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Physics 173

mRNA Expression Lab Manual

Introduction

When doing electrophysiology experiments with the oocytes it may be desirable to investigate the properties of particular protein channels not native to the oocyte. To facilitate this one needs to express the particular protein in the egg. This is done by obtaining the cDNA template of the protein, transcribing the protein into mRNA, and injecting the mRNA into an oocyte. Utilizing the translation machinery of the oocyte the mRNA is then translated into the protein and, through processes not at all clearly understood, the protein folds into its appropriate conformation, finds its way into the membrane of the egg and orients itself in the appropriate way so as to function properly (truly miraculous indeed!)

Overall Description

The template DNA you obtain will be embedded in plasmid DNA. This is circular DNA used in cloning and other recombinant DNA techniques. To transcribe only the template DNA you will need to linearize the circular DNA so that when the transcription machinery reaches the end of the template it will just fall off instead continuing around and around giving you a bunch of unwanted RNA. This is done by cutting the plasmids with restriction enzymes in a process called “linearization”.

This will give the desired linear DNA, but now you have DNA plus unwanted enzymes. You will need to “clean” the DNA by extracting out the unwanted stuff with phenol/chloroform and then precipitating the DNA with ethanol and centrifugation.

Once DNA is thus obtained, you will use the Ambion mMessage RNA kit to transcribe the DNA into mRNA. But again you will have unwanted material along with the mRNA. So you will perform phenol/chloroform extraction and ethanol precipitation once again.

With the RNA in hand you will need to figure out how much you have. This quantification is done using a spectrophotometer.

Injection of oocytes with the RNA will be done using the nanoinjector. It will take about 48 hours for expression of proteins to be complete after injection of mRNA. The last step is to take electrical measurements at a electrophysiology station to try and detect the signature current response of the protein channel expressed. Successful detection of the signal is the final indication of successful expression.

General precautions when handling mRNA!!

RNAses are ruthless, ubiquitous, and uncompromising. Their sole purpose is to destroy all your mRNA, cutting it to pieces, rendering it, and your work, useless. They exist on your fingers; they travel in your spit droplets expunged by sneezes or overzealous conversation. So to guard against them, only use sterile equipment (e.g. tubes, pipette tips etc.). Always wear gloves when handling mRNA. Don't sneeze on the mRNA or equipment holding mRNA. Try not to talk in the direction of the stuff. I know this is a bit paranoid, but you don't want to waste the hours of boring, tedious lab work.

Chemicals needed

- 4 M NaCl
- chloroform
- phenol/chloroform
- Ethanol
- 70% Ethanol
- Ambion mMessage Kit
- Deionized water
- TE (10mM Tris-base + 1mM EDTA at 7.8pH)

Linearization

Note: the amount of materials you use will depend on your circumstances. However, as reference, in italics is an example of an expression procedure of shaker potassium channel.

- Turn on centrifuge, and set temperature to 4 deg C
- Turn on water bath and set temperature to 37 deg C
- Add 5µl of X10 reaction buffer from the Ambion kit
- Add 1-2µl EcoR1 restriction enzyme from Ambion kit
- Add desired concentration of template DNA
For example, add 3µl of 0.75µg/µl ShBt7 DNA for a total of about 2µg of DNA.
- Add enough water to get 50µl of solution
- Incubate in 37 deg C bath for 2 hours

Purification and Precipitation

Purify with phenol/chloroform

- Add an equal volume of phenol/chloroform (50µl)
- Vortex for 20sec or until mixture looks milky white

- Centrifuge for 30sec at 4 deg C and at 14000 rpm
- Remove supernatant (the top layer of liquid containing the DNA) into a new Eppendorf tube
- Repeat above 4 steps one time
- Add an equal volume of chloroform (50 μ l)
- Vortex for 20 sec
- Centrifuge for 30 sec at 4 deg C and at 14000 rpm
- Remove the supernatant into new Eppendorf tube

Precipitate with EtOH

- Add 2.5 volumes of Ethanol (EtOH) (125 μ l)
- Add 52 μ l or 4M NaCl for each ml of solution (3 μ l)
- Vortex for 20 sec
- Store at -20 deg C for 45 minutes
- Centrifuge for 20 minutes at 4 deg C, 14000 rpm

Note: after centrifuging, the pellet of DNA will probably be too small to see. Thus to get your bearings to where the DNA may be, place the Eppendorf tube in the centrifuge so that the hinge of the tube's cap is pointing up. This way you know that the DNA pellet is somewhere on the back of the tube in line with the hinge. It is important to keep that in mind when removing the supernatant – keep the tip of the pipette clear of the area where the DNA is suspected to be, you don't want to suck out the DNA!

- Remove and discard supernatant
- Add 500 μ l of 75% EtOH
- Centrifuge for 10 minutes at 4 deg C, 14000 rpm
- Repeat last three steps one time
- Remove and discard supernatant
- Dry DNA pellet in air for about 15 minutes or until liquid is gone
- Resuspend pellet in 3 μ l TE (*expect to have ~1 μ g/3 μ l*)
- Store DNA at -20 deg C.

Note: expect to end up with about half of the DNA that you started with. So in our case we assume that we have about 1 μ g of DNA. There is no way to actually quantify the amount of DNA at this point; and there really is no need.

You can stop here and store the DNA for an indefinite period of time, continuing with transcription at a later time if need be.

Transcription of mRNA from Template DNA

See Ambion mMessage Instruction Manual page 7-8, section B, parts 1 – 4.

In our case we scaled up the reaction by a factor of 3 by making a 60µl mixture instead of 20µl. We obtained a good yield with this, so you may want to try this also. If you do this be sure to add three times the given amounts indicated in the instruction manual and three times the values given below.

Note: when working with the RNA only use individually wrapped sterile tubes and prepackaged, sterile pipette tips. Use each tip only once. All this is needed to avoid contamination by RNAses.

Recovery of the RNA

Facilitate removal of template DNA by cutting it up with DNase I

- Add 1µl DNase I from kit
- Incubate mixture in 37 deg C bath for 15 minutes to allow for digestion of DNA

Purification of mRNA with phenol/chloroform

- Add 115 µl nuclease free water (*now that you are working with RNA be sure to use the nuclease free water provided with the kit*)
- Add 15 µl Ammonium Acetate stop solution
- Vortex for about 20 seconds
- Add 500µl (i.e. equal volume) of phenol/chloroform
- Vortex for about 20 seconds
- Centrifuge for 30 seconds at 4 deg C at 14000 rpm
- Remove supernatant and put into new sterile Eppendorf tube
- Add 500µl (i.e. equal volume) of chloroform
- Vortex for about 20 seconds
- Centrifuge for 30 seconds at 4 deg C at 14000 rpm
- Remove supernatant (aqueous phase) and put it into new sterile tube

Precipitate mRNA with EtOH

- Add 1 ml EtOH
- Store at -20 deg C for 30 minutes to allow for precipitation
- Centrifuge for 20 minutes at 4 deg C at 14000 rpm

At this point you should see a large pellet of RNA stuck to the side of the tube. If so, congratulations, it worked!!!!

- Remove and discard supernatant
- Add 500µl of 70% EtOH washing the sides of the tube as you add
- Centrifuge for 3 minutes at 4 deg C at 14000 rpm
- Dry pellet in air for about 15 minutes
- Resuspend in 15ul nuclease free water

- Store RNA at -80 deg C. Can store RNA indefinitely at this point.

Quantification

Use the spectrophotometer to determine how much mRNA you have. Nucleic acids have a peak absorbance at 260nm. A peak value of 1.00 corresponds to $40\mu\text{g/ml}$ of RNA. Thus the concentration of RNA in a diluted sample is

$$A_{260} * \text{dilution factor} * 40 \mu\text{g/ml}$$

The spectrophotometer in the lab will make all the calculations for you. Read pages 59 – 60 and page 67 in the Biophotometer manual for the steps needed to make the measurements. Be sure to include the dilution in your measurements following the instructions on page 67.

You can also determine the purity of your sample. The absorbance at 280nm has exactly half the value of that at 260nm. Thus if sample is pure the ratio A_{260}/A_{280} should be 2. You can accept a value as low as 1.5; but if it is lower, then your sample is contaminated and you get to start over. The Biophotometer also gives a read out for this ratio.

Injection of mRNA Into the Oocyte

Preparation of micropipettes

- Pull some glass micropipettes (*this is same procedure as pulling electrodes*)
- Bake pipettes in the oven for 2 hours at 180 deg C to get rid of RNAses
- Put on gloves and get one of the pipettes
- Get a pair of forceps and fire the tips
- Under microscope, barely break the tip of the pipette with the forceps

To get this right will require plain old practice and trial and error. The tips cannot be too big or they will damage the oocytes, on the other hand, the hole in the tip has to be big enough to allow for injection of fluid. The only way to figure out what is “too big” or “too small” is to screw up a couple of times.

- Back fill pipette with oil using the syringe
Be sure not to allow air bubbles to get in.
- Lower the nanoinjector tip by pushing the empty button on controller box. Lower it until it sticks out a couple of centimeters or so.
- Push pipette over injector tip, ensuring it fits snugly into screw holder
- Once in, push empty button to see if oil comes out of tip
- Get 15 or 20 or so eggs and put them and some Bart’s solution into a dish which has a mesh on the bottom. The mesh will help hold the eggs.
- Line up eggs, each egg in a square, so that the vegetable pole (white half) of the egg is facing up. You will be injecting into the vegetable pole

Getting RNA for injection

- You should probably change gloves at this point
- Get a sterile Eppendorf tube; break off its cap. You will use the cap to put the RNA into.
- Get a tube of RNA from the freezer
- Vortex tube to thaw the mixture
- Centrifuge tube for 10 seconds to get mixture to the bottom of tube and prevent contamination.
- Using a 10 μ l pipette labeled “RNA only”, pipette desired amount into the cap

E.g., using a solution of 2ng/10nl solution, put 0.2 μ l in cap to inject 20 eggs at 2ng per egg (10nl per injection). The normal amount per egg should be about 0.2ng per egg. We used 10 times that amount to exaggerate expression, to increase the hope of getting expression. But once confident that your procedure is working, 0.2ng per egg should suffice.

- Position cap under microscope at injection station, move injection tip close to the “bubble” of RNA solution with course controls, then insert tip into the “bubble” with microcontroller.
- Push fill button to fill injection tip with RNA. *Be sure not to suck up air by letting the tip come out of the “bubble” while still filling.*
- You are now ready to inject oocytes
- Position oocytes such that you can easily move from one egg to the next by just the moving dish. Once you get the hang of this, injecting should become efficient.
- Position first egg under injection tip.
- Slowly stick egg with tip by turning microcontroller clockwise
- Make sure settings on controller box are set for desired volume of injection. (10nl is a good starting value *see manual of nanoinjector for detail on how to do this*)
- Push the inject button on controller box
- Pull out the tip by turning microcontroller counter clockwise. *(try to be somewhat quick when pulling tip out to increase probability that egg can heal from the trauma of being impelled)*
- Position next egg, and repeat above
- When done transfer eggs into another dish with Bart’s solution and store at 16 deg C
- Allow at least 48 hours for complete expression

Using Electrophysiology To Detect If Expression Occurred

By applying a command voltage which starts at the resting potential of the particular ion channel expressed and increasing by 10mV steps, you should see a typical current response of the protein channel. E.g., -90mV is the resting potential for the shaker

potassium channel (Johnston 17). The verification of such a response gives an indication that expression was successful.

- Set up an egg at an electrophysiology station for electrical measurements
- Once you have electrodes stuck in an egg and are ready to take measurements, open the Winwcp program on the computer.
- Look under **help, contents** for the section entitled “Things to do before making a recording”
- Read through the section entitled “Creating a Stimulus Protocol” to familiarize yourself with setting up the appropriate command signals

Below I shall use the shaker potassium channel as an example. If you expressed a different channel, adjust voltages accordingly

- Create a new file by going to **New** under **File**
- Go to **Stimulus protocol editor** under **Setup**
- Drag step voltage command onto the voltage command protocol area
- Enter the following settings

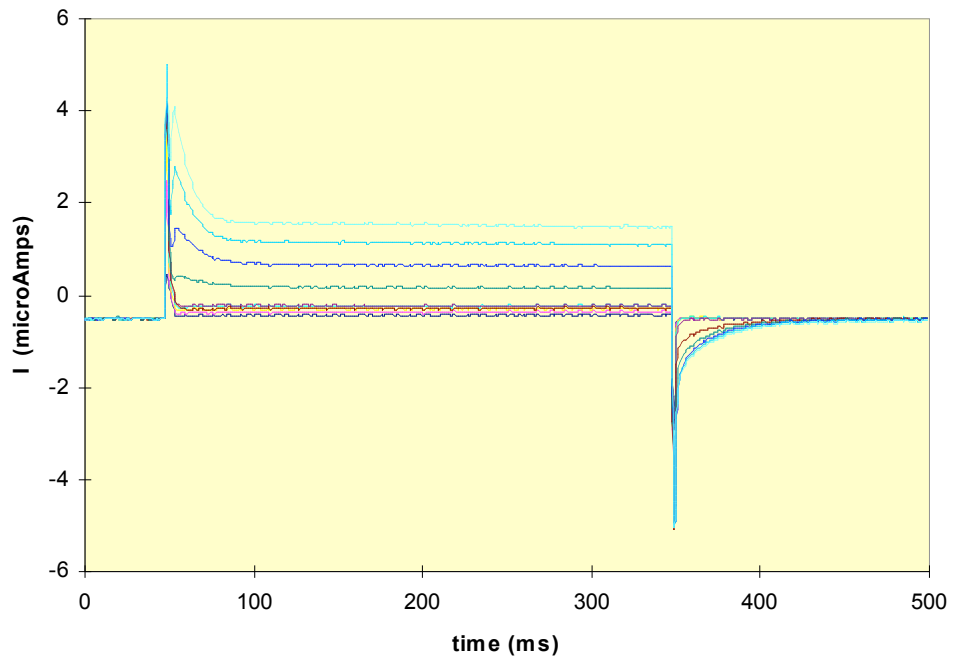
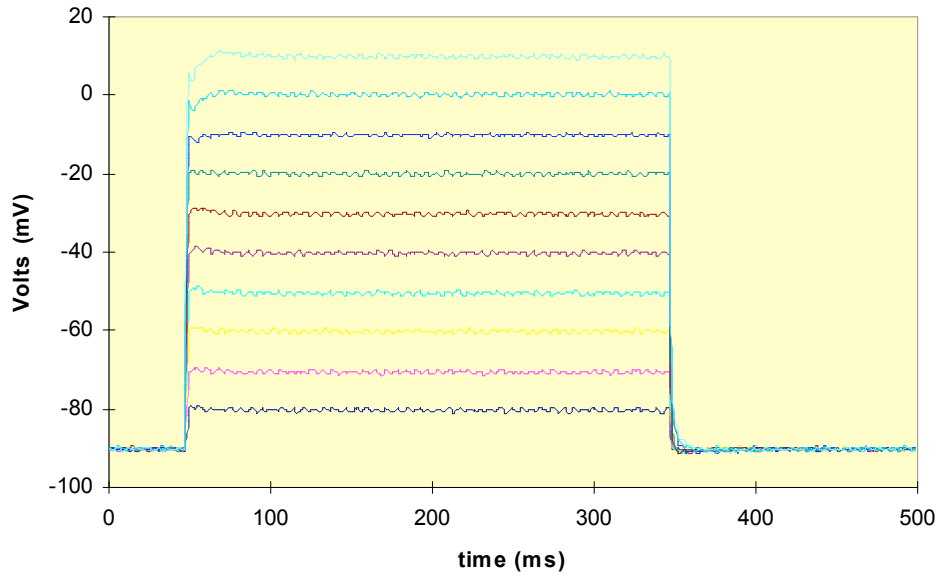
Delay	50.00ms
Initial Amplitude	-90.00mV (<i>if shaker</i>)
Increment	10.00mV
# of Increments	10
Duration	300.00ms

- Click on **Recording Sweep** and enter the following settings

Interval between Sweeps	1000.00ms (only doing one sweep)
Duration	500.00 ms
No. of waveform repetitions	1
Delay	0.00ms
Delay increment	0.00 ms
Holding Voltage	0.00 mV

- **Save** settings
- Go to **Record to Disk** under **Record**
- Ensure your saved stimulus protocol is the one listed
- When ready, click record
- If expression occurred, the current response should look something like that in fig. 1.

Figure 1:
Current Response of Shaker Potassium Channel



References

Johnston, D., Miao-Sin Wu, S. (1999) *Foundations of Cellular Neurophysiology*. MIT Press, Cambridge.