Gene 642 (2018) 474–482

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Methodological paper

A novel and versatile dual fluorescent reporter tool for the study of gene expression and regulation in multi- and single copy number

Indra Bervoets, Daniel Charlier*

Research Group of Microbiology, Department of Bioengineering Sciences, Vrije Universiteit Brussel (VUB), Pleinlaan 2, 1050 Brussels, Belgium

ARTICLE INFO

Keywords:
Fluorescent reporter
In vivo
High throughput
F′ episome
Homologous recombination
Real time measurement

ABSTRACT

To unravel intricate mechanisms of gene regulation it is imperative to work in physiologically relevant conditions and therefore preferentially in single copy constructs, which are not always easy to manipulate. Such *in vivo* studies are generally based on enzymatic assays, microarrays, RNA-seq, qRT-PCR, or multicopy reporter gene systems, frequently with *β*-galactosidase, luciferase or a fluorescent protein as reporter. Each method has its advantages and shortcomings and may require validation. Enzyme assays are generally reliable but may be quite complex, time consuming, and require a (expensive) substrate. Microarrays and RNA-seq provide a genome wide view of gene expression but may rapidly become expensive and time consuming especially for detailed studies with large numbers of mutants, different growth conditions and multiple time points. Multicopy reporter gene systems are handy to generate numerous constructs but may not provide accurate information due to titration effects of *trans*-acting regulatory elements. Therefore and in spite of the existence of various reporter systems, there is still need for an efficient and user-friendly tool for detailed studies and high throughput screenings. Here we develop and validate a novel and versatile fluorescent reporter tool to study gene regulation in single copy mode that enables real-time measurement. This tool bears two independent fluorescent reporters that allow high throughput screening and standardization, and combines modern efficient cloning methods (multicopy, *in vitro* manipulation) with classical genetics (*in vivo* homologous recombination with a stable, self-transmissible episome) to generate multi- and single copy reporter systems. We validate the system with constitutive and differentially regulated promoters and show that the tool can equally be used with heterologous transcription factors. The flexibility and versatility of this dual reporter tool in combination with an easy conversion from a multicopy plasmid to a stable, single copy reporter system makes this system unique and attractive for a variety of applications. Examples are *in vivo* studies of DNA-binding transcription factors (single copy) or screening of promoter and RBS libraries (multicopy) for synthetic biology purposes.

1. Introduction

Microorganisms have developed complex mechanisms to alter their gene expression profiles in response to signals related to the cell cycle and the ever-changing physical and geochemical parameters of open systems. Gene regulation can occur at any step from transcription initiation to RNA processing and post-translational modification. Even though regulation at the level of transcription initiation is rather slow compared to enzymotropic control, it is energetically favorable as it occurs at the top of the regulatory cascade and, consequently, it is ubiquitously present in nature. Regulation of gene expression is frequently performed by multiple regulatory proteins called transcription factors (TFs), which may form intricate regulatory networks. Furthermore, regulation by DNA-binding TFs may occur in combination with other regulatory mechanisms such as small regulatory RNAs, attenuation control or still ppGpp mediated global stringent control (Dalebroux and Swanson, 2012; Maisonneuve et al., 2013; Morris and Mattick, 2014; Naville and Gautheret, 2009; Ross et al., 2013; Waters and Storz, 2009; Yakhnin et al., 2015).

Current strategies for understanding and unraveling gene regulation at the transcriptional level are amongst others, DNA microarray and RNA-seq that provide a global, genome-wide view of differences in expression, and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) that explores changes in expression of a limited number of selected genes in more detail. Still for these techniques differences are measured at a single time point and thus differential

Abbreviations: ECF, extracytoplasmic function; OD, optical density; MCS, multiple cloning site; FRT, flippase recognition target; FU, fluorescence unit; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance

* Corresponding author.
E-mail addresses: Indra.Bervoets@vub.be (I. Bervoets), dcharlie@vub.be (D. Charlier).

https://doi.org/10.1016/j.gene.2017.11.061
Received 16 June 2017; Received in revised form 20 November 2017; Accepted 24 November 2017
Available online 27 November 2017
0378-1119/

expression is generally studied from a static viewpoint while regulation of gene expression is a dynamic process. It is therefore important to characterize changes in gene expression over time. This can be achieved by comparing gene expression levels over a number of time points, however, this may rapidly become expensive and time consuming. Additionally these techniques reveal only the effect on transcription, not on translation. Microarray results are often further validated and examined in more detail with other established methods as real-time PCR or a reporter assay (Kothapalli et al., 2002; Marguerat and Bähler, 2010; Trapnell et al., 2013; VanGuilder et al., 2008; Wang et al., 2009; Zhou and Yang, 2006).

Reporter genes are widely used for analysis of up and down regulation of gene expression both at the transcriptional or translational level. One of the most common reporter genes used is the *E. coli lacZ* gene, which codes for *β*-galactosidase. A transcriptional or translational fusion of the investigated promoter/operator with the reporter gene is generated *in vitro* and the recombinant vector is subsequently transformed in various genetic backgrounds. This assay will ultimately demonstrate whether a particular promoter is regulated by a specific TF and measurements with this reporter are accurate. However, they represent only a specific point in time as they often depend on destruction of cell integrity to obtain crude cell-free extracts or permeabilized cells (Miller, 1972; Silva-Rocha and de Lorenzo, 2012; Thibodeau et al., 2004).

Other widely used reporters for studying gene expression are luminescent and fluorescent reporters, which generate a signal that is easy to capture and allows real-time monitoring. In addition measurements can be performed both at the level of individual cells and cell populations. As for *β*-galactosidase, luciferase is dependent on a substrate (luciferin) while fluorescent reporters have no need for supplements. With single-cell fluorescence, fluctuations in gene expression due to internal and external noise can be measured and this has led to new insights. Additionally, microplate readers measure expression of cell populations instead of individual cells but the lower resolution is compensated by a higher throughput as many genes and constructs can be analyzed in parallel at higher precision and sampling number than presently possible with DNA microarrays (Adams et al., 2017; Rao et al., 2002; Silva-Rocha and de Lorenzo, 2012; Wilkinson, 2009).

Notwithstanding the fact that fluorescence is a very attractive reporter to work with, all reporter constructs still encounter some inherent problems of working with plasmids. These can be titration of TFs due to copy number of the plasmid bearing the fusion construct (Brewster et al., 2014), growth phase-dependent alteration of plasmid copy number or read-through of endogenous plasmid promoters into the reporter gene. A solution to these problems is to convert the reporter fusion from a multicopy to a more stable single copy construct by introducing the reporter into the genome. The latter is a more physiological relevant condition but most conventional strategies for insertion into the genome, often based on homologous recombination, are still cumbersome and time-consuming (Datsenko and Wanner, 2000; DeBoy and Craig, 2000; Ellermeier et al., 2002; Kulakauskas et al., 1991; Platt et al., 2000; Sabri et al., 2013; Sukhija et al., 2012; Yang et al., 2014). Still these strategies are continually used notwithstanding the appearance of new approaches such as mobile group II introns and CRISPR/Cas genome editing (Choi and Lee, 2016; Enyeart et al., 2013; Gasiunas and Siksnys, 2013; Jiang et al., 2013; Nakashima and Miyazaki, 2014; Song et al., 2015; Zhang et al., 2017; Zhao et al., 2016). In bacteria, the latter is presently more frequently applied for the creation of gene knock-outs and knock-downs rather than knock-ins although recent developments appear promising (Zhang et al., 2017; Zhao et al., 2016).

Here we develop a versatile tool for the detailed study of gene expression both on transcriptional and translational level that combines the advantages of different approaches described above. This results in a novel reporter tool bearing two independent fluorescent reporters that allow for high throughput screening of both single cell as populations. Moreover, by combining modern efficient cloning techniques and classical methods from bacterial genetics the tool can be utilized as a multicopy plasmid (≈ 10–20 copies) or can easily and efficiently be converted to a stable, single copy reporter system on a F′ episome, which is self-transmissible and can therefore be easily transferred to various genetic backgrounds by conjugation.

2. Material and methods

2.1. Media

Complex culture medium (853) consisted of 10 g bacto-tryptone, 5 g yeast extract, 0.1% glucose, 5 g NaCl, 0.7 g K_2HPO_4 and 0.3 g KH_2PO_4 in 1 l water. Minimal culture medium consisted of 2 g NH_4Cl, 5 g $(NH_4)_2SO_4$, 2.99 g KH_2PO_4, 7.32 g K_2HPO_4, 8.37 g MOPS, 0.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$ and 16.5 g glucose per liter. The pH was set to 7.0 using NaOH and HCl. Glucose solution, together with the $MgSO_4$ was autoclaved separately. 1 ml vitamin and trace element solution and 0.1 ml molybdate solution (both filter sterilized) were added afterwards per liter medium. The vitamin and trace element solution consisted of 3.6 g $FeCl_2 \cdot 4H_2O$, 5 g $CaCl_2 \cdot 2H_2O$, 1.3 g $MnCl_2 \cdot 2H_2O$, 0.38 g $CuCl_2 \cdot 2H_2O$, 0.5 g $CoCl_2 \cdot 6H_2O$, 0.94 g $ZnCl_2$, 0.0311 g H_3BO_4, 0.4 g $Na_2EDTA \cdot 2H_2O$ and 1.01 g thiamine·HCl per liter water. The molybdate solution comprises 0.967 g $Na_2MoO_4 \cdot 2H_2O$ per liter. Antibiotics were added to the media when needed as indicated, kanamycin (60 μg/ml), chloramphenicol (25 μg/ml), streptomycin (25 μg/ml) and ampicillin (100 μg/ml). L-arginine·HCl was supplemented at 120 μg/ml and L-proline at 100 μg/ml (added to minimal medium for growth of F^- strains).

2.2. Bacterial strains and plasmids

E. coli FW102 [F^- *araD* Δ(*gpt-lac*)5 *rpsL*] and *E. coli* CSH100 [F′ *lac proA*$^+$ *B*$^+$ (*lacI*^{q} *lac*PL8)/*araD* Δ(*gpt-lac*)5] were generously provided by F. Whipple (Whipple, 1998). The *lrp*::Tn*10* and *lrp*::Tn*10*Δ*argP* derivatives of strain FW102 have been described by (Peeters et al., 2009). *E. coli* MG1655 [λ$^-$ F^- *rph*-1 *rfb*-50 *ilvG*$^-$ *fnr*$^-$] was obtained from the Netherlands Culture Collection of Bacteria (NCCB, Utrecht, The Netherlands). A streptomycin resistant derivative thereof was constructed by P1vir-mediated generalized transduction with a lysate prepared on strain FW102.

Plasmids pIB3 and pIB5 were constructed by assembly of four PCR fragments in a seamless ligation reaction, protocol adapted from (Zhang et al., 2012b). One reaction consisted of 1 μl 10 × buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM ATP, 10 mM DTT), 1 μl cell extract prepared from *E. coli* DH10B transformed with plasmid pKD46 expressing the lambda red recombination system (Datsenko and Wanner, 2000), PCR fragments (with overlapping sequences of 40–50 nucleotides) in equal molar concentrations and ddH_20 was added to a total volume of 10 μl in PCR tubes. The reactions were incubated for 1 h at 37 °C in a water-bath and 1–2 μl reaction mix was subsequently transformed into 100 μl *E. coli* MG1655 competent cells. PCR fragment 1 was amplified from pFW11-null (Whipple, 1998) with primers IB0402 and IB0403 (Supplemental files Table S1 for list of primer sequences). PCR fragment 2 was amplified from pSC101Kan_ProB_BBa_B0032_mKATE2_TFAB391 (kindly provided by Prof. Marjan De Mey, UGhent) with primers IB0443 and IB0404 for pIB3 and primers IB0447 and IB0404 for pIB5. PCR fragment 3 was amplified from pKD4 (Datsenko and Wanner, 2000) with primers IB0444 and IB0389 for pIB3 and primers IB0448 and IB0450 for pIB5. PCR fragment 4 was amplified from pB-YFP (kindly provided by Prof. Marjan De Mey, UGhent) with primers IB0382 and IB0405 for pIB3 and primers IB0405 and IB0449 for pIB5. Full sequence information of plasmids pIB3 and pIB5 with annotations is available in Supplemental file 1.

Plasmid pTrc99a was obtained from Pharmacia Biotech (Uppsala, Sweden).

All derivatives of pIB3 and pIB5 with an insert in one or both