EXPERIMENTAL BIOLOGY 2008
TODAY'S RESEARCH: TOMORROW'S HEALTH

1028.1

Mammalian Luciferase Detection Vectors and Applications

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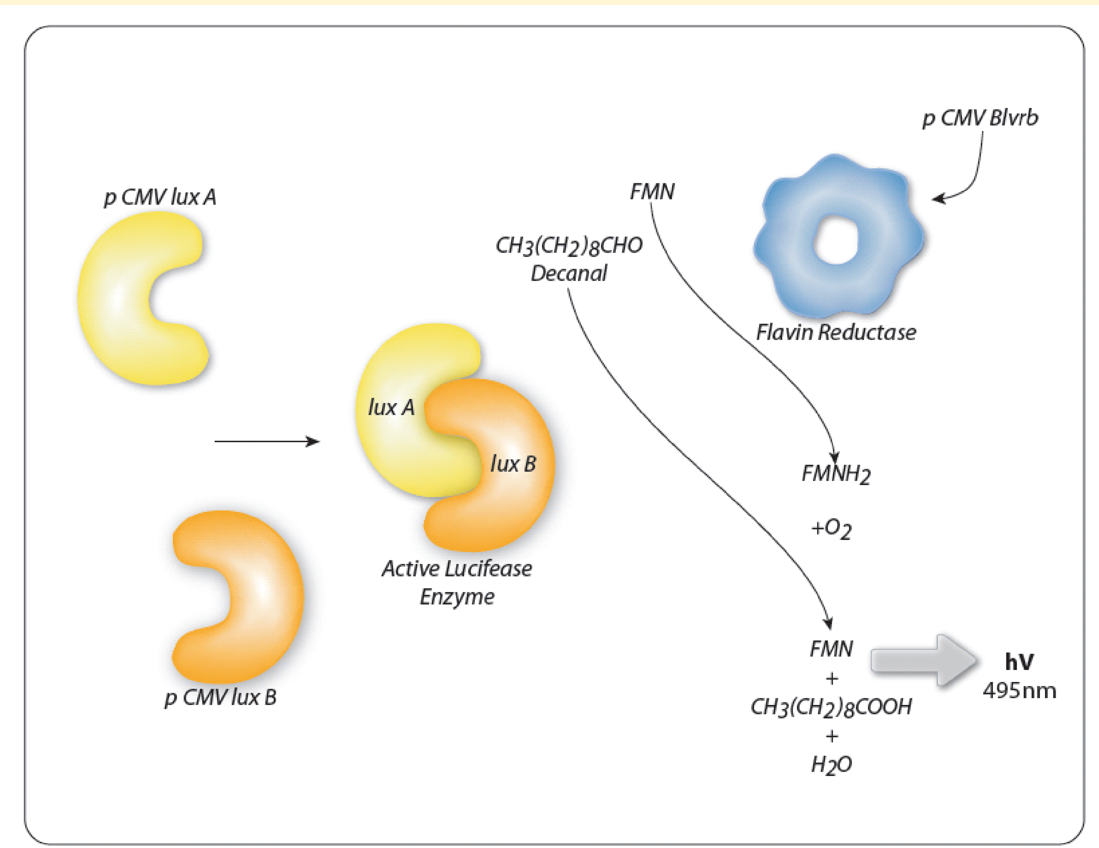
Abstract

The light-emitting reaction of the marine bioluminescent bacterium *Vibrio harveyi* is catalyzed by the bacterial luciferase enzyme which exists as an alpha-beta heterodimer encoded by the *luxA* and *luxB* genes with subunit molecular weights of 42K and 37K respectively. The enzyme catalyzes a reaction with FMNH2, oxygen and a long-chain aldehyde as substrates to yield visible light at 490 nm. A new luciferase marker gene detection system has been developed based upon this bacterial luciferase isolated from *Vibrio harveyi*. Sequences encoding the two luciferase subunits, luxA and luxB have been cloned into two separate vectors. These vectors also include a CMV promoter for expression in mammalian cells as well as an ampicillin resistance gene (amp) for selection and amplification, the SV40 polyadenylation sequence and the SD/SA-RNA splice donor and acceptor sequence for maximum expression. The vectors were tested for light emission parameters in a live mammalian cell format and with cell lysate samples from NIH3T3 murine fibroblasts in conjunction with transfection using vector GC:11726 *Mus musculus* biliverdin reductase B (flavin reductase (NADPH)) sequence for production of the reduced flavin mononucleotide ($FMNH_2$) cofactor intracellularly. These systems are being developed to monitor regulation of expression for two independent vector constructs, upon the dual expression.

Schematic Representation of the luxA:luxB Light Producing System.

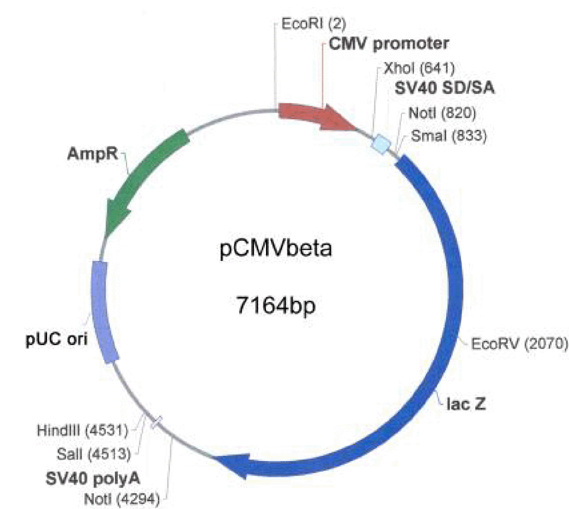


Cloning Methods

- The *lacZ* gene was excised from *pCMVbeta* using *NotI* endonuclease .
- *LuxA* was amplified directly from vector pQF110 from ATCC (contains *LuxAB* and *phoA)* using specific primers engineered to contain the NotI restriction site.
- The *LuxA* gene was subcloned into the *pCMVbeta* vector base using restriction *NotI* sites.
- Sequencing using forward and reverse sequencing primers showed correct directional insertion.
- *LuxB* was also amplified directly from vector *pQF110* using a set of specific primers engineered to contain the *NotI* restriction site.
- PCR amplification of the *LuxB* gene was performed using primers engineered to contain the restriction sites for *NotI*.
- The *LuxB* gene was subcloned into vector *pCMVbeta* using *NotI* restriction sites at both ends.
- The *luxB* insert repeatedly ligated in backwards orientation into the *pCMVbeta* vector base.
- Shrimp alkaline phosphatase digestion and extensive screening finally gave the correct insert orientation.
- Cells were transformed with the three vectors *pCMVLuxA, pCMVluxB and pCMVBlvrb* (flavin reductase).

Cloning Strategies for pCMVluxA and pCMVluxB Vector Construction.

NotI excision of lacZ



M0951: pCMVß Mammalian lacZ Expression Vector

Primers Analysis

Lux A 5'
ATATAAGAATGCGGCCGCCACCATGAAATTTGGAAACTTCCT
Junk RE site Kozak Coding Region

Lux A 3'
GTGTCGGGGTGCGGCCGCTTACTGTTTTTCTTTGAGATATGG
Junk RE site Coding Region

Lux B 5'
GTGTCGAAGTGCGGCCGCCACCATGAAATTTGGATTATTCTT
Junk RE site Kozak Coding Region

Lux B 3'
GTATCGAATTGCGGCCGCTTACGAGTGGTATTTGACGATGTT
Junk RE site Coding Region

Results — *Vector Analysis*

Sequencing Primers

bps 750 -769 in front of the NotI RE site at bp 820
5' ATGTTGCCTTTACTTCTAGG

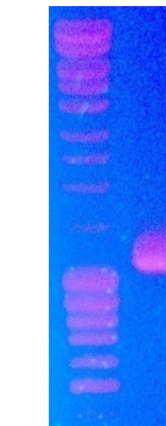
bps 4350 – 4369 after the NotI RE site at bp 4294
3' ATTTTTTTCACTGCATTCTA

NotI RE Digest of M0951 and pQF110/Lux A PCR

Ligation/Transformation with JM109

PCR Screen using sequencing primers

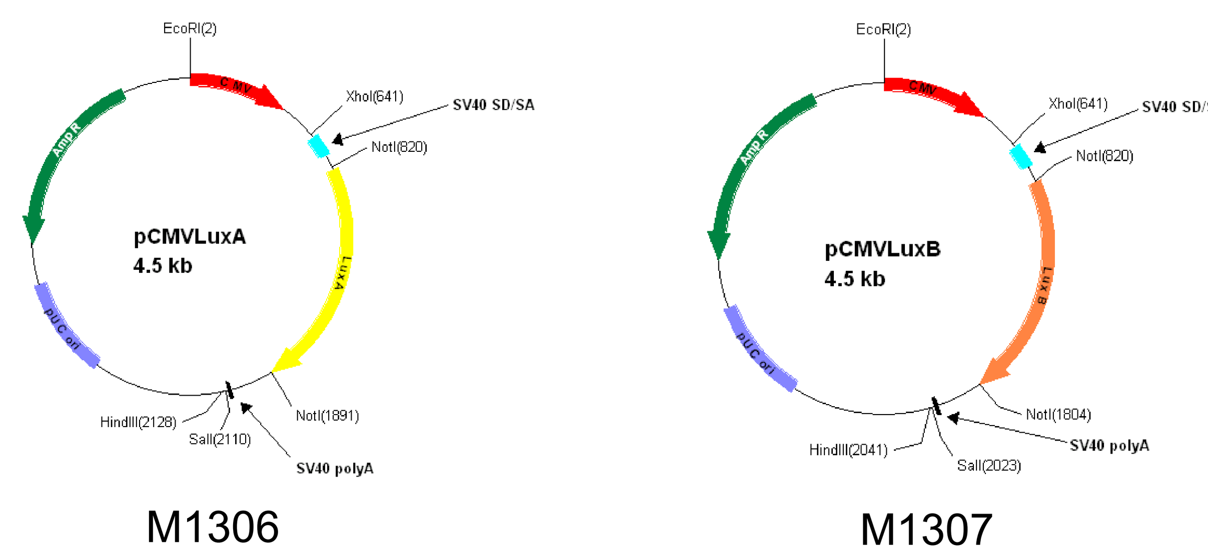
Directionality Screen
Forward primer of gene
Reverse primer of vector

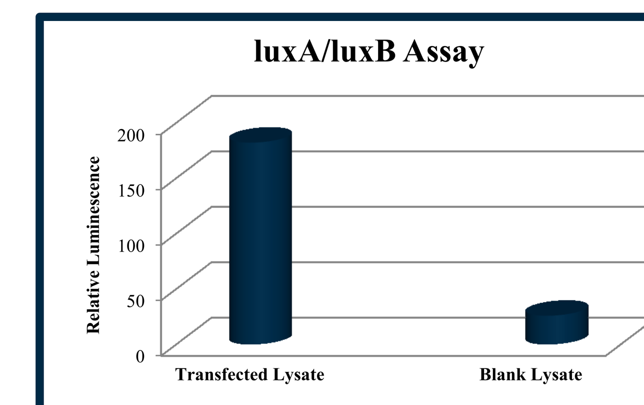


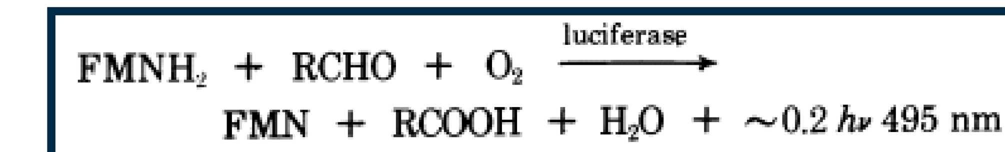
Luminescence values

Sample	RLU
Lysase 1	231
Lysase 2	133
No enzyme	85.5
Blank	59.8

pCMVluxA and pCMVluxB vectors







Transfection Methods and Analysis

$FMNH_2$ not available (unstable).

Purchased vector pCMVBlvrb from ATCC containing mouse flavin reductase

Transfect cells w/ LuxA, LuxB, and reductase vectors simultaneously.

Addition of FMN + transfection with reductase should produce $FMNH_2$.

Lysed cells w/ freeze-thaw method (3 cycles).

Added cell lysate to reaction mixture containing decanal, NADPH, DTT, and FMN.

Luminescence produced!

Discussion and Conclusions

In an effort to produce a mammalian vector system for use with the bacterial luciferase enzyme, we have developed a set vectors, *pCMVluxA* and *pCMVluxB* for use in measuring activity of secondary cloned genes or fragments. With this system, the expression of two genes can be monitored simultaneously in a particular cell line. Luminescence will occur only if both genes are cloned into the cell.

The luminescence-based activity assays described here (a) use a simple cloning method and (b) can be done with cell and tissue extracts from various mammalian species.

This new luciferase gene system, also utilizes a third vector (pCMVBlvrb) to generate flavin reductase intracellularly, which produces the unstable reduced dihydroflavinmononucleotide ($FMNH_2$) required as a cofactor for enzyme activity.

Triple transfection of NIH3T3 mouse fibroblast cell lines with all three vectors, utilizing several lipofection transfection reagents, produced a luminescent signal.

Addition of partially purified flavin reductase to the enzyme assays also produced luminescence using the *pCMVluxA*/*pCMVluxB* system.

Lysis of transfected cells using a freeze-thaw method was important to retain activity of the enzyme. The use of detergents or other lysis buffer systems caused denaturation and loss of activity in parallel assays.

The active enzyme requires proper intracellular folding for activity. The presence of *luxA* has been reported to be important for proper folding and activity of the *luxB* subunit. Hence, the same assays can be used in ultra-sensitive detection of complementation protein binidng studies intracellularly. These systems will be available commercially from Marker Gene.