Transfectionof constructs in diplonemids to block NHEJ pathway using the inhibitors of KU70/80 proteins.

Version 2

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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-chloro1-naphthalenesulfonamide) inhibits the cofactor of inositol hexakisphophate which effects the regulatory factor of Ku heterodimers.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS TEXT

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) beforeelectroporation. EC value of W7 was calculated by Alamar blue assay

2 Count the cells and plan to 1 nucleofection with 5x 10^7 cells for each construct.

3 Harvest thecells by centrifugation at 1300xg for 5 min at room temperature in Swing BucketRotor

4 Resuspendthe cell pellet in 100ul of AMAXA Human T- cell solution at 4C (from refrigerator combine 81.8ul of Human T-cell nucleofectorsolution + 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 microscopeand 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 x 10^2 cells/ml and let them grow untill 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.