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🌐 Transfection of constructs in diplomids to block NHEJ pathway using the inhibitors of KU70/80 proteins. V.2

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Protocol status: Working

We use this protocol and it's working

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Abstract

The process of NHEJ is completed when the DNA ligase complex ligates the broken ends of the DNA. Ku70-Ku80 are the DNA-dependent protein kinase heterodimeric regulatory factor that forms a link between two broken DNA ends and structurally support, align and protect them from further degradation. W7 inhibitor (W7(N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) inhibits the cofactor of inositol hexakisphosphate which effects the regulatory factor of Ku heterodimers.

Materials

Nucleofector™ 2b Device , Human T Cell Nucleofector™ Kit, Cell counter, Normal growth medium, Tissue culture plates, Microcentrifuge tubes

- 1 Step 0: Pre-incubation of Diplonema cells for 4 hours with W7 (5 mg/ml) before electroporation. EC value of W7 was calculated by Alamar blue assay
- 2 Count the cells and plan to 1 nucleofection with 5×10^7 cells for each construct.
- 3 Harvest the cells by centrifugation at 1300xg for 5 min at room temperature in Swing Bucket Rotor
- 4 Resuspend the cell pellet in 100ul of AMAXA Human T- cell solution at 4C (from refrigerator combine 81.8ul of Human T-cell nucleofector solution + 18.2ul Supplement).
- 5 Add 5-10ug of (PCR) (linearized DNA) into the cuvette (resuspend in 10ul of H₂O).
- 6 : Put everything into the cuvette, close the cap and place in the electroporator, cuvette should only fit in one direction, but metal sides should face towards you.
- 7 Press for the Program X-001 to electroporate.
- 8 Transfer the entire cell- DNA transfectant into 10ml of Diplonema media (chloramphenicol) with the supplied disposable micropipettes
- 9 Immediately observe the flasks containing transfectants under a microscope and place them in the incubator at 27C + shaker and let them grow for 6-8h.
- 10 After 6-8 hours took out the flasks and make controls which should be without antibiotics - put 1ml of the electroporated cells in the first well of 24 well plates.
- 11 In the rest - 9ml -transfectants add Puromycin antibiotics in the flasks.
- 12 Add 1.5ml of electroporated cells in the first row (6 wells) of 24 well plates. Put 900ul of the media (+ selectable drug) in the rest of the wells. Take 100ul from first well and transfer to the next to make 10x serial dilution and so on...up to the lowest concentration 5×10^2 cells/ml and let them grow until selection of clones is done.



- 13 Result: Unfortunately, targeting to the planned position (N-terminal tagging of alpha-tubulin with mCherry under puromycin^R selection) did not work in any of the obtained clones.