Tips for Yeast *CLuc* Reporter Assay

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1. Assay alternatives to β-Gal

Since Cypridina luciferase (CLuc) is a secretory luminescent enzyme, the yeast reporter assay using CLuc has the following features compared to the reporter assay using β-galactosidase (β-Gal) that has been used in yeast.

Feature	Why
Quick and high sensitivity	Measuring bioluminescence
No need to collect and lysis cells	Secreted enzyme
Traceable the time course in the same sample	Measured by sampling a small amount of medium

In addition, when comparing several promoter activities, the relative values correspond to the results of β-Gal (Reference 1), which can be expected as an assay alternative to β-Gal.

2. Notices for medium and substrate solution

CLuc is produced in yeast and secreted into the culture medium, so it is affected by the culture medium environment. Therefore, pay attention to the following points in culture and measurement.

(1) pH of culture medium

The stability of CLuc depends on pH, and CLuc becomes unstable in acidic conditions below pH 5. Generally, the pH of the culture medium of yeast is about 6 at the time of preparation, but it gradually decreases when yeast is inoculated and cultured, and drops to about pH 2-3 in the stationary phase (especially remarkable in minimal medium such as SD). For this reason, it is necessary to maintain the pH of the culture medium and prevent the inactivation of CLuc secreted in the culture medium by adding a final concentration of 0.2 M potassium phosphate buffer (pH 6.0) to the medium beforehand (Fig. 1).

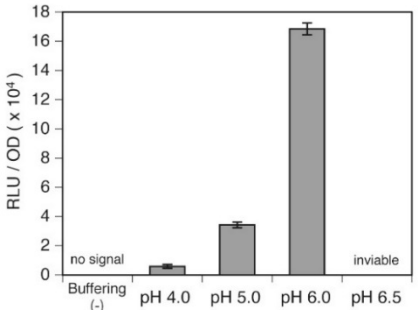

Fig. 1 pH of medium and activity of CLuc expressed in yeast
CLuc activity in the medium (measured at pH 7.5) in transformed yeast cultured in minimal medium without buffer or maintaining pH with buffer. At pH 5 or lower, CLuc activity in the medium decreases.

(2) Components of culture medium

The bioluminescence reaction of CLuc secreted in the culture medium can be affected by chemical substances contained in the culture medium. As far as we have investigated so far, the addition of high concentrations of ethanol and copper slightly inhibit the bioluminescence reaction (Fig. 2). When adding chemical substances that have not been investigated yet to the culture medium, it is recommended to study the effect of chemical substances on the CLuc bioluminescence reaction in advance using purified CLuc enzyme.

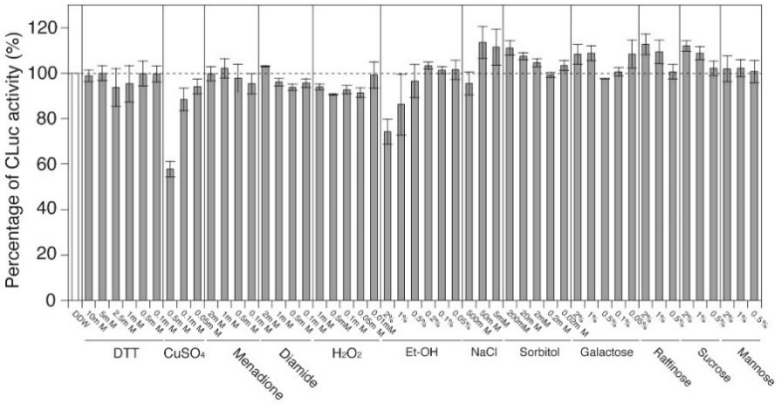

Fig. 2 Effect of chemicals on CLuc activity
CLuc activity is measured by adding various chemical substances to the medium containing CLuc

(3) pH during bioluminescence reaction

The CLuc bioluminescence reaction is detected between weakly acidic and weakly alkaline, but it shows the strongest bioluminescence reaction in the neutral region (Fig. 3). 0.2 M Tris-HCl (pH 7.4) is included to the luciferin dilution solution ("Solution B") attached to the ATTO CLuc substrate in order to obtain an optimum pH of around 7.4 during measurement.

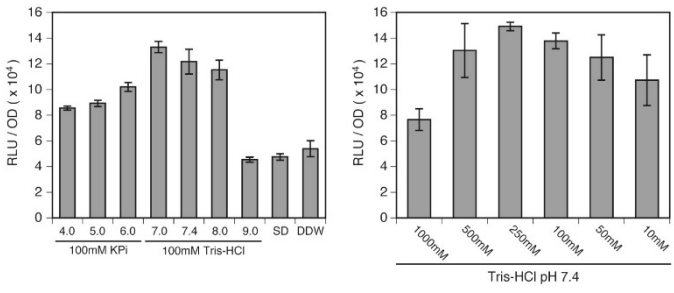

Fig. 3 Effect of buffer pH and concentration during measurement on CLuc activity
For CLuc produced in a medium containing a buffer solution (pH 6), CLuc activity was measured when the pH was adjusted again by adding a buffer solution (SD; medium, DDW; purified water).

3. CLuc activity measurement and normalization

(1) Normalization by OD_{600}

When measuring the transcription activity of a promoter using an intracellular reporter enzyme such as β-Gal, the protein concentration of the cell lysate is generally measured, and the reporter enzyme activity is divided by the protein concentration to normalize. On the other hand, CLuc, a secreted protein, can normalize CLuc activity by cell number, which can dramatically simplify the assay system. The turbidity OD_{600} (or 660 nm etc.) is measured as a value representative of the number of cells, and the CLuc activity is divided by OD_{600} for normalization.

[Transcriptional activity] = [CLuc luminescence] / OD_{600}

Although the conditions may vary depending on the composition of the culture medium and the activity of the promoter, it has been shown in the previous studies that the correlation between CLuc activity and OD_{600} is linear in the logarithmic growth phase (Fig. 2a in Reference 1).

(2) Centrifuge can be omitted

Generally, when measuring enzyme activity in culture medium, it is necessary to remove cells by centrifugation and prepare the supernatant. On the other hand, the yeast CLuc reporter assay can detect CLuc activity in the culture medium containing the transformed yeast. The CLuc luminescence value when using the culture medium containing the transformed yeast is slightly higher than the CLuc luminescence value measured by removing the cells by centrifugation, but both have a correlation with the cell number (OD_{600}). Therefore, CLuc activity can be measured in the culture medium containing the transformed yeast. The higher CLuc activity in the culture medium containing the transformed yeast is considered to be because CLuc in the process of secretion also catalyzes the bioluminescence reaction.

(3) Measuring time course of transcription activity

Since CLuc is secreted into the culture medium, a single sample can be used to measure the time course of transcriptional activity by collecting a portion of the culture medium and measuring CLuc enzyme activity and microplate OD_{600}.

In this case, the culture solution is collected at each time and measured, but it is necessary to be careful when measuring all at once after the collection of all time. Even after collecting the culture medium containing yeast, the yeast secretes CLuc, and the enzyme activity of the medium gradually increases. For this reason, if you want to perform a batch measurement after collecting all the samples, it is recommended to remove the yeast cells by centrifugation immediately after collection and store the supernatant in a refrigerated or frozen storage until the measurement.

4. Culture method for ensuring quantitativeness

(1) Quantitative OD_{600} range

As mentioned above, CLuc is relatively stable in pH-controlled culture medium, therefore CLuc accumulates in the culture medium over time. For the reason, even if normalization is performed by dividing by OD_{600} in the latter half of the culture, the luminescence value tends to increase due to CLuc that has already accumulated in the culture medium (Fig. 2a in Reference 1). Therefore, to measure within the quantitative range (range in which normalization is possible by dividing by OD_{600}), it is recommended that 200 µl was dispensed in a 96-well microplate and measured with a plate reader (hereinafter referred to as "microplate OD_{600}") and then check the value to be 0.1-0.4 to measure CLuc enzyme activity (corresponding to approximately 0.2-0.8 when measuring absorbance using a 1 cm square cuvette).

If the qualitative or semi-quantitative assay is acceptable, there is no problem if the microplate OD_{600} exceeds 0.4.

(2) Culture for adjusting OD_{600}

As described above, in order to measure CLuc activity within the quantitative rang (range in which normalization is possible by dividing by OD_{600}), inoculate for “microplate OD_{600}“ at the time of measurement to 0.1-0.4.

When measuring constitutive promoter activity, it is recommended to first carry out preculture until stationary phase, add an appropriate amount of preculture solution to new medium (at this time, inoculate with adjusting for “microplate OD_{600}” to 0.1-0.4 after culturing for the scheduled time (such as overnight)), and measure CLuc enzyme activity and “microplate OD_{600}” after culture.

When assaying the effect of an inducer using an inducible promoter, first perform preculture up to the stationary phase, add an appropriate amount of preculture medium to a new medium(at this time, inoculate with adjusting for “microplate OD_{600}” to 0.05-0.2 after culturing for the scheduled time (such as overnight)), then add the inducer, and after a suitable time, measure CLuc enzyme activity and “microplate OD_{600}”. Although it depends on the promoter activity, it is recommended to measure the activity 2 hours after adding the inducer. If the CLuc activity before induction is already high, you can remove the culture medium by centrifugation when adding the inducer, add a new medium and inducer, and then culture again.

5. Experimental example

5.1 Constitutive transcriptional activity measurement (for test tube culture)

(1) Multiple medium-sized colonies are collected from the yeast *S. cerevisiae* plate transformed with the CLuc reporter vector[1], and inoculate into medium (final concentration 0.2 M potassium phosphate buffer (pH 6.0) is included)[2] in a test tube.

(2) Pre-culture under optimal temperature conditions until reaching stationary phase (eg, 30 °C, 1-2 nights)[3].

(3) Inoculate a portion of the preculture (eg 25 µl) into a new tube of medium (eg 5 mL)[4]. The amount of the preculture is adjusted so that the value of "microplate OD_{600}" is about 0.1-0.4 after culturing for the determined time[5].

(4) After culturing, transfer 200 µl of the culture to a clear 96-well microplate and measure the OD_{600} ("microplate OD_{600}") with a plate reader. The OD_{600} of the microplate suitable for measuring the luminescence value is about 0.1-0.4.

(5) Transfer 20 µl of the culture medium to a 96-well microplate (black plate is recommended), add 80 µl of diluted luciferin solution[6], and measure CLuc activity with a luminometer (such as ATTO AB-2350 Phelios).

5.2 Inducible transcriptional activity measurement (for test tube culture)

(1) Multiple medium-sized colonies are collected from the yeast *S. cerevisiae* plate transformed with the CLuc reporter vector[1], and inoculate into medium (final concentration 0.2 M potassium phosphate buffer (pH 6.0) is included)[2] in a test tube.

(2) Pre-culture under optimal temperature conditions until reaching stationary phase (eg, 30 °C, 1-2 nights)[3].

(3) Inoculate a portion of the preculture (eg 25 µl) into a new tube of medium (eg 5 mL)[4]. The amount of the preculture is adjusted so that the value of "microplate OD_{600}" is about 0.05-0.2[7] after culturing for the determined time[5].

(4) After culturing, transfer 200 µl of the culture to a clear 96-well microplate and measure the OD_{600} ("microplate OD_{600}") with a plate reader. The recommended OD_{600} of the microplate is about 0.05-0.2[8].

(5) The inducer is added to the culture medium and the culture is continued. It usually takes about 2 hours before induction can be detected.

(6) Transfer 20 µl of the culture medium to a 96-well microplate (black plate is recommended), add 80 µl of diluted luciferin solution[6], and measure CLuc activity with a luminometer (such as ATTO AB-2350 Phelios).
At the same time, take 200 µl of the culture medium and measure "microplate OD_{600}". The OD_{600} of the microplate suitable for measuring the luminescence value is about 0.1-0.4.
It is also possible to repeat sampling to trace time course of transcription activity[9].

1 The expression status varies slightly from colony to colony. Mixing multiple colonies of medium size to inoculate will yield more stable data.

2 To maintain the pH at which CLuc becomes stable, add a final concentration of 0.2 M potassium phosphate buffer (pH 6.0) to the medium. We recommend autoclaving the potassium phosphate buffer separately from the culture medium before mixing (some amino acids may precipitate at pH 6.0).

3 It is important for quantitativeness to set the microplate OD_{600} at the time of measurement to 0.1-0.4. Since it is difficult to control "microplate OD_{600}" within this range at a scheduled time without seeding the colonies in medium and pre-culturing, we recommend pre-culturing and re-planting.

4 The amount of inoculum is regulated depending on the growth rate of the transformed yeast.

5 One night is convenient.

6 Use the substrate dilution solution (Solution B) included with the CLuc substrate product for dilution.

7 After adding the inducer, it is necessary to culture for a while until the transcription activity can be detected, but yeast grows during that time, so adjust "microplate OD_{600}" to a low level.

8 If you place importance on linearity, keep "microplate OD_{600}" of the culture medium with the addition of an inducer lower than 0.1 so that "microplate OD_{600}" does not exceed 0.4 by culturing for a long time.

9 If you want to measure all at once after collecting samples for all the time, it is recommended to remove the cells by centrifugation immediately after collecting and store the supernatant refrigerated or frozen until measurement.

5.3 Constitutive transcriptional activity measurement (for 96-well plate culture)

(1) Add 1 ml of medium (final concentration 0.2 M potassium phosphate buffer solution (pH 6.0))[2] to each well of 96 deep well plate, and inoculate medium size colonies from yeast *S. cerevisiae* plate transformed with CLuc reporter vector.

(2) Cover with a seal[10] and pre-culture under optimal temperature conditions until a stationary phase is reached (eg, 30 ° C, two nights)[3].

(3) Add 1 ml of medium (final concentration 0.2 M potassium phosphate buffer (pH 6.0))[2] to each well of a new 96 deep well plate, and inoculate the part of the preculture solution (eg 5 µl) into each well of the plate. The amount of the pre-culture solution is adjusted so that the value of the microplate OD600 is about 0.1-0.4[11] after culturing for the determined time[5].

(4) After culturing, transfer 200 µl of the culture medium to a transparent 96-well plate and measure the OD_{600} ("microplate OD_{600}") with a plate reader. Recommended value is about 0.1-0.4.

(5) Transfer 20 µl of the culture medium to a 96-well microplate (black plate is recommended), add 80 µl of diluted luciferin solution[6], and measure CLuc activity with a luminometer (such as ATTO AB-2350 Phelios).

10 Use a material that has good air permeability and does not allow water to easily penetrate. For example Gas Permeable Adhesive Seal AB-0718 (Thermo Scientific)

11 If the transformants differ from well to well, it is recommended to adjust the amount of pre-culture added for each well so that "microplate OD_{600}" of culture in many wells is 0.1-0.4.

5.4 Inducible transcriptional activity measurement (comparison of inducers using the same transformants, etc.) (For 96-well plate culture)

(1) Multiple medium-sized colonies are collected from the yeast *S. cerevisiae* plate transformed with the CLuc reporter vector[1], and inoculate into medium (final concentration 0.2 M potassium phosphate buffer (pH 6.0) is included)[2] in a test tube.

(2) Pre-culture under optimal temperature conditions until reaching stationary phase (eg, 30 °C, 2 nights)[3].

(3) Inoculate a portion of the preculture (eg 500 µl) into a new flask of medium (eg 100 mL)[4]. The amount of the preculture is adjusted so that the value of "microplate OD_{600}" is about 0.05-0.2[7] after culturing for the determined time[5].

(4) After culturing, transfer 200 µl of the culture medium to a transparent 96-well plate and measure the OD_{600} ("microplate OD_{600}") with a plate reader. Recommended value is about 0.05-0.2[8].

(5) Add 1 ml of the above culture medium to each well of a 96 deep well plate and add the inducer. It usually takes about 2 hours before induction can be detected.

(6) Transfer 20 µl of the culture medium to a 96-well microplate (black plate is recommended), add 80 µl of diluted luciferin solution[6], and measure CLuc activity with a luminometer (such as ATTO AB-2350 Phelios).
At the same time, take 200 µl of the culture medium and measure "microplate OD_{600}". The OD_{600} of the microplate suitable for measuring the luminescence value is about 0.1-0.4.
It is also possible to repeat sampling to trace time course of transcription activity[9].

<Reference>

1 Y. Tochigi *et al.* : Sensitive and Convenient Yeast Reporter Assay for High-Throughput Analysis by Using a Secretory Luciferase from *Cypridina noctiluca*. *Anal. Chem.* 82, 5768–5776 (2010).

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