Protocol for induction of expression and cell lysate production

AV-04 Doxycyclin induction and cell lysate

1.0 Introduction / Description

This method is intended for the treatment of the previously transfected Tet-ON HeLa cells with 3 different concentrations of doxycycline:

- 0 ng/ml: to analyze the recognition of the protein of interest by the antibodies to be validated at endogenous levels of the protein of interest (if the protein of interest is expressed in HeLa cells). This treatment also provides the baseline for the doxycycline inducible expression of the protein of interest from HuEV-A plasmid.
- 50ng/ml: to analyze the IP efficiency of the antibody to be validated at a low expression level of the protein of interest.
- 1µg/ml: to analyze the IP efficiency of the antibody to be validated at maximum expression level of the protein of interest. Also, overexpression of the protein enables to use an anti-FLAG antibody.

After treatment, cells are lysed to produce enough cell lysate to be used for IP assays with 3 different antibodies.

2.0 Materials

Glassware/Plasticware/Instruments and tools

Inverted fluorescence microscope (Leica DM IL)
autoclaved 1.5 ml tubes Axygen scientific cat.# 22-281
96 deep well plates Fisher cat.#12-566-121
Axygen mini tube 96-well rack system- Axygen cat#MTS-11-12-C-R

Reagents

DMEM 1X (Dulbecco modified Eagle medium) GIBCO cat.# 11965
Opti-MEM (Reduced serum medium)- GIBCO cat.# 31985
Tryple 1X GIBCO cat.# 12604
Tet system approved FBS Clontech cat. #631106
PBS 1X (Phosphate buffered saline pH7.4) GIBCO cat.# 10010
Doxycycline Clontech cat.# 631311

Lysis Buffer: 100mM Tris-HCl pH 7.4 (made from Tris-Base US Biological cat.# 18600)
150mM NaCl (Sodium chloride SIGMA cat.# S5886)
25mM NaF
50µM $ZnCl_2$
15% glycerol (Fisher cat.# G33-500)
1% Triton X-100 (Sigma-Aldrich P1379)
Supplement at the time of use with protease inhibitors tablets (Roche cat.# 11 836 145 001)

3.0 Doxycyclin induction and cell lysate

After cells have been plated for transfection (section 2) doxycycline is added as follow:

- ✓ Freshly prepare a two working solution (WS) of 100ug/ml and 5ug/ml doxycycline from a 1mg/ml stock solution
- ✓ Add in the respective wells 100µl of doxycycline WS to obtain a final concentration of 1000 and 50ng/ml doxycycline respectively (10ml final volume). Add 20µl OptiMEM to the 0ng/ml doxycycline wells.
- ✓ Incubate for 48-72 hrs to induce the expression of the gene of interest.
- ✓ Before lysis the YFP expression in live cells is checked using a fluorescence inverted-microscope (Leica DM IL). Pictures to observe the pattern of expression and localization of the protein if interest are collected
- ✓ Aspirate the media from all wells and add 1 ml of Tryple solution to each plate.

- ✓ Incubate with rocking at RT for 10 minutes
- ✓ Add 1.5 ml of PBS supplemented with 20% FBS
- ✓ Collect and distribute the cells into the four replicate sets wherein each set comprises of three mini tubes. Each mini-tube in a set (labeled A-C) corresponds to cell-pellets obtained from the different doxycycline inductions (see section 3). The approx. number of cells in a given tube per set is:

Tube A.	0ng/ml doxycycline cells (~62,500 cells)
Tube B.	50ng/ml doxycycline cells (~125,000 cells)
Tube C.	1000ng/ml doxycycline cells (~187,500 cells)

- ✓ Spin cells at 3000 rcf for 2 minutes (cell pellet are stored at -80°C until use).
- ✓ Lyse the cells with lysis buffer supplemented with proteases inhibitor freshly added. Add 180µl of lysis buffer in tube 1, 360µl in tube 2 and 540µl in tube 3.
- ✓ Pellets are resuspended by vortexing for 2mins followed by incubation at RT for 10mins to ensure lysis.
- ✓ Spin the lysates at 4000 rcf for 15 min.
- ✓ Transfer the supernatant into 96 deep well plates in this order:

	1	2	3	4	5	6	7	8	9	10	11	12
Lysate from tube=>	A	B	C	A	B	C	A	B	C	EMPTY		
A	Lysate Plate-1			Lysate Plate-9			Lysate Plate-17					
B	Lysate Plate-2			Lysate Plate-10			Lysate Plate-18					
C	Lysate Plate-3			Lysate Plate-11			Lysate Plate-19					
D	Lysate Plate-4			Lysate Plate-12			Lysate Plate-20					
E	Lysate Plate-5			Lysate Plate-13			Lysate Plate-21					
F	Lysate Plate-6			Lysate Plate-14			Lysate Plate-22					
G	Lysate Plate-7			Lysate Plate-15			Lysate Plate-23					
H	Lysate Plate-8			Lysate Plate-16			Lysate Plate-24					

N.B. Only 150µl/300µl /450µl of the cleared lysates from 0/50/1000 ng/ml reactions is recovered and the rest is discarded. Clear lysates are directly processed for IP.

AV-05 Total lysate preparation

1.0 Introduction / Description

This method describes the preparation of total lysate to be used for IP and as INPUT in Western blotting analyses described in the protocol for immunoprecipitation and protocol for western blot analysis.

2.0 Materials

Reagents

NuPAGE® LDS sample buffer (Life technologies corp. cat.# NP007)
β-mercaptoethanol (SIGMA-Aldrich cat.# M6250)
96 deep well plates Fisher cat.#12-566-121

3.0 Total Lysate preparation

- ✓ Mix 100ul of lysate from each sample to 33 µl of 4X LDS sample buffer (Invitrogen) supplemented with 5% β-mercaptoethanol (this will be the 'Input' sample to be used as for Western blot together with the immunoprecipitation samples)
- ✓ Denature the lysates in 1XLDS sample buffer at 90°C for 10 minutes