

Looking for a strong promoter for *Physcomitrella patens*

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- GFP and mCherry used as reporter proteins to research promoter strength in living protoplasts in 96-well format
- Using the LVF monochromator, the CLARIOstar® can detect < 100 protoplasts per well

Introduction

The 35S promoter of the cauliflower mosaic virus is a strong and constitutive promoter that is widely used in plant systems. However, in the moss *Physcomitrella patens*, its promoter strength is weak or all but silent in the dark. Therefore, it is necessary to find promoters that show higher expression levels and that have a more ubiquitous expression over time, tissues and treatments. Of further interest are cell or tissue specific promoters that can be used for targeted gene expression. With expression data available for an increasing number of treatments it is possible to select suitable candidates based on such data.

To test promoter strengths, the protoplasts of the moss *Physcomitrella patens* can be transiently transfected to allow several days of ectopic expression of circular DNA. This moss system can be used to test promoters without the need to produce mutant lines with stable integration. We show that the CLARIOstar can be used to measure the GFP fluorescence within living moss protoplasts and that this assay can be used to compare promoter strengths. To normalize for transformation efficiency a second reporter (mCherry) was introduced into the system.

Assay Principle

The validation of possible new promoters is often done by a promoter-reporter gene construct. A commonly used system fuses the promoter of choice with GFP and then measures the levels of fluorescence after insertion of the construct into the plant. mCherry was selected to be a reporter for transfection efficiency. The GFP/mCherry ratio will be used for further promoter strengths analysis.

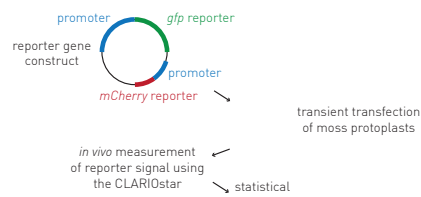


Fig. 1: Standard procedure for validation of new promoters.

Plasmids, carrying promoterA::GFP or promoterB::GFP or promoterC::GFP constructs as first reporter as well as promoter 35S::mCherry-constructs as second reporter, are transiently transfected into moss protoplasts. For

mCherry-constructs the double 35S promoter was used. The reporter signal is measured after six days of incubation in a CLARIOstar microplate reader. Statistical analyses of the GFP/mCherry-ratio allow the determination of the promoter strength.

Materials & Methods

- CLARIOstar microplate reader from BMG LABTECH
- Greiner CELLSTAR® medium binding 96 well half area polystyrene plates, black with transparent flat bottom
- Stable and transiently GFP and mCherry transfected protoplasts of *Physcomitrella patens* in regeneration medium
- Regeneration medium contains the following substances: 250 mg/L KH_2PO_4 , 250 mg/L KCl, 250 mg/L $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 1000 mg/L $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$, 12.05 mg/L $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$, 50 g/L glucose and 44 g/L mannitol]

Fluorescence intensity measurements were performed with transfected *Physcomitrella patens* protoplasts. Sample volumes of 100 μL (up to 30,000 protoplasts) were placed into black 96 well microplates with transparent bottom. After wavelength optimization the monochromator of the CLARIOstar was set up to measure GFP and mCherry fluorescence using one test protocol (ex/em for GFP: 480-12/519-15 nm; for mCherry: 562-12/603-20 nm). The samples were detected using the bottom optic, orbital averaging with 2 mm diameter and 15 flashes per well. Regeneration medium was used as the blank. All promoter measurements were background subtracted. The ratio of GFP and mCherry signals was calculated to normalize the data for the transfection efficiency.

Well Scanning

With the well scanning function of the CLARIOstar it was possible to check the distribution of protoplasts in the well. The instrument was set up to scan from the bottom using a scan matrix of 20 x 20 data points.

Results & Discussion

The well scan figure shows that the protoplasts are not evenly distributed in the well (Fig. 2).



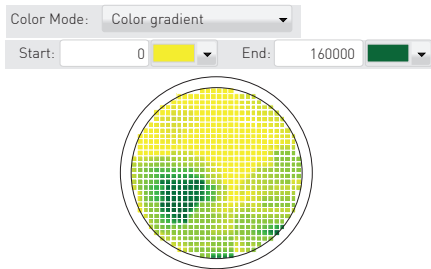


Fig. 2: Well scanning figure of protoplasts that express GFP. Measured at 480/519 nm.

Because of this finding we have assumed that it is more useful measuring data points in a small circle and average them afterwards instead of only measure in the middle of the well. The orbital averaging function in the BMG LABTECH control software allows for such a measurement, a diameter of 2 mm was found to give the most stable results.

After optimal wavelength settings were determined a protoplast dilution of single expressing cell lines was carried out in order to find the optimal number of protoplasts per well. Figure 3 shows standard curves for GFP and mCherry expressing cells.

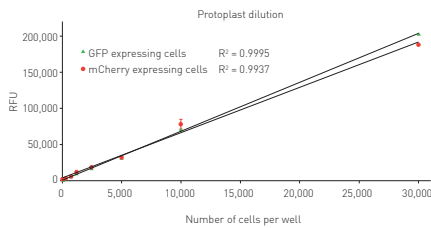


Fig. 3: GFP protoplast expression measured at 480/519 nm. mCherry protoplast expression measured at 562/603 nm.

Both protoplasts dilution curves show a good linearity over a broad number of cells per well range.

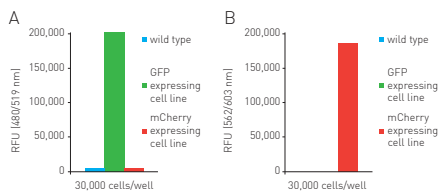


Fig. 4: A GFP fluorescence measured for wild type, GFP and mCherry expressing cell lines. B mCherry fluorescence measured for wild type, GFP and mCherry expressing cell lines.

Figure 4 and B compare the fluorescence intensity values obtained for wild type, GFP and mCherry expressing protoplast samples. In the wavelength range of GFP (480/519 nm) there is a certain amount of background fluorescence present in both, the wild type and the mCherry expressing cell line (Fig. 4A). In the wavelength range of mCherry (562/603 nm) there is nearly no background signal in wild type and GFP expressing cell line (Fig. 4B). It was possible to detect a significant signal in as low as 150 GFP expressing protoplasts and resp. < 100 mCherry expressing protoplasts per well.

Promoter strength analysis

For promoter strength analysis 4,000 protoplasts per well were used that stably expressed both GFP and mCherry. Figure 5 shows results for different promoter::GFP-constructs.

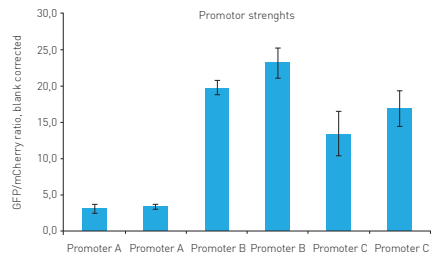


Fig. 5: GFP/mCherry ratio of transiently transfected protoplasts. Error bars show the standard deviation of technical replicates.

Transfection experiments using different promoters were evaluated with the help of the GFP/mCherry ratio. From Figure 5 it can be followed that the Promotor B is the strongest promoter tested.

Conclusion

We introduce a transient assay using BMG LABTECH's CLARIOstar that can be used to measure GFP and mCherry fluorescence in living protoplasts in a microplate format. With such an assay, it is possible to perform expression studies in transiently transfected cells. It is a really fast and simple method compared to the time-consuming process of post transfection selection using antibiotics. In the future, this assay format could be used as a standard method for screening transiently transfected protoplasts.

