Palmenberg lab protocols for growing cardioviral plasmids (pEC9, pMC0, pMwt), linearizing the cDNAs, making RNA transcripts, transfecting HeLa cells with transcripts, and/or infecting cells with virus. Buffer recipes are at end of file.

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Qiagen maxiprep protocol summary

1. For Mengo cDNAs start a 2 ml culture in the morning from an isolated colony from an agar plate with the appropriate selective antibiotic. For EMC cDNAs skip over night culture go directly from plate into 100 mls of LB.

2. Inoculate 1 ml of the starter culture in 100 ml of LB medium plus ampicillin and grow over night.

3. Harvest the cells by centrifugation at 6000 x g (6,000 rpm in a JA-20 rotor) for 5 min. Remove all the supernate.

4. Resuspend the pellet in 10 ml of **buffer P1.** (Qiagen Plasmid Maxi kit #12163). Transfer suspension into 30 ml Oakridge tube.

5. Lyse the cells with 10 ml of **buffer P2**. Mix gently by inverting the tube 4-6 times and incubate at room temperature for exactly 5 min.

6. Add 10 ml of **buffer P3,** mix gently, incubate on ice for 15-20 min.

7. Spin at 20,000 x g (16,0000 rpm JA-20) for 30 min at 4°C. Transfer supernate to fresh 30 ml Oakridge tube

8. Spin at 20,000 x g (16,0000 rpm JA-20) for 15 min at 4°C.

9. Equilibrate tip 500 by applying 10 ml of **buffer QBT**

10. Apply supernatant to the column. Wash the column twice with 30 ml of **buffer QC**. Elute with 15 ml of **buffer QF**. Collect eluate in a 50 ml falcon tube.

11. Precipitate DNA with 0.7 volumes of isopropanol at room temperature. Transfer to a corex tube. Spin immediately (16,000 rpm JA-20) for 30 min at 4°C. Carefully remove supernatant by inverting the tube. Let the tube sit for one minute and aspirate off any remaining isopropanol. Resuspend the pellet in 400 µl of sterile dd water, vortex the tube to resuspend the DNA that's adhered to the tube walls. Transfer to a 1.5ml eppi.

12. Precipitate the DNA with 1 ml of 100% ETOH do not use salts. Wash the DNA twice with 1 ml of 70% ETOH. Centrifuge and air dry the pellet for 5 min.

13. Resuspend the pellet in 300 µl of TE buffer pH 8.0. Spec DNA for conc. at 260/280.

Linearization of DNA

5 µg of DNA
18.1 µl of Sterile ddH_20
3 µl of BSA with enzyme Sal 1 (NEB)
3 µl of 10X buffer
0.9 µl of Sal1
30.0 µl total reaction volume.

1. React at 37°C for 5 hrs.

2. Run a agarose gel to ensure DNA is completely linearized.

3. Cleanup DNA, by phenol/chloroform extraction, followed by ethanol precipitation.

4. Alternatively, use Qiagen QIAquick PCR purification kit #28104. Speedvac 50 µl from column to a volume of 10 ul.

5. Check integrity on agarose gel.

IMPORTANT: For pEC_9 use Sal1 to linearize**. For pMC_0 and pMwt use BamH1.** NEB is the vendor for all enzymes.

RNA Transcription

Before beginning transcription, heat DNA aliquot for 8 min at 55°C. Have everything ready as master mix to add to DNA.

1. Mix
   ~ 2 µg linearized DNA template (Clean up with PCR kit)

5X T7 RNA Polym. buffer	5 µl
7.5 mM rNTPs	3 µl
0.7 M DTT	1 µl
RNasin	1 µl
T7 RNAPolym.	1.5 µl
H_2O	to 25 µl

2. Incubate at 37 °C (air) for two hours

3. Add:

RNase-free DNase	1 µl
H_2O	74 µl

4. Incubate at 37°C (air) for 20 minutes

5. Use Qiagen RNeasy Kit for clean-up (Qiagen Kit # can be used for cleanup)

6. See page 48 in Qiagen Kit booklet: our changes: add 355 µl of RLT in step 1, 255 µl ethanol step2, Step 6 an additional spin for 15 sec, with 45 µl Tris. Wait 1 minute before transfer.

7. Resuspend in 45-50 µl of 10 mM Tris pH 8.0

8. Speedvac to dry, without heat for 45 min, check after 30 min.

9. Remove 1 µl for gel analyses, 3 µl for spec at 260 (697 µL of Tris).

DNase is from Roche cat. #1 119 915 (500 µg/ml)
T7 RNA Polymerase is Life Technologies cat. #18033-019

Transfection of HeLa cells

1. Prewarm PBS and HBSS to 37°C

2. Dilute out your RNA. 50 ng/µl, 5 ng/µl, and 0.5 ng/µl should give a good range of plaques, so some plates will be able to be counted.

3. Set up RNA's to transfect onto plate. For 2 plates of 250 µl each use:
   - 488 µl HBSS
   - 10 µl Transfectase
   - 2 µl RNA
   - 0.2 µl rRNAsin

   Hold on ice until cells are ready.

4. Aspirate medium off of cells. Wash with about 2.5 ml 1X PBS.

5. Plate 250 µl of RNA sample onto each plate. Let sit at room temperature for 30 minutes.

6. Make agar and liquid overlays.

Agar overlay	Liquid overlay
50% P5	50% P5
50% 1.6% Noble Agar	48% ddH2O
	1.0% 100X GOP
	1.0% 20% Dextrose
	warm to 37°C

7. Wash plates with about 2.5 ml of 1X PBS.

8. Add 2.5 ml agar overlay on each plate. Let solidify. Add 2.5 ml liquid overlay to each plate. Place at 37°C for 29 hours for EMCV (31 hours for Mengo), under 5% CO_2

9. Aspirate off medium when time is up. Look for plaques and pick any if desired. (Place plaques in 1 ml of P5 or in PBS, let elute at 4°C overnight).

10. After plaques are picked, flip agar off of the plate. Add 1X crystal violet. Set at room temperature 15 minutes to overnight.

11. Aspirate off crystal violet. Wash with dH_2O. Look at and count plaques.

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Infecting HeLa cell plates

1. Check monolayers of HeLa cells under microscope to make sure they are healthy and confluent.

2. Make desired dilutions of virus into PBS

3. Aspirate medium A from plates and rinse with 2.5 ml PBS (for 6-well plates use 1.5 ml, for 100mm plates use 5 ml). Once you have removed the medium you want to work quickly to avoid desiccation of the monolayer.

4. Infect the plate with 250 µl of each viral dilution. (For 6-well plates use 1.25 µl and for 100mm plates use 500 µl). Set at room temperature for 30 minutes to allow the virus to attach.

5. During the attachment period, make up the overlays. If you are doing a plaque assay you will need 2.5 ml of each overlay per plate. (6-well plates use 1.5 ml of each, 100 mm plates use 5 ml of each). Warm the liquid overlay and the P5 for the agar overlay at 37°C before use. Add the agar to the warm P5 and let cool so you can touch it, then place it on the cells. Make sure to swirl each plate after adding the agar. Then when the agar layer has solidified, add the liquid overlay.

Agar overlay	Liquid overlay
50% P5	50% P5
50% Noble agar, 1.6%	48% ddH_2O
	1.0% 100X GOP
	1.0% 20% Dextrose

6. Incubate for 28 hours, for EMCV, (for Mengo go 29 hours) at 37°C under 5% CO_2.

7. After 28 hours take plates out of the incubator and look for plaques. Aspirate off the medium carefully. Then flip off the agar into a biohazard bag.

8. Stain with 1X crystal violet for 15 minutes to overnight.

9. Aspirate off the crystal violet and wash with ddH_2O. The plaques can now be visualized easily and counted. Count plaques for each dilution, average, multiply by the dilution factor and 4 to determine PFU/ml of the viral stock.

$$\text{avg number of plaques} \times \text{dilution} \times 4 = \text{PFU/ml}$$

•Reference - Rueckert et al., *Methods in Enzymology*, **78**, 315, (1981)

P5 (2x)

Amount:	**Storage:**	**Sterilization:**
2500 ml	**450 ml/ 500 ml bottle**	**millipore**

Protocol

1. MEM*	5 pouches (9.5 g each)
2. Penicillin-G	0.3 g
3. Streptomycin sulfate	0.5 g
4. $NaHCO_3$	11.1 g
5. DEAE dextran	0.375 g
6. $MgCl_2$	40.5 g
7. Bovine Albumin Serum	5.0 g
8. NEAA	50 ml
9. ddH_2O	2300 mls

*with Earle's salts, with L-glutamine, without $NaHCO_3$

Mix all ingredients except the NEAA until dissolved. Once dissolved add the NEAA. Take a reading using the pH meter. The pH should be 7.1. If it is not, add HCl or 10M NaOH drop-wise until the desired pH is reached. Add ddH_2O to bring final volume to 2500 ml.

Filter sterilize using the Millipore apparatus. To check for contamination aliquot 10 ml of media into 50 ml falcons at the beginning, middle, and end of bottle filling. Take 200 µl aliquots from the falcons and inoculate them into 2 ml of 2xYT in 5 ml falcons. Store the samples at 37˚C with 5% CO_2 for 3 days and then check for bacterial growth.

This media is used for plaquing virus. See Plating cells protocol.

20% Dextrose

Amount:	**Storage:**	**Sterilization:**
100 ml	**100 ml/100 ml bottle**	**filter sterilization**

Protocol

Dextrose 20 g
ddH_2O 100 ml
Stir until dissolved.

100x GOP

Amount:	**Storage:**	**Sterilization:**
100 ml	**100 ml/100 ml bottle**	**filter sterilization**

Protocol

L-Glutamine (Sigma G3126)	2.92 g
Pyruvic acid (Sigma P2256)	1.1 g
Oxalacetic acid (Sigma O4126)	2.64 g
ddH_2O	100 ml

Stir until dissolved.