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Hypoxia Reporter Element Assay

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[Abstract] Hypoxia is a condition in which there is a decrease in oxygen supply to the cellular environment. Changes to cellular oxygen levels can lead to transcriptional changes of oxygen-regulated genes. Reporter assays are used to study gene expression alteration and modifications in response to environmental changes. Dual-reporter assays allow the simultaneous measurement of two different genes within a single cell, thus improving experimental accuracy. Within this protocol, we describe the utilization of the LightSwitch Dual Assay System to measure BMX expression in response to hypoxic conditions.

Keywords: Luciferase, Reporter assay, Hypoxia, Regulation, Transcription

[Background] In our recent publication (van Oosterwijk *et al.*, 2018), we sought to examine the regulation of BMX, a nonreceptor tyrosine kinase, in response to sorafenib treatment. BMX, a Tec kinase family member, is known bind to tyrosine-phosphorylated proteins and mediate membrane targeting by binding to phosphatidylinositol 3,4,5-triphosphate (PIP3; Chen *et al.*, 2013). We showed that direct treatment of sorafenib in acute myeloid leukemia (AML) cells lines with or without stromal cell support did not contribute to the upregulation of BMX. Previous studies have shown that BMX expression can be induced by ischemia and that sorafenib has antiangiogenic activity (He *et al.*, 2006; Davis *et al.*, 2011). Therefore, we hypothesized that the antiangiogenic activity of sorafenib causes a hypoxic environment within the bone marrow, thus contributing to a hypoxia-dependent BMX upregulation in AML. Under hypoxic conditions, we were able to show a significant increase in BMX expression in a number of different cell lines. Further analysis of the BMX promoter identified a putative hypoxia-responsive element (HRE; 5'-ACGTG-3') at -5005. To test whether this HRE was involved in the hypoxia-induced promoter activation of BMX, we developed a hypoxia element reporter assay.

Reporter assays are extensively used throughout the scientific community to study alterations and modifications to gene expression in response to environmental changes. One type of reporter assay that is gaining in acceptance are the dual-reporter assays. This type of reporter assay allows the simultaneous expression and measurement of two different reporters within a single cell and had been widely proven to improve experimental accuracy by reducing extraneous influences. One such dual assay system is the LightSwitch Dual Assay System. This system utilizes the RenSP luciferase reporter gene, a novel luciferase developed by SwitchGear Genomics and the Cypridina luciferase reporter gene. RenSP and Cypridina employ different substrates, thus eliminating cross-reaction between proteins and

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their substrates. RenSP is used with your favorite gene as the reporter signal, and Cypridina is utilized with a constitutively active promoter as the control signal.

Here, we used the LightSwitch Dual Assay System to evaluate the involvement of the BMX HRE to its upregulation in response to hypoxic conditions.

Materials and Reagents

1. Pipette tips
2. White 96 well plates (CELLSTAR® Tissue Culture Plates, Greiner Bio One International, catalog number: 655083)
3. HEK293 (293[HEK-293]; ATCC, catalog number: CRL-1573)
4. pLightswitch_Prom_BMX (BMX promoter construct; SwitchGear Genomics, catalog number: S701154)
5. pLightswitch_Prom control vector (SwitchGear Genomics, catalog number: S790005)
6. pTK-Cluc (Cypridina TK control construct; SwitchGear Genomics, catalog number: SN0322S)
7. FuGENE® HD (Promega, catalog number: E2311)
8. DMEM, high glucose, pyruvate (Thermo Fisher Scientific, Gibco™, catalog number: 11995065)
9. QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, catalog number: 200516)
10. Terrific Broth Powder (BD, Difco™, catalog number: 243820)
11. Glycerol (Sigma-Aldrich, catalog number: G9012)
12. Poly-D-Lysine (Sigma-Aldrich, catalog number: P6407-5MG)
13. Opti-MEM (Thermo Fisher Scientific, Gibco™, catalog number: 31985070)
14. Fetal Bovine Serum (Thermo Fisher Scientific, Gibco™, catalog number: 10437028)
15. LightSwitch Dual Assay System (SwitchGear Genomics, catalog number: DA010)
16. Poly-D-Lysine hydrobromide (MP Biomedicals, catalog number: 02150175)
17. Terrific Broth (see Recipes)
18. 5x Poly-D-lysine Solution (see Recipes)
19. 1x Poly-D-lysine Solution (sterile) (see Recipes)
20. Growth media (see Recipes)

Equipment

1. Pipettes
2. Thermocycler (Eppendorf, model: Mastercycler® pro S, catalog number: 950030020)
3. Table-top centrifuge (Eppendorf, model: 5810 R, catalog number: 022625101)
4. Swinging bucket rotor (Eppendorf, model: S-4-104, catalog number: 5820755008)
5. High-performance centrifuge (Thermo Fisher Scientific, model: Sorvall™ RC 6 Plus, catalog number: 36-101-0816)

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6. Laminar flow hood (Thermo Fisher Scientific, model: 1300 Series Class II, Type A2, catalog number: 1323TS)
7. 37 °C, 5% CO_2 water-jacketed incubator (Thermo Fisher Scientific, model: Heracell VIOS 160i, catalog number: 51030285)
8. 37 °C non-humidified incubator (Labnet International, model: 311DS, catalog number: I5311-DS)
9. Hypoxia chamber (Coy Lab, model: O_2 Control InVitro Glove Box with no upgrades)
   Standard features include:
   a. Humidified incubation box
   b. Temperature control
   c. Control of O_2 and CO_2 in 0.1% increments
10. Plate luminometer (Molecular Devices, model: SpectraMax i3x, with no upgrades)
    Standard features include:
    a. Read modes: Absorbance, Fluorescence, Luminescence
    b. Wavelength ranges include: Abs: 230-1,000 nm, Fl Ex: 250-830 nm, Fl EM 270-850 nm, Lumi: 300-850 nm

Software

1. GraphPad Prism (GraphPad Software, Version 6)
2. QuikChange Primer Design (https://www.genomics.agilent.com/primerDesignProgram.jsp)

Procedure

A. Site-directed mutagenesis of pLightswitch_Prom_BMX to mutate the putative HRE within in the BMX promoter
   1. Primers are designed to mutate 2 base pairs (ACGTG → ATATG) within the HRE of the BMX promoter using the QuikChange Primer Design website (referenced above).
      The CG →TA bp change has been described in the literature to effectively disrupt the HRE.
   2. pLightSwitch_Prom_BMX is mutated using the QuikChangeXL Site-Directed Mutagenesis kit per manufacturer's protocol.
      a. Extension time: 4.5 min
      b. Substitute Difco™ Terrific Broth for NZY+ broth
   3. Mutated plasmids are confirmed by sequencing using promoter insert sequencing primers (Forward: 5'-TCCATCAAAACAAAACGAAACAA-3', Reverse: 5'-AGTCGAGCACGTTCATCTGCTT-3') listed on the Switchgear Genomics website (http://switchgeargenomics.com/resources/vector-maps).

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B. Hypoxia reporter assay (LightSwitch Dual Assay System Manual)

Day 1 (Carry out under a Laminar flow hood)

1. Coat two white 96-well plates with 1x Poly-D-lysine (50 µl/well) for 2 h at room temperature.
   a. Plate enough wells per plate to carry out in triplicate.
   b. Example samples:
      i. Empty vector (pLightSwitch_Prom) + pTK-Cluc
      ii. Wild-type Bmx promoter (pLightSwitch_Prom_BMX) + pTK-Cluc
      iii. HRE mutated BMX promoter (pLightSwitch_Prom_BMXmHRE) + pTK-Cluc
   c. Use one plate per condition (*i.e.*, 2 plates for comparison normoxia to hypoxia).
2. Wash the plates once with sterile H_2O (200 µl/well); plate 5 x 10^3 HEK293 cells per well in 100 µl of growth media and cultured overnight at 37 °C in 5% CO_2.

Day 2

Preparation of constructs and reagents

1. Allow all reagents and plasmids to equilibrate to room temperature.
   Note: Plasmids should be purified using a kit or protocol that will remove endotoxin, such as PureYeild Plasmid System from Promega.
2. Pre-warm Opti-MEM to 37 °C.

Transfection (Carry out under a Laminar flow hood)

1. Carry out transfections according to protocol details (LightSwitch Dual Assay System; link to protocol found above) for a 96-well plate with minimal changes.
   Cypridina TK control construct, pTK-Cluc: 1 ng (One nanogram is used instead of 10 ng due to luciferase saturation)
   pLightSwitch_Prom, pLightSwitch_Prom_BMX or pLightSwitch_Prom_BMXmHRE: 30 ng
   The ratio of FuGENE® HD to plasmid: 3:1 (0.3 µl FuGENE:100 ng DNA per well)
2. Briefly, the transfection protocol is:
   a. Combine reagents as indicated in the following table and mix well.

Reagents	Amounts
FuGENE® HD Transfection Reagent	0.09 µl
Opti-MEM	2.24 µl
pLightSwitch constructs (30 ng/µl)	1.67 µl
pTK-Cluc (1 ng/µl)	1 µl
Total	**5 µl**

   b. Let sit at RT for 30 min.
   c. Gently drop onto seeded wells.
   d. Gently shake.
   e. Plates should be incubated at 37 °C for 24 h in normoxia (5% CO_2).

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Day 3 (Under a Laminar flow hood)

1. Remove the transfection reagents by changing out the media on both plates.
2. Transfer one plate into hypoxia (5% CO_2, 94% N_2, and 1% O_2) at 37 °C and maintain another one in normoxia.
3. Incubate both plates for an additional 24 h.
   Note: Incubation time can be varied depending on the induction rate of your favorite gene due to hypoxia.

Day 4

Follow the manufacturer's instructions (LightSwitch Dual Assay System) for measuring Renilla (pLightSwitch_Prom vectors) and Cypridina (pTK-Cluc) luciferase activity.

1. No alterations were made to the protocol detailed by SwitchGear Genomics. Plates were always measured fresh and never frozen.
2. For hypoxia conditions (all done within the hypoxia chamber):
   a. Adding reagents and incubations are all done within the hypoxia chamber.
   b. Remove the plates from hypoxia and immediately read the luminescence on a plate reader.
3. For normoxia conditions: Adding reagents and incubation are all done at room temperature on the bench top.

Data analysis

1. Renilla (pLightSwitch_Prom) luciferase activity is normalized to Cypridina (pTK-Cluc) luciferase activity and adjusted for background luciferase for each condition.
2. Data can then be graphed as relative luciferase activity or as fold change. (see Figure 1)
   Note: If fold change is determined, the comparison should be to the wild-type promoter under normoxia conditions.

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Raw Data					
Promoter Construct (Renilla; values x10^4)					
	WT			Mut	
Replicates	Normoxia	Hypoxia		Normoxia	Hypoxia
1	1.362	7.048		1.863	2.443
2	1.296	6.309		1.617	2.564
3	1.486	7.770		1.623	2.788
Average	1.382	7.043		1.701	2.598
Control Construct (Cypridina TK; values x10^4)					
	WT			Mut	
Replicates	Normoxia	Hypoxia		Normoxia	Hypoxia
1	3.629	2.834		3.788	3.372
2	3.017	3.928		3.263	3.841
3	3.370	3.030		3.560	3.403
Average	3.339	3.264		3.537	3.539
Calculated data					
Calculation =Renilla/Cypridina*10					
	WT			Mut	
Replicates	Normoxia	Hypoxia		Normoxia	Hypoxia
1	3.754	24.871		4.917	7.245
2	4.295	16.062		4.955	6.675
3	4.411	25.647		4.560	8.192
Average	4.153	22.193		4.811	7.371
Final Normalized Data					
Fold Change to WT Normoxia Average					
	WT			Mut	
Replicates	Normoxia	Hypoxia		Normoxia	Hypoxia
1	0.904	5.988		1.184	1.744
2	1.034	3.867		1.193	1.607
3	1.062	6.175		1.098	1.972
Average	1.000	5.344		1.158	1.775

Figure 1. Example data

Notes

While there may be variation in transfection efficiency from experiment to experiment, the LightSwitch Dual Assay system is designed to improve experimental accuracy by reducing extraneous influences. Thus your reproducibility and variability should be minimal after the Renilla luciferase activity has been normalized to the Cypridina luciferase activity.

Recipes

1. Terrific Broth
   47.6 g Terrific Broth powder
   4 ml glycerol
   1 L H_2O

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Autoclaved at 121 °C for 15 min

2. 5x Poly-D-lysine Solution
   Dissolve 5 mg Poly-D-lysine in 1 L ddH_2O
3. 1x Poly-D-lysine Solution (Sterile)
   Dilute 200 ml 5x Poly-D-Lysine solution w with 800 ml ddH_2O and filter sterilize
4. Growth media
   DMEM
   10% FBS

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