Yeast
Yeast 2003; **20**: 1109–1113.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/yea.1024

Yeast Functional Analysis Report

One-step measurement of firefly luciferase activity in yeast

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Received: 19 December 2002
Accepted: 10 June 2003

Abstract

Firefly luciferase is often used as a sensitive genetic reporter in various cell types. The pitfall in yeast, however, has been the need to break down the rigid cells in order to measure the enzyme activity. In this study we have removed the peroxisomal targeting codons from the *Photinus pyralis* luciferase gene (*luc*) and shown that in the yeast *Saccharomyces cerevisiae* this modified luciferase gives high levels of light emission that is easy to measure from intact living cells. Furthermore, cells with the modified luciferase grew essentially faster than those with the wild-type luciferase, indicating that peroxisomal targeting of a foreign enzyme puts some constraints to cellular viability. As a model system we used two different reporter constructs. In the first, expression of the luciferase gene is under control of *CUP1*-promoter, a well known yeast promoter that is inducible by copper ions. In the second, luciferase activity is dependent on activation of the human oestrogen receptor and its interaction with oestrogen-responsive elements incorporated in a yeast promoter. The luciferase activity measurement could be done on a 96-well plate by simple addition of the substrate, D-luciferin, at a moderately acidic pH of 5.0. The ease of use of the non-peroxisomal luciferase makes it an interesting alternative for reporter genes that are conventionally used in yeast, such as *lacZ*. **Copyright © 2003 John Wiley & Sons, Ltd.**

Keywords: ***Photinus pyralis*; *luc* gene; copper-inducible; peroxisome-targeting; luciferase expression; *Saccharomyces cerevisiae***

Introduction

The ease of cultivation and genetic manipulation of the yeast *Saccharomyces cerevisiae* are among the properties that have made it a suitable organism for a wide range of biological studies. As a simple eukaryotic organism it has often been used for analysing eukaryotic gene functions, with the help of different reporter genes. Although luciferases have gained popularity in assays using bacterial or mammalian cells, β-galactosidase is still the most commonly used reporter gene in yeast. However, assays using β-galactosidase are time consuming and less sensitive than luciferase assays (de Wet *et al*., 1987; Pazzagli *et al*., 1992). There are only few reports of the use of the firefly luciferase as a reporter gene in yeast and even fewer are the reports of its use *in vivo*. Vieites *et al*. (1994) found that the *in vivo* light emission from firefly luciferase in yeast is highest when the pH of the assay medium is below 3, which is probably the result of an increase in the amount of uncharged D-luciferin that facilitates its diffusion through the cytoplasmic membrane and cell wall. Above pH 3 the light emission dramatically decreased and at pH 5 the signal was rather weak. This fact, together with a complicated measurement protocol with centrifugations, has hampered the *in vivo* use of luciferase in yeast.

The *in vivo* luminescence reaction is dependent on the availability of ATP, O_2 and D-luciferin. In eukaryotic cells such as yeast, insect luciferase is transported into peroxisomes (Gould *et al*., 1990) due to the peroxisomal targeting

signal Ser–Lys–Leu (skl) at the C-terminus of the luciferase (Gould *et al*., 1989). When the luciferase is transported into the peroxisomes the concentration of externally added D-luciferin may be a limiting factor, as it has to penetrate across both cytoplasmic and peroxisomal membranes to find its target.

In this study we modified the luciferase gene by removing the three peroxisomal targeting codons and tested the performance of the modified luciferase in the yeast *S. cerevisiae*. We also show that it is possible to measure luciferase activity in yeast by simply adding D-luciferin at pH 5.0 to the culture medium.

Materials and methods

Plasmid constructions

The wild-type luciferase gene of *Photinus pyralis* was cut with a *Not*I–*Sal*I digestion from pBluc* (described in Bonin *et al*., 1994) and the 1774 bp fragment was cloned under the *CUP1* promoter of *pSal1* (described in Mascorro-Gallardo *et al*., 1996), yielding *pSalluc*. Vector *pSal1* is a medium-copy plasmid which carries an *ars–cen* origin of replication and a gene coding for leucine auxotrophy. The C-terminally truncated luciferase lacking the SKL-tail was constructed by PCR. The sequences of the primers used in the PCR reaction are shown in Table 1. The *Nco*I and *Sal*I restriction were used for the insertion of the luciferase into vector *pSal1*, yielding *pSalluc-skl*. The cDNA of human *ERα* (oestrogen receptor alpha) was amplified by PCR from plasmid *pSP72hER/Sp6* (a kind gift from Dr Pettersson). The PCR product was isolated from an agarose gel, cut with *Nhe*I, blunted and then cut with *Xho*I. The *pSal1* vector was cut with *Sac*I, blunted and then cut with *Xho*I before being ligated with the *ERα* fragment, yielding *pSalERα*.

YIpMELα2 (described in Melcher *et al*., 2000) was digested with *Sph*I, blunted and then cut with *Sac*I. The 5 kb fragment with the vector backbone was isolated from an agarose gel. The PCR product *luc-skl* (primer sequences in Table 1) was ligated into the cloning site of vector pGEM-t easy (Promega). The yielded *pGEMluc-skl* was cut with *Aat*II, blunted and then cut with *Sac*I. The 1.7 kb fragment containing the *luc-skl* was isolated from an agarose gel and ligated into the vector backbone of *YIpMELα2*, yielding *YIpluc*. The ERE (oestrogen responsive element) sequences were inserted in the truncated promoter of *MELα*, which lacked its original UAS (upstream activation sequences). Oligos (see Table 1) containing two tandem ERE sequences were annealed in 72 °C for 7 min. *YIpluc* was digested with *Xho*I, dephosphorylated and then ligated with the ERE, yielding *YIpEREluc*.

All DNA manipulations were performed using standard techniques (Sambrook *et al*., 1989). All plasmids were constructed using the bacterial host MC1061 (Casadaban and Cohen 1980). After verifying the right constructs by restriction enzyme digestions and sequencing, the plasmids were transformed into yeast BMA64-1A (*MAT***a** *ura3-52; trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*, wild-type strain W303; Baudin-Baillieu *et al*., 1997), using the lithium acetate method (Gietz and Schiestl, 1995). The chromosomal integration of *YIpEREluc* was directed into the URA locus by linearizing with *Eco*RV.

Table 1. Sequences of the primers used in the constructions

Gene	Primer sequence
Luc-skl	5′ GGG *CCA TGG* ATG GAA GAC GCC AAA AAC ATA 3′
	5′ GGG *GTC GAC* TTA AAG CTT CTT TCC GCC CTT 3′
HERα	5′ CCC *TCG AGG* CTA GCA TGA CCA TGA CCC TCC AC 3′
	5′ CTG *GGC TAG* CTC AGA CTG TGG CAG GGA AAC CC 3′
ERE	5′ CCC *CTC GAG CTC* CGT CAG GTC ACA GTG ACC TGA TCA AAG TTA ATG TAA CCT CAG TCA GGT CAC AGT GAC CTG ACG *AGC TCG AGC* CC 3′

Restriction sites are shown in italics in the sequence. The ERE tandem sequences are underlined.

Cell culture

For copper inductions a 5 ml preculture was grown overnight in 30 °C and 280 rpm in synthetic complete (SC) medium (Burke *et al*., 2000) lacking leucine. In the morning the culture was diluted with SC-leucine to $OD_{600} \cong 0.6$. The diluted culture was grown at 30 °C and 280 rpm until it reached mid-logarithmic phase ($OD_{600} = 1.4$). Then 95 l aliquots of cell culture were pipetted into a 96-well

plate, into wells that contained 5 l $CuSO_4$ dilutions. The plate was briefly shaken for 20 s and then incubated without shaking in 30 °C for 1 h. After the incubation the plate was shaken for another 20 s.

For oestrogen inductions a 5 ml preculture was grown overnight in 30 °C and 280 rpm in SC medium lacking uracil and leucine with 50 M $CuSO_4$. Next morning the culture was diluted with the same medium to $OD_{600} = 0.6$ and then grown at 30 °C and 280 rpm until $OD_{600} = 1.0$. Of this culture, 100 l aliquots were pipetted into a 96-well plate and 1 l 17-β-oestradiol (E_2) diluted in ethanol was added. The plate was shaken for 20 s and then incubated in 30 °C for 2.5 h. After the incubation the plate was shaken for another 20 s.

Luminescence measurements

D-luciferin (1 mM, 100 l) in 0.1 M Na citrate buffer (pH 3.0 or 5.0) was pipetted into the wells containing induced cultures. The plate was briefly shaken and then immediately measured using a Victor multilabel counter (Perkin-Elmer Wallac, Turku, Finland) in the luminescence mode, using 1 s counting time. The light emission levels are expressed as RLU (relative light units = luminescence value given by the luminometer) and the normalized luminescence was calculated by dividing the RLU value of the induced culture by that of the blank solvent.

Results and discussion

We constructed two vectors where firefly luciferase gene (*luc*) expression is under the control of copper ion-inducible elements. *pSalluc* contained a wild-type gene, and *pSalLuc-skl* a truncated version lacking the peroxisome-targeting codons. Both plasmids were expressed in the yeast *Saccharomyces cerevisiae*. Throughout our experiments, the strain with the wild-type luciferase grew slower than the one with the modified luciferase (Figure 1). This is probably caused by the accumulation of the luciferase into the peroxisomes during the growth. The expression level of luciferase was high enough to disturb the growth, even without addition of copper as the growth media contained traces of copper. In all measurements the modified luciferase construct gave at least two orders higher light emission levels than the wild-type luciferase (Figure 2a). Although the basal level of luminescence (without addition of copper) from the modified luciferase was rather high, the induction coefficients for copper were still higher than with the wild-type luciferase (Figure 2b). Changing the pH of D-luciferin buffer from pH 5.0 to pH 3.0 gave higher luminescence levels for both wild-type and modified luciferases but, as it also raised the basal level of luminescence, the induction coefficients were similar at pH 3.0 and pH 5.0 (data not shown). We also estimated the performance of the modified luciferase in a system where it is placed after a promoter that is inducible with oestrogen. With this promoter the background luminescence was very low and the normalized luminescence values were considerably higher than those with the *CUP1* promoter (Figure 3).

Although the luminescence levels were higher if the induction had been done at a later phase of culture, the normalized luminescence did not show dependence on the cell number, so long as both the induction and luminescence measurements had been done during the logarithmic phase of growth (data not shown). The results were easily reproducible and the differences between separate measurements were small.

Luciferases allow the detection of reporter activity directly from intact living organisms or cells (Colin *et al.*, 2000; Loimaranta *et al.*, 1998; Wood *et al.*, 1987). The basis for the detection is that

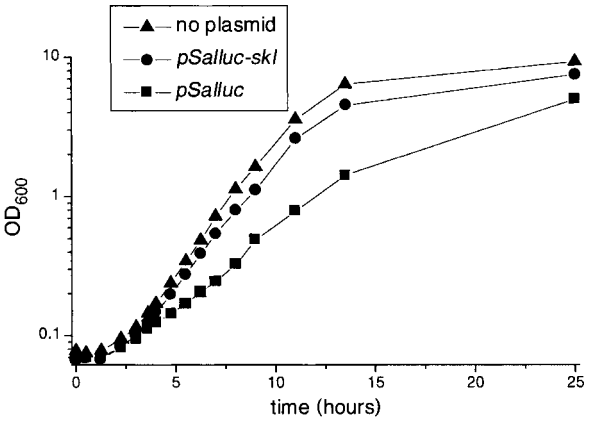

Figure 1. Effect of the peroxisomal luciferase on the growth of the yeast. An overnight culture was diluted to 1 : 100 at time zero and then grown in a shaking incubator at 30 °C, 220 rpm. The control is the BMA64-1A strain without plasmids

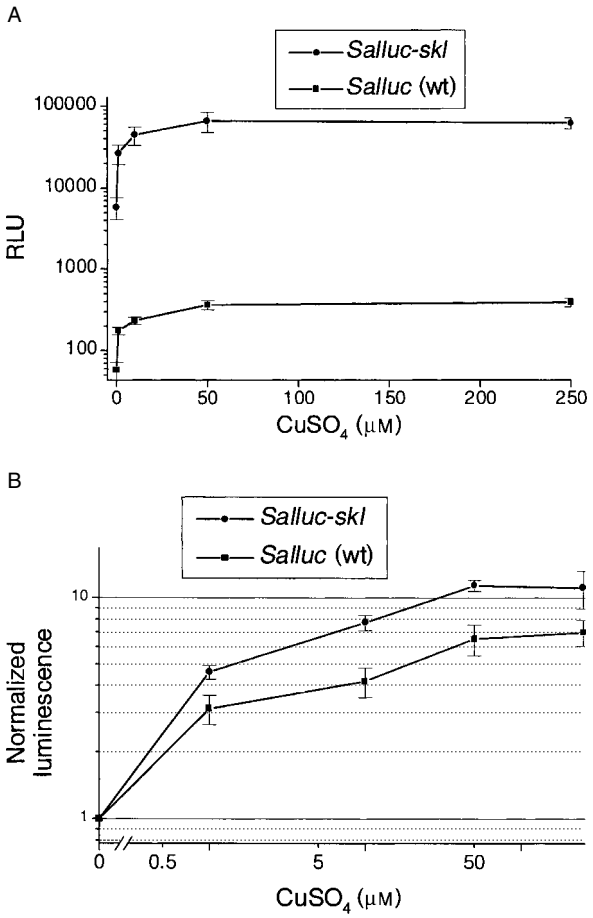

Figure 2. The effect of peroxisomal transport on the light signal in yeast. Peroxisomal (*Salluc*) or cytosolic (*Salluc-skl*) luciferase activity under *CUP1* promoter in living yeast cells. (A) Relative luminescence levels and (B) normalized luminescence as the function of $CuSO_4$ concentration after 1 h copper induction. The curves represent the average of four independent repeats which were all performed in triplicate. The error bars represent the standard deviation of the four experiments

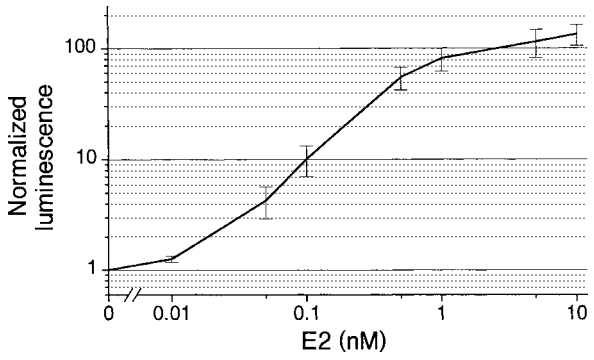

Figure 3. Luciferase activity under oestrogen-inducible promoter. Normalized luminescence measured after 2.5 h incubation in the presence of 17-β-oestradiol (E_2). The curve represents the average of four independent repeats, which were all performed in triplicate. The error bars represent the standard deviation of the four experiments

the D-luciferin substrate is in protonated form at pH 5.0 and therefore diffuses easily through the cell membrane (Wood *et al.*, 1987). We have previously shown that it is in fact more reliable to measure the activity *in vivo* than *in vitro* (Taurianen *et al.*, 1999) when *Escherichia coli* cells are used. However, when the luciferase is located in the peroxisomes of eukaryotic cells such as yeast, diffusion of the substrate to the cytoplasm is not enough to reach the luciferase molecule and the availability of the substrate may become a limiting factor for the luminescence reaction. Furthermore, the transport of luciferin to peroxisomes severely disturbs the growth of the yeast cells.

So far, the detection of luciferase activity of living yeast cells has required a somewhat elaborate protocol, which has included centrifugation and cell resuspension steps (Vieites *et al.*, 1994). In this study we wanted to simplify the luciferase activity measurement in yeast and especially avoid centrifugation–resuspension steps, since they are incompatible with high-throughput screening (HTS) applications.

In this study we have described how some major problems that have earlier been associated with the use of firefly luciferase in yeast can be avoided by removing the peroxisomal targeting signal from the *luc* gene. We have shown that it is possible to measure luciferase activity from intact living yeast cells just by adding D-luciferin solution. The protocol is suitable for high-throughput screening applications as it contains no centrifugation steps and can be done on a multi-well plate. Some of the vectors described in this paper can easily be converted for use in other studies. *YIpluc* has a truncated promoter lacking the UAS in front of the luciferase and an *Xho*I site is convenient for inserting UAS of interest. Also, the reporter plasmid *pSal1luc-skl* that was constructed for this study can be used for studying activities of different promoters by substituting the *CUP1* promoter with another. The ease of use of the non-peroxisomal luciferase makes it an interesting alternative for reporter gene in yeast.

Acknowledgements

We would like to thank Dr K. Pettersson, Dr Covarrubias Robles and Dr Bujard for their generous gifts of plasmids *pSP72hER/Sp6, pSal1* and *pBluc**, respectively. Plasmid *YIpMELα2* was acquired from EUROSCARF. A. Ivask and E. Michelini are thanked for their help with the experiments. This work was supported by the Maj and Tor Nessling Foundation and partly by the Academy of Finland (Grant No. 80278).

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