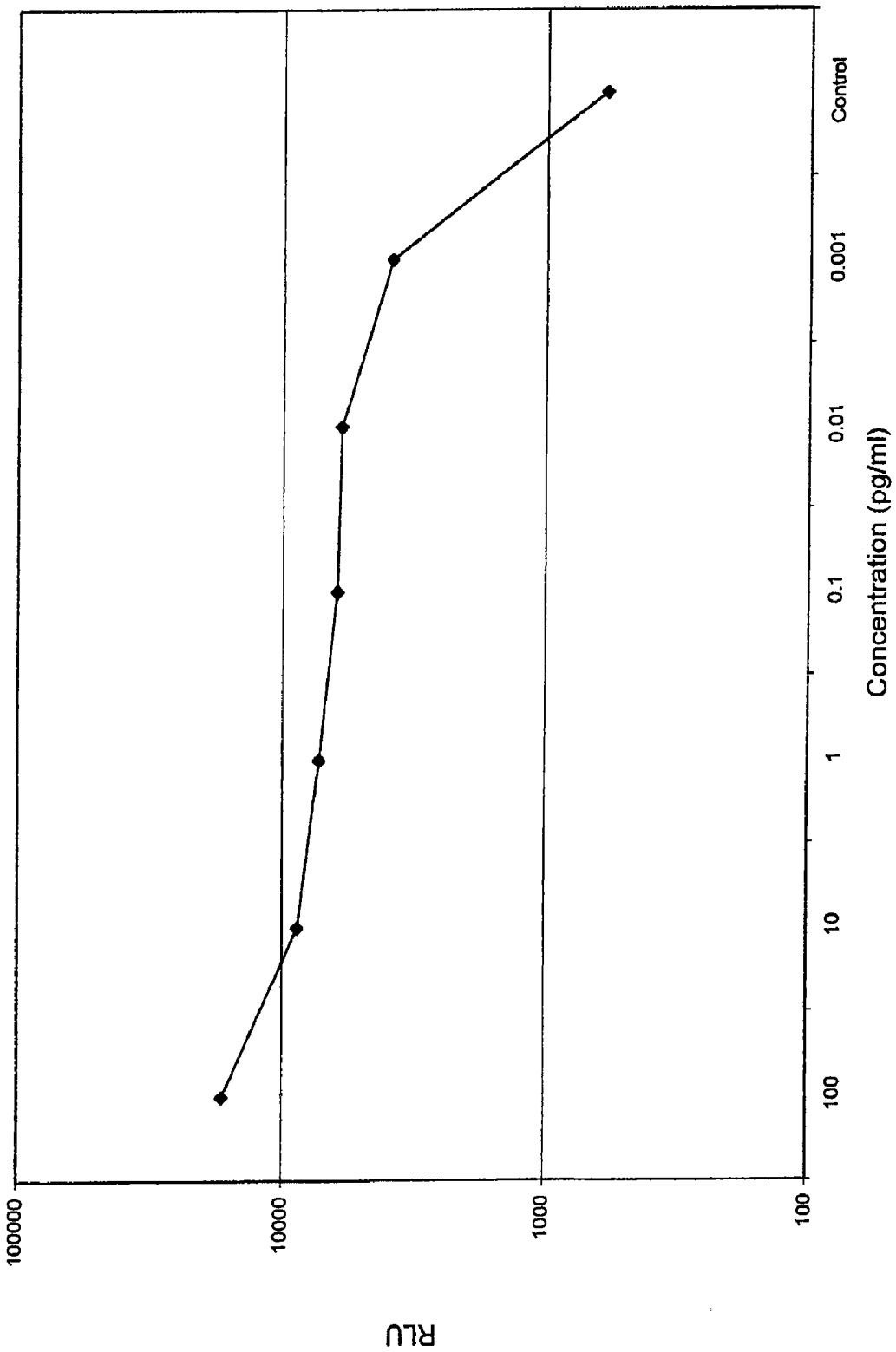

RLU
100000
10000
1000
100
Concentration (pg/ml)
100
10
1
0.1
0.01
0.001
Control

Fig. 6B

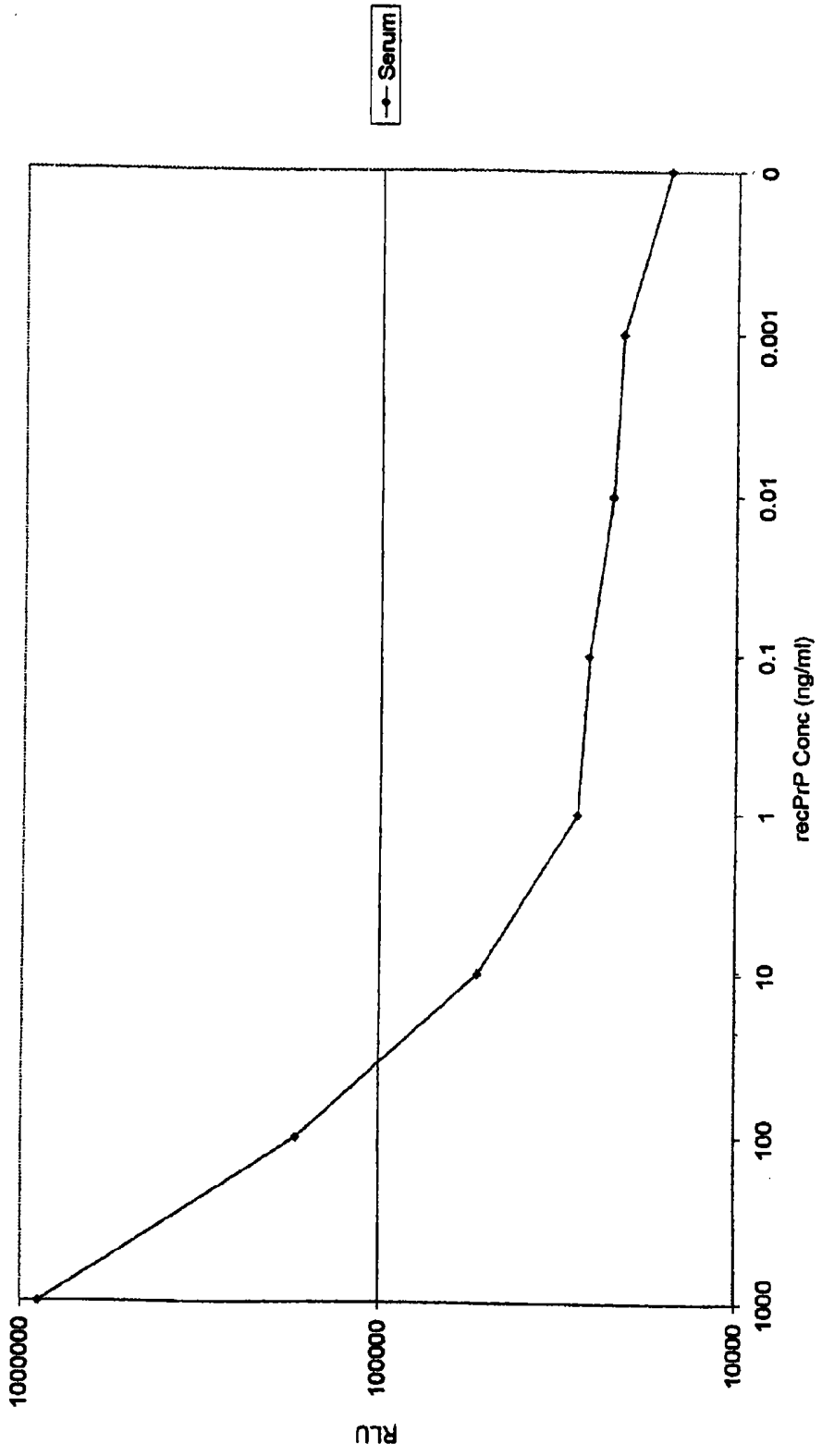

recPrP Assay
Serum
RLU
1000000
100000
10000
1000
100
10
1
0.1
0.01
0.001
0
recPrP Conc (ng/ml)

Fig. 7

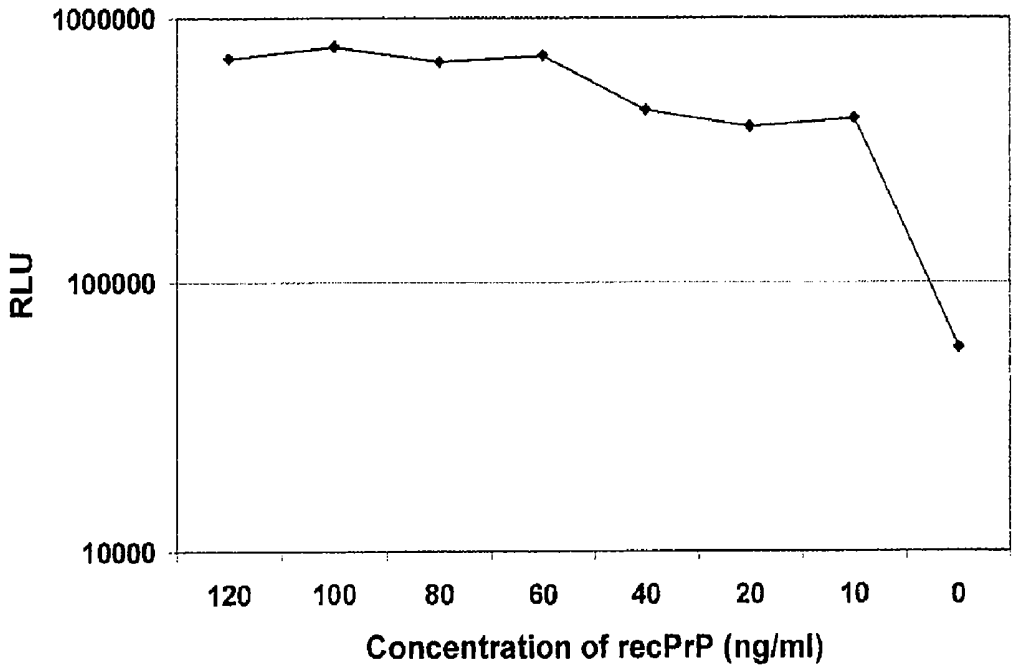

1000000
100000
10000
RLU
120
100
80
60
40
20
10
0
Concentration of recPrP (ng/ml)

Fig. 8

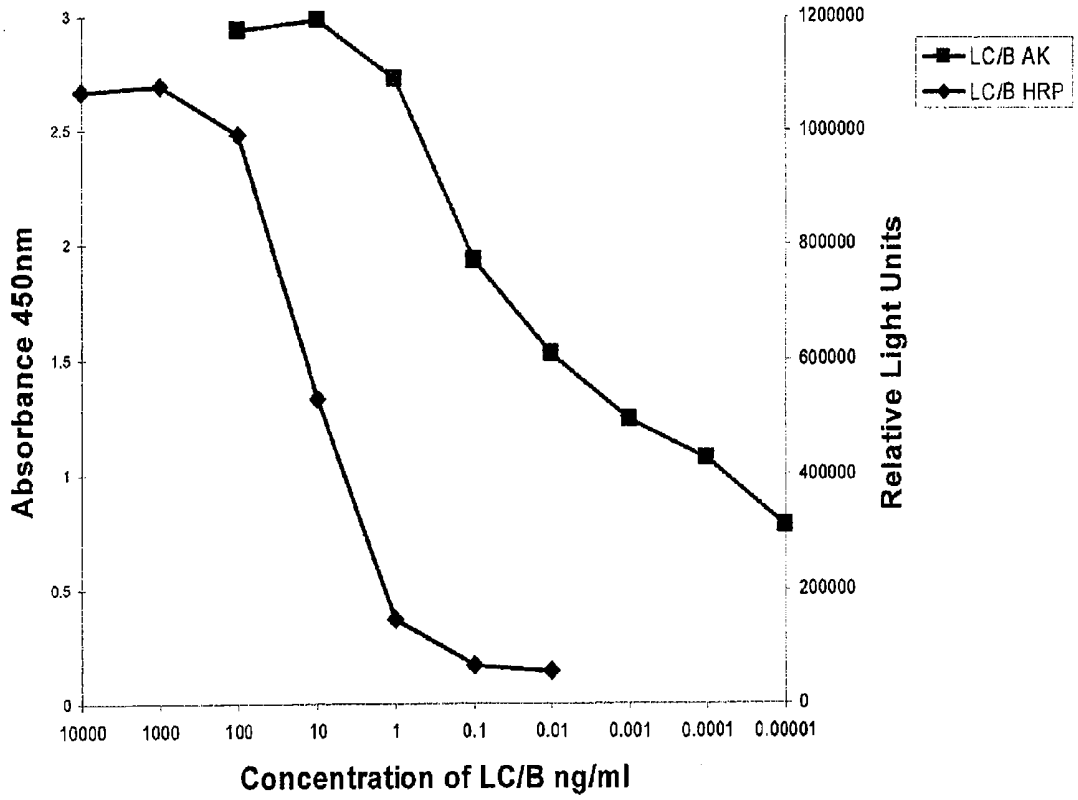
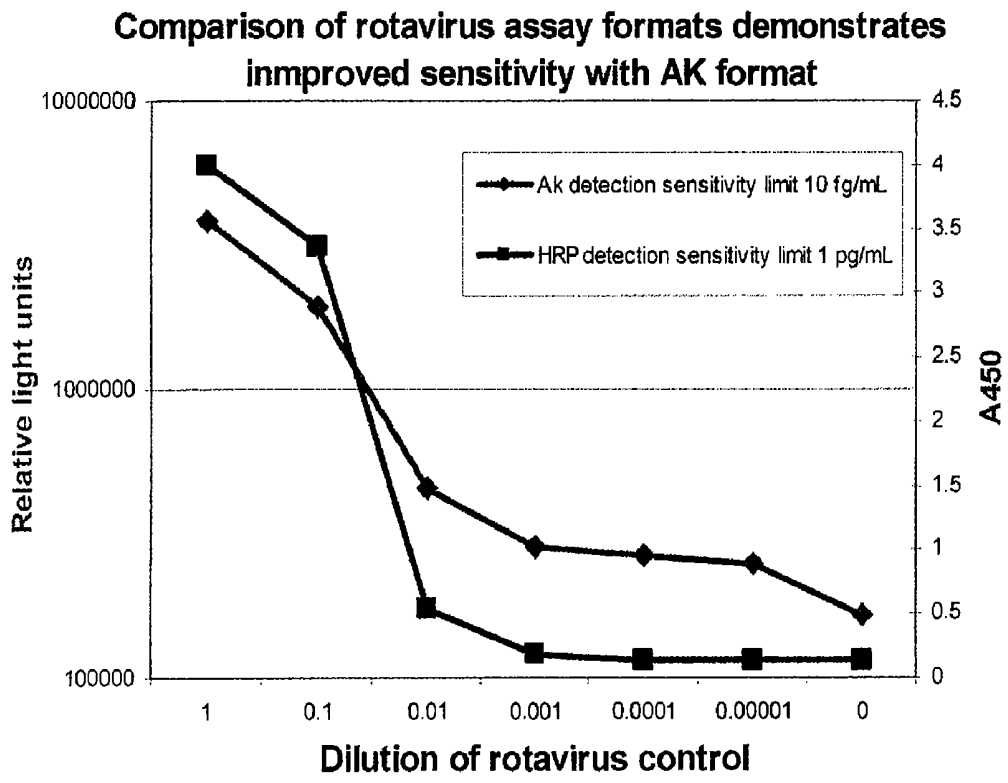

Absorbance 450nm
Relative Light Units
Concentration of LC/B ng/ml
LC/B AK
LC/B HRP
3
2.5
2
1.5
1
0.5
0
1200000
1000000
800000
600000
400000
200000
0
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001

Fig. 9


Comparison of rotavirus assay formats demonstrates inmproved sensitivity with AK format
Ak detection sensitivity limit 10 fg/mL
HRP detection sensitivity limit 1 pg/mL
Relative light units
10000000
1000000
100000
A450
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
0.1
0.01
0.001
0.0001
0.00001
0
Dilution of rotavirus control

ASSAY WITH REDUCED BACKGROUND

[0001] The present invention relates to an assay with reduced background, a method of assaying for an analyte, a method of reducing background in an assay and apparatus, in particular a test kit, for carrying out such an assay.

[0002] ATP bioluminescence has rapidly become the method of choice for hygiene and cleanliness monitoring due to its combination of sensitivity and ease of assay. A luciferin-luciferase bioluminescence assay can detect as little as 10^{-15} moles of ATP. Since an average microbial cell contains approximately 10^{-18} moles of ATP, this gives a detection limit of only 10^3 cells.ml^{-1}.

[0003] For most operations this detection level is sufficient, however, there are applications where even greater sensitivity is required, even down to a single microbial cell. GB-A-2304692 describes such an assay using the ATP forming enzyme adenylate kinase (AK). An average cell contains several hundred-fold less AK molecules than ATP molecules, however, in a 10 minute incubation, a typical 400,000-fold amplification is achieved by detecting AK through the ATP it produces. This corresponds to the level of single cell detection, although in practice 10 cells.ml^{-1} is more readily achieved due to background AK and ATP contamination. It also corresponds to a detection level of down to at least 10^{-20} moles of AK.

[0004] The commercial use of this extreme sensitivity is, therefore, under investigation. There are, however, some problems with more widespread use of this known AK-based assay, One is that while the assay detects the presence of micro-organisms, it does not differentiate between one organism and another. This has been overcome to a degree by the use of bacteriophage to release AK from specific bacteria (Blasco R, Murphy M J, Sanders M F and Squirrell D J (1998) Specific assays for bacteria using phage mediated release of adenylate kinase. *J. Appl. Microbiol* 84: 661-666).

[0005] Each micro-organism, however, requires a specific phage and contains an AK with different buffer requirements, plus temperature and pH optima. The second problem is more fundamental and is a problem for its use as a generalised reporter enzyme. Whereas in hygiene and cleanliness monitoring the ubiquity of ATP and AK is beneficial, in an enzyme reporter assay any unwanted background activity is detrimental. This is especially so where the sample is greatly concentrated to maximise potential detection.

[0006] A further problem is that the known assay is only effective for microorganisms which contain AK; the known assay will not work with other biological material, such as viruses or other analytes, including other biological such material that does not contain AK.

[0007] Transmissible Spongiform Encephalopathies (TSEs) is the term given for a spectrum of diseases associated with an unconventional transmissible agent. The agent displays many virus-like features, such as strain variation and mutation, but differs from conventional viruses in being exceptionally resistant to heat, ultraviolet and ionising radiation and to chemical disinfectants. The TSEs are a heterogeneous group of fatal neurodegenerative disorders occurring in humans, mink, cats and ruminant herbivores. The endemic occurrence of the TSE "scrapie" in many sheep populations and more rarely human TSEs, such as Creutzfeldt-Jakob Disease (CJD), has been known for some time. The occurrence of novel TSEs in wild populations of mule deer and elk in the United States and an outbreak of "Bovine Spongiform Encephalopathy" (BSE)" in cattle in the United Kingdom and Europe has, however, emphasised the need for sensitive and reliable diagnostic tests and detection systems for these diseases. More recently, however, it has become apparent that BSE has crossed the species barrier to the human population giving rise to a new variant TSE, generally known as "new variant CJD" (nvCJD) or "variant CJD" (vCJD).

[0008] The highest native concentrations of TSE infectivity are found in 263K scrapie-infected hamster brain where titres as high as 10^{10} infectious units per gram of tissue are frequently reported.

[0009] Current immunoassays give positive signals for PrP^{Sc} from as little as 1-10 g of TSE infectious brain tissue, e.g. by Western blotting or ELISA. ELISA, however, is considerably more suitable than Western blotting for the development of a fast and practical PrP (PrP^{C}+PrP^{Sc}) detection system. This level of detection is approximately 10^{-14} moles of PrP^{Sc} but insufficient to detect the presence of still infectious quantities of PrP^{Sc}. Where PrP^{c} is also included, however, the differential between the current and required level of sensitivity is significantly reduced. This brings current immunoassays potentially into the appropriate range, but with an inadequate margin of safety.

[0010] There is currently great uncertainty regarding the numbers of individuals in the UK potentially or actually infected with new variant Creutzfeldt-Jakob Disease (nvCJD). As a result there have been calls that all surgical procedures should be carried out using disposable instruments as a safeguard. Implementation has severe cost and procedural implications, consequently an alternative means to validate decontamination would be extremely beneficial, and would also be of benefit to other equipment such as meat processing equipment. Therefore, it remains a problem to provide an alternative assay for biological material, especially prior protein, preferably of increased sensitivity.

[0011] The present invention is aimed at addressing and overcoming or at least ameliorating these problems. A further object of specific embodiments of the present invention is to develop a rapid and sensitive method for assay of biological material, in particular for the detection of prion protein PrP (PrP^{c} and PrP^{Sc})—as the presence of either isoform in a sample is indicative of the presence of residual PrP-expressing tissue and the potential for transmissible infectivity. A still further object of specific embodiments of the present invention is to provide a method for assay of prion proteins that may be used in the screening of cleaning protocols to determine their suitability for the removal of TSE agents from surfaces and delivery of recovered material for immunoassay.

[0012] Accordingly, a first aspect of the invention provides an assay for an analyte, comprising specifically associating the analyte with a reporter kinase, adding ADP and testing for formation of ATP wherein, prior to addition of ADP, kinase other than reporter kinase is substantially removed.

[0013] Thus, in use of an assay of the present invention, a reporter adenylate kinase is specifically associated with the analyte so that the amount of reporter adenylate kinase is substantially in proportion to the amount of analyte present. In the absence of analyte there will be no reporter adenylate kinase associated and no signal generated. By substantially removing adenylate kinase other than reporter adenylate kinase, the present invention has the advantage that the signal obtained is not contaminated or otherwise adversely affected by any endogenous adenylate kinase that might have been

present in a sample being tested. By reference to removing adenylate kinase it is intended to refer to removing adenylate kinase activity, such as by removing the adenylate kinase, or denaturing or otherwise inactivating it in situ. Furthermore, by addition of reporter adenylate kinase, the assay is of application for detection of substantially any analyte and, unlike the prior art, is not limited to detecting analytes that comprise their own adenylate kinase.

[0014] In an embodiment of the invention there is provided a method of determining presence and/or amount of an analyte in a sample, comprising:—

[0015] exposing the sample to a reporter adenylate kinase coupled to a binding agent specific for the analyte, so that the reporter adenylate kinase is specifically associated with any analyte present in the sample;

[0016] removing reporter adenylate kinase that is not specifically associated with analyte;

[0017] exposing reporter adenylate kinase specifically associated with the analyte to ADP; and

[0018] testing for formation of ATP,

[0019] wherein prior to addition of ADP adenylate kinase other than reporter adenylate kinase is substantially removed.

[0020] Typically, the reporter adenylate kinase is coupled to an antibody that binds specifically to the analyte under investigation. The antibody may be obtained using conventional techniques for identification and isolation of specific antibodies, and the assay of the present invention is thus of application to substantially all analytes against which an antibody can be raised. This confers the advantage that the present invention is of considerably wider application compared to the known AK/ATP-based assays, as the previous assays were restricted to target analytes that contained their own adenylate kinase.

[0021] The reporter adenylate kinase is suitably coupled to the specific binding agent by conventional techniques. For example, there are numerous ways of labelling immunoreactive biomolecules with enzymes (conjugation).

[0022] Antibodies, the majority of antigens, and enzymes are all proteins and, therefore, general methods of protein covalent cross-linking can be adapted to the production of immunoassay reagents, The preparation of antibody-enzyme conjugates requires mild conditions to ensure the retention of both the immunological properties of the antibody and the catalytic properties of the enzyme. Common methods include, glutaraldehyde coupling, the use of periodate oxidation of glycoproteins to generate dialdehydes capable of forming Schiff-base linkages with free amino groups on other protein molecules, and the use of heterobifunctional reagents, for example, succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC).

[0023] Endogenous adenylate kinase present in the analyte is substantially removed or destroyed or otherwise inactivated before testing for formation of ATP is carried out. This removal step can conveniently be achieved by heating the endogenous adenylate kinase to a temperature at which it is denatured. Alternatively, other treatments might be appropriate to destroy the activity of the endogenous adenylate kinase, such as the use of ultrasound or extremes of pH or salt concentration. In an embodiment of the invention, the reporter adenylate kinase is a thermostable enzyme and endogenous adenylate kinase is removed by heating. In a specific embodiment of the invention described in more detail below, this denaturing step is carried out at about 90° C. for a period of about 10 minutes, though other temperatures and durations will be appropriate so long as the endogenous adenylate kinase is rendered incapable of catalysing the formation of ATP and the reporter adenylate kinase retains its activity.

[0024] It is a further, preferred, step in the assay of the present invention for any ATP present prior to addition of ADP to be removed, thereby further decreasing the background noise in the assay. The removal of endogenous ATP may be achieved by addition of an ATPase and incubation prior to adding ADP. More preferably, a thermolabile ATPase is used to remove ATP and then the thermolabile ATPase is itself destroyed by use of elevated temperature, to avoid the presence of the ATPase adversely influencing the signal obtained using the thermostable, reporter adenylate kinase.

[0025] The precise order of carrying out the steps of the present invention is not critical, provided that endogenous adenylate kinase is destroyed before addition of ADP and testing for the formation of ATP. Thus, the method of the present invention can be carried out by treating a sample to destroy its endogenous adenylate kinase, adding reporting adenylate kinase coupled to an antibody specific to the analyte, isolating reporting adenylate kinase that is specifically associated with analyte and then adding ADP and testing for formation of ATP. Alternatively, the assay can be carried out by adding a reporter adenylate kinase coupled to an antibody specific for the analyte to a sample, isolating reporter adenylate kinase that is specifically associated with analyte, destroying any endogenous adenylate kinase that may be present and then adding ADP and testing for formation of ATP. A further alternative is to add reporter adenylate kinase coupled to an antibody specific for analyte to the sample, treating the sample to destroy endogenous adenylate kinase, isolating reporter adenylate kinase specifically associated with analyte and then adding ADP and testing for formation of ATP.

[0026] In a specific embodiment of the invention described in more detail below, an assay is carried out by following the steps:—

[0027] 1. An antibody specific to the analyte is immobilised on a solid phase.

[0028] 2. A sample is combined with the solid phase so that analyte present in the sample can bind to the antibody.

[0029] 3. The solid phase is washed, thereby washing away components of the sample and retaining on the solid phase only any analyte that has bound to the immobilised antibody.

[0030] 4. A reporter composition is added to the solid phase, the reporter composition comprising an antibody which is specific to the analyte and which is coupled to a thermostable adenylate kinase.

[0031] 5. The solid phase is washed, thereby washing away unbound components of the reporter composition and retaining reporter composition that has specifically bound the analyte, the analyte being itself bound to the immobilised antibody.

[0032] 6. The solid phase is heated to denature any endogenous adenylate kinase that may be present but so as not to denature the thermostable adenylate kinase.

[0033] 7. Optionally, a thermolabile ATPase is added to the solid phase to remove any endogenous ATP.

[0034] 8. Optionally, the solid phase is heated to destroy the thermolabile ATPase of step 7.

[0035] 9. ADP is added to the solid phase which is then tested for presence and/or amount of ATP.

[0036] 10. If ATP is detected, this indicates that adenylate kinase in the reporter composition was bound to the solid phase, ie that analyte was present in the sample.

[0037] The solid phase is suitably selected from conventional solid phases used in immunoassays, and can for example be a microtitre well, a column, a dip-stick or a bead, such as a latex or a magnetic bead. Examples of further suitable solid supports are nitrocellulose, polyvinylchloride, polystyrene, diazotized paper, activated beads having a range of appropriate linking agents and *S. aureus* protein A beads. More thermostable supports are provided by plastics such as polypropylene, polycarbonate, polyphenylenine oxide polymethylpentene and fluoropolymers (e.g. PTFE, PFA, FEP and EFTE). The solid support can have several forms dependent upon the type of support and the conditions required. Commonly these will be microtitre plates, where each individual well serves as an independent incubation chamber. Similarly, membranes or sheets can be used providing lateral diffusion is limited. Alternatively, beads can be used, which enable the separate reactions to be performed in different tubes under different conditions. These individual matrix materials can be purchased in a variety of forms, as appropriate for the particular type of assay.

[0038] Firefly luciferin catalyses the oxidation of D(−) luciferin in the presence of ATP-Mg^{2+} and O_2 to generate oxyluciferin and light. The quantum yield for this reaction (0.88) is the highest known for bioluminescent reactions (Gould and Subramini, 1988). Firefly luciferase, however, is relatively unstable and has, therefore, not proved readily adaptable as an immunoassay label (Kricka, 1993). By contrast, in the present invention, the luciferase enzyme can be operated under its optimal conditions and is not exposed to harsh treatments such as antibody-coupling.

[0039] A number of extremely thermostable adenylate kinases have now been characterised (Ki and Takahisa, 1988; Lacher and Schäfer 1993; Rusnak et al., 1995) and are suitable for use in the present invention. One has been cloned and overexpressed in *E. coli*(Bonisch et al., 1996) and the full sequences of a range of others are now available as a result of genome sequencing programmes. A rapid and simple purification scheme is thus available to produce homogenous adenylate kinase. Initially a thermal denaturation step can be employed to denature the bulk of *E. coli* proteins (~90-95%) while retaining the thermostable activity in solution.

[0040] This procedure has been successfully employed in embodiments of the present invention with several recombinant thermostable enzymes.

[0041] Subsequently a generally applicable affinity purification procedure can be utilised to yield the purified enzyme. This involves binding of the enzyme to a mimetic dye matrix and selective desorption with the adenylate kinase inhibitor P^1, P^5-di(adenosine-5') pentaphosphate (Rusnak et al., 1995). The use of stable enzymes overcomes problems associated with inactivation upon antibody-coupling, and also provide other benefits. Since the activity is extremely thermostable, once substrate binding and removal of unbound components has occurred, the temperature can be increased to e.g. 70-90° C., denaturing and inactivating any residual contaminating mesophilic adenylate kinase. Additionally, on cooling, a mesophilic ATPase (or apyrase) can be added to remove any residual ATP. This ensures that no ATP or AK background is now present. A further heat incubation inactivates the mesophilic ATPase and ADP is added in order to generate ATP derived exclusively from the thermostable adenylate kinase. This ATP is then available for conventional luciferin-luciferase bioluminescence detection. A potentially contaminating ATP signal is now only possible from three sources: non-specifically bound thermostable AK, ATP-contaminated ADP and AK contaminated luciferase. The latter two can be eliminated by the use of high purity reagents and careful handing. In each case, however, contamination would result in a positive signal, i.e. a PrP-free sample might be determined to be PrP-containing but the opposite could not occur.

[0042] A known thermostable adenylate kinases, *Methanococcus jannaschii* has a very high specific activity, namely 89 μmol of ATP mg^{-1} min^{-1}. This corresponds to a turnover number in excess of 2000 min^{-1} and the potential to produce more than 1.2×10^5 molecules of ATP per molecule of AK in an hour's incubation. Since 6×10^8 molecules of ATP are detectable by ATP-bioluminescence then as few as 5×10^3 molecules of PrP would be detectable. This is 40-fold lower than the minimum number of PrP^{Sc} molecules identified as constituting a single infectious unit. An additional safety margin is provided by the presence of much higher quantities of PrP^c in relation to PrP^{SC} indicating that the present invention exceeds the required sensitivity by several orders of magnitude.

[0043] As an alternative to use of an analyte-specific antibody to immobilize analyte on the solid phase, the solid phase may be provided with analyte immobilised directly thereon without the presence of the first antibody. For example, the solid phase can itself be a substrate potentially contaminated by an amount, typically a trace amount, of analyte. This is the case in respect of medical equipment potentially contaminated by very small amounts of prion protein which are effectively immobilised on the surface of the equipment. The assay is of use in testing for the presence of the analyte for example following cleaning of the equipment. Analyte can also be immobilised non-specifically.

[0044] The method of the present invention may be carried out utilising relatively inexpensive equipment in a standard laboratory. Use of a method of the present invention to determine when the level of prion protein has been reduced to below detectable and, by extrapolation, infectious levels may be used to confirm the decontamination of instruments, equipment and other items potentially exposed to TSE infectious agents, permitting their safe use.

[0045] In use of a specific embodiment of the invention, the first washing step can be repeated a number of times, in accordance with conventional practice in this field, the object being to remove from the solid phase all components of the sample that have not bound specifically to the immobilised antibody. Thus, if there is no analyte present in the sample then the washing step will remove the whole of the sample and ultimately the assay will give no signal, indicating that no analyte was present. The antibody in the reporter composition binds to the same analyte as the antibody immobilised on the solid phase. The antibody and the reporter composition can in fact have the same binding properties as the immobilised antibody, though it is an alternative for the reporter antibody to bind to a different site on the same analyte. The reporter antibody is preferably selected so that the amount of reporter composition that binds to the analyte is substantially proportional to the amount of analyte present. The second washing step can, in line with the first, be repeated a number of times in accordance with conventional practice, the object of the

second washing step being to remove all components of the reporter composition that have not specifically bound to analyte which itself has specifically bound to immobilised antibody. Thus, if no analyte is present on the solid phase the second washing step is to remove all reporter composition, leading ultimately to no signal being generated in the assay, indicating no analyte was present in the sample under investigation.

[0046] This latter embodiment represents use of the principles of the invention in a two antibody capture assay, sometimes referred to as a sandwich assay. The invention is similarly of application in antigen capture assays and antibody capture assays.

[0047] Thus in a further embodiment of the invention, an assay for analyte comprises specifically associating an analyte with a reporter adenylate kinase, wherein the analyte is bound to a solid phase. This embodiment may be referred to as being of the antibody capture type. Binding of the analyte to the solid phase can be achieved by non-specifically binding the analyte to the solid phase and then treating the solid phase to prevent further non-specific binding thereto—in this way, a number of components from a sample are bound to the solid phase, which components include the analyte of interest if present in the sample, and subsequent treatment ensures that when an antibody is added to detect the analyte that antibody will only bind to the solid phase if analyte is present.

[0048] The use of heat to denature any endogenous kinase that may be present has been carried out in an embodiment above as step 6, though as mentioned this step can be carried out at an alternative juncture in the assay provided that it is carried out before addition of ADP. Further, ADP may be added before the ATPase provided the ATPase has no ADPase activity. The temperature and duration adopted are chosen so as to be sufficient to denature the endogenous adenylate kinase whilst leaving intact the reporter adenylate kinase, this reporter adenylate kinase preferably being a thermostable enzyme. In a specific embodiment described below, heating to a temperature of about 90° C. for about 10 minutes has been found effective, Sufficiently thermostable adenylate kinases may be found amongst a range of bacterial and archaeal genera and families. In the Bacteria, they may be produced, for example, by members of the genera *Alicyclobacillus, Ammonifex, Aquifex, Bacillus, Caldariella, Calderobacterium, Caldicellulosiruptor, Caldocellum, Caloramator, Carboxydothermus, Chloroflexus, Clostridium, Coprothermobacter, Dictyloglomus, Fervidobacterium, Geotoga, Hydrogenobacter, Hydrogenothermophilus, Meiothermus, Petrotoga, Rhodothermus, Rubrobacter, Thermoactinomyces, Thermoanaerobacter, Thermoanaerobacterium, Thermoanaerobium, Thermobacterium, Thermobacteroides, Thermobifida, Thermobispora, Thermobrachium, Thermochromatium, Thermocrispum, Thermodesulfobacterium, Thermodesulforhabdus, Thermodesulfovibrio, Thermohydrogenium, Thermomicrobium, Thermomonospora, Thermonema, Thermonospora, Thermopolyspora, Thermosipho, Thermosphaera, Thermosyntropha, Thermoterrabacterium, Thermotoga* and *Thermus*, Amongst the archaea, they may be produced, for example, by members of the genera *Acidianus, Aeropyrum, Archaeoglobus, Desulfurococcus, Desulfurolobus, Ferroglobus, Hyperthermus, Metallosphaera, Methanobacterium, Methanococcus, Methanopyrus, Methanothermus, Picrophilus, Pyrobaculum, Pyrococcus, Pyrodictium, Pyrolobus, Staphylothermus, Stetteria, Stygiolobus, Sulfolobus, Sulfophobococcus, Thermococcus, Thermofilum, Thermoplasma* and *Thermoproteus.*

[0049] It is preferred, though optional, also to carry out a step of removing endogenous ATP from the sample using a thermolabile ATPase and subsequently destroying this latter enzyme, again conveniently using heat. In a specific embodiment of the invention described below, an incubation of about 10 minutes has been effective using a thermolabile ATPase and this enzyme has been then denatured by temperatures of about 90° C. for 5 minutes. ATP can be released from cells or other cellular components after heating. Therefore, it is preferred that the step of removing ATP is carried out after an initial heating of the sample, for example after the step of using heat to destroy endogenous adenylate kinase.

[0050] It is further preferred to use ultrapure ADP, free of ATP, to avoid risk of background from contaminating ATP. As an alternative to the use of a pre-purified ultrapure form of ADP, ATP-free ADP can be generated in situ by the addition of an essentially irreversible and strictly ATP-dependent mesophilic kinase plus its substrate, for example, yeast hexokinase and glucose. ATP present is converted to ADP and the kinase is inactivated by heat prior to the incubation with thermostable adenylate kinase. Similarly, it is also preferred to use other reagents form of contamination by kinase or ATP. Luciferin and luciferase can contain adenylate kinase contamination and so it is preferred to use purified forms of these, or recombinant forms of luciferase. Luciferin is preferably the d-isomer as the I-isomer can inhibition the luminescence reaction.

[0051] The invention is of particular application to detection of diseases such as vCJD, which by December 1999 had resulted in approximately 50 deaths in the UK, with further cases reported in France and Ireland. Due to the long and variable incubation period for this new disease however, there is currently great uncertainty regarding the total numbers of individuals in the UK potentially or actually infected with vCJD. Affected individuals will frequently present with symptoms requiring neurological examination or may merely undergo common surgical procedures such as tonsillectomy or appendectomy along with the general population. A wide range of tissues, including tonsil and appendix, has been shown to harbour vCJD infectivity in addition to brain and spinal cord. This gives rise to a significant potential for transmission of infection by exposure to contaminated surgical instruments, since complete elimination of infectivity is not achievable using conventional sterilisation procedures.

[0052] Although the nature of the responsible agent is not fully understood, infectivity appears to be associated very closely with the abnormal conformation (PrP^{Sc}) of a normal central nervous system protein (PrP^{c}), designated the "prion" protein. Although the prion is not universally accepted as being solely responsible for infectivity, there is general agreement that it has an intimate association with it. Detection of prion protein is, therefore, considered to be an excellent measure of the potential presence of TSE infectivity. Prions have a tendency to form insoluble aggregates and are highly hydrophobic. There is, therefore, considerable doubt as to whether they can be reliably detached from surfaces and solubilised for detection by conventional enzyme-linked immunosorbent assay. This is particularly important for items like surgical instruments, where the presence of a very small amount of residual material after attempted decontamination, could give rise to iatrogenic transmission of vCJD infection. In a specific

embodiment, the invention describes an assay which permits in situ detection of the prion protein (Prion ELISA 1-3).

[0053] Since the presence of any residue containing either PrP^{c} or PrP^{Sc} indicates that the test item is not completely clean, the antibody selected need not discriminate between the different conformers. This greatly increases the range of antibodies available. The PrP^{Sc} conformation is, however, considerably more persistent and in general it is the form associated with infectivity which will be detected.

[0054] Thyroid stimulating hormone (TSH) is secreted by the anterior pituitary of the brain. This hormone acts upon the thyroid, stimulating the production of the hormones T3 and T4. The level of TSH is controlled by a negative feed-back system that maintains a constant level of free TSH. Hyperthyroidism is a condition caused by reduced levels of circulating TSH.

[0055] Diagnostic assays for the diagnosis of hyperthyroidism must be able to distinguish between hyperthyroidism and normal levels of circulating hormone. The assays should be able to monitor a very low signal without interference. In addition, assays for the measurement of circulating TSH should have a broad dynamic range. A specific embodiment of the invention, described below in more detail, provides an assay for a blood hormone.

[0056] Assays for drugs of abuse are routinely used by clinical laboratories, drug rehabilitation clinics, health officials and clinical justice facilities. The data obtained is often used to support medical-legal applications involving custody of children. A decision to renew custody of a child often rests on the results of urine drug analyses demonstrating prolonged abstinence of drug abuse, by the parent. In many countries random urine testing is mandatory in sensitive government posts, the armed forces and the transport industries. There is a requirement for more sensitive and rapid assays for drugs of abuse.

[0057] The principal agent produced by *Cannabis* saliva is δ-9-tetrahdrocannabinol (THC). Only a small amount of THC is excreted in the urine and the majority of assays are designed to detect the main inactive oxidation product, 11-nor-δ-tetrahydrocannabinol-9-carboxylic acid (11-COOH-THC). A specific embodiment of the invention, described in more detail below, provides an assay for *cannabis* metabolite.

[0058] Urine is a complex medium, which exacerbates the problem of distinguishing a signal from that of background instrument noise. This is overcome in commercial assays by assigning a threshold concentration, above which a sample is considered positive, that exceeds the detection limit by several orders of magnitude. In practice this results in a number of positive samples being assigned as negative as their signals are below the assigned threshold.

[0059] More sensitive assays make it easier to discriminate between positive and negative samples.

[0060] Many of the current commercial assays involve enzyme multiplied immunoassay (Emit). This ELISA involves competition between drug in the test sample and drug labelled with glucose-6-phosphate dehydrogenase (G6PDH). The G6PH drug conjugate is inactive when immobilised to a solid-phase comprising of an antibody specific for the drug of interest. On displacement the free drug-G6PH conjugate is detected by a change in the optical density at 340 nm, as NAD+ is reduced to NADH and the substrate is glucose-6-phosphate is oxidised. A further specific embodiment of the invention provides an assay for cocaine metabolites in urine.

[0061] It is known that human papilloma virus (HPV) infection is a prerequisite of the oncogenesis of many forms of cervical cancer. Currently cervical smears are screened for the presence of viral infection as a predictive precursor of oncogenesis. Another specific embodiment of the invention is a rapid screen for the presence of viral infection of cervical cells.

[0062] Combinational libraries are powerful tools for drug discovery. The sensitivity of the screening methodology is a major limit on the number of combinations that can be screened for in a combinational library. A library comprised of every combination of an hexa-peptide is composed of 206 possible combinations. More sensitive assays for the detection of target sequences would allow more extensive libraries to be screened. In a yet further specific embodiment of the invention a thermostable AK is used to screen a combinational peptide library for a sequence that binds a specific ligand of interest. This ligand may be a receptor or an enzyme.

[0063] Botulinum toxins are produced by the bacterial species *Clostridium botulinum* and are the causative agents of food-borne botulism. The most sensitive accepted method for the detection of botulinum toxins is the mouse lethality test. Few ELISA based assays using conventional amplification methodology have the sensitivity required. A yet further embodiment of the invention describes an ELISA based assay for the detection of botulinum neurotoxin in foods.

[0064] The present invention also provides, in a second aspect, apparatus for determining the presence and/or amount of analyte in a sample, comprising:—

[0065] a solid phase on which is immobilised the analyte or an antibody specific for the analyte;

[0066] a reporter composition comprising a thermostable kinase coupled to an antibody specific for the analyte; and

[0067] ADP plus, optionally, associated reagents for conversion of ADP into ATP by thermostable kinase.

[0068] An optional additional component of the apparatus is a thermolabile ATPase.

[0069] The components of the apparatus may be combined into a test kit for determining presence and/or amount of an analyte in a sample.

[0070] Testing for formation of ATP may be carried out using a number of conventional means, including formation of colour. Particularly preferred is the use of luciferin/iluciferase reagents in combination with calibration curves to determine both presence and amount of analyte. The presence of magnesium ions is usually required for formation of ATP, and further details are provided in the prior art publication GB-A-2304892, the contents of which are incorporated herein by reference.

[0071] The present invention has been described in relation to the use of kinases, in particular thermostable adenylate kinase, More generally, the invention also provides, in a third aspect, an assay for determining presence and/or amount of an analyte in a sample, comprising:—

[0072] exposing the sample to a detector composition, the detector composition comprising an antibody specific to the analyte coupled to a thermostable enzyme;

[0073] isolating (i) detector composition that has specifically bound to analyte from (ii) detector composition that has not specifically bound to analyte;

[0074] determining the presence and/or amount of detector composition that has bound to analyte by adding a substrate for the thermostable enzyme;

[0075] wherein prior to adding the substrate non-thermostable enzymes are destroyed by application of heat.

[0076] The thermostable enzyme is suitably a kinase, and may be selected from pyruvate kinase, adenylate kinase and acetyl kinase. All of these catalyse formation of ATP from ADP and may be used with reagent such as luciferin/luciferase.

[0077] It is preferred that prior to addition of the substrate background product is removed, which assists in reducing or limiting background in the assay.

[0078] Background product is suitably removed by the action of enzyme or by thermal inactivation.

[0079] The third aspect of the invention also provides apparatus for determining presence and/or amount of analyte in a sample, comprising:—

[0080] a solid phase on which is immobilised the analyte or an antibody specific for the analyte;

[0081] a reporter composition comprising a thermostable enzyme coupled to an antibody specific for the analyte; and

[0082] substrate for the thermostable enzyme.

[0083] This aspect of the invention confers the advantage that the signal obtained from the thermostable enzyme is substantially not contaminated by any background signals or background noise that may otherwise be obtained from the action of non-thermostable enzymes on the substrate.

[0084] Background signals and/or background noise are thus reduced and possibly even removed entirely. In use of a method of the third aspect of the present invention, an analyte is immobilised on a solid phase, a sample is combined with the solid phase and then the solid phase is washed, the solid phase is exposed to a detector composition including an antibody specific to the analyte coupled to a thermostable enzyme, the solid phase is then again washed, the solid phase is then heated to denature non-thermostable enzymes but so as not to denature the thermostable enzyme of the detector composition, and the amount of thermostable enzyme specifically bound to analyte which itself is specifically bound to the solid phase is determined by adding a substrate for the thermostable enzyme and determining how much product is then obtained. Immobilisation of the analyte can be through use of an analyte-specific antibody immobilised on the solid phase, or by directly binding the analyte to the solid phase.

[0085] A further aspect of the invention provides a conjugate comprising an antibody conjugated to a thermostable enzyme for use in the assay of any preceding aspect of the invention. In an embodiment of the invention, the enzyme an adenylate kinase. The antibody may suitably bind to an analyte selected from a protein, a microorganism, a peptide, a toxin, a hormone and a metabolite. In a specific embodiment, the antibody binds to a prion protein.

[0086] A still further aspect of the invention lies in use of the apparatus of the invention or the conjugate of the invention in an assay for an analyte.

[0087] The present invention is thus suitably employed to investigate the effectiveness of a range of agents with potential for surface cleaning of contaminated surfaces to remove cellular material and PrP. Steel, glass and plastic surfaces can all be investigated to determine whether any one is particularly recalcitrant to cleaning, and PTFE can be used as a control surface for comparative purposes.

[0088] Thermostable adenylate kinases may be purified from a number of thermophilic and hyperthermophilic microorganisms using a combination of ion exchange, gel filtration and affinity chromatography. The adenylate kinases may be cloned and expressed in *E. coli* in plasmid or phage libraries. Direct expression can be screened for (after replica plating) by examining pooled colonies for thermostable adenylate kinase activity by incubation with ADP, followed by ATP bioluminescence assay.

[0089] A range of commercially available coupling reagents is available for antibody-adenylate kinase conjugation. Both the antibody and the adenylate kinase can be repurified by affinity chromatography.

[0090] In certain uses of the invention, such as in the case that there is no endogenous adenylate kinase or no microbial contamination of the sample or if the risk of such contamination is removed, it is optional to dispense with the step of removing endogenous adenylate kinase. The method of the invention then comprises specifically associating the analyte with a reporter adenylate kinase, adding ADP and testing for formation of ATP. Preferably, prior to addition of ADP, ATP is substantially removed, for example by the use of an ATPase.

[0091] In one embodiment, the invention provides an assay for an analyte in a sample, comprising contacting the analyte with a thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, wherein a complex is formed, adding ADP and testing for the formation of ATP, wherein, prior to the addition of ADP, endogenous kinase and uncomplexed thermostable reporter adenylate kinase is substantially removed by washing, and residual endogenous kinase is inactivated by heating, wherein the amount of ATP correlates to the concentration of the analyte,

[0092] In another embodiment, the invention provides an assay for determining the presence and/or amount of an analyte in a sample, comprising exposing the sample to thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, so that the reporter adenylate kinase is specifically associated with any analyte present in the sample via the binding agent; removing the thermostable reporter adenylate kinase that is not bound to the analyte; exposing said thermostable reporter adenlyate kinase bound to the analyte to ADP; and testing for the formation of ATP, wherein prior to the addition of ADP, residual kinase other than thermostable reporter adenylate kinase is substantially removed by heating.

[0093] In a further embodiment, the invention provides an assay for determining the presence and/or amount of an analyte in a sample comprising, exposing the sample to a detector compound, the detector compound comprising an antibody specific to the analyte coupled to a thermostable enzyme; isolating (i) detector compound that has specifically bound to analyte from (ii) detector compound that has not specifically bound to analyte; determining the presence of and/or amount of detector compound that has bound to analyte by adding a substrate for the thermostable enzyme and measuring a product formed by conversion of said substrate to said product by said thermostable enzyme; wherein, prior to the addition of substrate, non-thermostable enzymes are destroyed by application of heat.

[0094] In a further embodiment, the invention provides an assay for an analyte comprising the steps of:

[0095] (a) specifically binding the analyte with a thermostable reporter kinase which has been coupled to a binding agent specific for the analyte forming a complex;

[0096] (b) washing to remove endogenous non-thermostable kinase and thermostable reporter kinase not bound to analyte;

[0097] (c) heating to inactivate endogenous kinase not removed by step (b); and

[0098] (d) adding ADP and testing for formation of ATP.

[0099] The assay of the invention is particularly suited to the analysis of samples having a high level of background activity. These samples may also be known as "complex" samples.

[0100] A high level of background activity can be caused by various factors, which are discussed in more detail below.

[0101] High background activity may be due to the presence of large amounts of material in suspension. This might represent fat (lipid), protein, carbohydrate or cellular debris derived from either host or bacterial contaminant. This material may directly interfere with the binding of, for example, an analyte with the solid phase used for the assay. The same would be true for samples where there are high levels of protein or other molecules, that, due to their solubility, do not make the sample turbid. Any reduction in the levels of analyte bound to the solid phase would effectively reduce the potential signal of the assays by limiting the amount of detectable substance.

[0102] Background activity may also result from the presence of contaminating enzyme activity. For example, endogenous peroxidase activity can interfere with horseradish peroxidase assays, endogenous phosphatases can interfere with alkaline phosphatase-based assays, and many cell/tissue extract have an endogenous fluorescence that may interfere with fluorimetric assays. In the assay of the present invention, enzymes such as endogenous adenylate kinase may increase the background activity of the samples. Any samples that contain intact cells or where cell debris is present are likely to have relatively high background activity.

[0103] The presence of endogenous ATP may also increase the background activity of the sample.

[0104] The background activity may be further complicated by the disease state of the individual. For example, the analysis of urine for the presence of cocaine metabolites might be complicated by a high level of protein or other metabolites present as a result of urinary tract infection, infection of the kidney or liver or with advanced kidney damage (perhaps with associated proteinuria). Similar considerations would be relevant for oral diagnosis of patients with disease (s) of the oral cavity, airway samples for patients with chronic obstructive pulmonary disorder (COPD), cystic fibrosis or other lung/airway disease.

[0105] One way of measuring the background activity of a sample (such as the background activity caused by endogenous kinase activity and the presence of endogenous ATP) is by measuring the Relative Light Unit value of the sample in the presence of luciferin/luciferase in a luminometer. Typical values for the background activity in complex biological samples (caused by e.g. endogenous adenylate kinase activity and the presence of ATP) may be in the range of about 300,000-500,000 RLUs for a 1 mg/ml sample of tissue homogenate, serum, oral sample or urine, up to a maximum of about 6,000,000 to 10,000,000 RLUs for a 1 mg/ml sample of whole blood.

[0106] Those familiar with the art will recognise that Relative Light Units (RLU) are a relative, not absolute, measurement. The figures given in the specification relate to measurements taken using a Berthold Orion 96-well microplate luminometer with injector system using a "flash" method of light measurement for 2 seconds immediately after the addition of the luciferase/luciferin reagents (technical specification photomultiplier measuring light emitted at a wavelength of 300-650 nm).

[0107] To address this issue, manufacturers have generated data for RLU "factors", which allow the data generated by a given luminometer to be normalised to a calibrated standard. Thus, comparisons can be made between different instruments. The RLU factor for the Berthold Orion 96-well microplate luminometer used in the experiments described in the present specification is 1. Accordingly, the RLU values given in the specification can be regarded as standardised/normalised RLU values.

[0108] In terms of absolute values, an RLU value can be related to the concentration of ATP required to give said value with the reagents as described in the method. As an approximate conversion, and given the linear relationship between RLU values and ATP concentration, the following values can be used:

RLU	Approximate concentration of ATP/μM
12,000,000	1000
1,200,000	100
120,000	10
12,000	1
1,200	0.1
120	0.01

[0109] In preferred embodiments of the invention, the sample used in the assay has an initial background activity (ie. before the sample is brought into contact with the reporter enzyme/detector compound) of at least 300,000 Relative Light Units per mg protein per ml sample when measured in the presence of luciferin/luciferase by a luminometer. Preferably, the sample has an initial background activity of at least 400,000, or at least 500,000, or at least 600,000, or at least 700,000, or at least 800,000, or at least 900,000, or at least 1,000,000, or at least 3,000,000, or at least 6,000,000, or at least 10,000,000 Relative Light Units per mg protein per ml sample when measured in the presence of luciferin/luciferase by a luminometer. More preferably, the sample has an initial background activity of between 300,000-500,000 or 800,000-1,000,000 or 6,000,000-10,000,000 Relative Light Units per mg protein per ml sample when measured in the presence of luciferin/luciferase by a luminometer.

[0110] The initial background activity of a sample may be significantly reduced by steps carried out during the assay. For example, the washing and/or heating and/or enzymatic steps of the assay described throughout this specification may result in a reduced background activity. In preferred embodiments of the invention, the sample, prior to the addition of the reporter enzyme substrate/detector compound substrate, has a reduced background activity when measured in the presence of luciferin/luciferase in a luminometer, said reduced background activity having a Relative Light Unit value that is 20-fold to 1000-fold lower than the initial background activity described above. Preferably, said reduced background activity has a Relative Light Unit value that is 50-fold to 800-fold lower than the initial background activity, more preferably 500-1000-fold lower than the initial background activity. Alternatively, the reduced background activity has a Relative Light Unit value that is 100, or 200, or 300, or 400,

or 500, or 600, or 700, or 800, or 900 or 1000-fold lower than the initial background activity.

[0111] Thus, samples used in the assay of the invention may have (i) an initial background activity as described above, and/or (ii) a reduced background activity after the background reduction steps of the assay have been completed.

[0112] Against the reduced background activity, the assay of the invention is capable of achieving a detection limit, based on a definition of 3 Standard Deviations above the control, that may be as little as 10-20% above the reduced background value. This compares to standard assay methods, which would require around a 100% greater value than the control background, based on the same 3SD definition of the detection limit.

[0113] As well as being particularly suited to "high background" samples, the assay of the invention may also be used to analyse samples that contain particularly low levels of analyte. The assay may also be used to analyse samples that have low levels of analyte and high levels of background activity, i.e. a low signal:noise ratio.

[0114] In a preferred embodiment of the invention, the analyte is present in the sample at a concentration of less than 10 ng/ml. Preferably, the analyte is present at a concentration of less than 1 ng/ml, or less than 500 pg/ml, or less than 100 pg/ml, or less than 10 pg/ml, or less than 1 pg/ml, or less than 100 fg/ml, or less than 10 fg/ml, or less than 1 fg/ml. Samples containing such levels of analyte may also have an initial and/or reduced background activity as described above.

[0115] A wide variety of sample types can be analysed using the assay of the invention. These samples may include a sample, or any combination of samples, independently selected from the group consisting of urine, faeces (stool), vomitus, blood components (including serum, plasma, whole blood, white blood cell fractions, buffy coat), airway samples (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates), oral samples (including crevicular fluid, parotid saliva, whole saliva), cerebrospinal fluid (CSF), tissue homogenates (including brain homogenate, tonsil homogenate), pus, swab samples (including those taken from throat, nose, ears, skin, and wounds), effluent samples, surface swabs, food, water, beverages, soil, air sampling and sewerage. Preferably, the sample is selected from the group consisting of faeces (stool), serum and whole blood.

[0116] Within such samples the detection of a wide range of agents/analytes/antigens provide the basis for a rapid and ultra-sensitive detection method to support diagnosis of an infection or disease. Similarly the methods can be applied to detect the presence of foreign material that may be present in the sample due to accidental or deliberate contamination. The method also allows for the development of methods to validate that an agentlanalyte/antigen has been effectively removed by a decontamination or disinfection procedure, such that the environment or sample is now safe.

[0117] Specific examples of agents/antigens/analytes that can be detected via the assay of the invention include the following:

Bacteria:

[0118] Any bacterial agent, either Gram-positive or Gram-negative that is associated or may be associated with disease, *Staphylococcus aureus* (in particular antibiotic resistant strains such as methicillin resistant *Staphylococcus aureus*; MRSA).

Toxins:

[0119] Botulinum toxin (including all botulinum neurotoxin serotypes A-G; BoNT A-G and tetanus neurotoxin; TeNT), Anthrax toxins (including lethal toxin, oedema toxin and their component parts; lethal factor (LF), oedema factor (EF) and protective antigen (PA), ricin, mycotoxins, afalatoxins, superantigens.

Viruses:

[0120] Rotavirus, Norwalk/Norwalk-like virus (alternatively termed norovirus), Measles, mumps, rubella, HIV, hepatitis (all forms).

Prion Agents:

[0121] Agents responsible for causing diseases such as Creutzfeldt Jakob Disease (CJD; including variant, familial, sporadic and iatrogenic forms of the disease), scrapie, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) and any other member of this family of disorders. The surrogate marker PrP^{Sc} (prion protein-scrapie associated form) associated with prion diseases may also be a valid means of identifying and/or diagnosing the presence of disease or infectious material.

Analytes:

[0122] B-lactamases, (including extended spectrum B-lactamases; ESBL), hormones, tumour markers, neurotransmitters, growth factors.

[0123] The ability of the invention to support the detection of antigen/antibodies/analytes at a lower level and in cruder samples than traditional formats has a number of advantages.

[0124] For example, the ability to detect a low level of a bacterial or viral antigen in eg. oral, faecal or urine samples may allow the earlier diagnosis of infection than would be supported by waiting for a general bacteraemia or viremia to be detected in blood. Further, the ability of the assay method to detect antigens directly in blood (rather than via an antigen-capture type assay) may facilitate detection and/or the diagnosis of disease. For example the early detection of circulating endotoxin in blood following infection by any one of a range of bacteria associated with human diseases would accelerate treatment and improve the prognosis.

[0125] The sensitivity of the assay also allows detection of a disease that might otherwise be undetectable due to the phase of the infection. For example diseases such as TB and HIV are characterised by extended dormant/latent phases where diagnosis may be difficult. The ability of the invention to assess very low levels of antigen in crude samples might support the diagnosis of such diseases despite the fact that they may be immunologically silent. In the case of TB for example the ability to work with very crude sputum, or bronchoalveolar aspirates would be very relevant to supporting diagnosis, even of very low levels of antigen. This same feature is true of a wide range of intracellular pathogens that evade recognition by the host by manipulating the immune response. The ability of these organisms to remain "immunologically silent" means that there may be little or no detectable immune response, and during early stages and/or specific phases of the infection the levels of antigen may be below

detectable limits. Again the nature of the samples that can be analysed according to the method of the invention, makes it more adaptable to measure such infections within, for example, the oral cavity, urinary tract, lower bowel or airways where samples are likely to be more complex and may have greater levels of contaminating activity and/or material.

[0126] The direct detection of analyte in a complex sample, whilst maintaining high sensitivity, is also an advantageous feature of the invention. For example, this would allow detection to be achieved where only a single antibody or equivalent reagent is available. Standardly, a pair of antibodies is used to provide a "capture" phase and a detection phase. In the absence of a suitable pair of antibodies (e.g. due to lack of epitopes on a particular antigen, where the binding of a single antibody prevents the binding of further reagents or where there is significant cross reactivity for one of the reagent pair) detection is currently difficult due to low sensitivity and high levels of interference. The assay method helps to reduce this as it is intrinsically more sensitive than most available assay methods and can eliminate background activity.

[0127] The invention also allows for reduced sample processing. The detection of low levels of an analyte/agent/antigen in complex biological samples may often require the enrichment and/or partial purification of the target molecule (s). This may be time consuming, require the collection and use of large amounts of sample and may pose a possible risk to the operators (e.g. during centrifugation of infectious samples). By being able to directly capture the antigen at sufficient levels for detection even in complex samples the method significantly reduces the time required for the assay, with a reduction in associated costs, can be used for assays where the amount of sample may be limiting (e.g. neonatal samples), and minimises any risks associated with processing of the sample.

[0128] The fact that the assay of the invention allows detection of low level of analytes in a sample means that it allows the detection of a disease at an earlier point than is currently possible. As an example the detection of immunoglobulins in oral samples is at the limit of current detection technologies with the level of immunoglobulin in oral samples typically 1/1000th that in blood. This is illustrated in the following table:

Concentrations of immunoglobulins relevant for supporting diagnosis in plasma and fractions of saliva.

Specimen	IgG mg/ml	IgM mg/ml
Plasma	14730	1280
Parotid Saliva	0.36	0.43
Crevicular Fluid	3500	250
Whole Saliva	14.4	2.1

(Adapted from McKie A, Vyse A, Maple C. *Novel methods for the detection of microbial antibodies in oral fluid. Lancet Infect Dis.* (2002) 2: 18-24. Original data from Brandtzaeg P, Fjellanger I, Gjeruldsen S T. *Human secretory immunoglobulins I. Salivary secretions from individuals with normal or low levels of serum immunoglobulins. Scand J Haematol Suppl* 1970; 12: 3-83, and Roitt I, Lehner T. *Oral immunity of oral diseases,* 2nd ed. Oxford: Blackwell).

[0129] The review article (McKie et al Lancet Infectious Disease 2002) describes many problems with the oral diagnosis of disease, but in particular suggest that lack of sensitivity is the most significant issue. The analysis of such samples is complicated by the high background of cellular debris, protein and possible contaminating microrganisms. Whilst the levels of IgG are sufficient to support the diagnosis of certain diseases/immune states, this often requires the use of radioimmunoassays. These have a number of advantages over traditional reporter enzyme detection formats, in terms of the dynamic range and sensitivity, but have serious disadvantages in that they require dedicated laboratories for the handling of radio-isotopes, are technically demanding, generate radioactive waste with disposal issues and have proved very difficult to transfer between laboratories.

[0130] The levels of IgM have, in the vast majority of cases, proved to be insufficient to support diagnosis via oral samples. For a number of infectious diseases the detection of IgM type antibodies as part of the initial infection prior to seroconversion to an IgG type response is a useful diagnostic indicator. For example in the diagnosis of West Nile disease, an emerging infectious disease in North America and elsewhere, levels of anti-virus serum IgM are used to support an initial presumptive diagnosis (as approved by the FDA as part of the PanBio West Nile Test kit). Aside from infection they are also an extremely valuable method for assessing the initial response to a vaccination, which after the first inoculation leads to an IgM response in most cases. By monitoring this initial priming response, by way of a simple non-invasive oral sample, it would be possible to predict likely efficacy of the vaccination schedule and curtail the vaccination if the person was not responding. This would be particularly useful for cases where there is significant risk of adverse effects from the vaccination. Such assays are described in Examples 18 and 19.

[0131] In other infections, IgM production is currently used as the diagnostic indicator by way of serum sampling. The ability of the invention to detect the relatively low levels of IgG or IgM in oral samples and to cope with the intrinsic high background of the sample, means that it supports both the early diagnosis of infections and the assessment of initial immune response following vaccination.

[0132] In one embodiment, the assay allows for the detection of a bacteraemia at an earlier stage than is currently possible, e.g. the detection of bacteria such as *Streptococcus pneumoniae* in urine as described in Example 17. It also offers the potential to discriminate against carriers of *S. pneumoniae* who are not actually infected.

[0133] In a further embodiment, the assay is used to detect Methicillin-resistant *Staphylococcus aureus* (MRSA) (see Example 16). MRSA is a significant public health issue with respect to hospital acquired infection. The number of cases of infection have increased in many countries over recent years and the spread of multiple antibiotic resistant strains is also of considerable concern. A method for the rapid and sensitive detection and diagnosis of MRSA from tissues swabs, without the time-consuming requirement to culture the bacteria, would significantly assist diagnosis. Those familiar with the art will recognise that a number of assays have been developed in which the bacteria are captured and lysed and the AK released from the cells is quantified. Some of these methods have been adapted to the detection of MRSA. The method of the invention has the advantage that by using serotype specific antibodies the method can provide information on both the presence and type of MRSA present in the sample. This is not possible with the alternative methods. The ability of the method of the invention to detect low levels of MRSA in tissue swabs and/or other samples makes it a valuable method

for detecting and controlling the transmission of MRSA in healthcare facilities. The emergence of community acquired cases of MRSA and other antibiotic resistant bacteria means that such an assay is likely to find widespread applications.

[0134] Toxins, such as eg. ricin and botulinum, can also represent a significant threat to public health. The assay of the invention is suitable for testing for the presence of these potent toxins in food, water or other environmental samples, even when the toxin is present at extremely low levels. Examples of toxin assays are provided in Examples 11 and 12.

[0135] The invention can also be used to assay for viruses. Noroviruses, sometimes termed Norwalk-like viruses (NLV), and associated with Winter Vomiting Disease are a major cause of viral gastroenteritis with high attack rates. Major outbreaks occur in a variety of settings associated with high densities of people, including hospitals, cruise ships, schools, and residential homes. Outbreaks of Norovirus can have a significant public health impact. Outbreaks in hospitals may be particularly severe due to general poor health of patients and hospital outbreaks often result in closure of wards and out-patient facilities, to control the spread of the disease, with consequences for the wider patient population. The relative ease with which the disease is transmitted between infected people and the enormous difficulties encountered with decontamination and disinfection means that secondary outbreaks are not uncommon. New methods to support the early diagnosis of NLV and the presence of NLV that remains after ineffective decontamination procedures would have a significant benefit for the public health management of these disorders.

[0136] The assessment of immune response to NLV infection offers the potential to identify patients at very early stages of infection, monitor people carrying the disease without symptoms and help to control and prevent onward transmission of the disease as part of outbreak control. Of particular use would be an assay with sufficient sensitivity to be able to detect the presence of IgM antibodies in oral samples at an early stage of infection. The value of IgM as a diagnostic marker of early phase NLV infection has been described by Brinker et al 1998, Detection of Norwalk virus and other genogroup I human calciviruses by a monoclonal antibody, recombinant antigen-based immunoglobulin M capture enzyme immunoassay, J. Clin. Microbiol. 171:p 1064-1069). An assay for the detection of NLV/Norovirus is described in Example 14.

[0137] The ability to effectively decontaminate surfaces, floors, walls etc in hospital wards and other sites of NLV outbreaks is critical in controlling the onward spread of the disease. To support this process a method to validate effective cleaning would be extremely valuable. The assay of the invention is ideally suited to be able to fulfil this role, being able to detect very low levels of antigen in environmental samples and surface swabs that may contain high levels of organic and inorganic matter. This type of assay is described in Example 15.

[0138] A further example of the use of the invention to assay for viruses is presented in Example 13, which describes the detection of rotavirus in a faecal sample.

[0139] One specific application of the assay of the invention is the detection of prion material in tissue homogenates, as described in Example 9. The diagnosis and detection of prion diseases is an important priority for public health management of this class of disorders. The assay method of the invention may be suitable for the detection of prion agents in blood fractions (Example 9), and/or on surgical instruments (Example 10) and/or urine, due to its high sensitivity and ability to eliminate background interference. One tissue that has been found to support the diagnosis of the variant form of Creuzfeldt Jakob Disease (vCJD), probably the form of CJD of most concern to public health scientists and the public, is the tonsil. Whilst a number of studies have examined the tonsil and used this as a basis for diagnosis, this has been done routinely by immunohistochemistry. This method is difficult and time consuming and does not lend itself to the high throughput required for routine diagnosis. The method of the invention is suitable for the rapid analysis of tonsil samples due to its ability to detect low levels of signal in complex samples. Hence the tonsil would be homogenised to generate a very crude sample containing a mixture of intact and lysed cells with high background activity, in terms of both total protein content and enzymatic activity, within which the prion is present at only low levels. Whilst the removal of tonsils requires surgery and as such could not be considered to be non-invasive, this is currently due to the nature of the immunohistochemistry assay used to detect the prion in tonsil tissue. With the method of the invention it might be possible, and preferable, to remove a small number of cells from the tonsil, either by swabbing the surface or using a fine needle to take the equivalent of a biopsy sample. Even if the tonsil does need to be removed to support diagnosis, given the difficulty in detecting the disease without analysis of brain tissue, this may still be a viable option. The assay would also be valuable for the diagnosis of the disease in prospective anonomysed tonsil tissue archives, being collected in the UK and elsewhere, for the assessment of the levels of the disease in the population. Cerebrospinal fluid samples might also be useful for the diagnosis of CJD and might be considered to be acceptably invasive if their use allows diagnosis leading to treatment of the disease. Again these would be complex samples with high levels of protein and cellular debris likely during the progression of neurodegenerative diseases such as CJD.

[0140] The invention is further described with reference to the following Figures in which:

[0141] FIG. **1** is a schematic representation of steps 1-4 of the assay described in Example 1;

[0142] FIG. **2** is a schematic representation of steps 5-8 of the assay described in Example 1;

[0143] FIG. **3** is a schematic representation of steps 9-10 of the assay described in Example 1;

[0144] FIG. **4** is a key, which explains the symbols used in FIGS. **1-3**;

[0145] FIG. **5** is a comparison of an AK-based assay and a traditional HRP based assay method for the detection of recombinant PrP. In Panel A, data is shown for the detection of recombinant PrP diluted in either phosphate buffered saline or in mouse brain homogenate (MBH) in PBS (data shown is for dilution in 0.5 mg/ml MBH but similar results have been shown for 0.05 mg/ml). Panel B shows the expanded range of the assay for ultrasensitive detection of recPrP;

[0146] FIG. **6** shows the detection limits for the detection of recPrP in complex samples. Panel A shows the sensitivity of the assay when recPrP is spiked into whole blood prior to plating. Panel B shows the similar data for recPrP spiked into serum;

[0147] FIG. **7** shows the detection of recombinant PrP on type 316 steel disks as a model for surgical instruments;

[0148] FIG. **8** is a comparison of an AK and a HRP-based assay for the detection of BoNT/B light chain domain. Similar results were obtained with full length BoNT/B (not shown);

[0149] FIG. **9** is a comparison of assay methods for the detection of rotavirus. The detection limit of an anti-rotavirus AK conjugate is compared with a commercially available Rotavirus assay kit (Dakocytomation, UK).

[0150] Specific embodiments of the invention are now described.

[0151] The assay of the present invention can involve the use of conventional equipment and reagents required for known ATP/AK bioluminescence assays, supplemented by a thermal cycler (widely and inexpensively available for PCR), plus two specific enzymes, a thermolabile ATPase and a thermostable adenylate kinase.

EXAMPLE 1

Assay for Prior Protein

Prion ELISA —1

(Reference is Made to the Attached Drawings)

1. Blocking

[0152] A standard item of potentially infectious equipment presents with a diverse range of biological material bound to the surface. This includes both free and cellular ATP and mesophilic adenylate kinases (mAK). A small area of the surface is sectioned off to form a chamber (not shown, 1 ml volume) into which reagents can be added and removed. To prevent non-specific binding of the antibody-thermostable adenylate kinase conjugate, the exposed surfaces, including the enclosed area of the surgical instrument, are "blocked" by incubation in the presence of buffer containing, for example, the non-ionic detergent Tween 20 (1% v/v) in 10 mM PBS pH7 for 1 hour. The chamber is then washed twice with 0.05% Tween 20 in 10 mM PBS pH7 prior to binding of the antibody-thermostable adenylate kinase conjugate.

2. Antibody Binding

[0153] The thermostable adenylate kinase from *Bacillus stearothermophilus* is coupled to an affinity-purified polyclonal antibody via a heterobifunctional thiol-cleavable cross-linking agent, N-Succinimidyl-3-(2-Pyridyldithio) Propionate (SPDP). The antibody is raised by standard procedures against a synthetic peptide corresponding to a conserved region of the prion protein, coupled to maleimide-activated keyhole limpet haemocyanin. Active conjugate (50 μl) is added to the buffer in the chamber and incubated for 30 minutes at room temperature.

3. Washing

[0154] The chamber is washed manually or by use of an automated washing device with six changes of buffer containing 0.2M NaCl, 0.05% Tween 20 in 10 mM PBS, pH7. These serve to remove unbound conjugate and any biological material only loosely attached to the surface.

4. Linker Cleavage

[0155] Dithiothreitol is added to the last wash to a final concentration of 25 mM and incubation at room temperature continued for 30 minutes. This cleaves the thermostable adenylate kinase moiety from the bound antibody providing a signal molecule in free solution proportional to the original amount of prion protein present.

Prion ELISA 2

5. Recovery/Transfer

[0156] At this stage the thermostable adenylate kinase-containing solution is aspirated by pipette and transferred to the wells of a thermostable luminometer microtitre plate. Transfer of non-specific background ATP and mesophilic adenylate kinase also occurs, giving the potential for over-estimation of prion protein present on the original instrument surface.

6. Thermal Inactivation

[0157] The adenylate kinase used is thermostable. The temperature is, therefore, increased to 80° C. and maintained at this temperature for 10 minutes in a microtitre plate thermal cycler. This thermally denatures and inactivates any residual contaminating mesophilic adenylate kinase leaving a preparation containing only the specific thermostable adenylate kinase proportional to the prion protein content of the sample.

7. ATP Hydrolysis

[0158] The plate is then cooled and 0.05 units.ml^{-1} of adenosine deaminase and *Solanum tuberosum* apyrase added prior to incubation at 30° C. for 30 minutes. This enzyme removes any residual ATP carried over from the original sample.

8. Thermal Inactivation

[0159] The combination of steps 6 & 7 ensures that no ATP or AK background is now present. A further heat incubation as in step 6 is then used to inactivate the mesophilic apyrase.

Prion ELISA —3

9. ATP Generation

[0160] Ultrapure ADP (0.1 mM) and free of ATP, is added along with magnesium ions (10 mM) in order to generate ATP derived exclusively from the thermostable adenylate kinase. Incubation is carried out at 80° C. for 30 minutes. The ATP is then available for D-luciferin-luciferase bioluminescence detection.

10. ATP Bioluminescence

[0161] The ATP-containing wells are cooled to 25° C. and synthetic ultrapure D-luciferin and adenylate kinase-free luciferase added to a concentration of 4 μM and 1 mg.l^{-1} respectively. Individual wells are read for ATP-dependent bioluminescence in a microtitre plate luminometer and the results recorded. The amount of light generated correlates directly with the original amount of prion protein in the sample.

EXAMPLE 2

An Assay for a Microorganism

[0162] A micro-organism is immobilized onto solid surface by non-specifically binding sample components including the microorganism to the solid phase, treating the solid phase to prevent further non-specific binding thereto and washing

(we use a microtitre well in this case but other known solid phases are suitable, such as a latex bead or a magnetic bead). An antibody specific to the micro-organism and coupled to a thermostable adenylate kinase is introduced and allowed to bind, prior to further washing/recovery.

[0163] (In the known AK assay, sensitivity would have been limited by the level of sample concentration possible before levels of background ATP and non-specific AK obscured any signal).

[0164] The sample is now heated to about 90° C. for about 10 minutes in a cell extraction buffer (in a thermal cycler) to denature any endogenous AK present and release any ATP that may be trapped within the micro-organism. The sample is then cooled to 37° C. and a thermolabile ATPase added. The sample is incubated for about 10 minutes to remove the background ATP, then the temperatures is raised to about 90° C. to denature the thermolabile ATPase.

[0165] Next, ADP is added and the temperature maintained at 90° C. so the thermostable adenylate kinase can convert ADP into ATP. This incubation generates ATP exclusively from the thermostable adenylate kinase. The ATP thus generated is then assayed by conventional ATP bioluminescence and is directly proportional to the concentration of the target present.

EXAMPLE 3

An Assay for a Microorganism

[0166] A micro-organism is captured by a conventional capture technique, using a specific antibody immobilised onto a solid surface (we use a microtitre well in this case but other known solid phases are suitable, such as a latex bead or a magnetic bead). After washing/recovery, a second antibody specific to the micro-organism and coupled to a thermostable adenylate kinase is introduced and allowed to bind, prior to further washing/recovery.

[0167] Thus, the method of Example 1 is repeated but using a microorganism immobilized using antibody.

EXAMPLE 4

A Blood-Hormone Assay

[0168] An antibody specific for the alpha subunit of TSH is immobilised onto a solid-phase. The solid-phase is treated to prevent further non-specific binding thereto. The solid-phase is washed with wash buffer, optionally containing detergent. A test sample of blood serum is added.

[0169] The sample is then incubated, e.g.: 37° C. for 60 mins, allowing the free TSH in the sample to bind to the capture antibody. The solid-phase is then washed to remove non-specifically bound material and an antibody specific for the beta subunit of TSH is added, to which a thermostable adenylate kinase reporter enzyme has been conjugated. The conjugate is then incubated at 37° C. for 60 minutes, or equivalent.

[0170] Non-bound material is then removed by washing and any endogenous ATP present on the solid-phase is removed by the addition of adenosine-5'-triphosphatase (an alternative is apyrase). The sample is then heated to 90° C., or equivalent, to denature and inactivate any mesophilic adenylate kinase that may be present.

[0171] Adenosine diphosphate (ADP) is added and the temperature is maintained at 90° C. so that the thermostable adenylate kinase can convert the ADP to ATP. This incubation generates ATP exclusively from thermostable adenylate kinase. The ATP generated is then assayed by conventional ATP bioluminescence technology using a luciferin/luciferase reaction. Signal from contaminating adenylate kinase in the luciferin/luciferase reagents may be quenched by the addition of a specific enzyme inhibitor. The ATP bioluminescence measured is directly proportional to the concentration of the TSH in the original test sample.

[0172] Whilst the solid-phase used in the above is a microtitre-plate, other solid-phases are suitable, such as latex or magnetic bead. The test sample may be whole blood or other body fluid, rather than blood, and the antibody may be a polyclonal or a monoclonal antibody.

EXAMPLE 5

An Assay for Cocaine Metabolites in Urine

[0173] A thermostable G6PDH is used as reporter enzyme. Test antibody specific for the class of drug of interest is immobilised onto a micro-titre plate as solid-phase. The solid-phase is treated to prevent further non-specific binding thereto. The solid-phase is washed with wash buffer, which may or may not contain detergent. A test sample of urine is added along with the drug-G6PDH conjugate. The drug-G6PDH is thermostable and is not active when bound to the antibody immobilised to the solid-phase.

[0174] The sample is then incubated, at 37° C. for 60 mins. The contents of the micro-titre well is then removed and heated to 90° C. to inactivate any mesophilic G6PDH present. The temperature is then maintained at 90° C. and the substrate glucose-6-phosphate and cofactor NAD+ is added in the appropriate buffer. The rate of change in the absorbance at 340 nm is measured and is directly proportional to the level of drug metabolite in the test sample.

[0175] Another reporter for this assay is a thermostable adenylate kinase. Test antibody specific for the class of drug of interest is immobilised onto a solid-phase. The solid-phase is treated to prevent further non-specific binding thereto. The solid-phase is washed with wash buffer, which may or may not contain detergent. A urine test sample is added along with the drug-adenylate kinase (AK) conjugate. The drug-AK conjugate is thermostable and is not active when bound to the antibody immobilised to the solid-phase.

[0176] The sample is then incubated, e.g.: 37° C. for 60 mins. The contents of the micro-titre well is then removed and endogenous ATP removed by addition of adenosine-5'-triphosphatase or apyrase and incubation at 37° C. The sample is then heated to 90° C. to inactivate any mesophilic adenylate kinase present.

[0177] Adenosine diphosphate (ADP) is added and the temperature is maintained at 90° C. such that the thermostable adenylate kinase can convert the ADP to ATP. This incubation generates ATP exclusively from thermostable adenylate kinase. The ATP generated is then assayed by conventional ATP bioluminescence using a luciferin/luciferase system Signal from contaminating adenylate kinase in the luciferin/luciferase may be quenched by the addition of a specific enzyme inhibitor. The ATP bioluminescence measured is directly proportional to the concentration of the drug metabolite in the original test sample.

[0178] Other solid-phases are suitable, such as latex or magnetic bead, and the test sample may be sera or other body fluid.

EXAMPLE 6

Assays for the Detection of Human Papilloma Virus DNA

[0179] Assay A: Cervical cells are collected and resuspended in phosphate buffered saline. PCR amplification of the HPV16, or equivalent sequence, is carried out as described in Lambropoulous et al. 1994) Journal of Medical Virology: 43, 228-230 using the consensus primers MY11 and MY09 and 30 rounds of amplification.

[0180] The PCR products are then transferred and immobilised on to a non-charged nylon coated microtitre plate, or equivalent. An oligonucleotide probe specific for HPV16 (MY14) conjugated to a thermostable adenylate kinase is then added and incubated. The oligonucleotide-AK conjugate is prepared following an identical method described the synthesis of DNA-antibody conjugates. This complex comprises of a biotinylated AK and an avidin-biotinylated DNA complex generated using available methodology: Ruzicka et al. Science 1993, 260, 698-699.

[0181] Non-bound material is then removed by washing and any endogenous ATP present on the solid-phase is removed by the addition of adenosine-5'-triphosphatase or apyrase. The solid-phase is then washed and the sample heated to 90° C., or equivalent, to denature and inactivate any mesophilic adenylate kinase that may be present.

[0182] Adenosine diphosphate (ADP) is added and the temperature is maintained at 90° C. such that the thermostable adenylate kinase can convert the ADP to ATP. This incubation generates ATP exclusively from thermostable adenylate kinase. The ATP generated is then assayed by conventional ATP bioluminescence using a luciferin/luciferase reaction. A positive signal is indicative of HPV infection.

[0183] Assay B: Cervical cells are collected and fixed onto a solid-surface, a non-charged nylon membrane contained within a microtitre plate. The cells are lysed and the endogenous ATP present on the solid-phase is removed by the addition of adenosine-5'-triphosphatase or apyrase. An oligonucleotide probe specific for HPV16 (MY14: 5'CATACACCTCCAGCACCTAA3') conjugated to a thermostable adenylate kinase is then added. The oligonucleotide-AK conjugate is prepared following an identical method described the synthesis of DNA-antibody conjugates. This complex comprises a biotinylated AK and an avidin-biotinylated DNA complex generated using available methodology: Ruzicka et. al Science 1993, 260, 698-699.

[0184] After incubation, 37° C. for 60 min, the sample is heated to 90° C., or equivalent, to denature and inactivate any mesophilic adenylate kinase that may be present. ADP added and the temperature is maintained at 90° C. such that the thermostable adenylate kinase can convert the ADP to ATP. This incubation generates ATP exclusively from thermostable adenylate kinase. The ATP generated is then assayed by conventional ATP bioluminescence using a luciferin/luciferase reaction. A positive signal is indicative of HPV infection.

EXAMPLE 7

An Assay to Screen Peptide Combinational Libraries

[0185] Peptides are synthesised on small beads (100 μm-200 μm) using standard solid-phase peptide synthesis methodology. The sequence corresponds to a combinational peptide library generated as described Lam. et al. (1991) *Nature* (*UK*). 354, 82-84.

[0186] The beads are split into 20 portions and a separate amino acid coupled to each portion. The beads are then recombined, randomised, and split into 20 for addition of the next amino acid. This process is repeated to build a peptide library of all possible combinations of amino acids. In theory each bead should have a different peptide sequence attached. After synthesis the beads are washed and any endogenous ATP is removed by addition of adenosine-5'-triphosphatase or apyrase. A ligand-thermostable AK conjugate is added and the sample heated to 90°, or equivalent, to denature and inactivate any mesophilic adenylate kinase that may be present.

[0187] Adenosine diphosphate (ADP) is added and the temperature is maintained at 90° C. such that the thermostable adenylate kinase can convert the ADP to ATP. The beads are split into portions and screened for the generation of light generated by a luciferin/luciferase reaction using a standard luminescence reader. Portions generating a positive signal are split into further portions and re-screened. This process is continued using a microscope equipped with a charge couple device camera, until the signal from a single bead is identified. The bead is removed and the sequence of peptide is then determined using standard micro-sequencing methodology.

EXAMPLE 8

An Assay for Botulinum Toxin

[0188] Antibody specific for the botulinum toxin is immobilised onto a solid-phase. The solid-phase may be a microtitre-plate but other solid-phases are suitable, such as latex or magnetic bead. The solid-phase is treated to prevent further non-specific binding thereto. The solid-phase is washed with wash buffer, which may or may not contain detergent. Test sample is added. The test sample is a food sample, but may be whole blood or body fluid. The sample is then incubated, e.g.: 37° C. for 60 mins, allowing the free toxin in the sample to bind capture antibody. The solid-phase is then washed to remove non-specifically bound material and an antibody specific for the botulinum toxin is added to which a thermostable adenylate kinase reporter enzyme has been conjugated. This antibody may be a polyclonal or a monoclonal antibody. The conjugate is then incubated at 37° C. for 60 minutes, or equivalent.

[0189] Non-bound material is then removed by washing and any endogenous ATP present on the solid-phase is removed by the addition of adenosine-5'-triphosphatase or apyrase. The solid-phase is washed and the sample heated to 90° C., or equivalent, to denature and inactivate any mesophilic adenylate kinase that may be present.

[0190] Adenosine diphosphate (ADP) is added and the temperature is maintained at 90° C. such that the thermostable adenylate kinase can convert the ADP to ATP. This incubation generates ATP exclusively from thermostable adenylate kinase. The ATP generated is then assayed by conventional ATP bioluminescence using a luciferin/luciferase reaction. Signal form contaminating adenylate kinase in the luciferin/luciferase may be quenched by the addition of a specific enzyme inhibitor. The ATP bioluminescence measured is directly proportional to the concentration of the toxin in the original test sample.

EXAMPLE 9

Detection of TSE Agents in Serum, Whole Blood, or Tissue (Brain, Tonsil) Homogenate

[0191] Recombinant human prion protein was used as a model for the detection of TSE agents in complex biological samples. In the model system the assay was performed essentially as described below.

[0192] A. Production and Purification of Recombinant Thermostable Adenylate Kinases

[0193] A clone expressing thermostable AK from the thermoacidophilic archaeon *Sulfolobus acidocaidarius* was generated in the expression vector pET3a and the protein expressed in JM109 host cells carrying additional tRNA genes for rarely expressed *E. coli* codons (specifically those encoding 11e codon ATA and Arg codon AGA) on a pACYC derived vector. Recombinant expression was carried out as follows. A primary inoculum of 100 ml was set up in Terrific broth supplemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol and grown overnight at 30° C. 160 rpm. The primary inoculum was subcultured by diluting 40 ml into 1 litre of fresh media with the same additions. The culture was grown at 30° C. 200 rpm for approximately 4-5 hours until the OD600 measurement reached at least 0.6 and typically around 0.8. The culture was induced by the addition of IPTG to a final concentration of 500 μM and the culture grown overnight at 30° C. Cells were harvested by centrifugation and stored at −80° C. until required.

[0194] A purification method was established using an initial heat treatment of incubation for 20 min at 80° C., to destroy proteins derived from *E. coli*, followed by centrifugation at 15000 rpm SS34 rotor in a Sorvall CL4B centrifuge to remove the degraded proteins. The thermostable nature of the AK enzyme, even in the presence of sequences derived from the pET3a vector, mean that it is unaffected by the treatment and will stay in solution under these conditions. An affinity chromatography step was then carried out by adsorption of the enzyme to Blue Sepharose in a buffer containing 50 mM Tris-HCl, pH 7.5, followed by specific elution with a low concentration of AK co-factors (AMP+ATP and magnesium ions). The ATP and AMP in the elution buffer were degraded by incubation with apyrase, which is readily inactivated by subsequent heat treatment. Gel filtration chromatography on a preparation grade Superdex column could be added to the protocol if required to add additional clean up of the enzyme.

[0195] Those familiar with the art will recognize that a number of further options are available for the production of adenylate kinase or other appropriate enzymes, e.g. purification of adenylate kinase from thermophilic bacteria or archaea using standard methods. The ability to overexpress the enzyme in *E. coli* does offer some advantages in terms of yield and according to our current studies is surprisingly effective at allowing the production and correct folding of the thermostable enzymes. A wide range of expression systems are available for the production of such enzymes. The use of plasmids expressing rare tRNA genes is a well described method for allowing the expression of AT-rich genes (or GC-rich as appropriate) in *E. coli* where the codon usage may be sub-optimal. Alternatively the use of *E. coli-codon* optimized synthetic genes, familiar to those with knowledge of the art may allow efficient production of the protein.

[0196] B. Development of Ultra-Sensitive ELISA with 6H4

[0197] The anti-prion monoclonal antibody, 6H4 (Prionics) was conjugated to purified recombinant thermostable *Sulfolobus acidocaldarius* adenylate kinase using the heterobifunctional thiol-cleavable linker N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce Chemicals) (Carlsson et al, 1978). In brief both the antibody and AK was derivatised with SPDP at a molar ratio of approximately 3 SPDP:I protein. The free SPDP was removed by either dialysis or gel filtration and the derivatised AK reduced to generate a reactive thiol group. This was reacted with the derivatised antibody either for 1-4 hours at room temperature or overnight at 4° C. The conjugate preparations were assessed by ELISA. A microtitre plate was coated with recPrP at a concentration of 0.2 μg/ml. The wells were washed 4 times with PBS+0.05% Tween 20 (all washing steps). Non-specific binding to the wells was blocked by incubation with a 3% solution of casein dissolved in PBS. After incubation with the sample, ADP was added to the wells at a concentration of 0.15 mM (0.01 volumes) diluted in 15 mM magnesium acetate buffer+1 mM EDTA, pH 6.7. 30 μl of D-luciferin-luciferase (Biothema) substrate was added to each well and the Relative Light Units (RLU) measured in a microtitre plate luminometer (Berthold Orion).

[0198] C. Detection of Recombinant PrP

[0199] A titration of RecPrP was coated onto a microtitre plate (Maxisorp, Nunc) by doubling dilutions from a starting concentration of 10 ng/ml. Dilutions were made in Coating Buffer (sodium carbonate buffer, pH 9.6) and 100 μl/well of each dilution was incubated overnight at RT. The unbound antigen was removed by washing with 4 changes of PBS containing 0.05% Tween-20 (All washing steps). 100 μl of a 1:2000 dilution of 6H4-AK conjugate was added to each well diluted in PBS containing 5% casein and 0.05% Tween-20 and incubated for 1 hour at RT. Thermostable AK was cleaved from the bound antibody by the addition of 100 ml of a 25 mM solution of (2-Mercaptoethanesulfonic acid) MESNA diluted in 50 mM Tris-HCl (pH 7.2) incubated for 30 minutes at 45° C. The contents of the microtitre well were transferred to a white thermocycler compatible microtitre plate. All further incubation steps take place in the thermocycler. 100 μl of 0.13 mM ADP substrate (Celsis) diluted in 15 mM MgAc, 1 mM EDTA buffer (pH 6.8) was added to each well and incubated at 70° C. for 20 minutes, cooled to RT and 30 ml of luciferin-luciferase reagent (Biothema) was added and the RLU read immediately on a plate luminometer.

[0200] The results of the assay are shown in Panel A of FIG. **5**, together with the results obtained when the assay was carried out using an HRP conjugated anti-mouse antibody to detect 6H4. The detection limit for the AK-based assay (defined as 3SD above the control background value) was calculated as 156 pg/ml, which equates to 6.75 pM of recPrP. This indicates a 100-1000 fold increase in sensitivity of the AK-based assay compared to the HRP-based assay.

[0201] D. Detection of RecPrP in Complex Backgrounds

[0202] A further requirement of the assay is that it can detect PrP in the presence of complex biological material. A titration of recPrP from a starting concentration of 10 μg/ml was diluted in either 0.5 or 0.05 mg/ml MBH in coating buffer, in sheep's blood, or in serum (Sigma) by placing 100 μl/well and incubated overnight at RT. The assay was continued exactly as described in sections IA-C.

[0203] Results are shown in FIG. **5** (for MBH in coating buffer) and in FIG. **6** (for sheep's blood and serum).

[0204] FIG. **5** shows the detection of recPrP spiked into non-infectious mouse brain homogenate (MBH). The data shown are for dilution in 0.5 mg/ml MBH but similar results were shown for 0.05 mg/ml. These values equate to a 5000-50000 fold excess of MBH. The detection limit for the assay (defined as 3SD above the control background value) was calculated as 156 pg/ml (which equates to 6.75 pM of recPrP). This detection limit appeared to be unaffected by the amount of MBH present (ie. within the 5000-50000 fold range).

[0205] In FIG. **6**, the assay of the invention was used to detect recPrP spiked into ovine sera (Panel B) and blood (Panel A). The assay was found to be capable of detecting PrP at levels of 100 pg/ml in sera and 10 fg/ml in whole blood.

[0206] A further example of the use of the assay is for detection of prion agents in tissue homogenates, principally in tonsil samples taken for diagnostic purposes or as part of surveillance studies. Tissue homogenate is prepared by disruption of the tonsil tissue, using any one of a variety of apparatus sold for such purposes, in a suitable buffer. If required, proteinase K is used to discriminate between the normal cellular PrP^{c} and the disease-associated protease-resistant form PrP^{Sc}. Whilst this forms the basis of most current tests for BSE, many experts in the field suggest that this may hide the presence of a protease-sensitive disease-associated form that may be important for assessing the presence of the disease. The detection of this form of the protein may increase the sensitivity of the assay if it can be detected alongside the protease-resistant form. Following homogenisation and protease digestion, if appropriate, the homogenate is applied directly to a solid support. Optionally, a specific capture reagent is used to increase the amount of the disease-associated PrP bound to the solid support. Many of these capture reagents will be familiar to those with knowledge of the art, but include derivatives of Congo Red (and related amyloid-binding reagents), heparin-derivatives, antibodies, peptides, nucleic acid derivatives (shown to bind PrP^{Sc}) or RNA aptamers. The plate is then washed and the bound prion material detected by the addition of AK-conjugated prion antibodies essentially as described above. Background reduction steps using heat treatment and/or apyrase are performed according to the protocol outlined above. The method allows the detection of the abnormal prion isoforms in the tissue as a presumptive confirmation of diagnosis.

EXAMPLE 10

Detection of Prion Protein on the Surface of a Surgical Steel Instrument

[0207] Surgical stainless steel disks of 5 mm diameter were purchased from Goodfellow; Cambridge Ltd. The diameter of the disks enabled them to be placed in the bottom of a microtitre well. These were coated by carefully placing a solution of recPrP or non-infectious mouse brain homogenate (MBH) diluted in coating buffer on the surface of the disc and incubating overnight at room temperature. The unbound antigen was removed by washing with 4 changes of PBS containing 0.05% Tween-20 (all washing steps). 100 µl of a 1:2000 dilution of 6H4-AK conjugate was added to each well diluted in PBS containing 5% casein and 0.05% Tween-20 and incubated for 1 hour at RT. Thermostable AK was cleaved from the bound antibody by the addition of 100 µl of a 25 mM solution of MESNA diluted in 50 mM Tris-HCl (pH 7.2) incubated for 30 minutes at 45° C. The contents of the microtitre well were transferred to a white thermocycler-compatible microtitre plate. All further incubation steps take place in the thermocycler. Thermal inactivation of contaminating AK was achieved by heating to 80° C. for 10 minutes. 5 µl of apyrase (Celsis) was added to each well and incubated at 37° C. for 30 minutes followed by inactivation of the apyrase by inclusion of a heat step to 60° C. for 10 minutes. 100 µl of 0.13 mM ADP substrate (Celsis) diluted in 15 mM MgAc, 1 mM EDTA buffer (pH 6.8) was added to each well and incubated at 70° C. for 20 minutes, cooled to RT and 30 µl of luciferin-luciferase reagent (Thermo Labsystems) was added and the RLU read immediately on a plate luminometer.

[0208] The results of this assay are shown in FIG. **7**.

[0209] A detection limit of below 10 ng/ml was achieved using the assay. At 10 ng/ml the assay values were significantly above the background value obtained suggesting that further serial dilutions below this value would be detectable via the assay method.

EXAMPLE 11

Detection of Botulinum Neurotoxin

[0210] An anti-guinea pig IgG antibody AK conjugate was generated as described in Example 9(A). The assay was then conducted as described below, using the "toxic" light chain (LC) domain as a model of the toxin activity.

[0211] A. BoNT/B Cleavage Assay with 1 mg/ml Vamp Substrate Standard Curve for Demonstration

1. A Nunc-MaxiSorp 96 well plate was coated with 100 µl/well of 5 µg/ml streptavidin in 50 mM Sodium Bicarbonate pH 9.5, incubated for 1 hr at 37° C., and then washed with 4 changes of PBS-Tween 0.1%.

2. The wells were then blocked with 200 µl/well of SuperBlock and shaken for 1 hr at 37° C. In addition, an additional plate was blocked in the same way but with 300 µl/well of SuperBlock for the cleavage reaction. The plates were then washed with 4 changes of PBS-Tween 0.1%.

3. Dilutions of toxin or isolated LC were then set up in cleavage buffer [5 ml HEPES 50 mM pH7.4 containing 2 mg/ml BSA; 50 µl DTT (1M); 5 µl ZnCl2 (20 mM)] at twice the required concentration by adding 1 µl of LC/B (2.5 mg/ml) to 125 µl of cleavage buffer producing a 20,000 ng/ml solution. 10-fold serial dilutions of toxin in cleavage buffer were then prepared.

4. 108 µl of 1 mg/ml VAMP (in 50 mM HEPES pH7.4) was then mixed with 108 µl of the required toxin concentration on the additional preblocked plate, and shaken for 2 hrs at 37° C. The cleaved VAMP samples were then transferred to the streptavidin-coated plate, incubated for 5 mins at 37° C., and the plate was then washed with PBS-Tween 0.1%.

5. 100 µl of 1 µg/ml Guinea Pig (GP) anti FESS antibody made up in 10% SuperBlock, PBS-Tween 0.1% was then applied to each well. The plate was then shaken for 1 hr at 25° C. or 4° C., and then washed with 4 changes of PBS-Tween 0.1%.

6. 100 µl of 1 µg/ml Goat anti GP-AK conjugated antibody made up in 10% SuperBlock, PBS-Tween 0.1% was then applied to each well. The plate was then shaken for 1 hr at 25° C. and then washed with 4 changes of PBS-Tween 0.1%.

7. 100 µl of MESNA buffer (25 mM MESNA, 50 mM Tris-HCl pH 7.2) was then applied to each well, and the plate shaken for 30 mins at 45° C. The content of the wells was then transferred to a white thermocycler plate (Costar) and 5 µl per

well of apyrase was added (Celsis Luminase). The plate was then shaken for 30 mins at 37° C., followed by heating for 10 mins at 70° C. to inactivate the apyrase.

8. 100 μl of 135 μM ADP (add 33 μl of 20.5 mM ADP to 5 ml of 15 mM MgAc, 1 mM EDTA buffer) was then added to each well, and the plate was incubated for 20 mins at 70° C. in the thermocycler.

9. Lastly, 30 μl of Luciferin/Luciferase (ATP) reagent (Biothema) was added to each well and the results were read on a luminometer immediately.

[0212] The results of this assay in comparison to a standard HRP-based assay are shown in FIG. **8**. The AK based assay shows a significant increase in sensitivity of between 1000 and 10,000 fold compared to the traditional HRP format. The detection limits of around 1 fg are significantly lower than the level of toxin that could be detected by mouse bioassay, currently the most sensitive method available for the detection of BoNT. Similar results were obtained with full length BoNT/B (not shown).

[0213] For use of the assay in detection of toxin in potentially contaminated food stuffs or in other samples the assay is performed essentially as described above. The suspected sample is homogenised in a suitable buffer to ensure dispersal of any toxin in the sample. A buffer such as the cleavage buffer outlined in the method described above, optionally supplemented with inhibitors for serine or cysteine proteases (the inhibitor is chosen so as not to interfere with substrate cleavage by BoNT and will be selected from a variety of options known to those familiar with the art) is used. Following homogenisation, the sample is optionally cleared by centrifugation and diluted if appropriate to the levels of toxin in the sample.

[0214] Further information on the detection of botulinum neurotoxin (BoNT) using the present assay can be found in the following papers: Wictome M, Newton K A, Jameson K, Dunnigan P, Clarke S, Gaze J, Tauk A, Foster K A, Shone CC. Novel assays for the detection of botulinum toxins in foods. 1999. Dev Biol Stand. 10 p 141-5; Wictome M, Newton K, Jameson K, Hallis B, Dunnigan P, Mackay E, Clarke S, Taylor R, Gaze J, Foster K, Shone C. 1999. Development of an in vitro bioassay for *Clostridium botulinum* type B neurotoxin in foods that is more sensitive than the mouse bioassay. Appl Environ Microbiol. 65 p 3787-92; Hallis B, James B A, Shone C C (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. J Clin Microbiol. 34 p 1934-8.), the content of which is incorporated herein by reference.

EXAMPLE 12

Method for the Detection of Ricin in Water or Other Environmental Samples

[0215] An assay for the presence of ricin in a sample is carried out using the method essentially as described in Example 9 above.

[0216] The sample is bound directly onto the solid support and probed with a monoclonal or polyclonal antibody directed against the ricin molecule and labelled with adenylate kinase. The background reduction steps of the invention are sufficient to eliminate any background activity associated with the sample and allow detection of the low levels of ricin that are expected in these samples.

[0217] Alternatively, the assay is performed as a more routine sandwich assay with ricin captured by an antibody bound to the solid support and detected with a second antibody-AK conjugate. If greater signal is required, an anti-species antibody-AK conjugate recognising the detection antibody is used.

EXAMPLE 13

Diagnosis of Rotavirus Infection by Analysis of Stool Samples

[0218] Using the protocol described below, the assay of the invention was used to detect the presence of rotavirus in stool samples.

1. A Nunc-MaxiSorp 96 well plate was coated with 100 μl/well of a 1:1000 dilution of rabbit anti rotavirus IgG in carbonate coating buffer; incubated overnight at room temperature; and washed with 4 changes of PBS-Tween 0.1%.

2. The plate was then blocked with 5% Skim Milk Powder in PBS-Tween 0.1%; shaken for 1 hr at 37° C.; and washed with 4 changes of PBS-Tween 0.1%.

3. 100 μl/well of the stool samples, including positive and negative controls, were added to the plate and incubated for 1 hr at room temperature.

4. 50 μl of 1:500 AK-conjugated or HRP conjugated, rabbit anti rotavirus Ab, made up in 5% Skim Milk Powder in PBS-Tween 0.1% was added to each well, shaken for 1 hr at room temperature and then washed with 4 changes of PBS-Tween 0.1%.

5. The HRP conjugated samples were developed and read on a plate reader at 450 nm.

6. 100 μl of MESNA buffer (25 mM MESNA, 50 mM Tris-HCl pH 7.2) was applied to each well, and the plate shaken for 30 mins at 45° C. 90 μl/well was transferred to a white thermocycler plate (Costar).

7. 5 μl apyrase (Sigma 100 units in 2 ml of Tris-HCl) was added to each well, and the plate was then shaken for 30 mins at 30° C. before being heated to 70° C. for 10 mins.

8. 90 μl of 135 μM ADP (add 33 μl of 20.5 mM ADP to 5 ml of 15 mM MgAc, 1 mM EDTA buffer) was added to each well, and the plate was then incubated for 20 mins at 70° C. in the thermocycler.

9. 30 μl of Luciferin/Luciferase (ATP) reagent (Biothema) was added to each well and the results read on a luminometer immediately.

[0219] The results from the assay are shown in FIG. **9** in comparison to a standard HRP-based assay as carried out in the Dakocytomation IDEIA Rotavirus assay. The AK-based assay shows at least a 100-fold greater sensitivity than the existing assay and allows the detection of low levels of rotaviral antigen in faecal samples to support diagnosis of infection.

EXAMPLE 14

Diagnosis of Norwalk/Norovirus Infection by Analysis of Oral, Faecal or Vomit Samples

[0220] Samples are collected and assayed using the method essentially as described in Example 9. The assay can be run in a variety of different formats depending on the precise strain (s) of virus to be detected, the reagents available and the phase of the disease.

[0221] The assay is carried out in the following ways:

[0222] A. Detection of Antigen in Faecal or Vomit Samples.

[0223] A hyperimmune serum from an immunised animal is coated onto a solid support such as a polyvinyl plate (typically for 16-18 hours at 4° C. in carbonate/bicarbonate buffer pH 9.6). The remaining sites on the plate are blocked to prevent non-specific binding using any one of a variety of agents known to those familiar with the art (e.g. skimmed milk, casein, bovine serum albumen, commercial blocking formulations). The faecal or vomit sample is diluted 1:10 with PNS and applied to the plate for a fixed period of time, typically 1 hour at 22° C. The plate is washed and a second hyperimmune serum, preferably from a different species of immunised animal is applied to the plate. Again the plate is washed to remove unbound signal. Preferably the second hyperimmune serum is labelled with the AK enzyme by conjugation as described above, but alternatively an anti-species AK conjugate is used to detect the presence of the second anti-NLV hyperimmune serum.

[0224] As an alternative, monoclonal antibodies directed against either a broad range of NLV genogroups (to give an overall diagnosis) or directed very specifically against a single virus type (e.g. for outbreak tracing or surveillance) are used to replace either or both of the hyper-immune serum in the described assay format. Again if a monoclonal is used for the detection antibody, then this is labelled with AK directly or an anti-mouse (for mouse monoclonal antibodies) AK conjugate used for the assay read out.

[0225] The use of this type of format for the detection of viruses is occasionally complicated by the presence of molecules that interfere with the interpretation of the assay giving rise to false positive signals. To reduce the chances of this happening the assay is optionally run using any one of the formats described above but with duplicate samples run on plates coated with a capture antibody (either polyclonal or monoclonal) derived from either a pre-immune serum, naïve animal serum or a species-matched non-relevant monoclonal antibody. The assay read-out is then determined by measuring a significant positive ratio (2:1 or better) between the test sample and the duplicate control.

[0226] B. Detection of Immune Response to NLV Infection in an Oral Sample

[0227] An NLV capsid antigen, recombinantly expressed form a suitable expression system (such as a baculovirus system as reviewed in Jiang et al 2000, Diagnosis of human calciviruses by use of enzyme immunoassay, Journal of Infectious Disease 181:pS 349-359) is used in this assay. The purified capsid, possibly in the form of a non-infectious virus like particle (VLP) is coated onto a solid support, such as a polyvinyl or polycarbonate plate, for 16-22 hours at 4° C. in carbonate/polycarbonate buffer pH 9.6. Plates are blocked to remove additional binding sites and incubated with an oral sample collected, for example, by one of the methods described by McKie et al (2002) and incubated for 1-2 hours at 22-37°. The plate is washed and an anti-human IgM-AK conjugate added. After incubation and subsequent washing the assay is developed with bioluminescent substrates as described in Example 9.

[0228] In an alternative format, designed to reduce the potential of anti-NLV IgG antibodies to interfere with the assay, the IgM is captured from the oral sample by incubation with a solid support coated with anti-human IgM antibodies. Detection of the bound anti-NLV IgM from the serum is then effected by incubation with recombinant capsid antigen conjugated directly to AK. As an alternative, recombinant capsid is added, followed by an anti-capsid monoclonal antibody-AK conjugate (or monoclonal antibody and anti-species antibody AK conjugate) to allow detection with bioluminescent substrates.

EXAMPLE 15

Detection of Norwalk-Like Virus/Norovirus in Environmental Samples and Use of the Method to Validate Decontamination Procedures

[0229] A. Detection of Norwalk-like virus/Norovirus in environmental samples Samples are assessed using swab-type devices as currently used for a wide variety of environmental sampling and hygiene monitoring. Essentially these are cloth, sponge or foam type devices moistened with a suitable buffer for the acquisition of surface contamination. These are "swabbed" over the solid surface for a defined period of time and transferred to a receptacle containing additional buffer/reagent designed to release the antigen from the swab and any associated tissue.

[0230] The swab is agitated in the buffer and this sample is then assayed using the methods as outlined in Examples 9 and 14.

[0231] B. Method to validate decontamination procedures

[0232] Recombinant NLV VLPs are used as a test antigen to ensure that surfaces are cleaned properly. A preparation of VLPs is sprayed onto a test surface to mimic the contamination of surfaces by oral-faecal transmission during the course of the disease.

[0233] The surface is then cleaned according to the facility protocol. The surface swab is then used to confirm whether the decontamination procedure has been effective at removing the VLPs from the surface, using the protocol as described in A. above.

[0234] Preferably, the VLPs are formulated in a material to mimic actual contamination, e.g. a mixture of egg yolk, purified mucin, dried blood and other components designed to mimic the physicochemical properties of human fluids. This "soil" would interfere with the vast majority of detection methodologies but would be an entirely acceptable matrix for carrying out the method of the invention.

EXAMPLE 16

Method for the Detection of MRSA or Other Antibiotic-Resistant Bacterial Strains in Throat or Tissue Swab Samples

[0235] Tissue/throat swab samples are obtained from a patient, and bacteria are then extracted from the swab samples.

[0236] The bacteria are bound directly to a suitable solid support, and the assay is then performed essentially according to the method described in Example 9. AK-conjugated antibodies specific for the bacteria, or specific for particular strain types, are used in the assay.

EXAMPLE 17

Method for the Detection of Pneumococcal Antigens in Urine

[0237] An indirect sandwich ELISA for the detection of pneumococcal C-polysaccharide in urine and other body fluids is described below.

[0238] In brief, plates are coated with rabbit polyclonal antibody to C-polysaccharide (CPS), urine (or alternative antigen source) is added, CPS-specific monoclonal antibody is bound to retained antigen and is detected by AK-conjugated rabbit anti-mouse antibody.

Assay Protocol:

[0239] 1. A Nunc-MaxiSorp 96 well plate is coated with 60 μl/well of polyclonal antibody diluted $\frac{1}{2000}$ in carbonate-bicarbonate. The plate is placed in the 'fridge overnight and discarded if not used within 7 days.

2. The plate is washed with 4 changes of PBS-Tween, and then blocked with 200 μl 1% skimmed milk in PBS, and incubated at room temperature for 1 hour. The plate is then washed again with 4 changes of PBS-Tween.

3. Urine is spun at 15,000 rpm for 1 min and then diluted 50.50 with PBS+1% milk. 60 μl is then added to each well of the coated and blocked plates, before incubation for 1 hour at room temperature and washing with 4 changes of PBS-Tween.

4. Anti-CPS monoclonal antibody, diluted in PBS Tween plus 1% skimmed milk, is added to each well at 70 μl/well and the plate is then incubated at room temperature for 1 hour, followed by washing with 4 changes of PBS-Tween.

5. Anti-mouse antibody-AK conjugate, diluted $\frac{1}{10,000}$ in PBS Tween plus 1% skimmed milk, is added to each well at 80 μl/well and the plate then incubated at room temperature for 1 hour, followed by washing with 4 changes of PBS-Tween.

6. The remainder of the detection procedure is carried out as described in Example 9B/C.

[0240] Suitable antibodies for use in this assay include, CPS specific rabbit polyclonal antibody —(e.g. as supplied by Statens Seruminstitut), CPS specific monoclonal antibodies —(e.g as supplied by Statens Seruminstitut) and Rabbit anti-mouse IgG (as supplied by Sigma) conjugated to AK as described above.

EXAMPLE 18

Ultrasensitive Method for the Detection of Immune Response to Vaccination

[0241] The assessment of immunisation against anthrax using an oral sample is described below.

[0242] Oral samples are collected using any one of the devices described in McKie et al 2002 (as cited previously above). The vaccinee is then assessed (i) for the presence of circulating IgM antibodies in an oral sample 7-14 days after the first vaccination, and/or (ii) for the presence of class-switched IgGs in oral samples approximately 7-10 days following the second immunisation. The assay is performed essentially as described in Example 9.

[0243] The sample is transferred directly to a suitable solid support. The support is then washed and the bound antibody probed with either an AK labelled antigen or with an unlabelled antigen, and a second AK labelled antibody. In the case of existing licensed anthrax vaccines or the new generation of recombinant protective antigen (rPA) based vaccines, the most appropriate antigen is anthrax PA. This is labelled directly with AK to act as a probe for the presence of PA-specific IgMs (or IgGs). An anti-PA monoclonal antibody labelled with AK is used to provide additional sensitivity.

[0244] In an alternative format, the solid support is coated with anti-human IgM (or IgG) and subsequently blocked prior to the application of the sample. The use of directly-labelled PA, or PA plus anti-PA antibody, are essentially as described above. Alternatively, the solid support is coated with PA, the sample applied, and the bound antibody detected with an IgM specific anti-human antibody.

[0245] In each case the presence of PA specific IgMs (or IgGs) following the initial vaccination is an extremely useful indicator that the vaccination method was capable of eliciting anti-PA antibodies that have proved to be protective in many anthrax challenge studies. By ascertaining the likelihood of successful vaccination at this early stage, compared to current methods that monitor immune response typically after the 3rd immunisation, it is possible to curtail or adjust the immunisation schedule.

EXAMPLE 19

Diagnosis of West Nile Virus Infection by Monitoring Levels of IgM in Oral Samples

[0246] An oral sample is collected from a patient using one of the methods as outlined in McKie et al 2002. The assay is then performed essentially as described in Example 9.

[0247] The sample is applied directly to the surface of a suitable solid support or to a solid support coated with anti-human IgM antibody. The presence of the captured anti-West Nile IgM is then detected with either AK-labelled West Nile antigen or unlabelled antigen together with a suitable antibody conjugate.

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1-16. (canceled)

17. An assay for an analyte in a sample, comprising contacting the analyte with a thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, wherein a complex is formed, adding ADP and testing for the formation of ATP, wherein, prior to the addition of ADP, endogenous kinase and uncomplexed thermostable reporter adenylate kinase is substantially removed by washing and, residual endogenous kinase is inactivated by heating, wherein the amount of ATP correlates to the concentration of the analyte, and wherein the analyte is present in the sample at a concentration of less than 10 ng/ml.

18. The assay of claim **17**, wherein said analyte is present in the sample at a concentration of less than 1 ng/ml.

19. The assay of claim **17**, wherein said analyte is present in the sample at a concentration of less than 100 pg/ml.

20. The assay of claim **17**, wherein said analyte is present in the sample at a concentration of less than 1 pg/ml.

21. The assay of claim **17**, wherein said analyte is present in the sample at a concentration of less than 100 fg/ml.

22. The assay of claim **17**, wherein said analyte is present in the sample at a concentration of less than 10 fg/ml.

23. The assay of claim **17**, wherein the sample is selected from the group consisting of urine, faeces (stool), vomitus, blood components (including serum, plasma, whole blood, white blood cell fractions, buffy coat), airway samples (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates), oral samples (including crevicular fluid, parotid saliva, whole saliva), cerebrospinal fluid (CSF), tissue homogenates (including brain homogenate, tonsil homogenate), pus, swab samples (including those taken from throat, nose, ears, skin, and wounds), effluent samples, surface swabs, food, water, beverages, soil, air sampling and sewerage.

24. The assay of claim **17**, wherein the sample is selected from the group consisting of faeces (stool), serum and whole blood.

25. An assay for an analyte in a sample, comprising contacting the analyte with a thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, wherein a complex is formed, adding ADP and testing for the formation of ATP, wherein, prior to the addition of ADP, endogenous kinase and uncomplexed thermostable reporter adenylate kinase is substantially removed by washing and, residual endogenous kinase is inactivated by heating, wherein the amount of ATP correlates to the concentration of the analyte, and wherein the sample is selected from the group consisting of urine, faeces (stool), vomitus, blood components (including serum, plasma, whole blood, white blood cell fractions, buffy coat), airway samples (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates), oral samples (including crevicular fluid, parotid saliva, whole saliva), cerebrospinal fluid (CSF), tissue homogenates (including brain homogenate, tonsil homogenate), pus, swab samples (including those taken from throat, nose, ears, skin, and wounds), effluent samples, surface swabs, food, water, beverages, soil, air sampling and sewerage.

26. The assay of claim **25**, wherein the sample is selected from the group consisting of faeces (stool), serum and whole blood.

27. (canceled)

28. An assay for determining the presence and/or amount of an analyte in a sample, comprising exposing the sample to thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, so that the reporter adenylate kinase is specifically associated with any analyte present in the sample via the binding agent; removing the thermostable reporter adenylate kinase that is not bound to the analyte; exposing said thermostable reporter adenlyate kinase bound to the analyte to ADP; and testing for the formation of ATP, wherein prior to the addition of ADP, residual kinase other than thermostable reporter adenylate kinase is substantially removed by heating, and wherein the analyte is present in the sample at a concentration of less than 10 ng/ml.

29. An assay for determining the presence and/or amount of an analyte in a sample, comprising exposing the sample to thermostable reporter adenylate kinase coupled to a binding agent specific for the analyte, so that the reporter adenylate kinase is specifically associated with any analyte present in the sample via the binding agent; removing the thermostable reporter adenylate kinase that is not bound to the analyte; exposing said thermostable reporter adenlyate kinase bound to the analyte to ADP; and testing for the formation of ATP, wherein prior to the addition of ADP, residual kinase other than thermostable reporter adenylate kinase is substantially removed by heating, and wherein the sample is selected from the group consisting of urine, faeces (stool), vomitus, blood components (including serum, plasma, whole blood, white blood cell fractions, buffy coat), airway samples (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates), oral samples (including crevicular fluid, parotid saliva, whole saliva), cerebrospinal fluid (CSF),

tissue homogenates (including brain homogenate, tonsil homogenate), pus, swab samples (including those taken from throat, nose, ears, skin, and wounds), effluent samples, surface swabs, food, water, beverages, soil, air sampling and sewerage.

30. (canceled)

31. An assay for determining the presence and/or amount of an analyte in a sample comprising, exposing the sample to a detector compound, the detector compound comprising an antibody specific to the analyte coupled to a thermostable enzyme; isolating (i) detector compound that has specifically bound to analyte from (ii) detector compound that has not specifically bound to analyte; determining the presence of and/or amount of detector compound that has bound to analyte by adding a substrate for the thermostable enzyme and measuring a product formed by conversion of said substrate to said product by said thermostable enzyme; wherein prior to the addition of substrate non-thermostable enzymes are destroyed by application of heat, and wherein the analyte is present in the sample at a concentration of less than 10 ng/ml.

32. An assay for determining the presence and/or amount of an analyte in a sample comprising, exposing the sample to a detector compound, the detector compound comprising an antibody specific to the analyte coupled to a thermostable enzyme; isolating (i) detector compound that has specifically bound to analyte from (ii) detector compound that has not specifically bound to analyte; determining the presence of and/or amount of detector compound that has bound to analyte by adding a substrate for the thermostable enzyme and measuring a product formed by conversion of said substrate to said product by said thermostable enzyme; wherein prior to the addition of substrate non-thermostable enzymes are destroyed by application of heat, and wherein the sample is selected from the group consisting of urine, faeces (stool), vomitus, blood components (including serum, plasma, whole blood, white blood cell fractions, buffy coat), airway samples (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates), oral samples (including crevicular fluid, parotid saliva, whole saliva), cerebrospinal fluid (CSF), tissue homogenates (including brain homogenate, tonsil homogenate), pus, swab samples (including those taken from throat, nose, ears, skin, and wounds), effluent samples, surface swabs, food, water, beverages, soil, air sampling and sewerage.

33. (canceled)

34. An assay for an analyte comprising the steps of:

(e) specifically binding the analyte with a thermostable reporter kinase which has been coupled to a binding agent specific for the analyte forming a complex;

(f) washing to remove endogenous non-thermostable kinase and thermostable reporter kinase not bound to analyte;

(g) heating to inactivate endogenous kinase not removed by step (b); and

(h) adding ADP and testing for formation of ATP,

wherein the analyte is present in the sample at a concentration of less than 10 ng/ml.

35. An assay for an analyte comprising the steps of:

(i) specifically binding the analyte with a thermostable reporter kinase which has been coupled to a binding agent specific for the analyte forming a complex;

(j) washing to remove endogenous non-thermostable kinase and thermostable reporter kinase not bound to analyte;

(k) heating to inactivate endogenous kinase not removed by step (b); and

(l) adding ADP and testing for formation of ATP,

wherein the sample is selected from the group consisting of urine, faeces (stool), vomitus, blood components (including serum, plasma, whole blood, white blood cell fractions, buffy coat), airway samples (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates), oral samples (including crevicular fluid, parotid saliva, whole saliva), cerebrospinal fluid (CSF), tissue homogenates (including brain homogenate, tonsil homogenate), pus, swab samples (including those taken from throat, nose, ears, skin, and wounds), effluent samples, surface swabs, food, water, beverages, soil, air sampling and sewerage.

36. The assay of claim **17**, wherein the analyte is selected from the group consisting of bacteria, viruses, toxins, prion agents, B-lactamases, hormones, immunoglobulins, tumour markers, growth factors, neurotransmitters, and metabolites of drugs of abuse.

37. The assay of claim **36**, wherein the analyte is a Gram-positive bacterium.

38. The assay of claim **37**, wherein the Gram-positive bacterium is *Staphylococcus aureus* (in particular antibiotic resistant strains such as MRSA), *Mycobacterium tuberculosis*, or *Streptococcus pneumonia*.

39. The assay of claim **17**, wherein the analyte is a virus selected from the group consisting of a rotavirus, a Norwalk virus (also known as Norovirus), a West Nile Virus, a measles virus, a mumps virus, a rubella virus, HIV, HPV and a hepatitis virus (all forms, especially Hepatitis C).

40. The assay of claim **17**, wherein the analyte is a toxin selected from the group consisting of botulinum toxin (including all botulinum serotypes A-G), tetanus toxin, anthrax toxins (including lethal toxin, oedema toxin and their component parts; lethal factor, oedema factor and protective antigen), ricin, mycotoxin, afalatoxin and superantigens.

41. The assay of claim **5**, wherein the toxin is botulinum toxin, tetanus toxin, or a superantigen.

42. The assay of claim **17**, wherein the analyte is a prion protein (including PrP^{Sc} and PrP^{c}) or an agent responsible for causing Creutzfeld Jacob Disease (CJD) (including variant, familial, sporadic and iatrogenic forms), scrapie, Bovine Spongiform Encephalopathy (BSE), or Chronic Wasting Disease (CWD).

43. The assay of claim **17**, wherein the sample is selected from the group consisting of faeces (stool), vomitus, effluent samples, food, water, sewerage, and environmental samples, and wherein the analyte is selected from the group consisting of Gram-positive bacteria, botulinum toxin, tetanus toxin, rotavirus, or Norwalk virus.

44. The assay of claim **17**, wherein the sample comprises a blood component (including serum, plasma, whole blood, white blood cell fractions, buffy coat) and wherein the analyte is selected from the group consisting of superantigens, West Nile Virus, HIV, prion proteins and hormones.

45. The assay of claim **17**, wherein the sample is an airway sample (including sputum bronchoalveolar lavage, endotracheal aspirates, nasopharyngeal aspirates) and wherein the analyte is selected from the group consisting of *Mycobacterium tuberculosis, Streptococcus pneumonia* and immunoglobulins.

46. The assay of claim **17**, wherein the sample is an oral sample (including crevicular fluid, parotid saliva, whole

saliva), and wherein the analyte is selected from the group consisting of a measles virus, a mumps virus, a rubella virus, and a hepatitis virus (all forms, especially Hepatitis C).

47. The assay of claim **17**, wherein the sample is cerebrospinal fluid and the analyte is selected from the group consisting of neurotransmitters, tumour markers and growth factors.

48. The assay of claim **17**, wherein the sample is pus or a swab sample (including those taken from throat, nose, ears, skin, and wounds), and wherein the analyte is *Staphylococcus aureus* (in particular antibiotic resistant strains such as MRSA).

* * * * *

专利名称(译)	背景减少的分析		
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[标]申请(专利权)人(译)	RAVEN NEIL DAVID哈蒙德 wictome马修帕特里克 SUTTON j选定 OBRIEN SUSAN 默多克希瑟		
申请(专利权)人(译)	RAVEN NEIL DAVID哈蒙德 wictome马修帕特里克 SUTTON j选定 奥布莱恩SUSAN 默多克希瑟		
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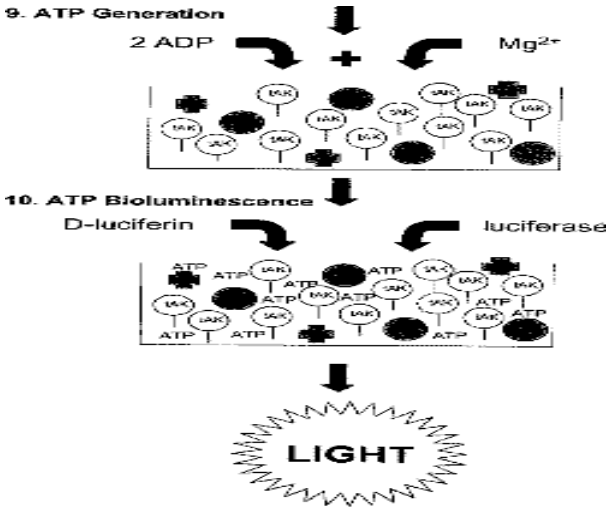
摘要(译)

在测定中，使样品中的分析物与热稳定的报告腺苷酸激酶接触，所述热稳定的报告腺苷酸激酶与对分析物特异的结合剂偶联，其中形成复合物。添加ADP，然后监测ATP的形成。在加入ADP之前，通过洗涤基本上除去内源激酶和未复合的热稳定的报告腺苷酸激酶，并通过加热使残留的内源激酶失活。在使分析物与热稳定的报告腺苷酸激酶接触之前，当在荧光素/荧光素酶存在下通过发光计测量时，样品具有至少300,000相对光单位/ mg蛋白质/ ml样品的背景活性。


9. ATP Generation
2 ADP
Mg^{2+}
10. ATP Bioluminescence
D-luciferin
luciferase
ATP
LIGHT