

Bacterial luciferase reporters

The Swiss army knife of molecular biology

Mark S. Waidmann, Fenja S. Bleichrodt, Tanja Laslo and Christian U. Riedel*

Institute of Microbiology and Biotechnology; Ulm University; Ulm, Germany

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Bioluminescence is a process during which light in the visible spectrum is emitted as a consequence of an enzymatic reaction catalyzed by luciferases. Luciferases have been identified mainly in marine organisms and are used for several biological purposes include camouflage, repulsion, attraction, communication and illumination. Some of the currently known luciferases have become indispensable tools in modern molecular biology and are used for diverse applications such as autoinducer-1 activity assays, promoter test assays in both prokaryotes and eukaryotes, imaging of bacterial infections in live animals, in vivo activity assays genes involved in host response and disease and monitoring of bacterial contaminations of food products. With the present review, the authors intend to give an overview on the currently used bacterial luciferase reporter systems, their methodologies and applications and compare them to other reporter systems.

Introduction

Although sunlight only penetrates the first few meters below the surface of the oceans, the deep sea is illuminated by a variety of marine organisms, crustaceans, cnidarians, cephalopods, echinoderms, annelids, dinoflagellates and bacteria that have evolved the ability to generate light from enzymatic reactions in a process termed bioluminescence.¹ Bioluminescent bacteria are mostly marine Gammaproteobacteria of the genera *Vibrio*, *Aliivibrio* and *Photobacterium*.² In some cases, these luminescent microorganisms are part of a symbiotic relationship with higher organisms such as the angler fish where they are contained in specialized organs for light production. The requirements for the generation of light are diverse and include attraction of prey and mating partners, repulsion of predators, communication with members of the same species, illumination of the environment and camouflage by counter illumination.¹

To date, more than 700 species are known to possess bioluminescence systems of which over 80% have been isolated from marine habitats.¹ However, while the majority of luciferases have been identified in marine organisms, two of the three most frequently used bioluminescent reporter systems are derived from

organisms that live in terrestrial environments, i.e., the luciferase of a North American firefly (*Photinus pyralis*) and the *lux* operon of *Photobacterium luminescens*, an enteric pathogen of nematodes belonging to the Enterobacteriaceae. The third most frequent used luciferase stems from the sea pansy (*Renilla reniformis*). More recently, a secreted luciferase from the marine copepod *Gaussia princeps* has been characterized and is beginning to be used as a tool in molecular biology.³ However, due to the limited data available for this system it will not be discussed in detail in the current review.

The best studied bacterial systems are the *lux* systems of *Photobacterium phosphoreum*, *P. leiognathi*, *Vibrio harveyi*, *V. fischeri* and *P. luminescens*. The genes responsible for bioluminescence in these organisms have been identified and cloned some 20–30 years ago.^{4–6} All bacterial luciferase systems are basically encoded by the *luxCDABE* operon (Fig. 1). The *luxAB* genes encode the α - and β -subunits of the heterodimeric luciferase, while the flanking *luxCD* and *luxE* genes encode a fatty acid reductase complex required for regeneration of the long-chain fatty aldehyde, which serves as luciferin.⁷ An additional *luxF* gene is located between *luxB* and *luxE* in most *Photobacterium* species but is absent in *Vibrio* sp. and *P. luminescens*.^{8,9} This gene encodes a flavoprotein homologous to the luciferase subunits with no apparent role in luminescence.^{8,9} Following *luxE*, all marine bioluminescent species possess *luxG* encoding a flavin reductase, possibly providing reduced flavin mononucleotide (FMN₂) for the luciferase reaction.^{9–11} In *V. harveyi*, with *luxH* another gene was identified as part of the *lux* operon. Its gene product LuxH might be involved in riboflavin biosynthesis, thus providing FMN.^{10,12,13}

Under normal circumstances, single bacterial cells never show bioluminescence. However, when grown in batch culture, light emission is induced in mid-log phase reaching a peak in stationary phase.^{4,6,14} In fact, for *Vibrio* sp. it has been shown that bioluminescence is regulated by a cell density-dependent mechanism termed quorum sensing (QS) in which the genes of the *lux* system are synchronically expressed after activation of an autoregulatory circuit.¹⁵

The LuxI/LuxR type of QS in Gram-negative bacteria is one of the best studied bacterial QS mechanisms.¹⁵ In *V. fischeri*, *luxI* encodes an N-acyl homoserine lactone (AHL-) synthase¹⁶ responsible for production of the autoinducing signalling molecule *N*-(3-oxohexanoyl)-homoserine lactone also termed *Vibrio* autoinducer 1 (VAI-1).¹⁷ In *V. fischeri*, *luxI* is located immediately upstream of *luxC* as part of the *lux* operon and is constitutively

*Correspondence to: Christian U. Riedel; Email: christian.riedel@uni-ulm.de
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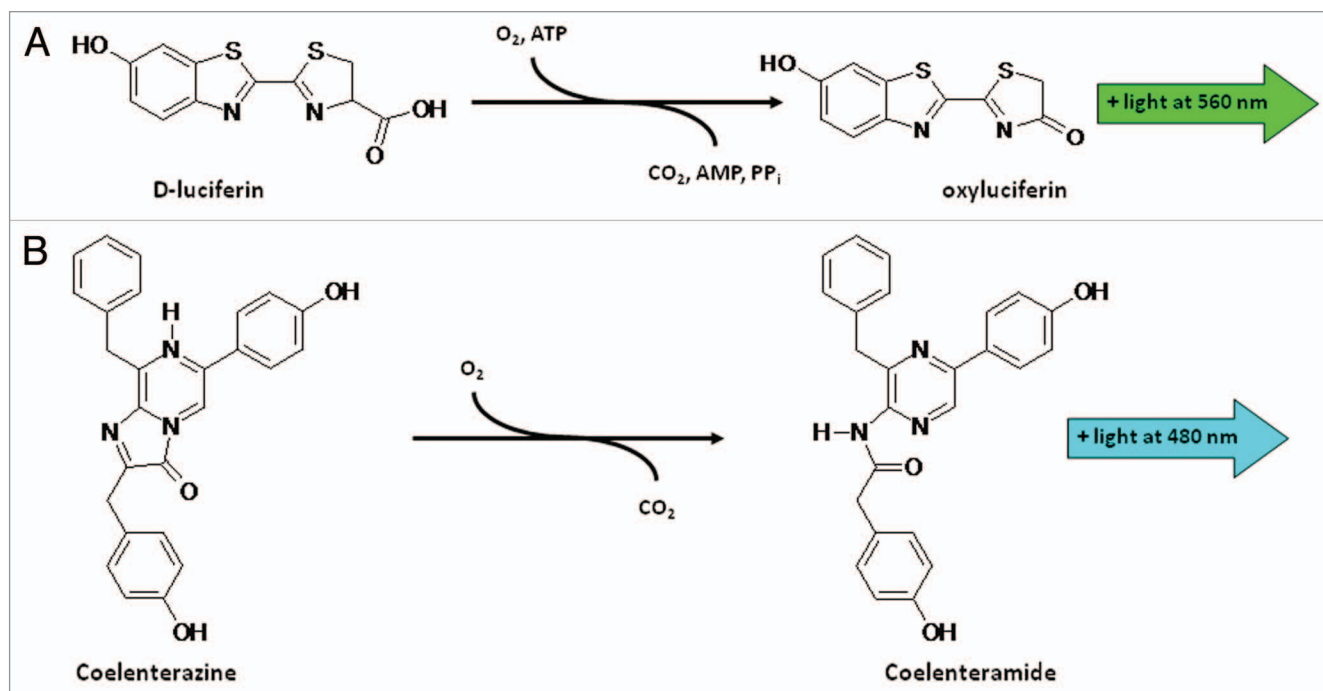


Figure 2. Light emitting reaction of the *P. pyralis* Luc (A) and *R. reniformis* Ruc (B) luciferases. Both luciferases require molecular oxygen but different substrates and co-factors. While Ruc requires Ca²⁺, Luc is dependent on Mg²⁺ and hydrolyzes ATP to AMP and PP_i.

serves as an attracting mechanism for mating partners. So far, the regulation of bioluminescence in the *P. pyralis* lanterns is not completely understood. The firefly luciferase is located in peroxisomes²⁸ of specialized cells of the lanterns called photocytes. It is assumed, that *P. pyralis* switches light emission on and off by regulating oxygen availability through a system of tracheae and tracheolae.²⁹ The Luc enzyme was first purified and characterized in 1978.³⁰ The *P. pyralis luc* gene was found to be a single-copy gene composed of seven exons and separated by six short introns.³¹ It encodes a 62 kDa protein which catalyzes the two-step enzymatic oxidation of D-luciferin to oxyluciferin and the simultaneous emission of light of a wavelength of 550–570 nm (Fig. 2A). D-luciferin is first coupled to the enzyme and activated by adenylation to luciferyl-adenylate and then oxidized to oxyluciferin. The catalyzed reaction requires molecular oxygen, ATP and Mg²⁺ as a co-factor and is highly efficient.²⁴ Competing with D-luciferin, oxyluciferin acts as a strong inhibitor on firefly luciferase.³² However, a luciferin-regenerating enzyme (LRE) was found in protein extracts of the firefly lanterns acting on oxyluciferin to regenerate D-luciferin in the presence of D-cysteine.³³

The *R. reniformis* luciferase (Ruc) is a 36 kDa protein and the corresponding *ruc* cDNA initially was isolated and expressed in *Escherichia coli*.³⁴ In *R. reniformis*, bioluminescence is triggered by mechanical stimulation.³⁵ It has been hypothesized that bioluminescence is directly coupled to a neuronal impulse.³⁶ Analysis of the light emitting process has revealed that this stimulus induces the uptake of Ca²⁺ to membrane-bound lumisomes in the photocytes.^{37,38} Further studies of the bioluminescent system of *Renilla* showed that Ca²⁺ triggers a luciferin binding protein to release the luciferin coelenterazine which then binds to Ruc.³⁶ Ruc then

catalyzes the oxidative decarboxylation of coelenterazine in the presence of molecular oxygen, forming the oxyluciferin coelenteramide and the emission of light with an emission maximum of 480 nm³⁹ (Fig. 2B). In vivo, Ruc is closely associated with the green fluorescent protein of *Renilla* which absorbs the energy of the luciferase reaction in a process called fluorescence resonance energy transfer and in turn emits green light with a maximum at 510 nm. The fact that Ruc, *Renilla*-GFP and the luciferin-binding protein all are located in the lumisomes suggests that bioluminescence takes place in these vesicles.³⁷

Applications of Luminescent Reporters

Starting with their initial cloning, the bacterial luciferase systems^{4,14,26} developed into various versatile tools for molecular biology. Applications of these luciferases include reporter gene assays both in bacteria and eukaryotic cell lines, promoter identification in bacteria by insertional mutagenesis, detection of bacteria in environmental samples or food matrices, in vivo imaging of bacterial and viral infections, analysis of gene expression in life animals and tumor imaging (reviewed in refs. 25, 40 and 41). For the remainder of this review we will focus on the applications and methodologies of bacterial luciferases and only give a very brief overview of eukaryotic reporter systems.

One of the major advantages of luminescent reporters over the more classical green fluorescent protein (GFP) is the dramatically shorter half-life of the luciferases. When expressed in mammalian cells, the half-life of GFP is ~24 h. By introducing a PEST sequence to the C-terminus of GFP thereby targeting the protein for degradation in the proteasome of mammalian cells,

the half-life could be reduced to about 5.5 h.⁴² For comparison, the half-life of *P. luminescens* luciferase is only ~240 min. Also, variants of the *P. luminescens* luciferase with a half-life of 46 min have been generated by introducing destabilization signals at the C-terminus of the proteins albeit at the expense of a reduced efficiency in the light emitting reaction.⁴³ Additionally, and even more importantly, GFP and other fluorescent reporters have to undergo posttranslational maturation, a process that, depending on the reporter can take several hours.⁴⁴⁻⁴⁶ Also, folding and maturation is even less efficient at 37°C for most of these fluorescent proteins.⁴⁴⁻⁴⁶ Lastly, fluorescent reporters are subject to photo-bleaching by the excitation.⁴⁴⁻⁴⁶ The dramatically shorter turnover of bacterial luciferases allows for the repeated online measurement not only of the onset of gene expression but also of decreased expression at a high temporal resolution.

Another advantage of luciferases compared to GFP is that the wavelength of light emitted is longer and thus the energy is higher. This allows for a better tissue penetration when the luciferase is expressed in living animals. Also, fluorescent reporters have to be excited prior to measurement resulting in a high background of autofluorescence in any biological sample. This problem is ruled out by the use of luciferase reporters.

Finally, luciferase reporter systems can be measured online during the experiment. By using entire bacterial *luxABCDE* operons, measurements can be performed even without adding an exogenous substrate. Thus, provided that the required instrumentation is available, luciferase reporters are faster and less laborious and expensive compared to more traditional promoter test assays such as chloramphenicol acetyltransferase, β -galactosidase, glucuronidase or secreted alkaline phosphatase, where samples have to be processed and enzyme tests have to be performed.

Since *P. luminescens* is a Gram-negative organism of the Enterobacteriaceae and its wild type *lux* operon has an A + T-content of about 70%, expression can be poor in Gram-positive bacteria or high G + C organisms. To overcome these problems, translational signals optimized for Gram-positive bacteria have been introduced in front of *luxA*, *luxC* and *luxE* resulting in enhanced expression in *Staphylococcus aureus*.⁴⁷ The same construct has been successfully used for bioluminescent labelling of *Listeria monocytogenes*,^{48,49} *Streptococcus pneumoniae*,⁵⁰ *Bifidobacterium breve*^{51,52} and *Mycobacterium tuberculosis* and *M. smegmatis*.⁵³ A similar approach, however introducing Gram-positive ribosome binding sites in front of all genes of the *luxABCDE* operon has been applied by others.⁵⁴ To address the issue of G + C content, a synthetic *lux* operon for use in high G + C bacteria such as *Streptomyces coelicolor* has been developed.⁵⁵

A major shortcoming of all luciferase systems is their requirement for oxygen in the light emitting reaction. This property strictly rules out the use of luciferases for online measurements under anaerobic conditions. Nevertheless, it has been shown that the *V. fischeri* luciferase is expressed at high levels in *Clostridium perfringens* under strictly anaerobic conditions and can be used to quantify gene expression in a discontinuous system where samples have to be withdrawn and exposed to oxygen to measure luminescence.^{56,57}

Autoinducer reporter assays. Soon after the discovery of the AI-1 and AI-2 QS systems of *V. harveyi*^{58,59} it became apparent that a significant number of bacteria employ similar or even identical systems for communication. This has fuelled the development of sensor strains of *V. harveyi* for both AI-1 and AI-2 molecules and has led to the identification of cross-species communication between different Vibrionaceae.⁶⁰ Since then these sensor strains for *V. harveyi* have been used in a large number of studies to identify AI-1 and/or AI-2 activity in the culture supernatants of a wide range of bacteria.

Bacterial gene expression. All three major bacterial luciferases have been used as reporter to measure activities of bacterial promoters. For example, the *V. fischeri luxAB* genes have been applied to monitor *plcA* and *hly* promoter activities in different *L. monocytogenes* strains during growth in vitro.⁶¹ A promoter fusion to an optimized *P. luminescens lux* operon was used to measure expression of a peptide sensing system of *S. aureus* in response to the signalling peptide in vitro and during phagocytosis and intracellular growth in macrophages.⁴⁷ The same system was used to quantify activities of various promoters of *L. monocytogenes* during growth in batch culture, infection of epithelial cells in vitro and in organs of infected mice ex vivo.^{48,49,62-64} (Fig. 3). Other studies have used the *V. harveyi* luciferase as a reporter for circadian promoter activity in cyanobacteria.^{65,66} Similar studies have been conducted with truncated or complete *lux* operons of *Vibrio* sp. or *P. luminescens* in *Escherichia coli*,⁶⁷⁻⁷⁰ *S. aureus*,^{71,72} *Yersinia pseudotuberculosis*,⁷³ *Acinetobacter baylyi*,⁷⁴ *Bacillus anthracis*,⁷⁵ *Haemophilus influenzae*⁷⁶ and the phytopathogen *Agrobacterium tumefaciens*.⁷⁷

A more specific application for luciferase reporters in the context of promoter activity in bacteria is their use as traps to identify DNA fragments with promoter activity in clone libraries. This has been successfully demonstrated for *Synechococcus* sp. and *Escherichia coli*.^{78,79}

Imaging of contaminations in environmental and food samples. Monitoring of environmental or food samples for bacterial contaminations is possible by using phages specific for the micro-organism in question. One possibility is to generate target-specific phages harbouring all genes required for bioluminescence. If the target bacterium is present in a sample the phage will infect it and, as a consequence, the target will transcribe the phage-encoded genes leading to luminescence. This approach has been used for enteric bacteria such as *Escherichia coli*, *Enterobacter* sp., *Citrobacter* sp. and *Klebsiella* sp. with a threshold of 10⁴ cfu/g of sample. By introducing an enrichment step the limit of detection can be lowered to as little as 10 cfu/g.⁸⁰ The same approach with similar low limits of detection has been applied for the detection of *L. monocytogenes* in various food matrices and environmental samples.⁸¹ Other authors have used a more sophisticated approach by generating bacteriophages harbouring the *luxI* gene. Upon infection the target bacterium will start to produce an AHL signalling molecule which then in turn induces luminescence by an additional sensor bacterium equipped with a *lux* operon and the system for detection of the AHL signal.⁸²⁻⁸⁴ A similar application is the efficacy testing of measures to prevent contaminations or growth of pathogens in food products.

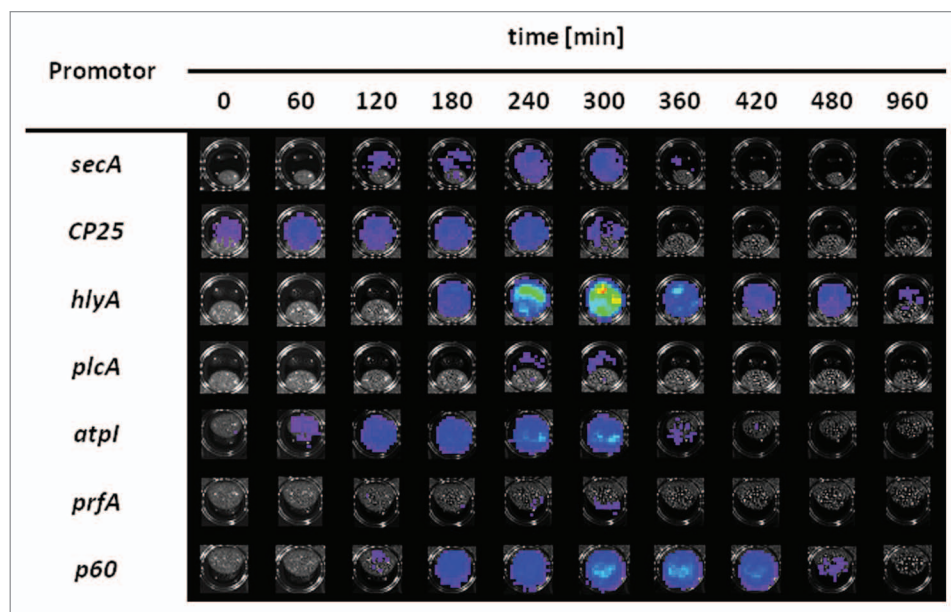


Figure 3. Lux reporter activities of seven different promoters expressed in single copy from the *L. monocytogenes* EGDe chromosome. Bacteria were grown in 96 well plates in LB medium pH 7.4 at 37°C. All strains harboring the different promoter-*lux* fusions had identical growth kinetics and did not differ from the wild type strain. Imaging was performed in an IVIS 100 imaging system. Representative wells of quadruplicate measurements on the same plate are shown and all images are on identical false color scales for bioluminescence intensity to allow for a comparison between promoters and time points (Riedel CU and Monk IR; unpublished data).

For example, a luminescent *L. monocytogenes* strain has been used to assess the efficacy of a recombinant *L. lactis* strain expressing enterocin A against *Listeria* contaminations in cottage cheese.⁸⁵

Interestingly, the *P. luminescens lux* operon was also used to monitor estrogenic compounds in the environment using a *Saccharomyces cerevisiae* reporter strain co-expressing Frp, the flavin oxidoreductase of *V. harveyi*, which provides the FMNH₂ required for the light emitting reaction.⁸⁶ These results demonstrate that in principal bacterial luciferases can also be expressed in eukaryotic hosts.

In vivo monitoring of bacteria-host interactions. One of the most important applications of bioluminescent reporter systems is their use to investigate interactions of bacteria with their hosts. Especially when infections of bacterial pathogens in for example mice are studied, bioluminescence imaging (BLI) has the striking advantage that live animals can be imaged several times over the course of an experiment. This allows for a dramatic reduction in the number of animals required for a given experiment. *Salmonella typhimurium* was the first bacterial pathogen for which in vivo BLI was reported. Infections of different strains labeled with the wild type *P. luminescens luxCDABE* operon by transposon insertion were monitored in live animals.⁸⁷ Similarly, a *luxABCDE* operon optimized for expression in Gram-positives was used to show efficacy of amoxicillin treatment against infections with *S. aureus*, *S. pneumoniae* and *Pseudomonas aeruginosa* in mice by in vivo BLI.^{54,88,89} Also, interactions of symbiotic bacteria with the rhizosphere of different plants have been monitored by BLI.^{90,91}

Hardy and colleagues have used in vivo BLI to monitor *L. monocytogenes* infections in mice.⁹² However, the transposon

system used for tagging in all these studies randomly integrates into the chromosome. This has considerable consequences: (1) first a significant number of transposon mutants have to be screened and (2) the insertion of the transposon could have an impact on the virulence of the selected strain. For example, in *L. monocytogenes* the strain selected for in vivo BLI was significantly attenuated as the LD₅₀ was 4-fold higher than the parental strain.⁹² By fusing P_{help}, a constitutive, synthetic promoter to the *lux* operon in pPL2*lux*,⁴⁸ an integrative plasmid with known integration site, our group was able to circumvent these problems and monitor *L. monocytogenes* during infection by in vivo BLI without interfering with the virulence properties of the strain.^{49,63} The same strain was used in a subsequent study to determine the mode of action of an anti-listerial probiotic *Lactobacillus* strain.⁹³ Moreover, we were able to use the P_{help} *lux* construct to tag various Gram-negative bacteria using a plasmid that integrates into a 16S ribosomal RNA gene.⁹⁴ This has allowed for the in vivo monitoring infections of *Citrobacter rodentium*, *P. aeruginosa* and *S. typhimurium*.^{94,95} It is worth mentioning, that the use of a defined integration site has several advantages over transposon-based systems besides avoiding problems with the virulence traits of a bacterium. By using a defined promoter and integration at a defined site a direct comparison of different strains is possible as shown for three different strains of *L. monocytogenes* (10403S, EGDe, F2365)⁴⁶ or the a Δ *agrD* mutant and its isogenic wild type parent strain.⁶³ Also, this system allows for the direct comparison of different promoters in the same strain^{49,63} (Fig. 3).

More recently, in vivo BLI has been applied to monitor infections with *M. smegmatis* and *M. tuberculosis* as well as *B. anthracis*

which would be required for stable plasmid replication, can be omitted thereby avoiding any side effects of antibiotics on gene regulation. Moreover, a defined integration site avoids extensive screening for suitable mutants and confirmation that the site of transposon integration does not interfere with the properties one wants to assay. In the rare case that the amount of luminescence is critical, multicopy plasmids can be a way to increase light emission. However, it is noteworthy that expression of the luciferase and accessory proteins and the process of light emission are a significant burden to the bacterial metabolism. As a consequence high level expression of luciferase systems might result in impaired growth characteristics.

Bacterial luciferase reporters have become valuable tools in modern molecular biology. Due to their short half-life and their independence of exogenous substrates or excitation they have considerable advantages over the classical reporter systems such as β -galactosidase, β -glucuronidase, chloramphenicol acetyl transferase or GFP. Moreover, they combine the ability to measure promoter activities with the possibilities to localize and quantify bacteria in batch cultures, cultured eukaryotic cells, ex vivo tissues or living animals in a method that can be rapidly and easily measured without extensive sample manipulation.

Although the luciferase has the advantage of being measurable without prior sample processing or excitation and, in case of entire bacterial *lux* operons, even without additional exogenous substrates it requires the appropriate instrumentation.

Luminescence can be measured in vitro by luminometers or multimode readers in almost any format from test tube to 384-well plates. Imaging in living animals requires a more sophisticated instrumentation. These instruments include an extremely light-proof dark chamber and charge-couple device (CCD) cameras with intensified photocathode detectors. The sensitivity of these CCD cameras is further increased by cooling to temperatures as low as -105°C thereby lowering the signal to noise ratio without altering the spectral sensitivity.¹⁰⁹ Luminometers, multi-mode plate readers and in vivo imaging systems are available from a number of commercial suppliers (Berthold Technologies, Caliper Life Sciences, Carestream Molecular Imaging) some of which also provide luminescent bacterial strains, cell lines and transgenic animal models.

In summary, bacterial luciferase reporters cover a wide range of applications allowing for the real-time measurement of biological processes including promoter assays, monitoring of bacteria in complex matrixes and in vivo imaging of infection and tumor targeting without the drawbacks of other reporter systems.

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