(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 July 2007 (26.07.2007)

PCT

(10) International Publication Number
WO 2007/082359 A1

(51) International Patent Classification:
C12N 15/12 (2006.01) *C07K 14/435* (2006.01)

(21) International Application Number:
PCT/BR2007/000018

(22) International Filing Date: 17 January 2007 (17.01.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PI0600263-3 18 January 2006 (18.01.2006) BR

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

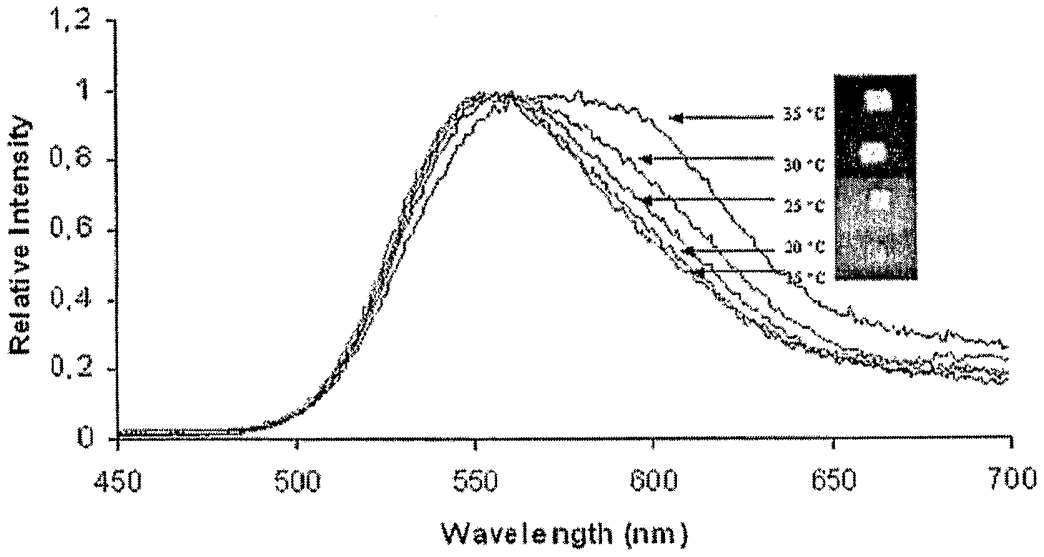
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: LUCIFERASE FROM MACROLAMPIS SP



(57) Abstract: This invention refers to a product from the area of biotechnology made up of recombinant Luciferase from Macrolampis SP with bimodal bioluminescence and high sensitivity to changes in Ph - and temperature, as dual reporter gene that encodes a bioluminous protein for use in simultaneous biosensors of gene expression and intracellular pH variations, with concentrations of phosphate and divalent cations from heavy metals, and presence of ATP and as intracellular biosensor of changes in pH and other homeostatic conditions.

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"LUCIFERASE FROM *MACROLAMPIS* SP"

This invention refers to a product from the area of biotechnology made up of Luciferase from *Macrolampis* SP as dual reporter gene that encodes a bioluminous protein for use in simultaneous biosensors of gene expression and PH intracellular variations, with concentrations of Phosphate and divalent cations of heavy metals, and as reporter gene for studies of gene expression in cells and tissues.

Recombinant Luciferase has a bimodal bioluminescence spectrum and high sensitivity to pH and temperature changes. Due to these pH and temperature sensitivity characteristics, this Luciferase is the object of patent as dual reporter gene for simultaneous analyses of gene expression and ATP presence and as intracellular biosensor of changes in pH and other homeostatic conditions.

Luciferases are the enzymes that catalyze the production of bioluminescence in different organisms. They catalyze the oxidation of molecules generically known as luciferins producing a visible light photon with high efficiency (Viviani, 2002). Luciferases from fireflies and other bugs catalyze the oxidation of a benzothiazolic luciferin activated by ATP.

In a first stage, these luciferases catalyze the activation of luciferin at the costs of ATP, producing luciferin adenylate. In the second stage, the luciferin adenylate is oxidized by molecular oxygen producing oxyluciferin, carbon dioxide and a light photon in the green-yellow range of the spectrum with a quantum efficiency of ca 90%.

Due to the sensitivity of the luciferin-luciferase system of firefly to ATP, this system has been used for lunimometric detection

of ATP in biological samples and enzymatic trials (Campbell, 1988). More recently, with the cloning of the cDNA that encodes the luciferase of fireflies, the gene that encodes the luciferase has been used as one of the most versatile and efficient reporter genes for study of gene expression in bacteria, yeast, plants and mammals (Greer and Szalay, 2002; Contag and Bachmann, 2002). Based on this principle, luciferase genes are used to analyze and quantify the activation of promoters in different cell types, as cell transformation and transfection markers by plasmid and viral vectors, as markers of progression and regression of bacterial, mycotic and viral infections (Contag and Bashmann, 2002) in clinical studies and tests for antimicrobicidal drugs, in cancer progression and regression studies (Yu et al., 2003), as biosensors of toxic agents like pesticides and heavy metals in the environment (Greer and Szalay, 2002), among many other biotechnological and biomedical applications. However, all this varied range of applications has mainly used the luciferase gene from the North-American firefly *Photinus pyralis,* which produces yellowish-green light in physiological conditions and some of its variants produced by genetic engineering.

Although sharing high degree of identity with the luciferase from *Photinus pyralis*, the luciferase from *Macrolampis* is unique in its DNA and amino acid sequence and due to its having differential spectral properties that makes it potentially useful as a reporter gene in biosensors for intracellular physical-chemical changes like changes in pH, phosphate and heavy metal concentration, using the spectral parameter in addition to the intensity parameter.

Although firefly luciferases are already widely used as reporter genes of gene expression using the light intensity

parameter for quantification, none of these luciferases has up till now used the property of spectral sensitivity to pH and other physical-chemical conditions to analyze intracellular homeostatic changes.

The luciferase from *Macrolampis* SP is used for bio-analytical purposes in the detection and quantification of ATP and microbiological contamination of various samples, through luminometric or photographic measurement of the bioluminescence.

Description of the invention

Cloning of cDNA for the luciferase from *Macrolampis*.

The DNA for the luciferase from *Macrolampis* was cloned as described.

The total RNA was extracted from 6 lanterns of the adult *Macrolampis* with Trizol. Next, the RNAm was isolated using dT oligo resin. The RNAm was used to construct the first and second tape of cDNA using Time Saver cDNA synthesis kit from Amersham. The cDNA was then connected to the ZAP lambda vector, generating a cDNA library. This library in the form of phage was amplified and then used to produce a library in the form of plasmid through an in vivo excision process, as described in the manufacturer manual (STRATAGENE).

The library in the form of plasmid was transformed into SOLR bacteria. The bacterial colonies were grown at night at 37C in dishes with LB medium and ampicillin and in the following day replicas were obtained in nitrocellulose membranes and transferred to LB/ampicillin dishes containing IPTG for expression of proteins during 12 hrs at room temperature. The dishes were then sprayed with acid luciferin solution to induce bioluminescence. The brilliant colonies were then selected by visual light detection.

The isolated brilliant colony was pealed and then the plasmid was extracted according to techniques described and subjected to the sequencing of the cDNA for the luciferase from *Macrolampis* sp2., according to techniques described.

CTCCCGGGCTGCCTGAATCGCGGCCGCTGTAACGCGCTGGTA
CTATTGTAAAGATGGAAGACGAAAAAAACATAATACACGGCCC
GGAGCCATTCTATCCTCTAGAGGATGGAACTGCCGGAGAGCAA
TTGCATAAGGCGATGAAAAGATATGCCCTAGTTCCAGGAACGA
TTGCCTTTACGGATGCGCATATCGAGGTAAATATCACGTACGC
CGAATATTTTGAAATGTCCTGTCGATTAGCCGAAGCTATGAANA
GGTATGGGCTTGGTTTAAAACACAGAATCGTTGTCTGCAGTGA
AAACTCTCTTCAATTCTTTATGCCGGTATTAGGCGCGTTATTTAT
TGGAGTTGCACTTGCGCCCGCAAATGACATTTATAACGAACGT
GAATTGCTCAACAGTATGACCATTTCGCAGCCCACCATAGTGTT
CTGCTCCAAAAAGGGACTGCAAAAGATTTTGAACGTACAAAAAA
AATTACCCGTCATCCAAAAAATTGTGATCATGGATTCCAAACCG
GATTACCAAGGGTTCCAGTCCATGTACACATTCATTGAGTCCCA
TTTACCTCAAGGCTTTAATGAATATGACTTCGTACCGGATTCCT
TTGATCGTGATGCAACAATTGCACTTATAATGAACTCCTCTGGA
TCTACTGGGTTACCTAAGGGCGTGGCGCTTCCGCATAAAAATG
CCTGTGTAAGATTCTCGCATGCCAGAGATCCTATTTATGGCAAT
CAAATCATTCCCGATACCGCTATTTTAAGTGTGGTTCCGTTCCA
TCATGGTTTTGGAATGTTCACAACCCTCGGATATCTGATATGCG
GATTTCGTGTTATTTTGATGTACAGATTTGAAGAAGAGTTGTTTT
TACGATGTCTTCAGGATTACAAAATTCAAAGTGCGATATTAGTA
CCCACGCTATTTTCGTTTTTTGCCAAAAGTACTCTGATAGACAA
ATACGACTTGTCTAATTTGCACGACTCCCGGGCTGCCTGAATC
GCGGCCGCTGTAACGCGCTGGTACTATTGTAAAGATGGAAGAC
GAAAAAAACATAATACACGGCCCGGAGCCATTCTATCCTCTAG

AGGATGGAACTGCCGGAGAGCAATTGCATAAGGCGATGAAAA
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TATCGAGGTAAATATCACGTACGCCGAATATTTTGAAATGTCCT
GTCGATTAGCCGAAGCTATGAANAGGTATGGGCTTGGTTTAAA
ACACAGAATCGTTGTCTGCAGTGAAAACTCTCTTCAATTCTTTA
TGCCGGTATTAGGCGCGTTATTTATTGGAGTTGCACTTGCGCC
CGCAAATGACATTTATAACGAACGTGAATTGCTCAACAGTATGA
CCATTTCGCAGCCCACCATAGTGTTCTGCTCCAAAAAGGGACT
GCAAAAGATTTTGAACGTACAAAAAAAATTACCCGTCATCCAAA
AAATTGTGATCATGGATTCCAAACCGGATTACCAAGGGTTCCA
GTCCATGTACACATTCATTGAGTCCCATTTACCTCAAGGCTTTA
ATGAATATGACTTCGTACCGGATTCCTTTGATCGTGATGCAACA
ATTGCACTTATAATGAACTCCTCTGGATCTACTGGGTTACCTAA
GGGCGTGGCGCTTCCGCATAAAAATGCCTGTGTAAGATTCTCG
CATGCCAGAGATCCTATTTATGGCAATCAAATCATTCCCGATAC
CGCTATTTTAAGTGTGGTTCCGTTCCATCATGGTTTTGGAATGT
TCACAACCCTCGGATATCTGATATGCGGATTTCGTGTTATTTTG
ATGTACAGATTTGAAGAAGAGTTGTTTTTACGATGTCTTCAGGA
TTACAAAATTCAAATGCGATATTAGTACCCACGCTATTTTCGTTT
TTTGCCAAAAGTACTCTGATAGACAAATACGACTTGTCTAATTT
GCACGAAATTGCTTCTGGTGGCGCGCCTCTATCAAAAGAAGTT
GGAGAAGCGGTCGCAAAACGCTTTCATCTTCCAGGTATACGAC
AAGGATATGGGCTCACCGAAACTACATCGGCTATACTCATTAC
ACCGAATGGAGATGATAAGCCAGGCGCGGTCGGTAAAGTTGTT
CCATTTTTTTCTGCAAAAGTGGTGGACCTTGACACTGGGAAAAC
GCTTGGCTGTAATCAAAGGGGTGAATTATGTGTCAGAGGGCCT
ATGCTTATGCACAGCTACGTAAACAACCCGGAGGCGACAAGTG
CTTTAATTGACAAGGATGGATGGTTACATTCTGGTGACATATCG
TACTGGGATGAAGACGGCCACTTTTTCATAGTTGATCGTTTGAA

GTCTTTAATTAAATACAAAGGCTATCAGGTACCCCCCGCCGAAT
TGGAATCGATACTGCTACAGCACCCCTGCATATTTGATGCGGG
CGTGGCAGGCATTCCTGACGAAGATGCCGGAGAACTTCCAGC
CGCCGTGGTTGTTTTGGAGCAAGGAAAAACATTGACGGAAAAA
GAAATCATGGATTACGTGGCAGGTATGGTGACAACAGCGAAAC
GGTTGCGCGTATTTGTCGACGAAGTACCTAAAGGTCTAACCGG
AAAACTCGACGCAAGAAAAATCAGGGAGATCCTCGTGAAGGCC
AAGATAGGCGGAAAGTCCAAATTATAAACTCTTGCTGTTTCGAA
CGCTGACGAAATTTTTAGCTATTGTAATATTATATGCAAATTGAT
GAATGGTAGAGGAAGCATTTAGGGAATTCATTATCGATATAGTT
TTCAATTTTTCGGAGGAGTTCATTGCACTGAACATTGGTAATTT
TGTAATTGAGGGTCACTGTGCTGTAATTTTCATTATGAGTGTTC
TAACGAATAATAAAATTCAGGTATAGTTAAAAAGCGGCCGCGAA

The invention has a sequence of amino acids from the luciferase from *Macrolampis* sp2, according to techniques described below.

MEDEKNIIHGPEPFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAH
IEVNITYAEYFEMSCRLAEAMXRYGLGLKHRIVVCSENSLQFFMPV
LGALFIGVALAPANDIYNERELLNSMTISQPTIVFCSKKGLQKILNVK
KLPVIQKIVIMDSKPDYQGFQSMYTFIESHLPQGFNEYDFVPDSFD
RDATIALIMNSSGSTGLPKGVALPHKNACVRFSHARDPIYGNQIIPT
AILSVVPFHHGFGMFTTLGYLICGFRVILMYRFEEELFLRCLQDYKI
QSAILVPTLFSFFAKSTLIDKYDLSNLHEIASGGAPLSKEVGEAVAK
RFHLPGIRQGYGLTETTSAILITPNGDDKPGAVGKVVPFFSAKVVD
LDTGKTLGCNQRGELCVRGPMLMHSYVNNPEATSALIDKDGWLH
SGDISYWDEDGHFFIVDRLKSLIKYKGYQVPPAELESILLQHPCIFD
AGVAGIPDEDAGELPAAVVVLEQGKTLTEKEIMDYVAGMVTTAKR
LRGGVVFVDEVPKGLTGKLDARKIREILVKAKIGGKSKL

Description of drawings

The examples below show the invention in greater details.

Figure 1 shows the effect of pH and phosphate buffer in the *in vitro* bioluminescence spectrum of the luciferase from *Macrolampis*: (a) Tris-HCl 0.1 M pH 8; (b) phosphate 0.1M pH 8; (c) phosphate 0.1 M pH 7 and (d) phosphate 0.1 M pH 6. .

Figure 2 shows the effect of temperature on the *in vitro* bioluminescence spectra. (Right panel) colonies of SOLR *E.coli* bacteria transformed with the pBluescript SK-Macroluc plasmid expressing the luciferase from *Macrolampis* and bioluminescence of heat-sensitive chromaticity when exposed to different temperatures after induction by IPTG and spraying of luciferin (pH 5 citrate buffer).

Finally, figure 3 shows the ratio between the luminescence intensities at 620 nm and 540 nm in function of the pH in different pH-sensitive luciferases.

Comparison of the primary structures of *Macrolampis* and *Photinus pyralis* linked to the studies of site-directed mutagenesis revealed that the N354, H310, E311 and Y227 residues make up an important part of the pH and temperature sensor (Viviani et al., 2005 no prelo). This data can serve as basis for the future engineering of this and other luciferases to produce mutants with different sensitivities to changes in pH, phosphate and concentrations of divalent cations of heavy metals.

Characteristics of the invention.

The luciferase from *Macrolampis*, once expressed under appropriate conditions in bacterial cells, extracted and purified, is catalytically active producing bioluminescence in the presence of luciferin, ATP, magnesium and oxygen. The bioluminescence

spectrum of luciferase is naturally bimodal in pH 8 and has pronounced sensitivity to pH and other conditions (Figure 1). Figure 2 shows the effect of temperature in the bioluminescence spectrum of this luciferase. The maximums of emission of the bioluminescence spectrum of this luciferase and some properties are given in Table 1.

Table 1.

Spectral properties of the luciferase from *Macrolampis* and its mutants.

Luciferase	λmax (nm) [Half-Bandwidth]	
	pH 8	pH 6
Macrolampis	569 [99]	606 [77]
C276S	569 [99]	606 [77]
N354E	558 [83]	606 [77]
H310A	564 [99]	607 [77]
H310R	573-579 [105]	607 [69]
E311A	600	611
Photinus pyralis	555 [74]	608 [62.5]
Cratomorphus distinctus	548 [71]	610 [95]

Method of expression of luciferases and detection of *in vivo* bioluminescence.

The pBluescript plasmid containing the cDNA for the luciferase from *Macrolampis* was used to transform the *E.coli* XI1-Blue cells (Stratagene), which were cultivated in LB medium containing ampicillin at 37°C at night and were then introduced at 25°C for 6 hrs in dishes containing LB/Amp medium complemented

with IPTG (Isopropylthiogalactoside) 1 mM for the luciferase expression.

The live colonies expressing luciferases were then sprayed with luciferin 1 mM in citrate buffer pH 5.0 to induce *in vivo* bioluminescence. The bioluminescence of the bacterial colonies induced can be detected visually, luminometrically, with light detection cameras and in photographic films.

The luciferases can also be expressed in bacteria in liquid cultures for later extraction and purification in the active form (produces bioluminescence in the presence of the substrates MgATP, luciferin in aerobic medium) according to protocols described (Viviani et al., 1999[a],b,c). The bioluminescence spectra were recorded in Spex Fluoromax spectrofluorimeter as described (Viviani et al., 1999a).

Qualitative analysis of intracellular physical-chemical changes in function of the bioluminescence spectra using luciferases from *Macrolampis* and from other fireflies.

To date, luciferases from fireflies have been used solely for luminescence intensity analyses. The quantification of two simultaneous events regulated genetically, for example, has been done through the use of two reporter genes of luciferases that emit in different wavelengths associated with different promoters (Wood; Nakajima et al., 2004).

Notably, although the sensitivity to pH and other factors in the luciferases of fireflies have been known for some time (Seliger, 1964), to date this dynamic property has not been used for analytical purposes.

There is a quantifiable relationship between the ratio of bioluminescence intensities at 620 nm and 540 nm in function of the

pH (Fig. 5) and of other physical-chemical variants like the concentration of certain divalent cations of metals (mercury, zinc, copper). This ratio depends on luciferase (Viviani et al., 2005 no prelo) and the more sensitive the luciferase, the higher it is, as in the case of the luciferase from *Macrolampis* sp2.

Therefore, using highly sensitive light detection equipment with cutoff filters, like the luminometer recently developed by the company ATTO (Tokyo, Japan; http://www.atto.co.jp/englishtop.html) for simultaneous detection of the green and red bioluminescence of the luciferases from *Phrixotrix* in live cells, we intend to introduce the luciferase from *Macrolampis* as the first dual reporter gene for simultaneous analyses of luminescence intensity (as but not limited to the gene expression, ATP quantification) and spectral variation (at 620 and 540 nm) in function of the pH and other intracellular homeostatic changes for bio-analytical purposes.

The patent has innovative characteristics, due to these advantages, it combines the necessary conditions to obtain the privilege.

References:

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Greer, L. F. and A. A. Szalay 2002. Imaging of light emission from the expression of luciferases in living cells and organisms: a review. Luminescence 17: 43-74.

Roda A., P. Pasini, M. Mirasoli, E. Michelini and M. Guardigli 2004. Biotechnological applications of bioluminescence and chemiluminescence. Trends in Biotechnology 22: 295-303.

Seliger, H. H., and McElroy, W. D. (1964) The colors of firefly bioluminescence: enzyme configuration ans species specificity. *Proc. Natl. Acad. Sci. USA* 52, 75-81.

Viviani V. R., Bechara E. J. H. and Ohmiya Y. (1999) Cloning, sequence analysis, and expression of active *Phrixothrix* railroad-worms luciferases: relationship between bioluminescence spectra and primary structures. Biochemistry 38: 8271-8279.

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Viviani, V. R, T.L. Ohelmeyer, F. G. C. Arnoldi, M. R. Brochetto-Braga (2005) A new firefly with bimodal spectrum: identification of a key structural determinant of spectral pH-sensitivity in firefly luciferases. *Photochem. Photobiol.*, (in press).

Yu, Young A. et al. 2003. Optical imaging: bacteria, viruses, and mammalian cells encoding light-emitting proteins reveal the location of primary tumors and methastases in animals. Anal. Bioanal. Chem.

Naylor L. H. (1999) Reporter gene technology: the future looks bright. Biochem. Pharm. 58:749-757.

The sequencing of the cDNA for the Luciferase from *Macrolampis* SP2:

CTCCCGGGCTGCCTGAATCGCGGCCGCTGTAACGCGCTGGTA
CTATTGTAAAGATGGAAGACGAAAAAAACATAATACACGGCCC
GGAGCCATTCTATCCTCTAGAGGATGGAACTGCCGGAGAGCAA
TTGCATAAGGCGATGAAAAGATATGCCCTAGTTCCAGGAACGA
TTGCCTTTACGGATGCGCATATCGAGGTAAATATCACGTACGC
CGAATATTTTGAAATGTCCTGTCGATTAGCCGAAGCTATGAANA
GGTATGGGCTTGGTTTAAAACACAGAATCGTTGTCTGCAGTGA
AAACTCTCTTCAATTCTTTATGCCGGTATTAGGCGCGTTATTTAT
TGGAGTTGCACTTGCGCCCGCAAATGACATTTATAACGAACGT
GAATTGCTCAACAGTATGACCATTTCGCAGCCCACCATAGTGTT
CTGCTCCAAAAAGGGACTGCAAAAGATTTTGAACGTACAAAAAA
AATTACCCGTCATCCAAAAAATTGTGATCATGGATTCCAAACCG
GATTACCAAGGGTTCCAGTCCATGTACACATTCATTGAGTCCCA
TTTACCTCAAGGCTTTAATGAATATGACTTCGTACCGGATTCCT
TTGATCGTGATGCAACAATTGCACTTATAATGAACTCCTCTGGA
TCTACTGGGTTACCTAAGGGCGTGGCGCTTCCGCATAAAAATG
CCTGTGTAAGATTCTCGCATGCCAGAGATCCTATTTATGGCAAT
CAAATCATTCCCGATACCGCTATTTTAAGTGTGGTTCCGTTCCA
TCATGGTTTTGGAATGTTCACAACCCTCGGATATCTGATATGCG
GATTTCGTGTTATTTTGATGTACAGATTTGAAGAAGAGTTGTTTT
TACGATGTCTTCAGGATTACAAAATTCAAAGTGCGATATTAGTA
CCCACGCTATTTTCGTTTTTTGCCAAAAGTACTCTGATAGACAA
ATACGACTTGTCTAATTTGCACGACTCCCGGGCTGCCTGAATC
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GAAAAAAACATAATACACGGCCCGGAGCCATTCTATCCTCTAGA
GGATGGAACTGCCGGAGAGCAATTGCATAAGGCGATGAAAAGA
TATGCCCTAGTTCCAGGAACGATTGCCTTTACGGATGCGCATAT

CGAGGTAAATATCACGTACGCCGAATATTTTGAAATGTCCTGTC
GATTAGCCGAAGCTATGAANAGGTATGGGCTTGGTTTAAAACA
CAGAATCGTTGTCTGCAGTGAAAACTCTCTTCAATTCTTTATGC
CGGTATTAGGCGCGTTATTTATTGGAGTTGCACTTGCGCCCGC
AAATGACATTTATAACGAACGTGAATTGCTCAACAGTATGACCA
TTTCGCAGCCCACCATAGTGTTCTGCTCCAAAAAGGGACTGCA
AAAGATTTTGAACGTACAAAAAAAATTACCCGTCATCCAAAAAAT
TGTGATCATGGATTCCAAACCGGATTACCAAGGGTTCCAGTCC
ATGTACACATTCATTGAGTCCCATTTACCTCAAGGCTTTAATGA
ATATGACTTCGTACCGGATTCCTTTGATCGTGATGCAACAATTG
CACTTATAATGAACTCCTCTGGATCTACTGGGTTACCTAAGGGC
GTGGCGCTTCCGCATAAAAATGCCTGTGTAAGATTCTCGCATG
CCAGAGATCCTATTTATGGCAATCAAATCATTCCCGATACCGCT
ATTTTAAGTGTGGTTCCGTTCCATCATGGTTTTGGAATGTTCAC
AACCCTCGGATATCTGATATGCGGATTTCGTGTTATTTTGATGT
ACAGATTTGAAGAAGAGTTGTTTTTACGATGTCTTCAGGATTAC
AAAATTCAAATGCGATATTAGTACCCACGCTATTTTCGTTTTTTG
CCAAAAGTACTCTGATAGACAAATACGACTTGTCTAATTTGCAC
GAAATTGCTTCTGGTGGCGCGCCTCTATCAAAAGAAGTTGGAG
AAGCGGTCGCAAAACGCTTTCATCTTCCAGGTATACGACAAGG
ATATGGGCTCACCGAAACTACATCGGCTATACTCATTACACCGA
ATGGAGATGATAAGCCAGGCGCGGTCGGTAAAGTTGTTCCATT
TTTTTCTGCAAAAGTGGTGGACCTTGACACTGGGAAAACGCTT
GGCTGTAATCAAAGGGGTGAATTATGTGTCAGAGGGCCTATGC
TTATGCACAGCTACGTAAACAACCCGGAGGCGACAAGTGCTTT
AATTGACAAGGATGGATGGTTACATTCTGGTGACATATCGTACT
GGGATGAAGACGGCCACTTTTTCATAGTTGATCGTTTGAAGTCT
TTAATTAAATACAAAGGCTATCAGGTACCCCCCGCCGAATTGGA
ATCGATACTGCTACAGCACCCCTGCATATTTGATGCGGGCGTG

GCAGGCATTCCTGACGAAGATGCCGGAGAACTTCCAGCCGCC
GTGGTTGTTTTGGAGCAAGGAAAAACATTGACGGAAAAAGAAA
TCATGGATTACGTGGCAGGTATGGTGACAACAGCGAAACGGTT
GCGCGTATTTGTCGACGAAGTACCTAAAGGTCTAACCGGAAAA
CTCGACGCAAGAAAAATCAGGGAGATCCTCGTGAAGGCCAAGA
TAGGCGGAAAGTCCAAATTATAAACTCTTGCTGTTTCGAACGCT
GACGAAATTTTTAGCTATTGTAATATTATATGCAAATTGATGAAT
GGTAGAGGAAGCATTTAGGGAATTCATTATCGATATAGTTTTCA
ATTTTTCGGAGGAGTTCATTGCACTGAACATTGGTAATTTTGTA
ATTGAGGGTCACTGTGCTGTAATTTTCATTATGAGTGTTCTAAC
GAATAATAAAATTCAGGTATAGTTAAAAAGCGGCCGCGAA

Sequence of amino acids from the Luciferase from *Macrolampis* SP2:

MEDEKNIIHGPEPFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAH
IEVNITYAEYFEMSCRLAEAMXRYGLGLKHRIVVCSENSLQFFMPV
LGALFIGVALAPANDIYNERELLNSMTISQPTIVFCSKKGLQKILNVQ
KKLPVIQKIVIMDSKPDYQGFQSMYTFIESHLPQGFNEYDFVPDSF
DRDATIALIMNSSGSTGLPKGVALPHKNACVRFSHARDPIYGNQIIP
DTAILSVVPFHHGFGMFTTLGYLICGFRVILMYRFEEELFLRCLQDY
KIQSAILVPTLFSFFAKSTLIDKYDLSNLHEIASGGAPLSKEVGEAVA
KRFHLPGIRQGYGLTETTSAILITPNGDDKPGAVGKVVPFFSAKVV
DLDTGKTLGCNQRGELCVRGPMLMHSYVNNPEATSALIDKDGWL
HSGDISYWDEDGHFFIVDRLKSLIKYKGYQVPPAELESILLQHPCIF
DAGVAGIPDEDAGELPAAVVVLEQGKTLTEKEIMDYVAGMVTTAK
RLRGGVVFVDEVPKGLTGKLDARKIREILVKAKIGGKSKL

CLAIMS

1. Luciferase from *Macrolampis SP* is characterized by sequence of encoding cDNA from the Luciferase from *Macrolampis* sp2.

2. Luciferase from *Macrolampis SP,* according to claim 1, is characterized by dual reporter gene that encodes a bioluminous protein for use in simultaneous biosensors of gene expression and PH intracellular variations, with concentrations of phosphate and divalent cations of heavy metals.

3. The gene from the luciferase from *Macrolampis* SP, according to claim 1, is characterized as a reporter gene for studies of gene expression in cells and tissues.

4. The Luciferase from *Macrolampis* SP, according to claim 1, is characterized by its use for bio-analytical purposes of detecting and quantifying ATP and microbiological contamination of various samples, by means of luninometric or photographic measurement of bioluminescence.

5. The sequence of amino acids from the luciferase from *Macrolampis sp2* specified and encoded by the cDNA sequence, according to claim 1, is characterized by sequences of amino acids with identity equal to or greater than 95% that contain the replacements Y227 and N354.

6. Luciferase from *Macrolampis* SP, according to claim 1, is characterized by being a catalytically active producer of yellow bioluminescence with bimodal bioluminescence spectrum.

7. Luciferase from *Macrolampis* SP, according to claims 1 and 3, is characterized by a method of expression and *in vivo* detection of the luciferase from *Macrolampis* that enables its expression in its catalytically active form and as producer of

bioluminescence in cells of bacteria, yeast, plants, insects, mammals, but not limited to the latter.

8. The dual reporter gene based on the luciferase from *Macrolampis sp2* and other luciferases from pH-sensitive fireflies is characterized by the simultaneous analysis of the gene expression through photometric/photographic/luminometric quantification of the bioluminescence and of the intracellular / extracellular variations of changes in pH, phosphate and concentration of divalent heavy metals (Hg+2, Zn+2, Cu+2) in function of the ratio of luminescence intensities at 620 and 540 nm. A prototype is the SOLR *E.coli* / pBluescript plasmid SK-Macroluc system.

9. Luciferase from *Macrolampis SP,* according to claim 1, is characterized by a mutant that produces red light consisting of the sequence of amino acids from the luciferase from *Macrolampis* specified and encoded by the cDNA sequence, containing the replacement E311A.

10. Luciferase from *Macrolampis* SP, according to claim 1, is characterized by being unique in its DNA and amino acid sequence and having differential spectral properties that make it potentially useful as a reporter gene in biosensors for intracellular physical-chemical changes like changes in pH and other homeostatic conditions, phosphate and heavy metal concentration, using the spectral parameter in addition to the intensity parameter.

FIGURE 1

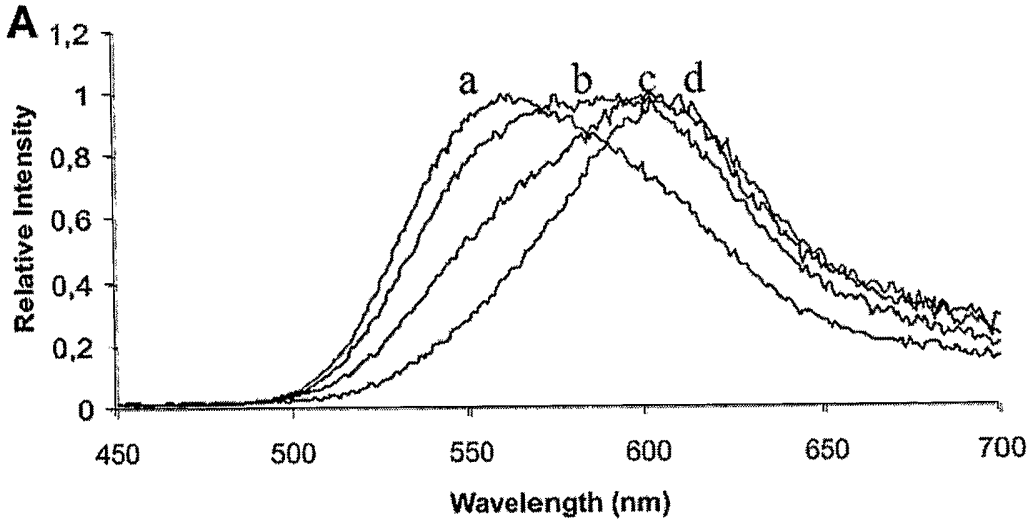

A
1,2
1
0,8
0,6
0,4
0,2
0
Relative Intensity
a
b
c
d
450
500
550
600
650
700
Wavelength (nm)

FIGURE 2

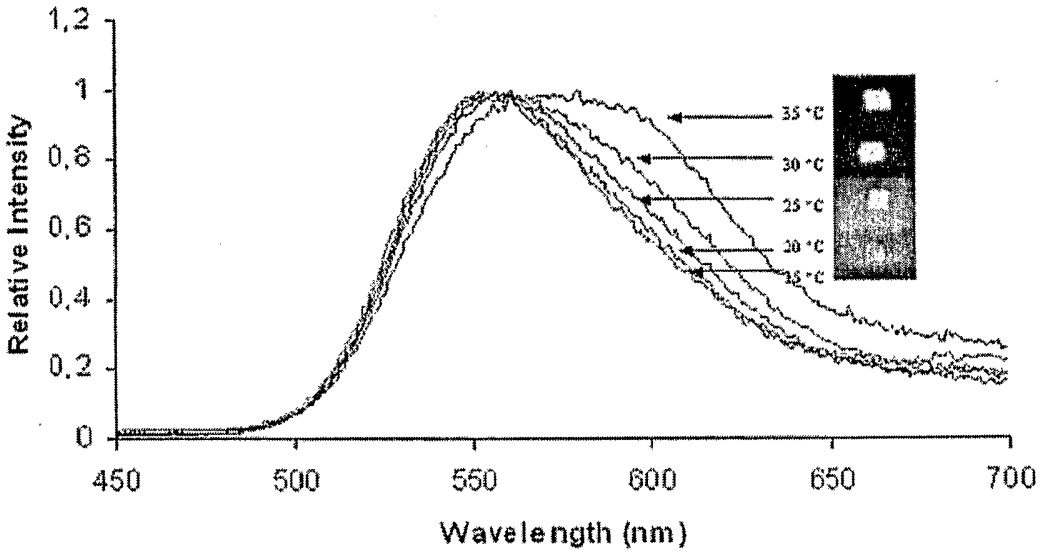
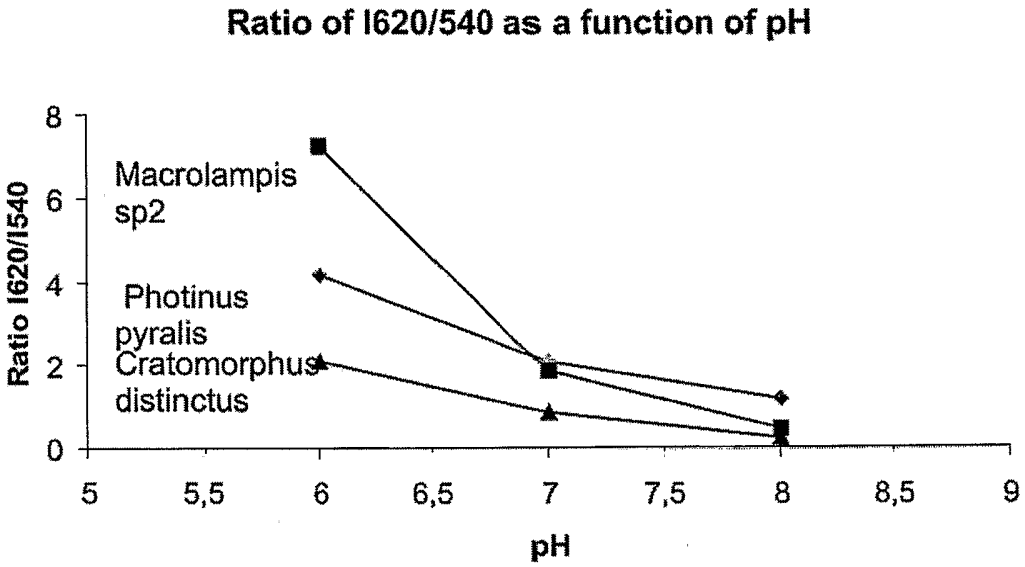

1,2
1
0,8
0,6
0,4
0,2
0
Relative Intensity
450
500
550
600
650
700
Wavelength (nm)
35 °C
30 °C
25 °C
20 °C
15 °C

FIGURE 3


Ratio of I620/540 as a function of pH
Ratio I620/I540
8
6
4
2
0
Macrolampis sp2
Photinus pyralis
Cratomorphus distinctus
5
5,5
6
6,5
7
7,5
8
8,5
9
pH

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 2007/000018

A. CLASSIFICATION OF SUBJECT MATTER
IPC[8]: ***C12N 15/12*** (2006.01); ***C07K 14/435*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[8]: C12N, C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN-registry, Fulltext, WPI, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIVIANI, V.R. et al. A new firefly luciferase with bimodal spectrum: identification of structural determinants of spectral pH-sensitivity in firefly luciferases. Photochemistry and photobiology, 2005, Vol. 81, No. 4, pages 843-848 *the whole document*	1-4, 6-10
Y	*table 2*	5
	--	
Y	VIVIANI, V.R. et al. The influence of the region between residues 220 and 344 and beyond in Phrixotrix railroad worm luciferases green and red bioluminescence. Protein engineering, design & selection, 2004, Vol.17, No. 2, pages 113-117 *abstract*	5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2007 (04.04.2007)	21 May 2007 (21.05.2007)

Name and mailing address of the ISA/ AT	Authorized officer
Austrian Patent Office Dresdner Straße 87, A-1200 Vienna	MOSSER R.
Facsimile No. +43 / 1 / 534 24 / 535	Telephone No. +43 / 1 / 534 24 / 437

International application No.
PCT/BR 2007/000018

Continuation of first sheet

Continuation No. I:

Nucleotide and/or amino acid sequence(s)

(Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material: table(s) related to the sequence listing

b. format of material: a sequence listing

c. time of filing/furnishing: filed together with the international application in computer readable form

furnished subsequently to this Authority for the purposes of search

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/BR 2007/000018

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
A		none	