**mRNA Synthesis for microinjection**

IS 100726

**Protocol Overview**

1. Linearization of template DNA at the 3’ end with restriction enzymes
2. Purification of DNA template with phenol/chloroform/IAA extraction and precipitation
3. Transcription of DNA
4. Purification of RNA
5. Analysis and storage of RNA

**Reagents, equipment and precautions**

1. Must use gloves throughout the protocol
2. Pipettes must be decontaminated with RNase Zap
3. Use autoclaved microcentrifuge tubes and filter pipette tips
4. All water and solutions must be RNase -free
5. All procedures are done at Room Temperature unless otherwise indicated

**Day 1. Linearization and purification of DNA template**

1. Digest 5-10 µg of DNA (about 20 µl of miniprep DNA) with an appropriate restriction enzyme that cuts at the 3’ end of the gene. Make sure that after restriction digest the gene retains its polyA tail. Digest DNA at the appropriate temperature for the restriction enzyme to work. Digest DNA for at least 1 hour.
2. Bring the volume of the DNA restriction digest to 200 µl with water.
3. Add 200 µl of phenol/chloroform/isoamil alcohol at pH 8-8.2. Vortex mixture hard for at least one minute. The solutions should make an emulsion. WASTE REMIDER: all phenol and chloroform waste must not be disposed in the drain or with the regular garbage. Store it to be picked up by Environmental Safety Office.
4. Spin tube for 5 minutes at 13-14K RPMs in a centrifuge.
5. Take the top aqueous phase to a new microcentrifuge tube.
6. Add 200 µl of chloroform and vortex the mixture hard for one minute.
7. Spin tube for 1 minute at 13-14K RPMs in a centrifuge
8. Take the top aqueous phase to a new microcentrifuge tube.
9. Add 20 µl of 3M Sodium Acetate ph 5.2 and 500 µl of Absolute Ethanol.
10. Mix solution gently and store overnight at -20°C (freezer)

**Day 2. Synthesis and Purification of mRNA**

*DNA resuspension and analysis*

1. Spin tubes for 15 minutes at 13-14K RPMs in a centrifuge.
2. Carefully remove supernatant leaving DNA pellet undisturbed at the bottom of tube.
3. Carefully add 180 µl of 70% Ethanol (in RNase free water).
4. Spin tubes for 5 minutes at 13-14K RPMs in a centrifuge.
5. Carefully remove supernatant leaving DNA pellet undisturbed at the bottom of the tube.
6. Invert tubes on a rack and allow DNA pellet to dry for about 30 minutes. Do not over dry pellet.
7. Add 20 µl of RNase free water to DNA pellet and incubate at 37°C for 5 minutes
8. Vortex DNA, tap tube down to bring liquid to the bottom of the tube and incubate at 37°C for an additional 5 minutes.
9. Vortex DNA and spin down at 13-14K RPM in a centrifuge
10. Verify that DNA is linear by taking 1 µl of DNA and running it on a 1% agarose gel. Remember to mix the 1µl of DNA with 8 µl of water and 1 µl of loading dye.
11. Measure the concentration of 1 µl of DNA using the Nanodrop.

*Synthesis of mRNA*

1. Thaw required buffers from the Ambion mMessage mMachine mRNA Synthesis Kit. Thaw at room temperature the 10X Reaction Buffer and the 2X Ribonucleotide Mix. Keep on ice the Enzyme Mix.
2. To an autoclaved 1.5 ml microcentrifuge tube at the following components in the indicated order at room temperature (i.e., not over ice):
	* 1. 10x Reaction Buffer 2 µl
		2. 2x Ribonucleotide Mix 10µl
		3. 1 µg of DNA + Water 6 µl
		4. Enzyme Mix 2 µl

FINAL VOLUME 20µl

1. Mix gently by pipetting the mixture up and down
2. Spin down at 13-14K RMP to collect liquid at bottom of tube
3. Incubate reaction for 2 hours at 37°C.
4. Spin down at 13-14K RMP to collect liquid at bottom of tube
5. Add 1 µl of Turbo DNase 1 to tube (enzyme is found in Ambion Kit)
6. Mix gently by pipetting the mixture up and down
7. Incubate reaction for 15 minutes at 37°C.

*Purification of mRNA*

1. Spin down at 13-14K RMP to collect liquid at bottom of tube
2. Add 80 µl of RNase free water to the tube
3. Add 350 µl of Buffer RLT to tube, and mix well. This and all subsequent buffers are part of the Qiagen RNeasy Minielute Cleanup Kit.
4. Add 250 µl of 100% Ethanol, and mix well by pipetting.
5. Transfer the sample (700 µl) to an RNasey MiniElute spin column placed in a 2 ml collection tube (supplied with the kit). Close the lid gently and centrifuge for 15 sec at 10,000 RPMs. Discard the flow-through, but reuse collection tube in next step.
6. Add 500 µl Buffer RPE to the spin column (make sure that ethanol was added to it). Close the lid gently, and centrifuge for 15 sec at 10,000 RPMs. Discard the flow-through, but reuse collection tube in next step.
7. Add 500 µl of 80% ethanol (in RNase free water) to the RNeasy MiniElute spin column. Close the lid gently, and centrifuge for 2 minutes at 10,000 RPMs. Discard the flow-through and collection tube.
8. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
9. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.
10. Retain the 10-12 µl of eluated RNA and discard spin column. In a new tube mix 1 µl of RNA and 9 µl of RNase free water. This diluted RNA will be used for subsequent analyses. Store concentrated and diluted RNA at -20°C.

**Day 3. Analysis of RNA**

1. Thaw diluted RNA solution. Take 6 µl of diluted RNA and put them in a new tube. Use the 4 µl remaining in the tube to quantify the concentration of RNA in the solution using the Nanodrop (make sure that you set up the machine to measure RNA instead of DNA).
2. To the 6 µl of diluted RNA add 6 µl of Ambion Gel Loading Buffer II, and heat sample for 3-5 minutes at 80-90°C.
3. Spin down at 13-14K RMP to collect liquid at bottom of tube. Keep tubes cold on ice.
4. Load sample in a 1% agarose gel in TBE. Run gel for 20-30 minutes at 100 V. Bands will not migrate too far but would be enough to distinguish the size of transcript. Expect to see band of expected size plus additional bands due to secondary structure of RNA.
5. If band on gel looks ok and you have a good concentration of mRNA based on spectrophotometric reading, adjust the concentration of your RNA to 500 ng/µl (=0.5µg/µl) and make 1.1 µl aliquots. Store aliquots at -80°C for long term storage.