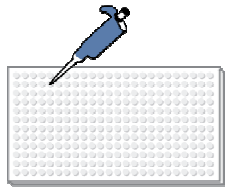
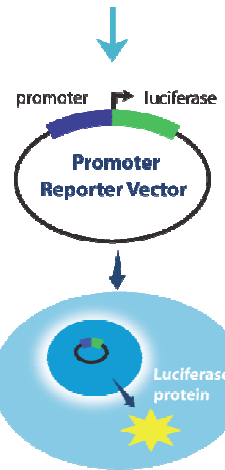


PROMOTER CONSTRUCTS: SAMPLE PROTOCOL FOR ADHERENT CELLS WITH TIPS FOR NF-KB PATHWAY

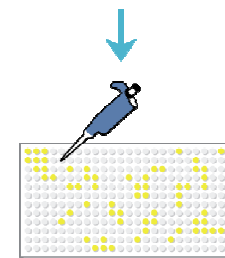
SwitchGear promoter reporter assay workflow



Step 1: Seed cells in plate format



Step 2: Transfect SwitchGear reporter constructs into the cells. Apply stimulus of interest.



Step 3: Add luciferase reagent and read on luminometer.

All SwitchGear reporter constructs utilize luc2P, a destabilized form of luciferase developed by Promega for its reporter gene assays. The optimized luciferase protein has a half-life of ~1 hour enabling a detailed analysis of kinetic responses with a highly robust signal.

DAY 1

Goal: Seed cells to yield 40-80% confluence after 24 hours

- Seed the appropriate number of cells for the assay (see Table 1).
- Seed the appropriate number of cells for assessing desired confluence in parallel (see Table 1).
 - Note: Use a 96- or 384-well white plate for the assay plate. Use a clear plate to assess confluency.

Tip: See specific recommendations for NF- κ B Pathway Promoters in Appendix.

Table 1

Number of wells in plate	Total volume for cell seeding*	Number of cells/well that yields target confluency of 40-80%*
96	100 μ l	5,000-10,000
384	30 μ L	2,000-5,000

* The recommendations in Table 1 were optimized using Fugene-6 (Roche) transfection reagents. Consult other manufacturer recommendations for optimal cell seeding conditions for target confluence.

DAY 2

Goal: Prepare reporter constructs and transfect into the seeded cells

Prepare constructs

- Thaw SwitchGear constructs (plasmid DNA) at room temperature. Mix well.
- Centrifuge the tubes or plates to remove condensation from lid.

Transfections

- Combine the reagents in Table 2 for each transfection. . Conduct at least three replicate transfections per construct in each condition (e.g. untreated and treated). Volumes listed in the table are for a single replicate transfection in a single well. We recommend at least 50ng of plasmid DNA per well for 96-well experiments and 30ng of plasmid DNA per well for 384-well experiments.

Tip: First, make Master Mix 1 containing enough transfection reagent and OptiMEM (in the appropriate ratios) for addition to all construct wells. (Add transfection reagent to OptiMEM without touching the sides of the tube.) Then, add an aliquot of Master Mix 1 to the DNA for each unique construct yielding Master Mix 2 (transfection reagent + OptiMEM + DNA for X replicate transfections). For each Master Mix step, make some extra volume to account for pipetting error and evaporation. See Appendix for the Experimental Design Example.

Table 2

Component	Per well (96-well format)	Per well (384-well format)
Transfection Reagent*	0.30 μ L	0.12 μ L
Opti-MEM (serum free media)	3.03 μ L	1.88 μ L
SwitchGear plasmid DNA construct (30ng/ μ L)	1.67 μ L	1.00 μ L
TOTAL	5.00μL	3.00μL

* Transfection reagents known to work with this protocol include Fugene-6 (Roche), TransIT-LT1 (Mirus), and Arrest-In (Open Biosystems). Other transfection reagents may also work well -- see appropriate manufacturer's instructions.

- Mix DNA, transfection reagent, OptiMEM combination well. Let sit at room temperature for 30-60 minutes.
- Gently drip 5µL or 3µL (96- or 384-well formats) onto seeded cells
- Shake plate gently, cover with lid or breathable sealing tape
- Put back in incubator for 16-24 hours.
- Apply stimulus of interest and return to incubator.

Note: Cells that transfect poorly may require the use of more DNA and/or longer incubation times. We recommend optimizing conditions for each cell line before beginning large-scale experiments.

DAY 3

Goal: Measure luciferase activity

Note: Luciferase assays may be conducted immediately or the plates may be stored at -80. If using frozen (stored) plates, thaw and bring to room temperature before assaying.

- Remove plate from incubator and bring to room temperature
- Add 100µL (96-well format) or 30µL (384-well format) Promega Steady-Glo Luciferase Assay Reagent, cover with lid or foil tape, and incubate for 15-30 minutes in a dark area.
- Read in a plate luminometer.

APPENDIX

Experimental Design Example (for a 96-well plate with triplicate transfections for each construct per condition):

Experimental design example: transfection set-up for hypoxia inductions in 96-well plate format (triplicate transfections for each construct in each condition)

Step 1: Calculations (including extra for pipetting error and evaporation)

Variables: X replicate transfections per construct and Y constructs (experimental + controls)

Here: 9 total replicates per construct (3 replicates per condition * 3 conditions - untreated, hypoxia, DFO)

24 total constructs (16 experimental + 8 controls)

Transfection mix calculations:

	Per replicate well (96-well format)	Per construct (9 replicates + 1.5 extra) x 10.5
Transfection Reagent*	0.30µL	3.15µL
Opti-MEM (serum free media)	3.03µL	31.81µL
SwitchGear plasmid DNA construct (30ng/µL)	1.67µL	17.54µL
TOTAL	5.00µL	52.5µL

Step 2: Make Master Mix #1 – OptiMEM + transfection reagent mix that will be added to each DNA aliquot

	Per construct (9 replicates + 1.5 extra)	Master Mix #1 (24 constructs + 10% extra) x 26.4
Transfection Reagent*	3.15µL	83.16µL
Opti-MEM (serum free media)	31.81µL	839.79µL
SwitchGear plasmid DNA construct (30ng/µL)	17.54µL	Unique to each
TOTAL	52.5µL	922.95µL

Step 3: Make Master Mix #2 – Master Mix #1 + DNA for all replicates of one construct

	Master Mix #2 Per construct (9 replicates + 1.5 extra)
Master Mix #1	34.96µL
SwitchGear plasmid DNA construct (30ng/µL)	17.54µL
TOTAL	52.5µL

Step 4: For each construct, gently drip 5uL of Master Mix #2 onto each of the 9 replicate wells

NF-kb Promoters Suggested Protocol Modifications

Transient transfection assays were conducted in HT1080 cells (ATCC, Manassas, VA) in 96-well white plates. First, 5,000 cells per well were seeded in standard culture medium for 24 hr. Next, 50 ng of reporter plasmid DNA was transfected with Fugene-6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to Fugene standard protocols. After 12 hrs, the transfection medium was removed and fresh culture medium with or without the addition of an inducing compound was added to each well. For the no treatment control, only fresh media was added to the wells. TNFa (Abcam) was added to the media of the treated samples at a final concentration of 20 ng/ml. Cells were incubated for 8 hrs after induction. After 8 hrs, 100uL of Steady-Glo (Promega) was added into each well, incubated at room temperature for 30 min, and then read in a standard plate luminometer (Molecular Devices, Sunnyvale, CA). Each treatment for each reporter construct was assayed in triplicate, and the average of the 3 replicates without treatment and the average of the 3 replicates with treatment were recorded.

EXAMPLE CATALOG NUMBERS

Item	Vendor	Catalog Number
White Tissue Culture Plates (96-well solid bottom)	VWR	82050-736
Clear Tissue Culture Plates (96-well)	VWR	353072
White Tissue Culture Plates (384-well solid bottom)	VWR	82051-278
Clear Tissue Culture Plates (384-well)	VWR	781186
Fugene-6 Transfection Reagent	Roche	11814443001
TransIT-LT1 Transfection Reagent	Mirus	MIR2310s
Arrest-In Transfection Reagent	Open Biosystems	ATR1740
Opti-MEM	Invitrogen	31985-070
Steady-Glo Luciferase Assay Reagent	Promega	E2510, E2520
Foil Plate Sealing Tape	E&K Scientific	T592100
Breathable Plate Sealing Tape	E&K Scientific	T896100-S
	VWR	47749-926
Plate Luminometer	Molecular Devices	LMaxII-384