Generate_spliced _RNA_from_DNA_library

Generate Spliced RNA from DNA

Objective

The objective of this protocol is to transfect DNA into HeLa cells and allow them to express and splice the RNA. Then to harvest and purify the RNA.

Steps

Seeding cells

4ug of library (Mandy did 6ug for her experiments)

10ul Lipofectamine 2000

Cell density is critical since high plasmid+Lipofectamine amounts can be toxic to cells. The higher the cell density the better. Recommended amount is 0.5-2×10^5 cells per 500ul of growth media. In 6 well plates, we use 2mL total so 1×10^5 cells to 8×10^5 cells needed per well. Mandy seeded 1×10^6 cells per well so let's stick with the amount she used

1. Remove plate from 37C incubator
2. Wash with 10mL PBS
3. Aspirate off all liquid
4. Add 1mL of 1x Trypsin if using 10cm plate, or 3mL 1x Trypsin if using 15cm plate
5. Put in 37C incubator for 5min. We want almost all the cells to detach from the bottom of the plate
6. Remove plate from incubator and add 9mL media for 10cm plate of 17mL media for 15cm plate. Mix well by pipetting up and down several times to collect all cells
7. Transfer cells to a clean glass bottle or 50mL conical (depending on how much cells+media are present) and pipet up and down once again several times. It's critical to break up clumps of cells so that we have an accurate count of cells for seeding
8. Take 10ul of the cell suspension and transfer into 1.5ml eppi tube. I make 3 tubes to do 3 separate counts to get a better idea of how many cells are present
9. Take tubes to the cell counting station and turn on the CytoSmart cell counting machine. Follow instructions for username and password as mentioned on tablet. Pick the 1:1 trypan blue:cell setting if already not done on the machine
10. Add 10ul of Trypan blue from the top (it gets clunky at the bottom and that interferes with the reading) and flick tube. Do this before every reading since Trypan blue will kill cells

11. Adjust the glass coverslip on the slide, tilt the pipette and eject the sample into the space between the coverslip and the slide
12. Put under camera and the cells will be visible on the tablet screen. The cells should be visible such that the outer rim of the cells are in focus. Adjust if necessary. Click on "Count" and the machine will generate the numbers. Do the second reading to make sure that it is within range of the second. If not, take the 3rd reading. Take average of the readings
13. Record the number of live cells present. Record the viability as well. Cell viability >90% is desirable
14. Count number of cells needed per well as follows: (1×10^6 cells needed/well) / 'x' cells/mL) = 'y' mL/well
15. Since the total amount of cells and media in each well will be 2mL for a 6-well plate: 2mL-'y' mL cells/well = (2-y) mL media per well. When seeding more than one well, make MasterMix (MM) of cells and media for the number of wells needed. Add 10% extra, so it will be for 2.2 wells media: (2-y)mL/well x 2.2 wells; cells: y mL/well x 2.2 wells. Make this MM in a new bottle/tube
16. Mix media and cells well and and using reverse pipetting, use the P1000 to pick up 1mL of mixture and add to a well on the 6-well plate, tilting to the side and eject liquid. Repeat for the other 1mL for each well
17. Mix well by shaking plate gently sideways and up and down a few times. Take plate to microscope to see what the cells look like. They should be floating and evenly distributed, and preferably not present in clumps, since we want to grow a monolayer of cells
18. Put plate in 37C incubator for the next 16-18 hours

Transfection

1. Warm up Opti-MEM and Lipofectamine 2000 to room temperature. It shouldn't take too long
2. Take plate out and look at the cells. The cells should be evenly spread out across the well in a monolayer, >90% confluency is desirable. For our experiments, 95% confluency works quite well and doesn't end up killing cells after 48h. After visualizing, put cells back in the incubator until needed in step 6
3. We will prepare our DNA-lipid mixes in 500ul Opti-Mem/well. Prepare everything in a MM as before, adding an additional 10%. Since we're doing two wells, it will be 2.2 wells for our calculations
4. Pipet 250ul x 2.2ul= 550ul Opti-MEM into a 1.5mL eppi We will be using 10ul Lipofectamine 2000 per well so 10×2.2= 22ul Lipofectamin2000. Add this amount to the Opti-MEM aliquoted above. Mix 2-3 times and let sit on bench for ~5min
5. In a separate 1.5mL eppi, add the same amount of Opti-MEM as above Since we'll be adding 4ug of the library per well, adjust again for 2.2 wells, so the amount needed is 8.8ug. 8.8ug/ 'concentration on maxiprep)= 'x' ul of DNA goes into the second 1.5mL eppi. Mix well and add the whole Opti-DNA mix to the Opti-Lipo mix above. Mix again several

times to ensure even distribution and then let sit for 20min for the DNA-lipid complexes to form
6. Bring the 6-well plate out of the incubator and using reverse pipetting, add 500ul of the Opti-DNA-Lipo mix to each well. Can be added directly on top of the media
7. Shake plate gently sideways and up and down a few times to distribute the mixture across the media
8. Put back in incubator and record time at which the transfection was done. After 48h, the cells will be harvested

Harvest RNA

1. After 48h, visualize cells under microscope. There will be plenty of dead cells floating but there should be a confluent layer of cells underneath
2. Aspirate off media
3. Add 1mL of PBS to each well
4. Aspirate
5. Add 1mL of TriZol to each well
6. Let sit for a few mins for the TriZol to dissolve the cells and peel them off the plate
7. Pipet up and down several times to collect the cells from the plate and put into 1.5mL eppis
8. Cells can be frozen at -20C at this point until needed further

Purify RNA

1. Add 200ul of chloroform to each sample.
2. Vortex each tube until it turns milky pink (~30sec)
3. Spin in 4C centrifuge at 12,000rpm for 15 minutes.
4. Meanwhile, prepare collection tubes.
5. Carefully pipette 500ul of the clear aqueous phase into collection tubes. Do not pick up the white precipitate or the pink solution. Those contains DNA and protein.
6. Add 500ul of isopropanol to precipitate the RNA. Mix by inverting. Can freeze samples at this point or proceed.
7. Centrifuge in 4C centrifuge at 12,000rpm for 10 minutes.
8. Carefully aspirate the liquid. Be very careful. The RNA pellet can get sucked up very easily. You do not need to aspirate absolutely all of the liquid. Can leave ~50ul.
9. Add 500ul 70% ethanol to wash. Spin for 5 minutes. Aspirate.
10. Add 500ul 70% ethanol to wash again. Spin for 5 minutes. Aspirate.
11. Spin for 1 minute to collect any residual liquid to the bottom. Carefully use P200 pipette to remove as much liquid as possible without disturbing pellet.
12. Let pellets air dry for 5 minutes.
13. Add 50ul of water. Immediately place on ice. RNA is not stable at RT. Must be kept on ice at all times from now on.
14. Measure the RNA concentration. Be sure to select RNA setting on the nanodrop. Good

RNA has 260/280 of 2.0 and 230/280 of greater than 2.0.

15. Continue with DNase I treatment or store RNA in -20C freezer.

DNase I treatment and clean-up

1. Prepare a MM with the following reagents per well for a 50ul total reaction volume:
    1. 5ul 10x Turbo Dnase buffer
    2. 1ul Turbo Dnase I (add last)
    3. 19ul NF water
2. Add all of the 25ul of the RNA into 8 strip reaction tubes
3. Add Dnase I to the MM and mix well by pipetting up and down several times
4. Add 25ul of the MM to the RNA and mix tubes by flicking them
5. Pulse briefly to collect liquid at the bottom of the tubes and run the DNase treatment program on the thermocycler for 1 hour at 37C
6. Once the run is finished, add 5ul of DNase Inactivation reagent to each reaction and mix well. Let sit for 5min. Flick 2-3 times during this time since the pellet will settle. This is meant to quench the DNase and other ions in solution
7. Spin down the samples in the centrifuge for 5min at 300rpm.
8. Transfer supernatant to another eppi, careful to not touch the white precipitate at the bottom
9. Clean samples with NEB Monarch RNA cleanup kit (I do this step, Yuma doesn't. cDNA can be used for synthesis right after collecting the supernatant from step 8, but in my hands it doesn't work as well so I do this additional clean-up step. Maybe you can skip this step if step 8 works well for you). Elute in 8ul NF water
10. Measure the RNA concentration. Be sure to select RNA setting on the nanodrop. Good RNA has 260/280 of 2.0 and 230/280 of greater than 2.0.
11. Continue with cDNA synthesis or store RNA in -20C freezer.

cDNA synthesis

1. Mix:
    1. 2ug RNA
    2. 1ul reverse primer
    3. Xul water to total v of 5ul

    for 1 sample, add a no RT control to check for genomic contamination
2. In PCR machine, incubate at:
    1. 70C 5min
    2. 4C hold
3. Meanwhile, prepare master mix. Per sample contains:
    1. 6.6ul water
    2. 4ul 5x buffer
    3. 2.4ul 25mM MgCl2

    4. 1ul 10mM dNTP
    5. 1ul Improm II RT
    6. total v = 15ul
4. Add to samples
5. In PCR machine, incubate at:
    1. 25C 5min
    2. 42C 1hr
    3. 70C 15min
    4. 4C hold

Notes

Protocol transcribed from SMN2 5'ss library analysis with LRS.pdf and RNA extraction .docx from Andalus Ayaz