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Amaxa Nucleofector Protocol

You are responsible for:

1. Hazardous waste removal (bring your own orange bag)
2. Your Amaxa kit
3. Supplies
    a. Plastic pipets (10mL, 5mL, 2mL)
    b. Pipet tips
    c. Sterile glass pipets
    d. Sterile 1.5mL tubes
    e. Sterile 15mL or 50mL conical tubes
4. Media and Trypsin
5. Cleaning up the hood when finished

We will provide:

1. The nucleofection machine
2. Rainin Pipettes
3. Centrifuge
4. Heat block
5. Hemocytometer

- Please bring your cells still attached to the plate – you will trypsinize and count them here.
- If you need to be trained, please give at least 2 days (no exceptions!) notice prior to the time you would like to use the nucleofector.
- If you are already trained, please sign up on the calendar in the tissue culture room at least 1 day in advance

Before you begin:

- Heat block set at 37°C
- 1.5mL tubes with 500µL of Ca++ free media @ 37°C
- 6 well plate/100mm plate with fresh media @ 37°C
- All reagent at RT
- Cells 60-80% confluency

1. Preparation:
    a. Place Nucleofector in hood
    b. Write up protocol and program sheet
    c. Place 1.5mL tubes in 37°C heat block
        i. Label with numbers corresponding to program
    d. Open all pipet packages
    e. Open all cuvettes and loosen caps
    f. Label plates
2. Add supplement solution to Amaxa Reagent (V, T, or R for cell lines)
3. Trypsonize cells, inactivate, and count cells
    a. For a 6 well plate – 1 x 10^6 cells per well
4. Spin for 10 min (900 RPM) at RT and remove all media

If all same DNA:

5. Make up master mix: 100μL of reagent x (# of wells) to the cells – mix gently
6. Set program number on the nucleofector
7. Control: add 100μL of mixture to cuvette – pulse – recover in warmed media and add to 1.5mL tube - 37°C heat block
8. Sample: Add DNA to master mix and carry out transfection using method described for control
9. Let the tubes sit for 5 minutes at 37°C – add to 6-well plates and incubate for 24 hours

If separate samples:

10. Label 1.5mL tubes – add DNA
11. Add 100μL of cell/reagent mixture to tube – add to cuvette and pulse
12. Recover with warm media - 37°C.

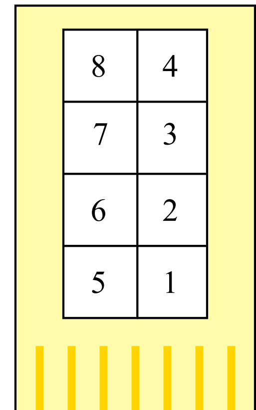
Important points:

- Fresh media – 2-3 days old at most
- Cells must be < 20 passages
- Recover cells in Ca++ free media
- No bubbles in the cuvette or transfection will not work (error 8)
- Rxn must be completed within 15 minutes of nucleofector reagent addition to the cells

ECIS Adhesion Assay Protocol

Use 8W-10E dishes for adhesion
DO NOT use PBS – the phosphates will interfere with the resistance readings

1. Cysteine-treat chamber wells for 15 minutes with 10mM cysteine. Rinse wells with sterile water
2. Coat wells with matrix diluted in HBSS O/N (~200µL)
    a. Rinse wells with HBSS
3. Add 400µL media to wells – let run in ECIS chamber for ~10 minutes.
4. Remove media and add cells – 5×10^5 cells per well in 350-400µL.
    a. Select "Pause data acquisition"
5. Computer settings:
    a. # of arrays
    b. specify 8W10E
    c. all wells (or wells containing samples/controls)
    d. 45 kHz
    e. 1 min. between readings
    f. stop time (~10-12 hours)
6. Select "new experiment" – save files to folder and label contents of each wells
7. Run an electrode check – save values to folder
8. Run ECIS for 8-10 hours 45 kHz 1 Volt



Lipofectamine Transfection Protocol

Day 1

1. Split cells so that they are ~30% confluent on the day of transfection

Day 2

1. For each well of the transfection, dilute .2μg of DNA in 25μL of serum free media
2. For each well of the transfection, dilute 2μL of Lipofectamine reagent into 25μL of serum-free media
3. Combine DNA and lipofectamine mixtures gently – incubate at RT for 45 minutes
4. While complexes are forming, replace media on cells with 200mL of serum-free media
5. For each transfection, ass 150μL of serum-free media to the tube containing the complexes – mix gently and overlay the diluted complexes onto the cells
6. Incubate the cells at 37°C with 5% CO_2 for 5 hours
7. After incubation, change media on the cells and replace with media containing serum
8. Assay for gene expression after 60 hours

Culture plate diameter (mm)	Lipid and DNA dilution volume (μL)	Lipofectamine volume range (μL)	DNA amount (μg)	Transfection medium volume (mL)
35	100	2-25	1-2	0.8
60	300	6-75	3-6	2.4
100	800	16-200	8-16	6.4

Recombinant Protein Generation and Purification

1. Start with cleared bacterial lysates (see generation of fusion proteins from bacterial preps protocol)
2. Prepare GST beads
    a. For 4mL of lysate, use 1mL bead slurry (500µL bed volume)
3. Spin at 4° for 2 hours in the cold room
4. Wash beads 2X with TBST
5. Wash beads 4X with cleavage buffer
6. Add enzyme
    a. 1µL protease / 100µg of protein
    b. if amount of protein is unknown, use 40µL of protease for each mL of GST bed volume
7. Spin tubes overnight in the cold room
8. Spin down and collect S/N; wash beads 3X in cleavage buffer – combine S/Ns
9. Dialyze in buffer
10. Quantitate on 8% gel with BSA standards

Cleavage Buffer (1L)
50mL 1M Tris-HCl, pH 7.0
30mL 5M NaCl
2mL 0.5M EDTA
1mL 1M DTT
1mL Triton X

Cleavage Buffer – final concentrations
50mM Tris-HCl, pH 7.0
150mM NaCl
1mM EDTA
1mM DTT
0.1% Triton-X

Src Kinase Assay

1. Make fusion proteins – quantitate levels
2. Lyse CEF cells – 1 flask ≈100pM of cSrc
3. Want a ratio of 1:100 – 1:1000 Src/AFAP110
4. IP Src from CEF lysates - 2μg Ab/tube
5. Put fusion protein on beads – 2 hours incubation
6. Wash Src 2X in RIPA, 2X in Src Kinase buffer
7. Wash GST-AFAP110 2X in MT-PBS, 2X in Src kinase buffer
8. Final wash of Src – resuspend in SRc kinase buffer so that you have equivalent amount for each tube of fusion protein
9. Mix fusion protein, Src beads, ATP, and Src reaction buffer to 80μL at various time points
10. Boil, and run out on SDS-PAGE

Time Lapse Movie Analysis

1. Open file for T = 0
2. Trace the edge of the lamellipodia using the trace button – double click to end
3. Open next image (ex. – T = 5)
4. Under the regions heading, select transfer regions. The region from the T = 0 picture should appear on the T = 5 picture
5. Under the measure heading, choose calibrate distances
    a. 60X
    b. Apply to images
6. Draw a line from the original line from T = 0 to the edge of the lamellipodium
7. Go to Regions, region measurements, display measurements
8. Information is displayed as μm/pixel

Adhesion-dependant tyrosine phosphorylation of FA proteins Protocol

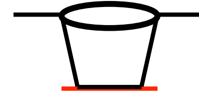
1. Trypsonize cells and place in suspension (_MEM + 2% BSA) for 60-90 minutes
2. Plate cells on coverslips coated with FN
3. For IF –
    a. Fix cells with 4% PFA in PBS for 10 minutes
    b. Stain as usual
4. For western blot analysis
    a. After ~1 hour, lyse cells, quantitate, and either use neat or IP

Staining Invasion Chambers Protocol

Wear gloves at all times!!!!!

Prep List:

- 4L of water
- cotton swabs
- Crystal Violet stain – see below
- Forceps



1. Rinse wells with 1XPBS 2x
2. Fix cells in 4% PFU – 15 minutes
3. Pull insert out of well
   a. Swab around bottom of well with Cotton swab
   b. Clean around rim of insert
4. Wet swab and clean inside of chamber – remove cells from both the bottom and the sides
5. Add 500µL CV stain to empty well
6. Add insert into CV stain – avoid bubbles and incubate 15 minutes.
7. Pull inserts out of CV stain and rinse all inserts in water
8. Repeat steps 3 and 4 until swab is no longer purple
9. Let dry and examine by microscopy

CV stain –

- 10mL dH20
- 200µL 100% EtOH
- 0.1g of Crystal Violet
  - Mix thoroughly and filter