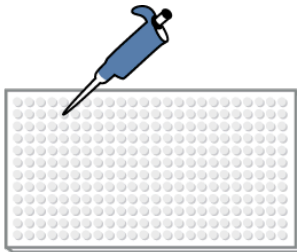
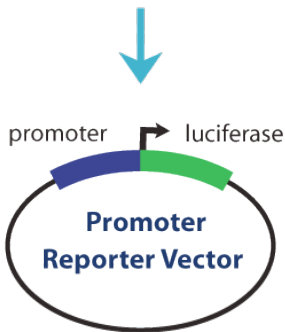


PROMOTER CONSTRUCTS: SAMPLE PROTOCOL FOR HYPOXIC CONDITIONS USING ADHERENT CELLS

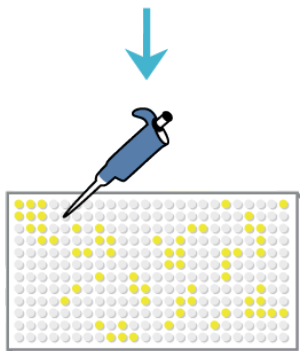
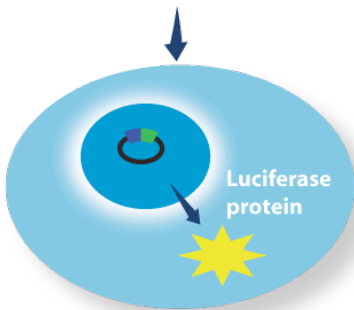
SwitchGear promoter reporter assay workflow



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.

DAY 1

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

- Seed the appropriate number of cells for the assay in a 96- or 384-well white tissue culture plate (see Table 1).
- Seed a few rows of the same number of cells in a clear tissue culture plate for assessing confluence.

Hypoxia experimental tip:
Propagate HT1080 cells in standard media recommended by ATCC. Set up enough replicate transfections for control and treatment conditions.

Table 1

Number of wells in plate	Total volume for cell seeding*	Number of cells/well that yields target confluency of 40-80%*
96	100µl	5,000-10,000
384	30µL	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 and target confluence.

DAY 2

Goal: Prepare reporter constructs and transfect into the seeded cells

Prepare constructs

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

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*	0.30µL	0.12µL
Opti-MEM (serum free media)	3.03µL	1.88µL
SwitchGear plasmid DNA construct (30ng/µL)	1.67µL	1.00µL
TOTAL	5.00µL	3.00µL

* Transfection reagents successfully tested 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. Cover and 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.

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. SwitchGear has optimized these experiments with HT1080 cells.

DAY 3

Goal: Perform treatments

Condition	Media	Treatment
Control	Fresh	Gently remove the transfection media and replace with 100 µL fresh, pre-warmed media. Incubate for 24 hours.
Hypoxia	Fresh	Gently remove the transfection media and replace with 100µL fresh, pre-warmed media. Move plates into hypoxia chamber. Flush with triple gas (1% O ₂ , 5% CO ₂ , 94% N ₂). Allow media in plate to de-gas for 2 hours and then repeat flush. Incubate for 24 hours.
DFO	DFO	Gently remove transfection media and replace with 100 µL fresh, pre-warmed media containing 100µM DFO (final concentration). Incubate for 24 hours.

DAY 4

Goal: Perform luciferase assays

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.

- For hypoxia treatment plates, remove from chamber and expose to normal air for 10 minutes before freezing or assaying.
- 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: 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

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 5µL of Master Mix #2 onto each of the 9 replicate wells

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