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(54) Titre : DOSAGES BIOLOGIQUES ENZYMATIQUES
(54) Title: ENZYME ASSAYS

(57) **Abrégé/Abstract:**

Enzymes and methods suitable for assaying ATP, and specific applications for such assays are described and claimed. In particular, there is described a recombinant mutant luciferase having a mutation (e.g. the amino-acid corresponding to amino acid residue number 245 in Photinus pyralis which is such that the K_m for ATP of the luciferase is increased e.g. five-fold with respect to that of the corresponding non-mutated enzyme such that it is of the order of 500 μM - 1mM. Also disclosed are luciferases having additional mutations conferring improved thermostability or altered wavelength of emitted light. Recombinant polynucleotides, vectors and host cells are also disclosed, as are methods of assaying the amount of ATP in a material (e.g. cells) optionally in real-time. Also disclosed are test-kits for in vitro assays.



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(21) International Application Number: PCT/GB98/01026 (22) International Filing Date: 7 April 1998 (07.04.98) (30) Priority Data: 9707486.8 11 April 1997 (11.04.97) GB (71) Applicant (<i>for all designated States except US</i>): THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB). (72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): SQUIRRELL, David, John [GB/GB]; CBD, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). WHITE, Peter, John [GB/GB]; The Babraham Institute, Babraham Hall, Babraham, Cambridge CB2 4AT (GB). LOWE, Christopher, Robin [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB). MURRAY, James, Augustus, Henry [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB). (74) Agent: SKELTON, S., R.; D/IPR, Formalities Section (Procurement Executive), Poplar 2, MOD Abbey Wood #19, Bristol, BS34 8JH (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 28 January 1999 (28.01.99)
(54) Title: ENZYME ASSAY FOR MUTANT FIREFLY LUCIFERASE (57) Abstract Enzymes and methods suitable for assaying ATP, and specific applications for such assays are described and claimed. In particular, there is described a recombinant mutant luciferase having a mutation (e.g. the amino-acid corresponding to amino acid residue number 245 in <i>Photinus pyralis</i> which is such that the K_m for ATP of the luciferase is increased e.g. five-fold with respect to that of the corresponding non-mutated enzyme such that it is of the order of 500 μM – 1mM. Also disclosed are luciferases having additional mutations conferring improved thermostability or altered wavelength of emitted light. Recombinant polynucleotides, vectors and host cells are also disclosed, as are methods of assaying the amount of ATP in a material (e.g. cells) optionally in real-time. Also disclosed are test-kits for <i>in vitro</i> assays.		

ENZYME ASSAYS**Technical Field**

The present invention relates broadly to enzymes and methods
5 suitable for assaying ATP. It further relates to specific
applications for such assays.

Background Art

Intracellular ATP concentrations can vary 10-fold or more
10 depending upon a cell's state of health or developmental stage.
It is of great value to be able to measure fluctuations in
intracellular ATP levels as a means of investigating e.g. the
effects of drugs, toxins, hormones, environmental agents or
disease on cells.

15 There is apparently at present no convenient method for
analysing the concentration of ATP *in vivo*. For instance, in
Dementieva et al (1996) Biochemistry (Moscow) Vol 61, No. 7.,
the intracellular concentration of ATP was measured in *E. coli*
by calculating the total amount of ATP present using a
20 recombinant luciferase, and dividing by an estimated total cell
volume.

Such an indirect approach can at best produce only an estimate
of the actual ATP concentration.

The measurement of ATP concentration in cells has also been
25 performed using an *in vitro* coupled assay, such as that
disclosed in the Sigma Diagnostic Kit Catalog No. 366, in which
Phosphoglycerate kinase is used to convert 3-phosphoglycerate
to 1,3 diphosphoglycerate in an [ATP]-dependent fashion. The
1,3 diphosphoglycerate is then converted to glyceraldehyde-3-P
30 concomitantly with conversion of NADH to NAD, which can be
monitored spectroscopically. The assay has a dynamic range up
to 1 mM; the expected range is 380-620 μ M when used with blood
cells.

However it can be seen that, as with all coupled assays, the test is inevitably cumbersome to perform. Additionally it could not readily be adapted for *in vivo* use. It would thus be a contribution to the art to provide materials and methods which
5 overcome some of the drawbacks of the prior art.

Disclosure of the Invention

In a first aspect of the invention there is provided a recombinant mutant luciferase having a mutation which is such
10 that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme. Preferably the K_m is at least double that of the non-mutated enzyme, and more preferably at least around five, ten, or twenty times higher than that of the non-mutated enzyme.

15 Luciferases are, of course, already known in the art. In the presence of Mg^{2+} , luciferase (originally obtained from fireflies) catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2 , pyrophosphate and light. This basic property (luciferin and ATP to produce light) is
20 hereinafter referred to as 'luciferase activity'.

The term 'luciferase' as used in relation to the invention is intended to embrace all luciferases, or recombinant enzymes derived from luciferases which have luciferase activity. This explicitly includes recombinant mutant luciferases which have
25 deletions, additions or substitutions to their amino acid structure provided that they retain luciferase activity. Such luciferases will typically have considerable homology (e.g. up to 70, 80, 90, or 99%) with wild-type enzymes. However the crucial technical feature of the luciferases of the present
30 invention which distinguishes them from those of the prior art is that they have a mutation which causes an increase in the K_m for ATP of the luciferase as compared with that measured for a corresponding enzyme which differs only in it that it lacks that same mutation.

This increase K_m may be measured by the person of ordinary skill in the art by conventional enzyme assays, as described in more detail in the Examples below.

It should be noted that in the prior art, luciferase has sometimes been used as a marker for gene expression (in vivo) where its production in a cell is linked to a particular genetic control element. Luciferin is added exogenously and intracellular ATP concentrations, under almost all conditions, will be such that the enzyme is saturated. Thus the switching on of gene expression is signalled by light that is emitted in a quantitative manner according to the amount of active luciferase that is generated.

However it should be stressed that in the previously known systems it is generally the concentration of luciferase which is measured; this concentration is then correlated with a different event e.g. the efficiency of a promoter. Indeed it has, on occasions, been an object of the prior art teaching on luciferases to reduce the K_m for ATP (see e.g. WO 96/22376) which ensures that changes in the ambient [ATP] does not interfere with the assay.

Similarly the assay disclosed by Dementieva et al (1996) discussed above requires that all of the ATP be efficiently converted to light so that the total ATP present can be calculated. This approach requires a low K_m luciferase so that the enzyme operates at near maximal velocity until all the ATP is hydrolysed.

By making available luciferases which have an increased K_m compared with those already known in the art, the present inventors have for the first time opened up the possibility of using these enzymes to measure steady state ATP concentrations over range which was previously unsuitable. This is because, generally speaking, the relationship between enzyme velocity (V , as measured by light intensity) and substrate concentration (of ATP, where luciferin is in excess) is as follows:

$$V = V_m \cdot [ATP] / K_m + [ATP]$$

It can therefore be seen that only when the K_m is greater than (or of a similar order as) the ambient [ATP] will there be a degree of proportionality between changes in [ATP] and changes in light intensity. Where the K_m is much less than the ambient [ATP], any changes in [ATP] will not tangibly effect the measured light intensity. Clearly the more sensitive the light detection is, the smaller the measurable changes in 'V' can be, and the smaller the K_m can be with respect to the [ATP] range being assessed.

For certain applications, e.g. *in vivo* measurements, it may be advantageous to have a luciferase wherein the K_m is of the order of between 400 μ M to 1.4 mM e.g. 500 μ M, 600 μ M, 1 mM etc. However, as can be appreciated from the discussion above, the main criterion is that the K_m is not much less than the expected [ATP] range to be assessed, and the phrase 'of the order of' should be construed accordingly.

A particular expected [ATP] range which is important for physiological assays of blood cells is between 300 μ M and 1 mM, or more particularly 380 μ M and 620 μ M, (cf. Sigma Diagnostic Kit, Catalog No. 366 discussed above). For other mammalian cells such as hepatocytes, the [ATP] range is 2.5 mM - 6 mM (see Dementieva et al (1996) discussed above. Use of the recombinant luciferases of the present invention for continuous assays in these ranges is particularly envisaged.

The disclosure of the present application makes such high K_m luciferases available for the first time. The prior art disclosures reveal only luciferases having a K_m of between 60 μ M and 150 μ M, which would be saturated in these ranges.

It is also advantageous, as with all enzymes used in assays, that the mutant enzyme retains sufficient activity (i.e. a high maximum turnover number, giving a high V_m) such that practical concentrations of enzyme can give detectable results.

5

Preferably the activity for ATP of the mutant is at least 5-100% of that of the corresponding wild-type; however reduced-activity as a result of the high K_m mutation can, if necessary, be compensated for by using more enzyme or more sensitive
5 detection if required.

In one embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue
10 such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.

It should be noted that the sequences of a number of luciferases from different sources have already been published in the literature, see e.g. WO 95/25798 for *P pyralis*; EP 0 524
15 448 for *Luciola cruciata* and *Luciola lateralis*. Other known luc genes include *Luciola mingrellica*, and *Lampyrus noctiluca* (see Newby et al (1996) Biochemical J 313: 761-767.)

Whether an amino-acid in a luciferase 'corresponds' to number 245 in *P pyralis* (which is His in the wild-type, non-mutated
20 enzyme) can be established by the person of ordinary skill in the art without difficulty as follows: the sequence of the luciferase is established (either from the literature or by sequencing); the sequence is aligned with *P pyralis*, for instance using commercially available software (e.g. "Bestfit"
25 from the University of Wisconsin Genetics Computer Group; see Devereux et al (1984) Nucleic Acid Research 12: 387-395) or manually such as to demonstrate maximal homology and align conserved amino acids; the amino acid corresponding to number 245 in *P pyralis* is identified. An example of this is shown
30 below using *L cruciata* - the corresponding amino acid in that case is number 247.

Once identified a mutant can be prepared e.g. by site directed mutagenesis by methods commonly used in the art and exemplified below.

6

Preferably corresponding amino-acid is substituted for an uncharged amino acid, for instance nonpolar (e.g. Ala) or uncharged polar (e.g. Asn, or Gln):

CLASS	EXAMPLES OF AMINO ACID
Nonpolar:	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged polar:	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic:	Asp, Glu
Basic:	Lys, Arg, His

It should be noted that WO 95/18853 (PROMEGA) lists a large number (over 80) of *Pyrophorus plagiophtalamus* mutants which are reported to have altered spectral properties. However the K_m for ATP of the mutants is not reported, nor indeed discussed at any point in the application.

In another embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 318 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme. Correspondence may be assessed as above; preferably the amino acid (Ser in the wild type) is substituted for a bulkier one (e.g. Thr).

In preferred forms the mutant luciferases of the present invention incorporate one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light. Some suitable mutations are already known to those skilled in the art; see e.g. WO 95/25798 and WO 96/22376 and EP 0 524 448 for thermostability improving mutations (e.g. at positions corresponding to 354 and 215 of *P pyralis*).

Preferably the mutation causing the increased K_m itself improves one or more of these properties, particularly thermostability. It should be noted that an enhanced stability at around 37°C is especially advantageous for enzymes which are to be employed in vivo.

In a further embodiment the luciferases may be in the form of fusion proteins or incorporate polypeptide extensions. This may improve the ease by which they can be produced, localised in vivo or extracted and purified.

- 10 In a second aspect of the invention there is disclosed a recombinant polynucleotide encoding a mutant luciferase of the present invention, as described above.

In a third aspect there are disclosed vectors comprising a polynucleotide of the second aspect. For instance vectors further comprising a replication element which permits replication of the vector in a suitable host cell and/or a promoter element which permits expression of said polynucleotide in a suitable host cell. The promoter may be a constitutive promoter. Optionally the promoter element may be tissue- or organ-specific.

In a fourth aspect there is disclosed a host cell containing, or transformed with, a vector of the third aspect.

Optionally the host cell of the fourth aspect may express one or more further luciferases which have a lower K_m for ATP than those of the present invention, and possibly emit light of a different wavelength, such as to extend the useful range of any assay, and/or allow the use of a ratiometric assay i.e. one in which the activity of the high K_m mutant is compared with that of a further luciferase. The further luciferases may be recombinant non-mutant luciferases or recombinant mutant luciferases having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme (see e.g. WO 96/22376 .

Coloured mutants are disclosed in WO 95/18853 and in Ohmiya et al (1996) FEBS Letters 384: 83-86.

In a fifth aspect there is disclosed a process for producing a luciferase of the present invention comprising culturing a host
5 cell as described in the fourth aspect.

In a sixth aspect there is disclosed a single cell organism consisting of a host cell as described above, or a multicellular organ or organism comprising it. The use of e.g. transgenic higher animals in which the luciferases of the
10 present invention are expressed could allow in vivo study of [ATP] in different types of cell or tissue as described in more detail below. In particular, as ATP is present in virtually all living cells, any type of cell into which luciferase could be cloned, from bacterial to plant or animal, could be studied
15 through the measurement of ATP changes.

Thus in a seventh aspect of invention there is disclosed a method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as described above.

Preferably the method comprises the following steps(a) the
20 luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

The measurement in step (b) may be compared with a control
25 value such as minimise base-line errors.

The assay can be *in vitro* or *in vivo*.

More preferably the material itself is a cell, in to which the luciferase is introduced e.g. by transforming the cell with a vector as described above. Alternatively the luciferase may be
30 introduced into the cell by direct injection.

Equally the material measured may be part of a synapse i.e. the ATP is neurotransmitter.

Generally the assay will be most useful for real-time analysis (on a time-scale of seconds e.g. using a CCD camera,
5 photomultiplier or photodiode) of events initiated by particular stimuli (e.g. addition of an active agent to the material). In this case the assay can monitor changes in [ATP] concentration over a relatively short time-scale. Such
10 measurement will not, therefore, be greatly affected by longer time-scale events, such as changes in the concentration of luciferase in the system. These changes can be correlated with cellular events e.g. tissue necrosis may be associated with falling [ATP], fatigue in muscle likewise. Such continuous assays have hitherto not been possible.

15 Other possible applications include measuring the effect of drug treatments on various tissues; toxins and uncoupling agents on oxidative phosphorylation; bacterial infection; metabolic processes and stress (e.g. obesity and exercise); studies of brain activity (e.g. memory function and mental
20 disorders) etc.

If appropriate the [ATP] can be measured periodically (rather than constantly) using photographic film.

Essentially the monitoring can be done in ways analogous to those already used in the art for other applications e.g. for
25 the photonic detection of bacterial pathogens in living hosts disclosed by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In that paper a Hamamatsu intensified CCD camera was used to visualise *Salmonella Typhimurium* expressing luciferase during infection of a mouse. Equally a system equivalent to PET
30 (positron emission tomography - as used in brain scans) could be used to achieve precise localisation of luciferase-generated light to allow the metabolism of specific body regions to be ascertained.

10

Generally speaking it will be necessary to introduce luciferin into the system being studied. By 'luciferin' is meant any co-factor which has luciferin activity i.e. can be used in conjunction with luciferase to cause light to be emitted in the presence of ATP. The manner by which this is introduced in to the system will depend on the system itself. For instance where animal cells are being studied, luciferin may be introduced by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part. Similarly when the system being studied is one or more plant cells, the luciferin may simply be introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

In a final aspect of the invention there is disclosed a test kit comprising a luciferase discussed above and further comprising one or more of the following (a) a buffer or dry materials for preparing a buffer; (b) ATP standards; (c) luciferin; (d) Dithiothreitol (e) instructions for carrying out an ATP assay.

The invention will now be further described with reference to the following none-limiting Figures, Sequence Listings and Examples.

Figures

Fig 1. shows plasmid pPW601a as described in Example 1.

Fig 2. for mutant H245A, (a) shows the plot of V against [ATP] and (b) shows 1/V against 1/[ATP] as described in Example 1.

Fig 3. shows a sequence comparison of one region of *P pyralis* (Pp) and the corresponding region of *L cruciata* (Lc) as described in Example 2.

Fig 4 is a graph showing light emission versus ATP concentration for mutant H245N.

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Fig 5 . shows the effect of the addition of nutrient broth to luciferase-expressing *E. coli* cells pre-charged with Luciferin as described in Example 6.

Fig. 6 shows Seq ID No. 1 which is the nucleotide sequence of the wild type *luc* gene from *P pyralis*.

Fig. 7 shows Seq ID Nos. 2-5 which are the primers used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4) and the equivalent wild-type sequence (Seq ID No 5).

Fig. 8 shows Seq ID No. 6 which is the amino acid sequence of a high K_m mutant H245Q of the present invention, wherein amino acid 245 has been changed to Gln.

Examples

15

EXAMPLE 1: PRODUCTION OF RECOMBINANT HIGH K_m MUTANT LUCIFERASE

Except where otherwise stated, the methods employed were as those used by White et al (1996) Biochemical Journal 319: 342-350, which is concerned with thermostable mutants.

STARTING MATERIALS: Mutants were generated by site directed mutagenesis of the plasmid pPW601a (Fig 1) comprising the luciferase gene, *luc*, from *P pyralis*. The wild type *luc* gene from *P pyralis* is shown at Seq ID No. 1. Plasmid pPW601a was created by cloning the *luc* gene *Bam*HI/*Sst*I fragment from pGEM-*luc* (available from Promega) into pDR540 (available from Pharmacia). The unique *Xho*I site in the polylinker of the plasmid was removed to simplify the following procedures.

SITE DIRECTED MUTAGENESIS: Three mutagenic oligonucleotides were used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4). The equivalent wild-type sequence is shown at Seq ID No. 5. The oligonucleotides also introduced a silent mutation which destroys a unique *Xmn* I site in the *luc* gene - this did not result in an amino acid

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substitution but facilitated mutant selection. The mutagenesis was carried out in accordance with the kit instructions of kit supplied by Clontech laboratories Inc, Palo Alto, California USA.

- 5 The amino acid sequence of H245Q is shown in Seq. ID No. 6.

ISOLATION OF PLASMID DNA & TRANSFORMATION: this was carried out by the method of Brinboim & Doly (1979) Nucleic Acids Research 7: 1513.

- 10 CELL CULTURE AND EXTRACTION: *E. coli* JM109 transformants were grown to an $OD_{600} = 1.0$. Aliquots of cells expressing mutant luciferases from plasmid pPW601a, were subjected to lysis as described in the Promega technical bulletin and the lysed extracts were then stored on ice prior to assay.

- 15 ASSAY OF K_m OF MUTANT LUCIFERASES: luciferase assays were performed at 21°C using 100 μ l of assay buffer (20 mM Tricine pH 7.8 containing 2.0 mM $MgSO_4$, 0.1 mM EDTA, 33mM dithiothreitol, 470 μ M D-luciferin and ATP in the concentration range 6.25 - 800 μ M). Each assay contained 5-10 μ l of crude cell extract.

- 20 The plots of V against $[ATP]$ and $1/V$ against $1/[ATP]$ for mutant H245A are shown in Fig. 2. Such plots can be used to determine the K_m .

The results of each mutation and the recombinant Wild Type are shown in Table 1:

Table 1

Luciferase	K _m MgATP (μ M)
r Wild Type	66
H245A	442
H245N	623
H245Q	1340
A215L*	65

* A215L is a thermostable mutant in which amino acid 215 is substituted with lysine (see WO 96/22376 - SECRETARY OF STATE FOR DEFENCE).

ASSAY OF THERMOSTABILITY OF MUTANT LUCIFERASES: the thermostability of H245N & H245Q was also tested, as compared with mutant A215L and the wild-type. Lysed crude extracts of cells containing luciferase activity were incubated at 37°C for set time periods. The thermostability of the mutant H245A was found to be very similar to that of the recombinant wild-type. The results are shown in Table 2:

Table 2

Enzyme	Remaining activity %			
	0	2	4	8 minutes
r Wild Type	100	64.8	36.6	26.6
A215L	100	101	88	84
H245N	100	96	61	46
H245Q	100	103	78.6	51.5

PURIFICATION: luciferases, e.g. incorporating the H245Q mutation, may be purified as described in White et al (1996) [supra]. Briefly, the cell lysates are centrifuged at 30000 g for 30 mins and the supernatant is fractionated with ammonium sulphate (30-55%). This fraction is resuspended and desalted. The desalted material was passed through a hydroxyapatite column and eluted with 10-200 mM sodium phosphate containing dithiothreitol. The luciferase containing eluant is dialysed and applied to a Mono Q anion-exchange column. The enzyme can be eluted with 0 to 500 mM NaCl.

EXAMPLE 2: IDENTIFICATION OF CORRESPONDING HIGH K_m MUTANTS

Fig 3. shows a sequence comparison of one region of *P pyralis* and the corresponding region of *L cruciata* as describe din Example 2. In this case it can be seen that amino acid 245 corresponds to 247.

EXAMPLE 3: EXPRESSION OF MUTANT LUCIFERASE IN MAMMALS

This can be achieved by methods analogous to those disclosed by Liu et al (1997) Nature Biotechnology 15: 167-173. In this method cationic liposomes are used to deliver plasmid DNA containing luciferase a gene to mice.

EXAMPLE 4: AN IN VIVO ATP ASSAY IN MAMMALS

This can be carried out by methods analogous to those used by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In this method luciferase expression in *S typhimurium* in mice is
5 monitored using a CCD camera.

EXAMPLE 5: A KIT FOR AN IN VITRO ATP ASSAY

This may be provided as follows: luciferase H245Q; buffer; or dry materials for preparing a buffer; ATP for standards; luciferin; and instructions for carrying out an ATP assay.

10 EXAMPLE 6 : Assay for determining cell behaviour

Using a luciferase assay as described in Example 1, a plot of the photon count versus the ATP concentration was prepared for the H245N mutant. The results are shown in Figure 4.

In order to demonstrate how the enzyme of the invention can be
15 used in studying cellular behaviour, a sample of recombinant *E.coli* cells which expressed the H245N mutant luciferase were rendered dormant by exhaustion of nutrients. The cells were charged with luciferin by 10 minutes immersion in p.H. 5.0 citrate buffer containing 1mM luciferin. They were then
20 centrifugally washed, resuspended in 1ml Nutrient Broth and the luminescence monitored. The results are shown in Figure 5.

Using the mutant luciferase of the invention, the revival and growth of functional cells could be monitored.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: THE SECRETARY OF STATE FOR DEFENCE
- (ii) TITLE OF INVENTION: ENZYME ASSAY FOR MUTANT FIREFLY LUCIFERASE
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
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 - (E) COUNTRY: CANADA
 - (F) ZIP: K1P 5Y6
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: ASCII (text)
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: CA 2,283,287
 - (B) FILING DATE: 07-APR-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: GB 9707486.8
 - (B) FILING DATE: 11-APR-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FETHERSTONHAUGH & CO.
 - (B) REGISTRATION NUMBER:
 - (C) REFERENCE/DOCKET NUMBER: 22762-797
- (ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1722 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Photinus pyralis*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 4..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAATGGAAG ACGCCAAAAA CATAAGAAA GGCCCGGCGC CATTCTATCC TCTAGAGGAT	60
GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA	120
ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG	180
TCCGTTCCGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC	240
GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA	300
GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC	360
ATTTCGCAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG	420
CAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG	480
GGATTTCACTG CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC	540
GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT	600
GGATCTACTG GGTTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC	660
TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT	720
GTTGTTCCAT TCCATCACGG TTTTGGGAATG TTTACTACAC TCGGATATTT GATATGTGGA	780

TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTTTACGATC CCTTCAGGAT	840
TACAAAATTC AAAGTGCGTT GCTAGTACCA ACCCTATTTT CATTCTTCGC CAAAAGCACT	900
CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGGGG CGCACCTCTT	960
TCGAAAGAAG TCGGGGAAGC GGTTGCAAAA CGCTTCCATC TTCCAGGGAT ACGACAAGGA	1020
TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCGAGGGGGA TGATAAACCG	1080
GGCGCGGTCG GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG	1140
AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC	1200
GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT	1260
TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG	1320
TCTTTAATTA AATACAAAGG ATATCAGGTG GCGGGCGCTG AATTGGAATC GATATTGTTA	1380
CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA	1440
CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG	1500
GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG	1560
GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC	1620
ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC	1680
GAAATTCTTA GCTATTGTAA TCCTCCGAGG CCTCGAGGTC GA	1722

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTTGTTCCAT TCCATGCCGG TTTCGGAATG TTTAC

35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTGTTCCAT TCCATCAGGG TTTCGGAATG TTTAC

35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTGTTCCAT TCCATAACGG TTTCGGAATG TTTAC

35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTAC

35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 550 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Photinus pyralis

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Glu	Asp	Ala	Lys	Asn	Ile	Lys	Lys	Gly	Pro	Ala	Pro	Phe	Tyr	Pro	1	5	10	15
Leu	Glu	Asp	Gly	Thr	Ala	Gly	Glu	Gln	Leu	His	Lys	Ala	Met	Lys	Arg	20	25	30	
Tyr	Ala	Leu	Val	Pro	Gly	Thr	Ile	Ala	Phe	Thr	Asp	Ala	His	Ile	Glu	35	40	45	
Val	Asn	Ile	Thr	Tyr	Ala	Glu	Tyr	Phe	Glu	Met	Ser	Val	Arg	Leu	Ala	50	55	60	
Glu	Ala	Met	Lys	Arg	Tyr	Gly	Leu	Asn	Thr	Asn	His	Arg	Ile	Val	Val	65	70	75	80
Cys	Ser	Glu	Asn	Ser	Leu	Gln	Phe	Phe	Met	Pro	Val	Leu	Gly	Ala	Leu	85	90	95	
Phe	Ile	Gly	Val	Ala	Val	Ala	Pro	Ala	Asn	Asp	Ile	Tyr	Asn	Glu	Arg	100	105	110	
Glu	Leu	Leu	Asn	Ser	Met	Asn	Ile	Ser	Gln	Pro	Thr	Val	Val	Phe	Val	115	120	125	
Ser	Lys	Lys	Gly	Leu	Gln	Lys	Ile	Leu	Asn	Val	Gln	Lys	Lys	Leu	Pro	130	135	140	
Ile	Ile	Gln	Lys	Ile	Ile	Ile	Met	Asp	Ser	Lys	Thr	Asp	Tyr	Gln	Gly	145	150	155	160
Phe	Gln	Ser	Met	Tyr	Thr	Phe	Val	Thr	Ser	His	Leu	Pro	Pro	Gly	Phe	165	170	175	
Asn	Glu	Tyr	Asp	Phe	Val	Pro	Glu	Ser	Phe	Asp	Arg	Asp	Lys	Thr	Ile	180	185	190	
Ala	Leu	Ile	Met	Asn	Ser	Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	Gly	Val	195	200	205	
Ala	Leu	Pro	His	Arg	Thr	Ala	Cys	Val	Arg	Phe	Ser	His	Ala	Arg	Asp	210	215	220	
Pro	Ile	Phe	Gly	Asn	Gln	Ile	Ile	Pro	Asp	Thr	Ala	Ile	Leu	Ser	Val	225	230	235	240

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Val Pro Phe His Gln Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
 245 250 255
 Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
 260 265 270
 Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
 275 280 285
 Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
 290 295 300
 Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
 305 310 315 320
 Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
 325 330 335
 Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
 340 345 350
 Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
 355 360 365
 Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
 370 375 380
 Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
 385 390 395 400
 Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
 405 410 415
 Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
 420 425 430
 Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
 435 440 445
 Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
 450 455 460
 Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
 465 470 475 480
 Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
 485 490 495
 Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
 500 505 510
 Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
 515 520 525
 Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
 530 535 540
 Gly Gly Lys Ser Lys Leu
 545 550

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile	Ile	Pro	Asp	Thr	Ala	Ile	Leu	Ser	Val	Val	Pro	Phe	His	His	Gly
1				5					10					15	
Phe	Gly	Met	Phe	Thr	Thr	Leu	Gly	Tyr	Leu	Ile	Cys				
			20					25							

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val	Ser	Pro	Gly	Thr	Ala	Val	Leu	Thr	Val	Val	Pro	Phe	His	His	Gly
1				5					10					15	
Phe	Gly	Met	Phe	Thr	Thr	Leu	Gly	Tyr	Leu	Ile	Cys				
			20					25							

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ile	Asp	Lys	Tyr	Asp	Leu	Ser	Asn	Leu	His	Glu	Ile	Ala	Ser	Gly	Gly
1				5					10					15	
Ala	Pro	Leu	Ser	Lys	Glu	Val	Gly	Glu	Ala	Val	Ala	Lys	Arg	Phe	His
			20					25					30		

Leu Pro Gly
35

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Asn Lys Tyr Asp Leu Ser Asn Leu Val Glu Ile Ala Ser Gly Gly
1 5 10 15

Ala Pro Leu Ser Lys Glu Val Gly Glu Ala Val Ala Arg Arg Phe His
20 25 30

Leu Pro Gly
35

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Arg Phe His Leu Pro Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr
1 5 10 15

Thr Ser Ala Ile Leu Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala
20 25 30

Val Gly Lys Val Val Pro Phe Phe
35 40

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 amino acids

(B) TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg	Phe	Asn	Leu	Pro	Gly	Val	Arg	Gln	Gly	Tyr	Gly	Leu	Thr	Glu	Thr
1				5					10					15	
Thr	Ser	Ala	Ile	Ile	Ile	Thr	Pro	Glu	Gly	Asp	Asp	Lys	Pro	Gly	Ala
			20					25					30		
Ser	Gly	Lys	Val	Val	Pro	Leu	Phe								
		35					40								

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CLAIMS:

1. A recombinant mutant luciferase wherein the amino-acid corresponding to amino acid residue number 245 or 318 in *Photinus pyralis* has been substituted with respect to the
5 corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.
2. A recombinant mutant luciferase according to claim 1 wherein the amino-acid corresponding to amino acid
10 residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue.
3. A luciferase as claimed in claim 1 or claim 2 wherein the K_m is at least double that of the non-mutated
15 enzyme.
4. A luciferase as claimed in claim 3 wherein the K_m is at least five times higher than that of the non-mutated enzyme.
5. A luciferase as claimed in claim 1 or claim 2
20 wherein the K_m is of the order of 500 μ m.
6. A luciferase as claimed in claim 1 or claim 2 wherein the K_m is of the order of 1 mM.
7. A luciferase as claimed in any one of claims 1 to 6 having a V_m for ATP which is at least 5-100% of that of
25 the corresponding wild-type.
8. A luciferase as claimed in claim 2 wherein said amino-acid has been substituted for an uncharged amino acid.
9. A luciferase as claimed in claim 7 wherein the amino-acid has been substituted for Ala, Asn, or Gln.

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10. A luciferase as claimed in any one of claims 1 to 9 which is derived from *Photinus pyralis* and wherein amino acid residue number 245 has been substituted.

11. A luciferase as claimed in any one of claims 1 to 9 which is derived from *Luciola cruciata* and wherein amino acid residue number 247 has been substituted.

12. A luciferase as claimed in any one of claims 1 to 11 incorporating one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light.

13. A fusion protein comprising a luciferase as claimed in any one of claims 1 to 12.

14. A recombinant polynucleotide encoding a luciferase as claimed in any one of claims 1 to 12.

15. A replication vector comprising a polynucleotide as claimed in claim 14 further comprising a replication element which permits replication of the vector in a suitable host cell.

16. An expression vector comprising a polynucleotide as claimed in claim 14 further comprising a promoter element which permits expression of said polynucleotide in a suitable host cell.

17. A vector as claimed in claim 16 wherein the promoter element is tissue or organ specific.

18. A host cell containing a vector as claimed in any one of claims 15 to 17.

19. A host cell transformed with a vector as claimed in any one of claims 15 to 17.

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20. A host cell as claimed in claim 19 which also expresses a second luciferase having a lower K_m for ATP.

21. A host cell as claimed in claim 20 wherein the second luciferase is selected from: (a) a recombinant
5 non-mutant luciferase; (b) a recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme.

22. A process for producing a luciferase comprising
10 culturing a host cell as claimed in any one of claims 19 to 21, so that said luciferase is expressed, and recovering said luciferase.

23. A cell of host organism comprising a vector as claimed in any one of claims 15 to 17.

15 24. Use of a recombinant luciferase as claimed in any one of claims 1 to 12 for assaying the amount of ATP in a material, wherein the concentration of the ATP is expected to be between 300 μ M and 6 mM.

25. Use of a recombinant luciferase as claimed in any
20 one of claims 1 to 12 for assaying the amount of ATP in a material.

26. A method of assaying the amount of ATP in a material, said method comprising the steps of (a) contacting a recombinant mutant luciferase having a K_m for ATP which is
25 higher than the non-mutated form and which is of the order of the expected ATP range in the material, with the material and luciferin; (b) measuring the intensity of light emitted by the luciferase; and (c) the measurement in step (b) is correlated directly with the amount of ATP in the material.

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27. A method as claimed in claim 26 wherein the measurement in step (b) is compared with a control value.

28. A method as claimed in claim 26 or 27 wherein the measurement in step (b) is made in real-time.

5 29. A method as claimed in any one of claims 26 to 28 wherein the material measured forms part of a synapse.

30. A method as claimed in any one of claims 26 to 29 wherein the material is a cell and the luciferase is introduced into the cell.

10 31. A method as claimed in claim 30 wherein the luciferase is introduced into the cell by transforming the cell with a vector comprising a polynucleotide which encodes a recombinant mutant luciferase according to any one of claims 1 to 12.

15 32. A method as claimed in claim 30 or claim 31 wherein the luciferase is introduced into the cell by direct injection.

33. A method as claimed in claim 31 or claim 32 wherein the cell is an animal cell and the luciferase is
20 introduced into the cell by ingestion of luciferase or a precursor thereof by an animal of which the cell is a constituent part.

34. A method as claimed in claim 31 or claim 32 wherein the cell is a plant cell and the luciferase is
25 introduced into the cell by applying a solution of luciferase or a precursor thereof to a plant of which the cell is a constituent part.

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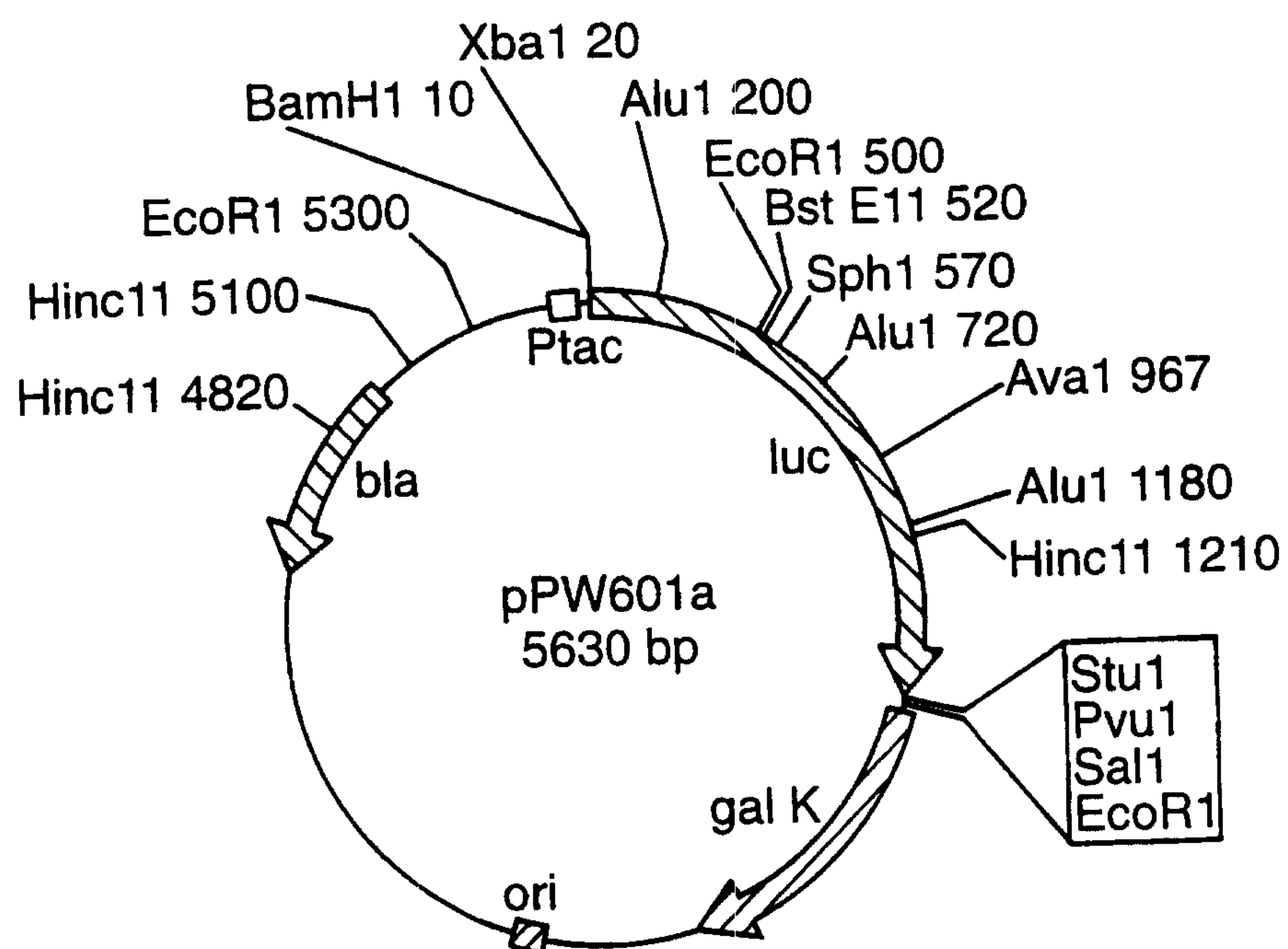
35. A method according to any one of claims 26 to 34 wherein the recombinant mutant luciferase is a luciferase according to any one of claims 1 to 12.

36. A test kit comprising a luciferase as claimed in
5 any one of claims 1 to 12 and further comprising one or more of the following (a) a buffer or dry materials for preparing a buffer; (b) two or more measured portions of ATP suitable for preparing standard solutions; (c) luciferin; (d) instructions for carrying out an ATP assay.

FETHERSTONHAUGH & CO.
OTTAWA, CANADA

PATENT AGENTS

Fig.1.



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Fig.2.

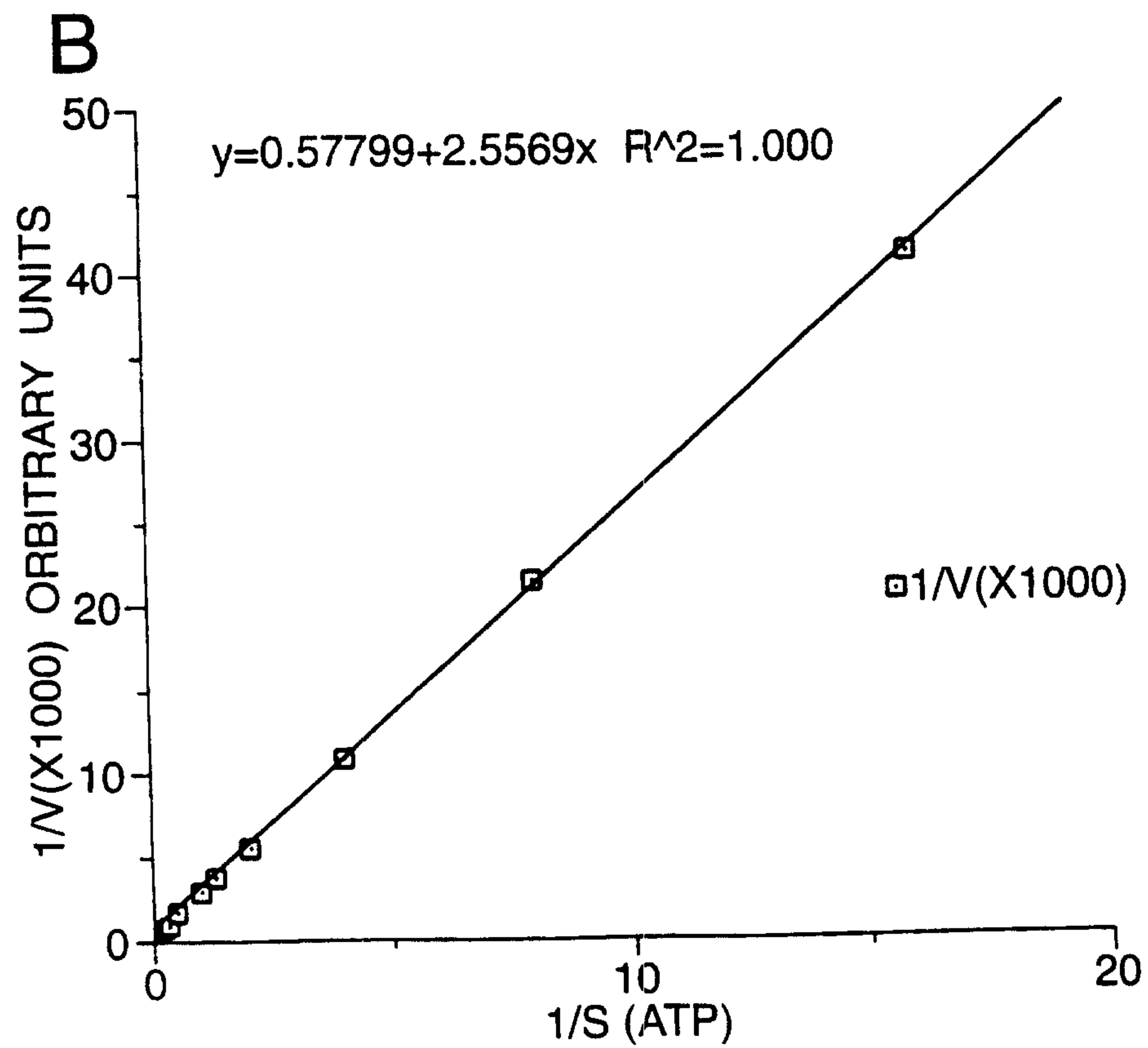
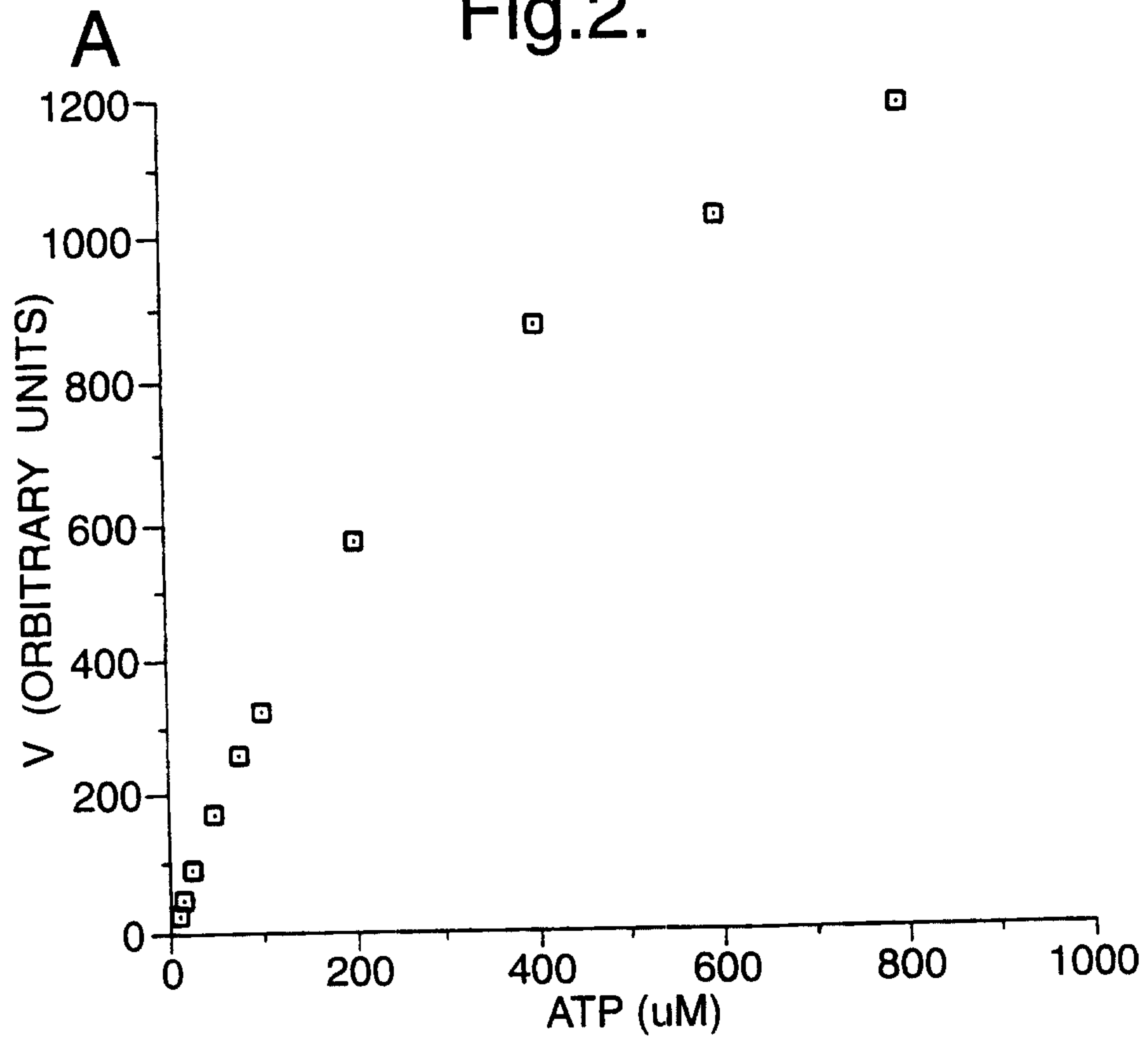


Fig. 3.

[illegible]

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Fig.4.

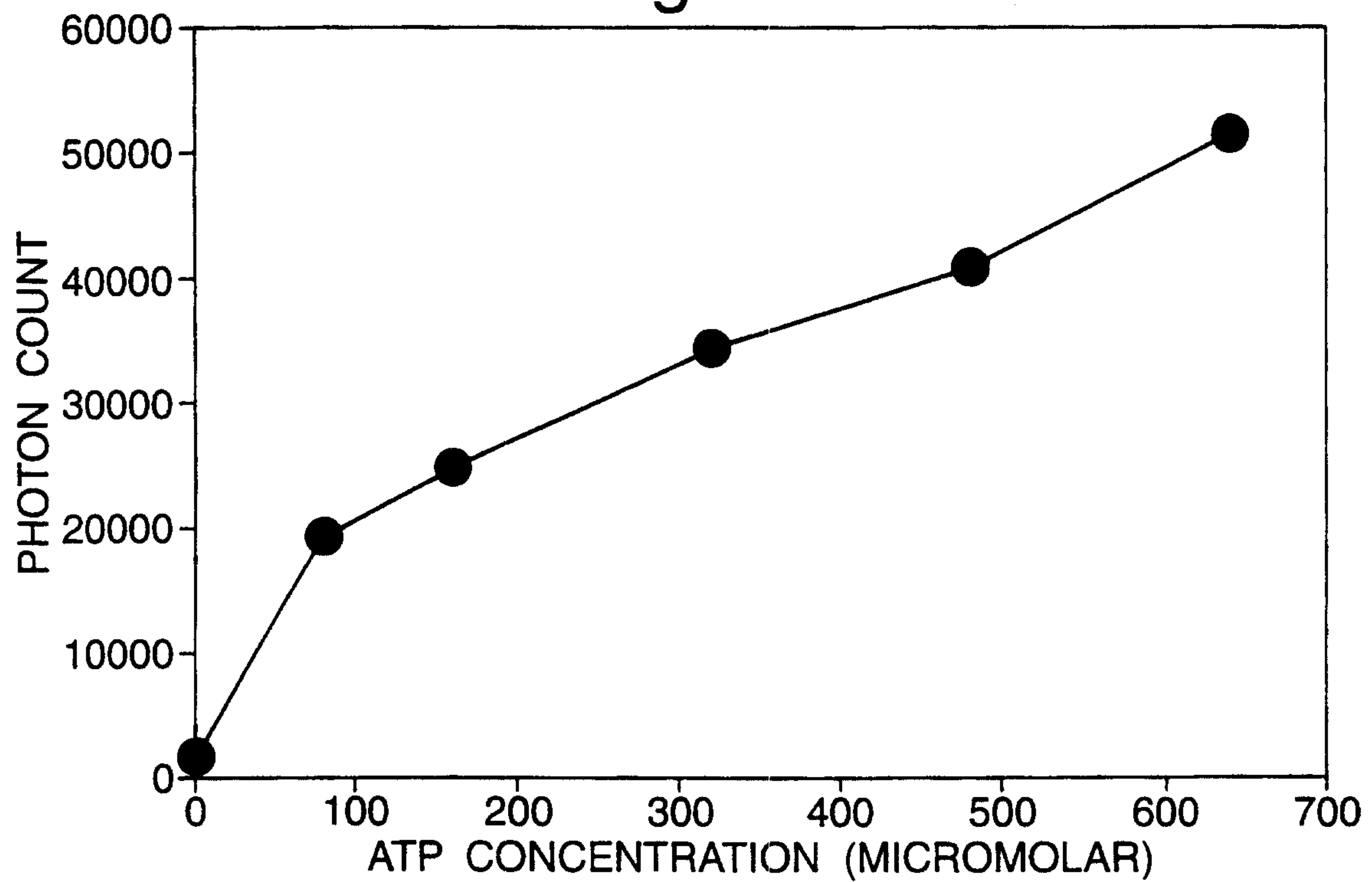
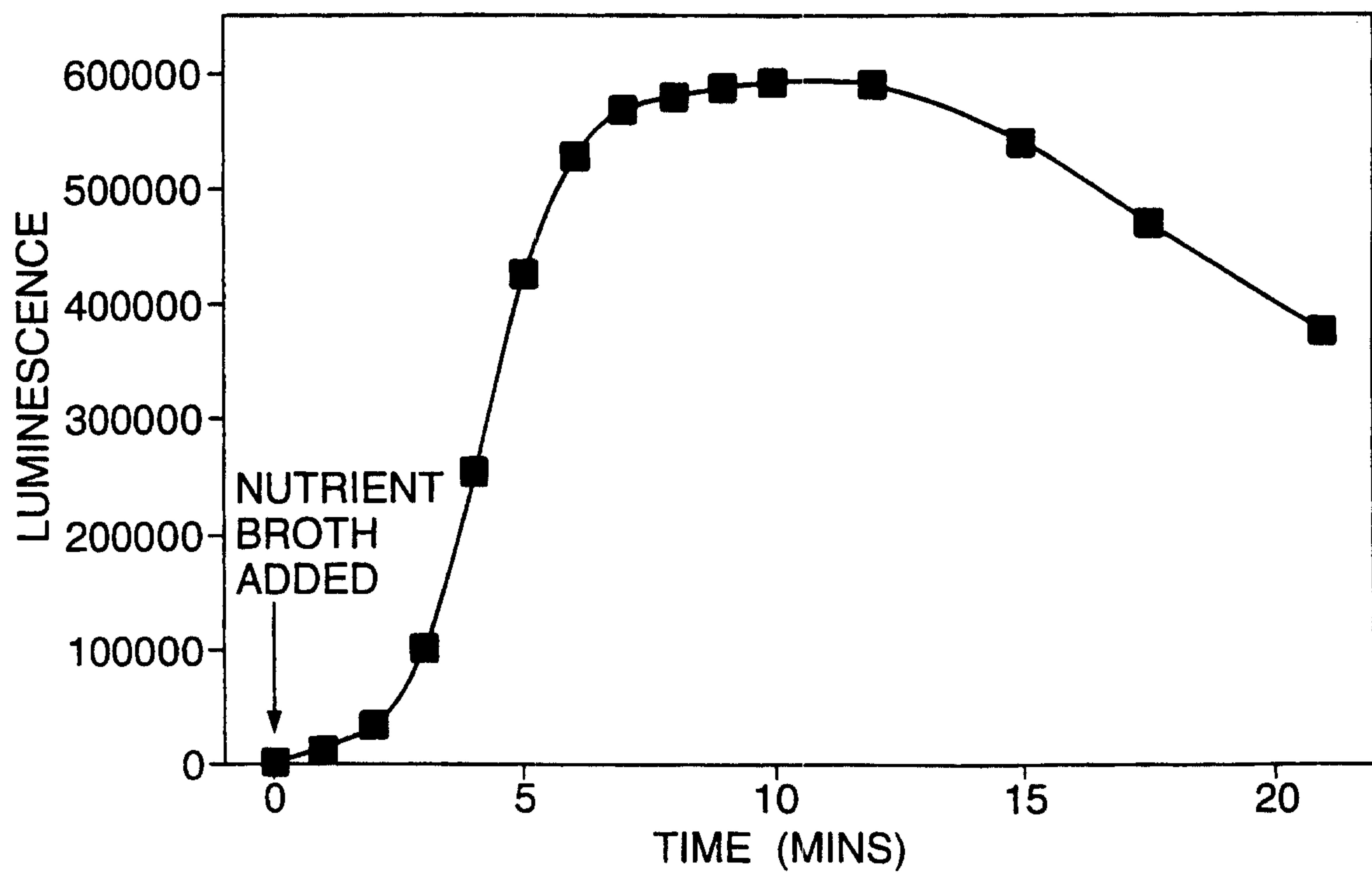


Fig.5.



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Fig.6.

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1722 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Photinus pyralis*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 4..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAATGGAAG ACGCCAAAAA CATAAGAAA GGCCCGGCGC CATTCTATCC TCTAGAGGAT	60
GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA	120
ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG	180
TCCGTTCCGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC	240
GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA	300
GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC	360
ATTTGCGCAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAT TTTGAACGTG	420
CAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG	480
GGATTTCACTG CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC	540
GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT	600
GGATCTACTG GGTTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC	660
TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAAGT	720

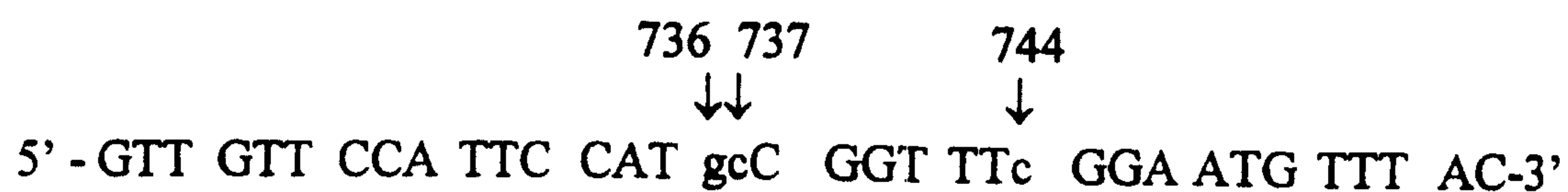
Fig.6 (Cont).

GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTACTACAC TCGGATATTT GATATGTGGA	780
TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTTTACGATC CCTTCAGGAT	840
TACAAAATTC AAAGTGCGTT GCTAGTACCA ACCCTATTTT CATTCTTCGC CAAAAGCACT	900
CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGGGG CGCACCTCTT	960
TCGAAAGAAG TCGGGGAAGC GGTTGCAAAA CGCTTCCATC TTCCAGGGAT ACGACAAGGA	1020
TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCGAGGGGGA TGATAAACCG	1080
GGCGCGGTCT GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG	1140
AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC	1200
GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT	1260
TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG	1320
TCTTTAATTA AATACAAAGG ATATCAGGTG GCCCCGCTG AATTGGAATC GATATTGTTA	1380
CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA	1440
CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG	1500
GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG	1560
GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC	1620
ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC	1680
GAAATTCTTA GCTATTGTAA TCCTCCGAGG CCTCGAGGTC GA	1722

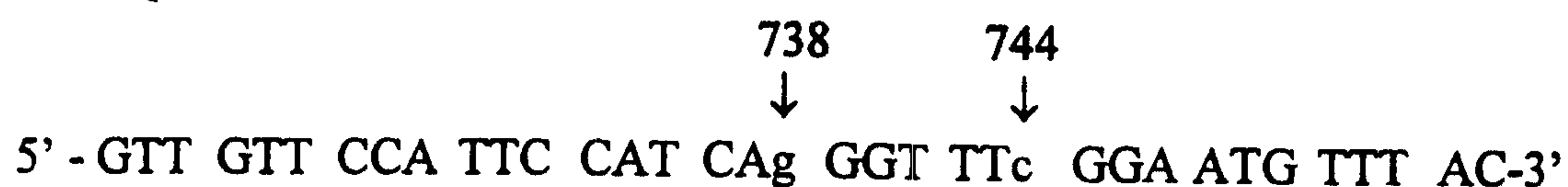
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Fig.7.**MUTAGENIC OLIGONUCLEOTIDES**Sequence ID No. 2

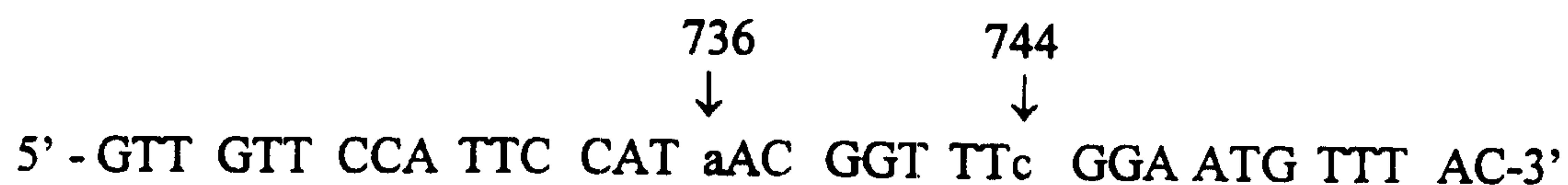
H245A:

Sequence ID No. 3

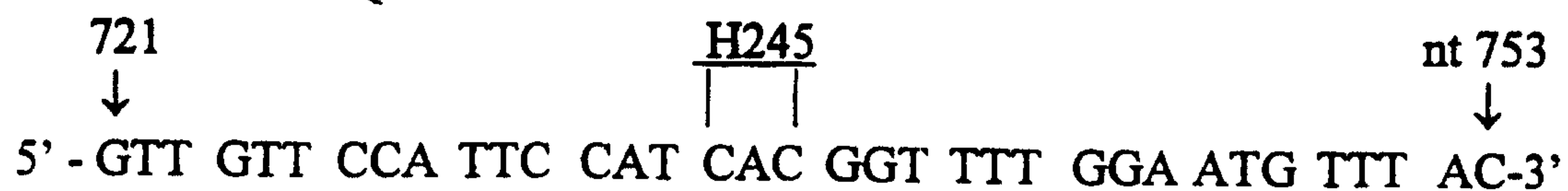
H245Q:

Sequence ID No. 4

H245N:

Sequence ID No. 5

WILD TYPE SEQUENCE:



nt numbering is from luc gene sequence,

CAA
|
nt 1

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 550 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

Fig.8.

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Photinus pyralis*

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Glu	Asp	Ala	Lys	Asn	Ile	Lys	Lys	Gly	Pro	Ala	Pro	Phe	Tyr	Pro	1	5	10	15
Leu	Glu	Asp	Gly	Thr	Ala	Gly	Glu	Gln	Leu	His	Lys	Ala	Met	Lys	Arg	20	25	30	
Tyr	Ala	Leu	Val	Pro	Gly	Thr	Ile	Ala	Phe	Thr	Asp	Ala	His	Ile	Glu	35	40	45	
Val	Asn	Ile	Thr	Tyr	Ala	Glu	Tyr	Phe	Glu	Met	Ser	Val	Arg	Leu	Ala	50	55	60	
Glu	Ala	Met	Lys	Arg	Tyr	Gly	Leu	Asn	Thr	Asn	His	Arg	Ile	Val	Val	65	70	75	80
Cys	Ser	Glu	Asn	Ser	Leu	Gln	Phe	Phe	Met	Pro	Val	Leu	Gly	Ala	Leu	85	90	95	
Phe	Ile	Gly	Val	Ala	Val	Ala	Pro	Ala	Asn	Asp	Ile	Tyr	Asn	Glu	Arg	100	105	110	
Glu	Leu	Leu	Asn	Ser	Met	Asn	Ile	Ser	Gln	Pro	Thr	Val	Val	Phe	Val	115	120	125	
Ser	Lys	Lys	Gly	Leu	Gln	Lys	Ile	Leu	Asn	Val	Gln	Lys	Lys	Leu	Pro	130	135	140	
Ile	Ile	Gln	Lys	Ile	Ile	Ile	Met	Asp	Ser	Lys	Thr	Asp	Tyr	Gln	Gly	145	150	155	160
Phe	Gln	Ser	Met	Tyr	Thr	Phe	Val	Thr	Ser	His	Leu	Pro	Pro	Gly	Phe	165	170	175	
Asn	Glu	Tyr	Asp	Phe	Val	Pro	Glu	Ser	Phe	Asp	Arg	Asp	Lys	Thr	Ile	180	185	190	
Ala	Leu	Ile	Met	Asn	Ser	Ser	Gly	Ser	Thr	Gly	Leu	Pro	Lys	Gly	Val	195	200	205	
Ala	Leu	Pro	His	Arg	Thr	Ala	Cys	Val	Arg	Phe	Ser	His	Ala	Arg	Asp	210	215	220	

Fig.8 (Cont).

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Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
 225 230 235 240
 Val Pro Phe His Gln Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
 245 250 255
 Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
 260 265 270
 Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
 275 280 285
 Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
 290 295 300
 Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
 305 310 315 320
 Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
 325 330 335
 Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
 340 345 350
 Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
 355 360 365
 Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
 370 375 380
 Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
 385 390 395 400
 Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
 405 410 415
 Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
 420 425 430
 Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
 435 440 445
 Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
 450 455 460
 Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
 465 470 475 480
 Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
 485 490 495
 Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
 500 505 510
 Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
 515 520 525
 Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
 530 535 540
 Gly Gly Lys Ser Lys Leu
 545 550