Protocol: Kinetic Reporter Gene Assay using the IncuCyte™ FLR or ZOOM

Reporter gene assays continue to be one of the simplest, most robust ways to analyze the activation of transcription factors and their associated signaling pathways. This generic protocol is intended to provide a framework around which one could plan reporter assays that fit any application. In addition to the use of stable clones, there are multiple strategies to transiently deliver a reporter gene into a cell including transfection, electroporation, and viral infection. All of these strategies are acceptable ways to deliver the reporter gene in this assay system. The main requirement is that the reporter gene reagent must fit within our fluorescence excitation/emission specifications of 450-490 nm/500-530 nm (GFP), respectively. A list of reagents and associated companies can be found at the end of this protocol. This list is not intended to be exhaustive, but illustrates the breadth of reagents that are currently available. Of course, multiple GFP based reporter plasmids containing multiple clone sites (MCS) also exist that can be used to clone in the promoter of your choice.

Sample Protocol using plasmid DNA and reverse transfection:

Day 0:

1) Prepare lipid complexes as recommended by the manufacturer of the transfection reagent
   *For example, using Lipofectamine™ 2000 (Invitrogen) with HEK 293 cells**
   a. Dilute 320 ng of reporter plasmid into a total of 25 µl Opti-MEM™ (Invitrogen) media per well of a 96-well plate. This dilution can be prepared in bulk for multiple wells.
   b. Dilute 0.4 µl of Lipofectamine™ 2000 into a total of 25 µl of Opti-MEM™ media per well of a 96-well plate and incubate at room temperature for 5 minutes. This dilution can be prepared in bulk for multiple wells.
   c. Combine the Lipofectamine™ 2000 reagent dilution with the DNA dilution within 30 min.
   d. Incubate at room temperature for 20 minutes to allow DNA-Lipofectamine™ 2000 complexes to form.
   e. Pipette 50 µl of the DNA-Lipofectamine™ 2000 mixture into each well of a 96-well poly-D-Lysine treated tissue culture plate.**

2) Prepare a cell suspension in antibiotic free medium containing serum so that 6×10^4 cells are plated in 100 µl (6×10^5 cells per ml).

3) Add 100 µl of cell suspension to each well containing DNA-Lipofectamine™ 2000 complexes.

4) Incubate at 37ºC in a CO_2 incubator until ready to use in the assay (24-48 h post transfection). It is not necessary to remove the complexes or change the medium. Cells will adhere as usual in the presence of complexes.***
   *NOTES: *Different cell types may require alternative transfection strategies. Preliminary experiments evaluating the optimal transfection protocol should be conducted using a matrix of transfection*

*reagent:DNA ratios per the instructions provided with the transfection reagent of choice. **poly-D-Lysine coated tissue culture plates are recommended when using the reverse transfection strategy. ***You can track the adherence of your cells and monitor cell morphology during transfection using the IncuCyte™ FLR or ZOOM.*

Day 1:

5) Treatment preparation
    a. Prepare treatments and controls in the appropriate growth medium. It may be advisable to serum starve your cells prior to stimulation. This will depend on the reporter assay, and the scientific question. We recommend using medium with low levels of riboflavin to reduce the fluorescence background. EBM, F12-K, and Eagles MEM have low riboflavin (<15 mg/L). DMEM and RPMI have high riboflavin (>30 mg/L).

    b. A volume of 100 µl per well is generally sufficient for the duration of the assay.

6) Add prepared treatments to cells

7) Place the plate within a microplate tray of the IncuCyte™ FLR or ZOOM inside your incubator

8) Set Scan Type to "Fluorescence & Phase-Contrast" if using the IncuCyte™ FLR or select the phase and green channels if using the IncuCyte™ ZOOM

9) Set IncuCyte data acquisition to automatically acquire images every 1-2 hours. At least 2 images per well is recommended
NOTE: A delay of 10-15 minutes before the first scan is recommended to allow the plate sufficient time to equilibrate to the incubator environment. Insufficient equilibration may result in condensation on the bottom of lid compromising image quality.

Sample Protocol using Lentiviral-based reporter infection:

Day 0:

1) Plate cells such they are approximately 60-80% confluent at the time of infection.

2) Infect cells with a pre-determined MOI based on manufacturer's recommendations and preliminary optimization experiments.* This often times will include the use of polybrene or other commercially available reagents that facilitate Lentivirus infection.

3) Incubate at 37°C in a CO_2 incubator until ready to use in assay (24-48 h post transfection).**
*NOTES: * Different cell types may require alternative infection strategies. Preliminary experiments evaluating the optimal infection protocol should be conducted using a matrix of cells:MOI ratios per*

*the instructions provided with the viral reagent. **You can track the adherence of your cells and monitor cell morphology during infection using the IncuCyte™ FLR or ZOOM.*

4) Continue with Day 1 protocol as laid out above.

Ending the assay and data analysis

Assay duration will vary depending on the reporter construct as well as the stimulation. It is recommended to track the experiment's progress by either performing an Open Ended analysis job, which can be initiated after the first scan is complete, when using the IncuCyte™ FLR or ZOOM, or to apply an analysis job at the time of scheduling when using the IncuCyte™ ZOOM. Both the IncuCyte™ FLR and ZOOM will automatically collect and store data until the plate is removed from the instrument, and therefore the "end" of the assay may be determined retroactively. Data analysis is best done using the object counting analysis built into the IncuCyte™ FLR software or the IncuCyte™ ZOOM basic analyzer processing definition, although the specific metric used to measure reporter activity can also vary based on the experimental setup is. Commonly used metrics include Object Confluence, Avg. Object Summed Intensity, and Avg. Object Mean Intensity. For more complete information, see the CellPlayer 96- well Kinetic Reporter Gene Assay Application Note at http://www.essenbioscience.com/reportergene.html.

Reporter Genes and Pathway Summary Table

	Transcription Elements/Promoter Available	
Pathway/Therapeutic Area	System Biosciences	SA Bioscience
C/EBP	C/EBPa	C/EBP
EGR1		EGR1
SP1	SP1	SP1
p53	p53	
PI3K/Akt		FOXO
MAPK/ERK	Elk-1/SRF	Elk-1/SRF
MAPK/JNK	AP-1	AP-1
Wnt	TCF/LEF1 & c-Jun	TCF/LEF
Hypoxia	HIF-1	
PPAR-gamma	PPARy	
cAMP/PKA	CREB	CREB
MEF2	MEF2	
Notch	RBP-Jk	RBP-Jk
TGF-beta	SMAD	SMAD2/SMAD3/SMAD4

NF-κB	NFKB	NFKB
Type I Interferon	ISRE & STAT1	STAT1/STAT2
Interferon Gamma	GAS	
Stem Cells/Nuclear Reprogramming	Oct4 & Nanog	KLF4
PKC/Ca++	NFAT	
Retinoic Acid Receptor	RAR	
Sterol Response	LXRE in SREBP-1c	
Xenobiotic Responses/Toxicity (CYP1 proteins, etc.)		AhR
Antioxidant Responses		Nrf2 & Nrf1
Heavy Metal Stress		MTF1
Oculogenesis	Pax6	

System Biosciences- http://www.systembio.com/lentiviral-technology/transcription-reporter vectors/#product_20_tab_1_1

SA Biosciences – Cignal Reporter Systems – http://www.sabiosciences.com/cellassay.php

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