7. Organ, tissue and cell culture

v-Reporter assays

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Abstract

Transcriptional feedback loops are at the core of the molecular clockworks. As single clock genes were cloned it was compelling to develop an assay that allowed simple and direct functional testing of putative activators or repressors of transcription. This chapter includes a general description and guidelines to carry out transcriptional assays in transiently transfected Schneider's cells.

Keywords: transcriptional assays; Firefly *luciferase*; Renilla *luciferase*; S2 cells

1. Introduction

Reporter assays are widely used to study gene expression and other aspects of cellular function. Among the most common uses are the characterization of transcription factors [1,2,3] and the dissection of signaling pathways [4,5]. Transcriptional assays usually employ two reporter genes to improve experimental accuracy. This implies simultaneous expression and measurement of two reporters within a single experimental set. One of them reports the effect of the specific experimental conditions that are under investigation, meanwhile the second reporter provides an internal control that reflects the baseline response. The latter is usually under the control of a constitutive promoter which is anticipated not to be affected by the experimental condition. Determining the expression of both reporter genes allows to normalize the activity of the 'experimental'

reporter to the internal control, minimizing variability due to differences in cell viability or transfection efficiency. Other sources of variability, such as differences in pipeting volumes, cell lysis efficiency and assay efficiency, are also under control.

Normalization against other parameters such as total protein (see Note 1) takes into account the discrepancy arising from a imperfect technique, but does not reflect the variability stemming from differential transfection efficiencies.

How to choose the ideal reporter assay.

Initially, chloramphenicol acetyl transferase (CAT) and ß-galactosidase (ß-*gal*) were the most commonly used. Later on, the green fluorescent protein (GFP) and luciferase opened the possibilities further as they allow *in vivo* monitoring of gene expression. Depending on the nature of the question the most appropriate reporter gene can be selected. ß-galactosidase and *luc* are the ones of choice when quantitation is an issue, as both fluorometric and colorimetric substrates are available. On the other hand, GFP is by far the ideal one for *in vivo* spatial reporting. When it comes to reporting *in vivo* circadian expression, most reporter genes fail due to protein stability issues (they stay around far too long!). Only *luc* and the unstable version of GFP are useful in this regard.

Reporter genes.

The bacterial CAT enzyme transfers acetyl groups to chloramphenicol from acetyl coenzyme A (acetyl CoA). In a typical assay this reaction is monitored by ^{14}C- labeled chloramphenicol, where acetylated and non-acetylated forms can be separated by thin layer chromatography and quantitated in a scintillation counter. Since CAT is very stable (a couple of days in culture) is not the reporter of choice when temporal changes in gene expression are studied.

The green fluorescent protein is encoded in the genome of the jellyfish *Aequorea victoria*. The intrinsic fluorescence of the protein is due to a unique covalently-attached chromophore which is formed post-translationally within the protein upon cyclisation and oxidation of residues 65-67, Ser-Tyr-Gly [6]. GFP is a relatively small protein (~27kDa) and diffuses freely within a cell. It is widely employed as an amino or carboxi-terminal fusion to the protein of interest, to follow its destination within the cell as well as patterns of expression.

Firefly luciferase catalyzes a bioluminescent reaction in which its substrate (luciferin) is oxidized, and in doing so it generates light as a by-product. This light (or bioluminescence) can be quantitated with a luminometer or a scintillation counter (i.e. Packard Top Count). This bioluminescence has been measured in *Drosophila* tissue culture extracts [7] and in whole flies and isolated body parts [8-11]. Luciferase has a relatively short half-life (about 4 hs in flies as measured in [11]), which is an absolute requirement if it is to report *in vivo* rhythmic transcriptional oscillations.

Transcriptional assays.

To analyze in depth the function of putative transcription factors a reporter construct needs to be generated. Usually it takes the form of a full length promoter or a portion therein where the major target sequences are included, driving the reporter gene selected (i.e. *luciferase*). This native regulatory region can also be replaced by a multimerized version of the core motifs (i.e. the E-box [12]) flanked by the native sequences (from the *per* or *tim* promoters[1] –see Note 2-). These constructs require the addition of a minimal promoter for basal transcription. Most of the circadian reporter constructs derive from the pGL3 basic vector (Promega), which carries the *luc* gene

downstream a multiple cloning site (see Note 3). Likewise, most labs perform reporter assays in *Drosophila* Schneider's cells ([1,4,13,14]); when this is the case, the minimal promoter employed derives from the heat shock protein 70 (*hsp 70*) gene (see Note 4).

To test the ability of a putative transcription factor to activate/repress transcription in cultured cells it has to be subcloned into an expression vector such as pAct5C (Fig. 1), under the *actin* promoter[15]. A more recent version of this vector (pAct5.1, available from Invitrogen) allows cloning in frame with different tags (his, V5), simplifying the subsequent identification of the protein of interest (see Note 5). These type of assays benefit from constitutive and rather strong expression where the *actin* promoter is the one of choice.

As stated earlier it is highly recommended to include a non related transfection control. Originally *β-gal* was employed, but given the disparity between the test conditions for the *β-gal* and *luc* assays, it is increasingly common to use a second *luc* reporter (Renilla *luciferase* under a constitutive promoter such as *copia* or *actin 5C*) which can be assayed together with the firefly one (see Promega's dual reporter assay system).

Additional controls.

When running transcriptional assays a number of controls need to be included.

-transfection efficiency: a second reporter not affected by the experimental condition (discussed earlier in this chapter).

-binding specificity: a mutated version of the regulatory region controlling reporter expression to highlight non-specific binding of the putative transcription factor(s) under analysis.

-baseline reporter activity: determined in the absence of any putative transcriptional regulator; usually an empty pAct5C vector is sufficient to determine the leakiness of the reporter system in use.

Materials

-Schneider's S2 cells.

-*Drosophila* S2 medium supplemented with 10% and 20% FCS and antibiotics (50 units penicillin G and 50µg/ml streptomycin sulfate), filter sterilized (serum containing medium, SCM).

-Serum free S2 medium (SFM).

-Plasmid DNA purification columns (such as Mini or Midipreps from Qiagen).

-Lipid-based reagents: Lipofectin and Cellfectin (Invitrogen) or Effectene (Qiagen).

-Tissue-culture treated Corning (Costar) 6 and 12 well culture clusters, sterile pipets and tips.

-PBS buffer (To make one liter of 10X: 11.5g Na_2HPO_4, 2g KH_2PO_4, 80g NaCl, 2g KCl. The pH of 1X PBS will be 7.4).

-Dual luciferase reporter assay kit (Promega, catalog number E1910).

-Luminometer.

-BCA protein assay reagent kit (Pierce cat #23227).

-ELISA plate reader (or spectrophotometer).

Methods

A more detailed transfection protocol is provided in a previous chapter (7.4). Only a brief overview will be included here.

3.1 Transient transfection employing Lipofectin.

1-Under the laminar flow seed twice as many wells as needed in a 12-well culture cluster (see Note 6), adding 0.8 ml of cells/well at a density of $1x10^6$ cells/ml.

2-Let them sit for 12-24hs.

3-Clean up the plasmid DNAs with Qiagen columns (see Note 7).

The following steps will greatly vary depending on the lipid-reagent chosen; a protocol employing Lipofectin (Invitrogen) will be described in more detail.

4-Right before transfection activate the lipid reagent by diluting it in SFM. Use 8μl lipofectin per well diluted 1/5 in SFM (40 μl in total). Let it sit for 30-45 min at room temperature inside the hood.

5-Prepare the DNA solutions in an equal volume of SFM (40 μl). It is convenient to prepare a DNA mix for each of the reporter vectors to be tested mixed with the internal control (i.e. Promega's pRL). Take into account to prepare 10-20% of extra solution to account for pipeting errors!

Reporter vectors are usually employed at a 0.1-0.2 μg/well. According to the manufacturer's recommendation the transfection control (internal control) should be used at 1:10 (to even 1:50) ratio of the experimental one, to help ensure independent genetic expression (see Note 8). Typically 10-25ng/well of pRLcopia can be employed. Expression vectors alone or in combination need to be tested at a range of concentrations. Potent transcriptional activators such as *dClock* (from a pAct-*dClk* construct) are included at a concentration of 0.5-1 ng/well (see Note 9). On the other hand, *timeless* or *period* are usually tested in a wider range of concentrations (10ng/well as stated in Darlington *et al.*, 1998; 10, 100, 600 ng/well as reported by Chang &

Reppert (2003); 10 and 50 ng/well as reported by Weber & Kay (2003). It is important to keep the total amount of DNA/well constant (see Note 10).

6-Combine the lipofectin and plasmid solution(s) and let it stand for 10 min at room temperature. In the meantime get a sterile cotton-plugged Pasteur pipet ready in an aspirator. Since Lipofectin is inhibited by serum, the SCM in each well needs to be removed prior to transfection (see Note 11). Keep in mind the S2 cells do not adhere tightly to the plastic surface so be careful to avoid discarding some of them in the process.

7-Dilute the lipid-DNA complexes up to 400 μl/well in SFM and quickly add to each well drop wise.

8-Let the transfection proceed for a minimum of 8hs (typically overnight) and then add an equal volume (400 μl/well) of 20% FCS.

9-Cover with parafilm. Place in an incubator (or quiet drawer) at room temperature (22-25^{o}C).

10-A time course is recommended to determine the optimal harvesting time. Usually the cells are collected 24-48h after transfection.

3.2 Harvesting the S2 cells

1-Resuspend the S2 cells by gently pipeting up and down with a P1000 micropipette, and transfer to an Eppendorf tube. If too many cells were left in the well, add PBS and repeat the operation.

2-Spin down at 100g for 2 min.

3-Remove the SCM and add and equal volume of PBS to wash out residual SCM. Resuspend the cells carefully not to break them open.

4-Spin down at 100g for 2 min and remove most of PBS.

5-Add 250 μl of freshly diluted Passive Lysis Buffer (Promega), gently resuspend the cells and keep them for 15 min at room temperature before vortexing to break the cells open (see Note 12). The efficiency of this operation can be enhanced by a freeze-thaw cycle.

6-Take 1mλ of each lysate to determine firefly and *Renilla* LUC activity according to the manufacturer's recommendations, and keep the remaining at –80°C. Subjecting cell lysates to more than 2–3 freeze-thaw cycles may result in gradual loss of luciferase reporter enzyme activities.

3.3 Performing firefly and *Renilla* LUC assays

1-Add 10μl prepared cell lysate to 50μl of Luciferase Assay Reagent II (Promega) predispensed into luminometer tubes; LAR II is light sensitive so prepare only as many tubes as needed; mix by pipeting up and down 2-3 times (avoid vortexing) and initiate reading.

2-Quantify firefly luciferase activity.

3-Add 50μl of Stop & Glo Reagent (Promega) and quickly mix (see Note 13).

4-Quantitate *Renilla* luciferase activity.

3.4 Normalization against protein content

1-Prepare the BSA standard curve following manufacturer's recommendation.

2-To perform this quantitation in microtiter plates (96 wells) dispense 250μl of Pierce reagent/well. Keep in mind the reagent is light sensitive, so only prepare as many wells as needed for the calibration curve and samples.

3-A calibration curve needs to be included in each determination (within the same microtiter plate). Add 10μl of each of the BSA standards together with 5μl of PLB to the Pierce reagent dispensed (see Notes 14 and 15).

4-To determine the protein concentration in the transfected samples add 5μl of each lysate (diluted in 10μl of deionized water) to the Pierce reagent.

5-Take the microtiter plate to an ELISA reader setting the filter to 562nm (wavelenghts between 540 and 590 can be successfully used).

Notes

1-In some instances the normalization reporter does not stay constant throughout the experiment; instead, it appears to follow the same trend as the experimental control. Under those circumstances normalization against total protein is recommended to avoid diluting the experimental effect, although it is advisable to resort to a different normalization reporter for future experiments. Total protein can be determined using a modified version of Bradford's (BCA protein reagent kit, see Note 14).

2-Each multimer reporter consisted of four 18-bp elements containing the E box from either the *per* or the *tim* promoters. The multimers were made piecing together two 50-mers containing half of the multimer each. The core sequence included the 6 nt palindrome (GAGCTC) flanqued on each side by the 6 naturally occurring nucleotides; novel sequences introduced (between each monomer, 6nt) were chosen so that they

would not match with the corresponding ones in the native promoters. Each fragment was first annealed to its complementary sequence, and then ligated to the other half. A novel restriction site was created in the hinge to simplify the screening process. Additionally, two different restriction sites on each end were introduced to allow for directional cloning into the reporter vector (pGL3hs).

3-To test for binding specificity it is convenient to generate another reporter construct carrying mutations within the consensus site. The mutated E box contained the central two nucleotides swapped (CG instead of GC).

4-When employing mammalian cells conditions will vary greatly depending on whether they adhere or not to the plastic surface; follow manufacturer's recommendation with regard to the ideal plating density depending on the lipid reagent used. Other aspects worth mentioning are: mammalian cells grow at 37^{o}C and 5% CO2; they require a more refined sterile practice. Mammalian tissue culture is carried out under a biosafety hood. Promega has different series of firefly and *Renilla* luciferase vectors that may be used to co-transfect mammalian cells with any experimental and control reporter genes.

5-As biological observations may vary to a certain extent depending on the levels of expression accomplished (compare [1] to [16]) it is recommended to setup the conditions for the constructs in hand and perform dose response curves at a number of concentrations (see text).

6-Running transcriptional assays in duplicate is recommended, although this repetition should not be taken as a statistically meaningful one.

7-Plasmid DNA used for transfection should be of high quality. Impurities present in the DNA preparations may lower the transfection efficiency. DNA should be purified

employing Qiagen columns or similar. Ideally, final concentration should be higher than 0.1-0.2 μg/ml, to reduce the amount of impurities present during the transfection.

8-The extreme sensitivity of both firefly and *Renilla* luciferase assays, and the very large linear range of luminometers (typically 5–6 orders of magnitude), allows accurate measurement of both reporters. The advantage is that it is possible to add relatively small quantities of a control reporter vector which will aid greatly in suppressing the occurrence of *trans* effects between promoter elements.

9- The cognate CLK partner (CYCLE/ dBMAL) does not need to be included since it was reported to be expressed in naive Schneider cells (K. Wager-Smith & S. Kay, unpublished results).

10- Usually this is accomplished employing the empty expression vector (i.e. pAct5C).

11- To avoid excessive dissecation that will be detrimental to cell viability remove SCM from a maximum of 4 wells at a time.

12- Generally, it is unnecessary to clear lysates of residual cell debris prior to performing the LUC assays. However, if subsequent protein determinations are to be made clear the lysate samples for 30 seconds by centrifugation in a refrigerated microcentrifuge. Transfer the cleared lysates to a fresh tube prior to reporter enzyme analyses.

13- Quenching of firefly luciferase luminescence and concomitant activation of *Renilla* luciferase are accomplished by adding this reagent to the sample tube immediately after quantitation of the firefly luciferase reaction.

14- Certain chemicals present in the PLB may interfere with the colorimetric reaction in a concentration dependent manner. When performing this reaction in microtiter plates it is advisable to use a small volume (5-20μl) of cell lysate and to include an equivalent volume of PLB in the calibration curve.

15- In the microtiter plate format the BCA kit works in the 20-1500μg/ml range.

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Legend to Figure 1. Original version of pAct5C.
Additional description: p*actin*, *actin* promoter region; *actin* pA, poly adenilation signals from the *actin* locus. *Ampicillin*, gene conferring ampicillin resistance in *E coli*; pBR322 ori, origin of replication in *E coli* derived from plasmid pBR322. Arrows indicate the direction of transcription. Asteriscs highlight single cutters.