

***Note: This protocol is specific for a project that I did in my previous lab. However, this should work for generally any immunohisto/cytochemistry project, with the appropriate modifications.***

## **Isabel's Magical Co-transfection and Immunofluorescence Protocol**

### **Transfection**

#### Reagents and Materials

- 1:10 poly-L-lysine (Sigma), dilute with sterile ddH2O
- Sterile ddH2O
- 6-well plates
- coverslips, 22mm x 22mm
- Superfect transfection reagent (Qiagen)
- DMEM (serum-free)
- DMEM + 10% FBS
- 1x PBS (sterile)
- COS-7 cells, P9 (in Pachman's cryofreezer, top rack).
- Fusion-tag expression plasmid preps (in pcDNA3.1 for example) at 1mg/ml, sterile-filtered w/Spin-X filters (Sigma). Note: 1mg/ml is optimal for Superfect transfection efficiency. However, preps as low as 0.5mg/ml can be used; transfection efficiency decreases w/lower concentrations.

#### Method

##### *Prepare coverslips in 6-well plates*

- 1) Place one 22mm<sup>2</sup> coverslip per well in 6-well plate.
- 2) Dilute poly-L-lysine 1:10 in sterile ddH2O.
- 3) Add 2 mls per well 1:10 PLL to coat each coverslip.
- 4) Incubate in hood x 30 mins. at RT.
- 5) Rinse once w/sterile ddH2O.
- 6) UV-radiate x 1-2 hrs. in hood.
- 7) Split one confluent 100 mm dish of COS-7 cells per one 6-well plate; so, about 1x10<sup>6</sup> cells divided over 6 wells (0.167x10<sup>6</sup> cells/well).
- 8) Grow overnight at 37 degra. C., 5%CO2.

##### *Co-Transfection set-up*

Co-transfection efficiency has been optimized for a baseline of 4 ugs. plasmid DNA per well on a 6-well plate or 10 ugs. per 100 mm culture dish. Qiagen recommends different ratios of ugs. plasmid: ul Superfect. I found the optimal ratio to be 1:5 ugs. plasmid: ul Superfect. The following set-up is for one transfection in one well on a 6-well plate. Transfections should be done in duplicate or triplicate; so scale-up amounts accordingly. Plasmid constructs are denoted "X" & "Y" with the amount of X being kept constant. Be sure to label wells **prior to** adding each transfection complex to prevent confusion.

<u>X:Y</u>	<u>plasmid DNA</u>	<u>sf DMEM</u>	<u>Superfect</u>	<u>DMEM+10%FBS</u>
1:0.5	4 ugs. X: 2 ugs. Y	Qs. to 100 uls.	30 uls.	900 uls
1:1	4 ugs. X: 4 ugs. Y	Qs. to 100 uls.	40 uls.	1200 uls
1:2	4 ugs. X: 8 ugs. Y	Qs. to 100 uls.	60 uls.	1800 uls
<u>Controls</u>				
X only	4 ugs. X	Qs. to 100 uls.	20 uls.	600 uls.
Y only	4 ugs. Y	Qs. to 100 uls.	20 uls.	600 uls.
Empty vector	4 ugs. empty vector *	Qs. to 100 uls.	20 uls.	600 uls.

- \*For a negative control, use a plasmid prep of empty vector (i.e., vector w/no insert), denoted below as plasmid Z.
- Note: Mock co-transfection controls are simply a duplicate set of co-transfections except with empty vector (Z).

Mock co-transfection controls

<u>X:Z</u>	<u>plasmid DNA</u>	<u>sf DMEM</u>	<u>Superfect</u>	<u>DMEM+10%FBS</u>
1:0.5	4 ugs. X: 2 ugs. Z	Qs. to 100 uls.	30 uls.	900 uls
1:1	4 ugs. X: 4 ugs. Z	Qs. to 100 uls.	40 uls.	1200 uls
1:2	4 ugs. X: 8 ugs. Z	Qs. to 100 uls.	60 uls.	1800 uls

Using the amounts noted above, follow the procedure below:

- 1) In sterile 5 ml tubes, add plasmid DNA first, serum-free DMEM (serum-free DMEM) second, and Superfect last. Incubate at room temp. x 5-10 minutes to allow complex formation.
- 2) While Superfect-DNA complexes are forming, wash Cos-7 cells (grown on coverslips) once with sterile 1xPBS. At this time, properly label each well.
- 3) Add DMEM+10%FBS to stop complex formation.
- 4) Transfer Superfect-DNA complexes in 10% FBS to washed Cos-7 cells.
- 5) Incubate at 37 degra. C., 5%CO2 x 5-6 hrs.
- 6) After incubation, wash once w/1xPBS. Then, add 2 mls. DMEM+10% FBS.
- 7) Grow 48-72 hours at 37 degra. C., 5%CO2 for protein expression (72 hours optimal).

**Two-color Immunofluorescence**

The following is the staining protocol for detection of three separate epitopes on one sample. I recommend fixing tranfectants and doing your antibody incubations right in the 6-well plate. Prior to each IF experiment, fill in a IHC form designating which antibody to use on which transfectants; It is VERY easy to get confused if you don't take time to plan your incubations.

*Fixing tranfectants*

- 1) Following the 72-hour incubation for protein expression, wash cells once w/cold 1xPBS.
- 2) Add 1 ml ice-cold 1:1 acetone/methanol (kept at -20 degra. C) to each well and incubate at -20 degra. C. x15-20 mins.
- 3) Pour off acetone/MeOH and briefly drain on paper towel; air-dry x 5-10 mins. at RT.
- 4) Incubate at -70 degra. C. x 30 mins.

*Immunofluorescence set-up*

Appropriate controls are extremely important when doing any kind of immunohistochemistry. See my experiment from 10/21/99 for an example of appropriate controls. It's important to have both positive and negative controls for comparative references when examining your results.

The following are the antibody concentrations I worked out for optimal staining of both *myc* and FLAG tags. All antibody dilutions should be made in blocking buffer (PBS+0.5% Tween-20). Remember that all fluorochromes are light-sensitive; therefore minimize exposure to light. Note that there are two different primary antibodies (which can be incubated simultaneously):

<u>Blocking buffer</u>	<u>Primary antibodies (final conc.)</u>	<u>Secondary antibody (final conc.)</u>
PBS+0.5%Tween-20	1) 10 ug/ml FITC-anti-FLAG (Sigma) 2) 5 ug/ml biotin-anti-myc (Berkeley Ab)	1) ----- 2) 1:200 Texas Red-Streptavidin or 1:20 Cy5-Streptavidin (Zymed)

*Procedure*

100 ul antibody is enough to cover one 20mm<sup>2</sup> coverslip. Important: Never let coverslips dry once you begin IHC! Of course, once you finish, you must let them dry in the appropriate fixative (Fluorsave, Calbiochem, for IF). All incubations are done at room temp. and should be done in the 6-well plates.

- 1) After fixing cells in acetone/MeOH, equilibrate x 5 mins. w/1xPBS.

- 2) Block x 15-20 mins. with PBS+0.5% Tween-20.
- 3) Add 50 ul 20 ug/ml FITC-anti-FLAG (for 10 ug/ml final conc.) and 50 ul 10 ug/ml biotin-anti-myc (for 5 ug/ml final conc.) and incubate x 45 mins.
- 4) Wash x 3 over 5 mins. w/1xPBS. Drain off excess PBS onto paper towel after last wash.
- 5) Add 100 ul 1:200 Texas Red-Streptavidin and incubate x 45 mins.
- 6) Repeat step 4 (wash step). During this step, label slides.
- 7) Rinse once w/ddH<sub>2</sub>O.
- 8) Wear gloves for this step. Add two drops of Fluorsave to center of prelabeled slide. With an exacto-knife razor blade, carefully lift off the coverslip holding a finger over the well to keep the coverslip from flipping out of the well. Place coverslip face down on slide. Gently press out air bubbles. Dry overnight in a covered chamber at RT.  
\*\* A couple notes about Fluorsave: This stuff dries really fast. Avoid lifting off or moving the coverslip around once you mounted it. Also, don't dry slides on paper (no matter how neatly you work, excess Fluorsave will make slides stick to paper); dry them on a plastic surface.

Store slides in -20 degra. C. for long-term storage.

### **Three-color Immunofluorescence**

For 3-color immunofluorescence with a third primary antibody being an unconjugated mouse monoclonal (like 5c8 Ab), follow this procedure. Block initially with PBS+0.5% Tween-20. Then, incubate with the unconjugated monoclonal first. Wash. Then, incubate with 10 ug/ml Texas-Red anti-mouse polyclonal (Vector labs). Wash. Before adding the anti-myc and anti-FLAG Ab's, block with PBS+0.5% Tween-20 + 50ug/ml mouse IgG<sub>1</sub> (Sigma). Now, continue with the procedure as outlined above.