Plasmid transfection and expression in P388 cells

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Materials and methods:

Invitrogen: DMRIE-C reagent, Lipofectamine 2000, Opti-MEM I reduced serum medium

Gibco/Invitrogen: RPMI 1640 glutamax, FBS, penicillin/streptomycin

Roche: FuGENE 6 reagent:

Biontex: Metafectene Pro:

Santa Cruz Biotechnology Inc: H300 topoisomerase I primary antibody (sc10783)

Abcam TBP primary antibody (ab818)

Dakocytomation: Polyclonal goat anti-mouse or anti-rabbit secondary antibodies

pcDNA3.1-HTwt: mammalian expression vector expressing human topoisomerase I

P388 45R cell line, Top1-deficient murine leukemia cells (a gift from Prof. Y. Pommier, Center for Cancer Research, National Cancer Institute, NIH, Maryland, USA) The cells were grown in RPMI 1640 glutamax with 10 % FBS and 1% penicillin/streptomycin.

Experimental procedures / transfection protocol:

All transfection were done according to the manufacturer's directions:

Invitrogen DMRIE-C reagent:

5 x 10^6 P388 45R cells are transfected with 10 µg of plasmid DNA (pcDNA3.1-HTwt) in a 6 cm dish.
Aliquot 0.5 ml Opti-MEM I reduced serum medium into the dish.
Mix the DMRIE-C transfection reagent and add 7.5 µl to the OPTI-MEM medium in each flask.
Dilute 5.9 µl (1.7 µg/µl) plasmid DNA in 1 ml Opti-MEM I medium and add to the dish.
Mix by gentle swirling and incubate for 20 min at RT.
Prepare single cell suspension from stock cells by pipeting gently up and down.
Wash the cells once with RPMI-1640 (without FBS or antibiotics)
Resuspend 5 x 10^6 cells in 0.4 ml Opti-MEM I medium and add to the dish drop wise.
Place in 37°C incubator
After a 5-h incubation at 37°C, 1 ml complete RPMI medium (RPMI 1640 + 10% FBS + 1% pen/strep) is added to the transfected cells in each flask.

Invitrogen Lipofectamine 2000:

5 x 10^6 P388 45R cells are transfected with 6.25 µg of plasmid DNA (pcDNA3.1-HTwt) in a 6 cm dish.
Prepare single cell suspension from stock cells by pipeting gently up and down.
Wash the cells once with RPMI-1640 (with FBS but without antibiotics)
Resuspend 5 x 10^6 cells in 2 ml RPMI-1640 (with FBS but without antibiotics) and add to the dish.
Place cells in 37°C incubator until transfection mix is ready.
Dilute 3.6 µl (1.7 µg/µl) plasmid DNA in 0.5 ml Opti-MEM I medium in the well of a 24 well cell culture plate and mix gently.
Mix the Lipofectamine 2000 gently and dilute 10 µl in 0.5 ml OPTI-MEM medium in the well of a 24 well cell culture plate.
Incubate for 15 min at RT.
Combine diluted DNA with diluted Lipofectamine 2000 by adding the DNA dilution to the Lipofectamine solution.
Mix gently and incubate for 20 min at RT.

Add the combined DNA and Lipofectamine 2000 to the 6 cm dish containing the cells by slowly dripping and mix by rocking the plate gently.
Incubate at 37°C.

Roche FuGENE 6 reagent:

5 x 10^6 P388 45R cells are transfected with 6.25 µg of plasmid DNA (pcDNA3.1-HTwt) in a 6 cm dish.
Prepare single cell suspension from stock cells by pipeting gently up and down.
Wash the cells once with RPMI-1640 (with FBS but without antibiotics)
Resuspend 5 x 10^6 cells in 4.5 ml RPMI-1640 (with FBS but without antibiotics) and add to the dish – place in 37°C incubator until transfection solution is ready.
Add 300 µl RPMI-1640 (without FBS or antibiotics) to the well of a 96 or 24 well plate.
Add 6 µl FuGENE 6 reagent to the medium
Mix by swirling plate.
Incubate for 5 min at RT.
Add 3.6 µl (1.7 µg/µl) plasmid DNA to the diluted FuGENE 6 reagent. Mix by swirling.
Incubate for 30 min at RT.
Take out cells from incubator and add transfection mix drop wise. Swirl plate to mix
Incubate at 37°C.

Biontex Metafectene Pro:

5 x 10^6 P388 45R cells are transfected with 5 µg of plasmid DNA (pcDNA3.1-HTwt) in a 6 cm dish.
Prepare single cell suspension from stock cells by pipeting gently up and down.
Wash the cells once with RPMI-1640 (with FBS but without antibiotics)
Resuspend 5 x 10^6 cells in 5 ml RPMI-1640 (with FBS but without antibiotics) and add to the dish.
Place cells in 37°C incubator until transfection mix is ready.
Prepare the DNA and transfection solutions in the wells of a 24 well plate,
Mix 5.9 µl (1.7 µg/µl) plasmid DNA in 0.3 ml PBS.
Mix the Metafectene Pro gently and dilute 25 µl in 0.3 ml PBS. Mix gently by pipeting one time.
Incubate for 15 min at RT.
Mix the DNA dilution with the Metafectene Pro solution by pipeting the DNA dilution into the well containing the Metafectene Pro solution. DO NOT MIX!!

Incubate for 15 min.
Add the DNA/Metafectene Pro mix drop wise to the dish containing the cells and swirl gently to mix.
Incubate at 37°C in incubator

Testing expression of topoisomerase I in transfected cells:

Harvest all cells 24 hrs after transfection.
Transfer cells to 15 ml tube; spin 2000 rpm for 2 min
Remove medium
Wash with 2 ml PBS, spin again at 2000 rpm, 2 min
Remove supernatant
From this step all samples are kept on ice and buffer should be ice cold. This is to prevent degradation and denaturation of proteins in the extract.
Resuspend cells in 1 ml Lysis buffer.
Incubate on ice for 10 min.
Spin 2000 rpm, 5 min, 4 °C
Remove supernatant
Add 100 µl nuclear extraction buffer and rotate tube for 1 hr, 4 °C
Spin 10 min at 11000 rpm, 4 °C
Transfer supernatant to new tube.

Use the nuclear extracts for Western blotting (NE from 1.5 x 10^6 cells per lane) using the H-300 topoisomerase I primary antibody and the TBP antibody for loading controls.

Results and discussion:

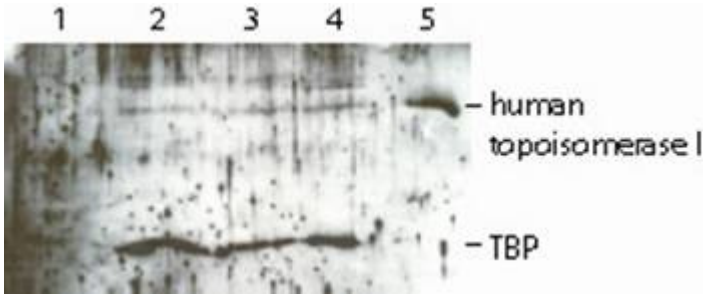

Figure 1: Western blot showing human topoisomerase I expression in P388 cells transfected with pcDNA3.1-HTwt using different transfection reagents. Expression in nuclear extract from cells

transfected with; lane 1: DMRIE-C reagent (Invitrogen), lane 2: Lipofectamine 2000 (Invitrogen), lane 3: FuGENE 6 reagent (Roche), lane 4: Metafectene Pro (Biontex). Lane 5: Recombinant purified human topoisomerase I. TATA binding protein (TBP) was used as loading control.

Lane 1 with nuclear extract from cells transfected using the DMRIE-C reagent does not show any topoisomerase I or TBP (loading control). However, the cells seemed very sick after the transfection so this may explain why no protein was detected in extract from those cells. Both lanes 2, 3, and 4 show a band corresponding to human topoisomerase I with lane 4 (Metafectene Pro (Biontex)) showing a slightly higher expression of the protein when comparing the loading control.