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In vivo processing assay based on dual luciferase reporter system to evaluate DROSHA enzymatic activity

Summary

Luciferase reporter assays are widely used to study promoter activity, transcription factors, intracellular signaling, protein interactions (Jia et al., 2011), mRNA processing (Allegra & Mertens, 2011) and targeting (Jin, Chen, Liu, & Zhou, 2013). Here we demonstrate the use of a dual luciferase reporter system to evaluate enzymatic activity of a key enzyme involved in RNA maturation - DROSHA. It is a simple and fast method to quantitatively detect DROSHA processing activity in live cells.

Keywords: microRNA, Luciferase, miRNA processing assay, DROSHA, Ribonuclease III

1. Introduction

A dual reporter assay using firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*, also known as sea pansy) luciferases allows improving experimental accuracy by normalizing the results and reducing technical error. Firefly and Renilla luciferases do not require posttranslational modifications and function as a reporter immediately after translation. Secondly, these two luciferases have dissimilar substrate requirements making it possible to measure them subsequently in a single sample (Bronstein, 1994). In such a dual reporter assay Renilla luciferase is normally used as an internal control to normalize for experimental variation (Stables et al., 1999). We developed a protocol that applies a dual luciferase assay to measure enzymatic activity of DROSHA enzyme.

DROSHA is a member of ribonuclease III family that catalyzes the cleavage of primary long miRNA transcript (pri-miRNA) to its precursor miRNA form. The process is critical for miRNA maturation except for a small subgroup of miRNA genes where this step is omitted (Winter, Jung, Keller, Gregory, & Diederichs, 2009). DROSHA has two RNase III domains and one double-stranded RNA binding domain. The recognition of miRNA targets occurs with the help of cofactors. The main cofactor is DGCR8 (also known as Pasha) that form a microprocessor complex together with DROSHA.

It is believed that DROSHA activity is not sequence specific, but instead it recognizes RNA structure. Pri-miRNA have a hairpin structure with a large, unstructured terminal loop and a stem of less than 40 bp and more than 26 bp in length (Zeng, Yi, & Cullen, 2005). It was shown that DROSHA preferentially cleaves 11 bp away from free ends of pri-mRNA at the base of the hairpin stem (Han et al., 2006).

Here we used that recognition feature of DROSHA enzyme to evaluate its enzymatic activity. As an exemplary pri-miR we used pri-16-1 because its recognition and processing by the microprocessor complex have been extensively studied. We constructed 2 plasmids: one containing the hairpin of pri-miRNA-16-1 including 25 bp on each side cloned to the 3'UTR of firefly gene and a second control

plasmid containing the identical sequence of pri-miRNA-16-1 with 4 point mutations introduced into the stem of the hairpin. These mutations destabilize the hairpin structure so that the control plasmid cannot be recognized and cleaved by DROSHA and can therefore serve as a normalization control for sequence dependent and independent effects of the 3'UTR. (Allegra & Mertens, 2011).

2. Materials

2.1. Cell culture

Medium should be stored at 4°C and warmed up to 37°C before use. We do not add penicillin to the medium.

1. Dulbecco's modified eagle medium (DMEM, Life technologies, Germany)
2. Fetal bovine serum (FBS, PAN Biotech, Germany)
3. 1x PBS (Biochrome AG, Germany).
4. 5x Trypsine (). 1x solution in PBS store at 4°C.
5. HEK293T cells (DSMZ, Germany, CRL-1573) maintained in DMEM supplemented with 10% FBS at 37°C with 10% CO_2 in 75cm^2 flasks.

2.2. Transfection reagents

6. TransIT®-LT1 Transfection Reagent (Mirus Bio LLC, USA). Store at -20.

2.3. Plasmids

1. pMir-Report Luciferase (Ambion, Austin, USA)
2. pSuper (Oligoengine)
3. pRL-CMV (Promega Inc., USA)
4. pMir-Report Luciferase pri-miR-16-1wt (amplified from genomic DNA with primers (see 2.5) and cloned with Hind III into pMir-Report Luciferase)
5. pMir-Report Luciferase pri-miR-16-1ss (mutated using primers (see 2.5) and cloned with Hind III into pMir-Report Luciferase)
6. pSuper_shEGFP (synthetic oligos (see 2.4) were cloned between the BglII and HindIII sites of pSuper plasmid, Oligoengine)
7. pSuper_shDROSHA (synthetic oligos (see 2.4) were cloned between the BglII and HindIII sites of pSuper plasmid, Oligoengine)

2.4. Oligoes for shRNA plasmids

Name	Sequence (Oligoengine)
shDROSHA_s	5'-GATCCCCGCGAGTAGGCTTCGTGACTTATATGACACCTGACCCATGTCATATAA GTCACGAAGCCTACTCGTTTTTA-3'
shDROSHA_as	5'-AGCTTAAAAACGAGTAGGCTTCGTGACTTATATGACATGGGTCAGGTGTCATAT AAGTCACGAAGCCTACTCGCGGG-3'
shEGFP_s	5'-GATCCCCGCTGACCCTGAAGTTCATCTTCAAGAGAGATGAACTTCAGGGTCAGC TTTTTA-

	3'
shEGFP_as	5'-AGCTTAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAACTTCAGGGTCA GCGGG-3'

2.5. Primers for cloning

Primer name	Sequence
pri-miR-16-1 FOR	5'- TGATAGCAATGTCAGCAGTG-3'
pri-miR-16-1 REV	5'- TGGTCAACCTTACTTCAGCA-3'
Mutagenesis	
pri-miR-16-1 ss_f	5'-TACCACCAGGTAAAAATTGGCGTTAAGATTCTAAAATTATC TCC-3'
pri-miR-16-1 ss_r	5'-CGCCAATTTTTACCTGGTGGTAAGGCACTGCTGACATTGCT-3'

2.6. In vivo processing assay

Luciferase buffers should be aliquot and stored at -20 °C. Repeated freezing-thawing cycles of these buffers may decrease assay performance.

1 5X Passive Lysis Buffer (Promega). Store at -20°C. 1x solution in ddH2O can be stored at 4°C for up to one month.
2 Firefly buffer pH(8.0): 25 mM glycylglycine, 15 mM Kx PO4 (pH 8.0), 4mM EGTA, 2mM ATP, 1mM DTT, 15mM MgSO4. 0.1mM CoA, 75µl luciferin. Note 1 and 2
3 Renilla buffer pH(5.0): 1,1 M NaCl, 2,2 mM Na2EDTA, 0,22M KxPO4 (pH 5.1), 0,44 mg/mL BSA, 1,3 mM NaN3, 1,43 µM coelenterazin
4 Luminometer GloMax 96 (Promega, Mannheim, Germany).

3. Methods

3.1 Transfection of adherent cells

3.1.1 Knowdown of DROSHA using shRNA

1 One day before transfection seed the cells into two 75cm^2 flasks. Cell density should reach 50-80% confluence on the day of transfection.
2 The day of transfection, replace the medium with 9 ml of complete growth medium.
3 Allow the transfection reagent to warm up to room temperature. Pre-warm serum-free DMEM medium to 37°C.
4 For each flask to be transfected prepare a *transfection mix* by mixing 8µg pSUPER-shEGFP or 8µg pSUPER-shDROSHA with 980µl serum-free DMEM. Add 20µl TransIT-LT1 transfection reagent, mix well and incubate for 20 min at room temperature. **Note 3**

5 Dropewise add 1 ml of transfection complexes to the cells and shake gently by rocking the plate back and forth.
6 Four day after transfection proceed with *in vivo* assay **Note 4**. Complexes do not have to be removed following transfection. **Note 5**

3.1.2 *In vivo* assay transfection

1 One day before transfection seed the cells into 12 well plates. Cell density should reach 50-80% confluence on the day of transfection.
2 The day of transfection, replace the medium with 1 ml of complete growth medium.
1 For each well to be transfected prepare *transfection mix* by mixing 200 ng Renilla+100_ng pMirLuc16-2ss +1µg_pSuper-shEGFP in 93,5µl serum-free DMEM **Note 6**. Add 4µl TransIT-LT1 transfection reagent, mix well and incubate for 20 min at room temperature.
2 Dropewise add 100µl of transfection mix to the cells and shake gently by rocking the plate back and forth.
3 Assay luciferase signal 18-24 hours after transfection.

3.2 Cell lysis

1 Prepare proper amount of 1x Passive lysis buffer by diluting 1part of 5x Passive lysis buffer (keep on ice) in 4 parts of ddH2O. **Note 7**
2 Gently remove the medium from the cell culture wells and rinse cells with 500µl 1xPBS.
3 Add 100µl of 1x Passive lysis buffer and shake at room temperature for 10 min.
4 Transfere the lysate to a 1.5-ml microcentrifuge tube and spin down the cells at 13000 rpm at room temperature for 1 min to remove cell debris.
5 Transfer a supernatant into a new 1.5-ml microcentrifuge tube and keep the samples on ice before measurement. **Note 8**
6 Pipet 15µl of each sample into 96 well plate. **Note 9**

3.3 Measurment of luciferase activity

5 Thaw proper amount of buffers referring to 100µl buffer/well. Let them warm to room temperature. Always mix the reagent prior to use because thawing generates density and composition gradients. **Note 10, 11**
6 For measurement we use a luminometer with two automatic injectors. The following parameters are set in our instrument settings: injection volume of buffer 75µl with 5 sec delay between injection and measurement. **Note 12**
7 Depending on the luminometer used it might be necessary to prime auto-injector systems with buffers before measurement.
8 Record the firefly luciferase activity measurement by adding FF buffer to the well.
9 Record the Renilla luciferase activity measurement by adding Renilla buffer to the well.

3.4 Evaluation of results

1 Luciferase assay results are normalized as follows:

$$\text{Normalized activity} = \frac{\text{Firefly Luciferase activity}}{\text{Renilla Luciferase activity}}$$

2 The processing efficiency of pri-miRNA construct can be calculated:

$$\text{pri} - \text{miRNA processing efficiency} = 1 - \frac{\text{normalized activity of wt construct}}{\text{normalized activity of ss construct}}$$

In this calculation the processing efficiency of ss construct will be equal to o.

Notes

1. DTT is highly unstable in water solutions, aliquote and store at -20°C, thaw the aliquots on ice.
2. The addition of Coenzyme A allows a more sustained plateau of activity facilitating subsequent analysis. CoA in micromolar concentrations produces a more intense and sustained light emission because of the more favorable kinetics of reaction of the enzyme with the luciferyl CoA substrate. In the presence of CoA, the luciferase assay yields stabilized luminescence signals with significantly greater intensities
3. Transfection reagent should be added at the end of master mix preparation.
4. The time estimates can vary for different cell lines. The optimization of the protocol should be performed before starting the experiments.
5. Depending on the reagent used for transfection it might be necessary to change the medium at certain time point. Follow the manufactory instructions when transecting the cells with different reagent.
6. The amount of the plasmids and reagent used for the experiments needs to be optimized for every cell type.
7. Presence of detergents in the lysis buffer (i.e. TritonX) is another method to increase the intensity and duration of light emission. An inherent property of coelenterazine is that it emits low-level autoluminescence in aqueous solutions. Originally this drawback prevented sensitive determinations at the lower end of enzyme concentration. Additionally, some types of nonionic detergents commonly used to prepare cell lysates (e.g., Triton® X-100) greatly intensify coelenterazine autoluminescence.
8. The stability of FF luc have been shown to be 6 weeks at 4°C in cell lysates.
9. The amount of extract required may vary depending on the luciferase expression level and the instrumentation used; the amount used should be adjusted to keep the signal within the linear range of the assay.
10. At low pH the wavelength of emmited light from FF reaction is shifted into the red region of spectrum.
11. Control of temperature is also important since the use of nonequilibrated reagents directly from the refrigerator can cause a 5-10% decrease in efficiency.
12. Depending on the luminometer used it might be necessary to to prime auto-injector systems with buffers. Calculate additional volume of the buffers for this aim.

Abbreviations

DTT - Dithiothreitol;

BSA - bovine serum albumin;

CoA - Coenzyme A;

ss – single stranded

Wt – wild type

PBS - Phosphate buffered saline

FF – firefly

DMEM - Dulbecco's modified eagle medium

Pri-miRNA – primary microRNA

DGCR8 - DiGeorge critical region 8 protein

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