qPCR assay to test for interferon induction in human cells

This protocol describes a qPCR assay that measures the abundance of endogenous interferon stimulated genes (ISGs) after the addition of exogenous DNA to human cells.

Required
Square plates (Thermo Scientific, cat. no. 166508)

1x Trypsin (Gibco, Life Technologies, Cat No. 25300 054)
1xPBS (Autoclaved)

DMEM (Gibco, Cat no. 52100-047)
+ 10% heat-inactivated FBS (Sigma, Cat No. F7524)
+ 2 mM L-glutamine (Sigma, Cat No. G7513)

Electroporation devise: MaxCyte STX
Electroporation buffer (Cat No. EPB1)
OC-100 100-µL (Cat No. GOC1)

RNeasy Mini Kit (Qiagen, Cat No. 74104)
Qiashredder column (Qiagen, Cat No. 79654)
β-Mercaptoethanol ((Sigma-Aldrich, Cat No. 63689)
Ambion rDNase I & Ambion buffer (Cat No. AM1906)

Oligo dT_{20} (50µM, Thermo Fisher, Cat No. 18418020)
RNase Inhibitor Murine (NEB, Cat No. M0314S)
GoTag Sybr Green Master Mix (2x) (Promega, Cat No. A6001)

pRL Renilla Luciferase Control Reporter Vector (Promega, Cat No. E2261)
pBluescript vector

Transfection by electroporation with the MaxCyte STX transfection system

This protocol describes 1x electroporation (EPO) for adherent cells (HeLa S3 & HCT116). To identify if a cell line has an INF response compare cells electroporated with DNA to cells mock electroporated without DNA. Use independent electroporations as replicates.

Prepare $5x10^6$ cells per electroporation for the day of transfection in square plates. Scale up or down the cell number according to the number of EPOs you want to perform.

- Remove Medium completely
- Wash cells carefully with 8 ml 1xPBS, remove PBS completely
- Add 8 ml 1xTrypsin to the cells to cover the plate completely
- Incubate cells at 37°C until they completely detach
- Add 12 ml Medium to the cells, resuspend and collect them in a 15 ml or 50 ml Falcon tube depending on the number of EPOs
- Count cells and aliquot $5x10^6$ cells per EPO

- Spin cells down, 125 g; 5 min
- Wash pellet once in 5 ml Electroporation (EPO) buffer
- Remove buffer and add ~ 50% of the final volume of EPO buffer to the cells (you need to reach a final volume of 100 µl at the end → see below)
- Prepare a separate tube with a mix of the Renilla and pBluescript plasmids, 1:10 ratio to reach a total of 20 µg → Renilla 2 µg : pbluescript 18 µg
- Resuspend the cells and add them to the plasmid DNA mix (be careful not to introduce air bubbles)
    - (Plasmid concentration should be > 1 µg/µl for all used plasmids)
    - For the control samples do not add DNA to the cells
- Measure volume of cells after addition of the plasmid DNA, fill up with EPO buffer to a total of 100 µl
- Add 100 µl of the cell suspension into 1x OC100 MaxCyte cuvette and use default settings for electroporation (e.g. Hela or HCT 116) or Optimization protocols for cell lines if no pre-set protocol is available (test before, which optimization protocol fits best for your cell line).
- Transfer the 100 µl of cell suspension immediately to a 1x75 pre-warmed flask without adding medium *(cells recover without the addition of any medium)*
- Incubate cells @ 37°C; 30min.
- Add an appropriate amount of pre-warmed medium without antibiotics to the cells, resuspend and plate at $1x10^6$ cells per well in a pre-warmed 6-well plate.

Harvesting after electroporation

- Harvest the cells 6h after addition of media
- For each well, harvest the medium of the cells (20% of cells are still not fully attached to the plates) in a 15ml Falcon tube
- wash once with 1ml PBS, harvest PBS
- add 1ml of trypsin
- incubate at 37°C until the cells fully detach, then add 1 ml of pre-warmed medium
- Harvest cell suspension
- Spin the cells down at 125g; 5min, take of S/N
- Resuspend cells in 1ml PBS
- Spin down cells 125g/5min, take of S/N

RNA isolation with RNeasy Mini Kit (Qiagen, Cat No. 74104)

- Resuspend the cells in 350µl RLT + 1% β-Mercaptoethanol (Cat No. 63689)
- Transfer lysate to Qiashredder column (Cat No. 79654), spin for 2 min at max speed
- Discard column, mix flow through with 1 volume 70% ethanol, mix by pipetting
- Transfer max 700 µl lysate to RNeasy mini column, spin for 30s at 10,000g
- Discard flow through
- Wash column with 700 µl RW1 (30s, 10,000xg), discard flow through,
- Wash column with 500 µl RPE (30s, 10,000xg), discard flow through,
- Wash column with 500 µl RPE (2 min, 10,000xg),
- Place column into new collection tube, spin for 1 min at full speed
- Elute into new 1.5ml tube with 50 µl RNase free H2O

Ambion rDNase I treatment (Cat No. AM1906)

Add the following to a RNase-free tube on ice:

2 µg RNA sample
2 µl Ambion buffer DNAse I (Cat No. AM1906)
2 µl Ambion rDNase I (Cat No. AM1906)
fill up with H_2O to 20 µl

- Incubate tube(s) for 30min at 37º.
- Inactivate the DNase I by the addition of 2 µl of inactivation solution to the reaction mixture (mix well before use). Incubate for 2min at RT, vortex occasionally
- Centrifuge at 10,000xg for 5min and transfer 10 µl of the RNA into a fresh tube for the following reverse transcriptase. The remaining 10 µl can be transfer into a separate tube and used as a negative –RT control.

Reverse Transcription (RT)

Add the following to the DNaseI treated RNA (prepare master mix, add 10µl):

1 µl of Oligo dT_{20} (50µM, Thermo Fisher Cat No. 18418020)
1 µl dNTPs (10 mM each)
4 µl 5X First-Strand Buffer (in the Superscript kit)
1 µl 0.1 M DTT (in the Superscript kit)
1 µl RNase Inhibitor Murine (Cat No. M0314S)
1 µl SuperScript III RT (SuperScript® III Cat No. 18080044)
1 µl H_20

Mix by gently pipetting up and down

RT program:

25ºC for 5 min
50ºC for 50 min
70ºC for 15 min

Bring RT reaction volume to 100 µl (add 80µl H2O)
(Dilution for qPCR)

qPCR

Reaction Setup

10 µl GoTag SYBR Green Master Mix (2x) (Promega; Cat No. A6001)
1 µl primer mix (10µM each)
2 µl cDNA
7 µl H2O

Distribute 18 µl MM into wells; add 2µl cDNA, seal, vortex and spin down.
Pipette each sample per primer pair in triplicate.

Program:

95ºC, 2 min
95ºC, 3s
60ºC, 30s
Read plate, go back to 2 for 39 times (40 cycles total)

Primers:

Gene	Sequence	Amplicon length
ACTB_fwd	GTTGTCGACGACGAGCG	93
ACTB_rev	GCACAGAGCCTCGCCTT	
TUBB_fwd	TCGATGCCATGTTCATCACT	97
TUBB_rev	TAACCATGAGGGAAATCGTG	
IFIT1_fwd	TGCTCCAGACTATCCTTGACCT	106
IFIT1_rev	TCTCAGAGGAGCCTGGCTAA	
ISG15_fw	AGCATCTTCACCGTCAGGTC	91
ISG15_rev	GCGAACTCATCTTTGCCAGT	
IFI27_fwd	GCCACAACTCCTCCAATCAC	105
IFI27_rev	ATCAGCAGTGACCAGTGTGG	
OAS3_fwd	GGTGAGGAGCCTCGAGTAGA	105
OAS3_rev	CTGAAGAGCTGGACGGATGT	
IRF7_fwd	AGGGTGACAGGTACGGCTCT	105
IRF7_rev	CTCCTGGAGAGGGACAAGAA	
MX1_fwd	GATGATCAAAGGGATGTGGC	102
MX1_rev	AGCTCGGCAACAGACTCTTC	

Analysis of the qPCR:
Ct values for each target gene can be normalized to a housekeeping gene (i.e. ACTB or TUBB) using the delta Ct method described in: Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. *Methods* **25,** 402–408 (2001). Delta delta Ct values can then be calculated between conditions (e.g. with or without DNA electroporation).