Chapter 18
RNA Encapsidation Assay

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Abstract Analysis of viral RNA encapsidation assay provides a rapid means of assaying which of the progeny RNA are competent for packaging into stable mature virions. Generally, a parallel analysis of total RNA and RNA obtained from purified virions is advisable for accurate interpretation of the results. In this, we describe a series of in vivo assays in which viral RNA encapsidation can be verified. These include whole plants inoculated either mechanically or by Agroinfiltration and protoplasts. The encapsidation assay described here is for an extensively studied plant RNA virus, brome mosaic virus, and can be reliably applied to other viral systems as well as with appropriate buffers. In principle, the encapsidation assay requires purification of virions from either symptomatic leaves or transfected plant protoplasts followed by RNA isolation. The procedure involves grinding the infected tissue in an appropriate buffer followed by a low speed centrifugation step to remove the cell debris. The supernatant is then emulsified with an organic solvent such as chloroform to remove chlorophyll and cellular material. After a low seed centrifugation, the supernatant is subjected to high speed centrifugation to concentrate the virus as a pellet. Depending on the purity required, the partially purified virus preparation is further subjected to sucrose density gradient centrifugation.

Following purification of virions, encapsidated RNA is isolated using standard phenol-chloroform extraction procedure. An important step in the encapsidation assay is the comparative analysis of total and virion RNA preparations by Northern hybridization. This would allow the investigator to compare the number of progeny RNA components synthesized during replication vs. encapsidation. Northern blots are normally hybridized with radioactively labeled RNA probes (riboprobes) for specific and sensitive detection of desired RNA species.

Keywords Encapsidation; RNA virus; RNA packaging

From: *Methods in Molecular Biology, Vol. 451, Plant Virology Protocols: From Viral Sequence to Protein Function*
Edited by G.D. Foster, I.E. Johansen, Y. Hong, and P.D. Nagy © Humana Press, Totowa, NJ

1 Introduction

Encapsidation or assembly of plant viral genome into stable particles by virus-encoded coat protein is considered to be a crucial event in the life cycle of a given virus (17). Although this process was initially envisioned to protect viral nucleic acids from extra cellular environment, advances made in handling viral genomes through reverse genetics revealed that encapsidation is obligatory to fulfill two important roles: cell-to-cell and long distance spread within a susceptible host plant and transmission by insect vectors to healthy hosts (10, 12, 18). Consequently, study of encapsidation provides fundamental knowledge concerning how viruses assemble into infectious particles and eventually help to develop strategies to curb spread of viral diseases.

Plant viruses exhibit a variety of genome organizations (mono, bi, or tripartite) and particle morphologies (icosahedral, flexious, and rod-shaped) (15). Literature is replete with review articles concerning the organization of plant viral genomes and their replication (1, 3, 9, 13). Following initial replication of a given viral genome, genes required to perpetuate the infection process are expressed either by proteolytic processing of the polyprotein translated from the genomic RNA (eg., Monopartite potyviruses; 11) or via subgenomic RNA synthesis (eg., Monopartite tobacco mosaic virus or tripartite cucumber mosaic virus; 16). Mature virions of viruses that express their genes via polyprotein processing contain only the genomic RNA (eg., Potyviruses; 5). By contrast, encapsidation in viruses that express their genes via subgenomic RNA synthesis are highly selective. For example, although TMV and CMV express their coat protein genes via subgenomic RNA, only in the later case the subgenomic genomic RNA is efficiently encapsidated (15, 17). By definition, subgenomic RNAs are genetically redundant and are always generated from the replicated genomic RNAs. Thus, reasons for their selective encapsidation in one case but not in the other are not well understood. Therefore, a comparative analysis of total and virion RNA profiles obtained from infected hosts will be informative. Although the procedures described later are optimized for RNA encapsidation analysis of brome mosaic virus (BMV), they are applicable to other plant viruses as well.

2 Construction of Biologically Active Clones (4)

2.1 Materials

T7/T3 vector, PCR machine, PCR reaction kit (Ambion, or Strategene or other kit), restriction enzymes, Rapid ligation kit (Promega, or Stratagene), competent cells, LB medium, and appropriate antibiotics for selection.

2.2 Preparation RNA Transcripts (In Vitro Transcription)

Construction of a cDNA clone from which biologically active RNA transcripts can be synthesized in vitro using either T7 or T3 polymerase promoter is almost a

prerequisite for many molecular biology-related experimentation. Because cDNA clones are not directly infectious (8), placement of the viral sequence downstream of a promoter of a RNA polymerase is essential for in vitro synthesis of infectious RNA transcripts (2, 14). Alternatively, desired gene of interest can also be amplified by PCR with specific primers that contain polymerase promoter site and these templates can be used for in vitro transcription. The methods for cloning the gene(s) of interest are available in most of the molecular biology books such as Sambrook and Russel (20). Later we describe a procedure that is routinely used in our laboratory for in vitro synthesis of RNA transcripts (19) that can be used for in vitro encapsidation assays. Note that these reactions can be set up without incorporating a 5′ cap-like structure, which is not required for in vitro assembly. Furthermore, absence of 5′ cap-like structure will give high yield of RNA transcripts.

1. Lineraize the plasmid DNA with an appropriate restriction enzyme downstream of the insert to be transcribed.
2. Once the plasmid DNA was completely digested, extract the reaction mixture with phenol/chloroform and precipitate the DNA with ethanol followed by a 70% ethanol wash and drying the sample in speed vacuum.
3. Resuspend the digested plasmid DNA in TE buffer at a concentration of 1 g l^{-1}.
4. Using a commercially available kit (for eg., MEGAscript, Ambion), set up the transcription reaction at 37 °C (approximately for 1–2 h). Follow the instructions according to the manufacturer of the kit.
5. After incubation, DNA template is removed either with DNase (20) or by LiCl precipitation (19).
6. Centrifuge the contents at 4 °C for 15 min at maximum speed (12,000–15,000 rpm) to pellet RNA.
7. Remove the supernatant and wash the pellet with 70% ethanol.
8. Dry RNA pellet by speed-vac and dissolve with known amount of RNase free water.
9. Determine RNA concentration by spectrophotometer. It is imperative to verify the integrity of RNA transcripts by agarose gel electrophoresis prior to using them for in vitro assembly assays. Store RNA transcripts at −20 °C or −80 °C.

3 Virus Purification

3.1 Purification of BMV Virus Particles

Materials

BMV extraction buffer: 0.5 M NaAc; 0.08 M MgAc pH 4.5 and add 1/100 volume of B mercaptoethanol just before use. Store the buffer at 4 °C.

BMV suspension buffer: Dilute BMV extraction buffer to 1/10 with sterile distilled water.

Sterile mortars and pestles, sterile centrifuge tubes, chloroform and acid-washed sand (Sigma).

1. Collect BMV-infected leaves either barley, *Chenopiodium quinoa*, or *Nicotiana benthamiana*.
2. Grind leaves thoroughly in extraction buffer (1.0 ml g^{-1} leaf) and add 0.5 g acid-washed sand to facilitate easy grinding and breaking of cells.
3. Filter the extract through muslin cloth and collect flurry. Again ground the portion retained on cheese cloth with extraction buffer. Repeat filtration through cheese cloth.
4. Transfer the solution to centrifuge tubes and add equal volume of prechilled chloroform and vortex for 5 min at room temperature.
5. Centrifuge the emulsified solution at 10,000 *g* for 15 min at 4 °C.
6. Transfer supernatant to clean sterile beaker and make sure that no chloroform is left in the supernatant.
7. Transfer supernatant to sterile ultracentrifuge tubes.
8. Centrifuge at 30,000 rpm for 2.5–3 h in a high speed Beckman centrifuge.
9. Remove the supernatant completely and suspend pellet in desired volume of (200–500 l) BMV suspension buffer.
10. Subject the above partially purified virus to 5–25% sucrose density gradient centrifugation. Finally, measure the concentration of the virus using spectrophotometer at OD at 260 nm.

3.2 Preparation of Coat Protein Subunits

Materials

Required stock solutions: 1 M Tris-HCl, pH 7.5, 50 mM EDTA, 100 mM DTT, and 100 mM PMSF in isoprophyl alcohol.

1x Dialysis buffer: 0.5 M $CaCl_2$, 50 mM Tris HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM DTT, and 0.5 mM PMSF.

Dialysis membrane (20) or dialysis cassettes.

3.3 Dissociation of Coat Protein from Purified Virus Particles

1. Prepare 1,000 ml of 1x dialysis buffer.
2. Prepare dialysis membranes according to Sambrrok et al. (20).
3. Dispense required concentration of purified virus into a dialysis bag. Test for any holes in the dialysis bag, prevent leakage during dialysis.
4. Place the virus containing dialysis bag or dialysis cassette in a beaker containing dialysis buffer.
5. Dialyze 24 h at 4 °C while stirring. A cloudy precipitate (representing viral RNA) will appear.
6. After 24 h, a cloudy precipitate (viral RNA) must appear in the dialysis bag. Collect the solution from the dialysis bag/cassette and centrifuge at 12,000 *g* for

15 min at 4 °C to pellet viral RNA. Cloudy precipitate will form a pellet and this pellet can be used to recover viral RNA by reextracting with phenol/chloroform followed by ethanol precipitation.
7. Collect supernatant and centrifuge at 220,000 *g* in a Beckman TL 100 centrifuge for 2 h at 4 °C to pellet any undissociated virus particles.
8. Collect the supernatant.
9. Determine the concentration of the coat protein subunits by measuring at OD 254 and 280 nm or by other methods such as Bradford assay.
10. Use coat protein subunits immediately or can be stored at 4 °C for 1–2 weeks.
11. Verify the authenticity and integrity of coat protein by 12–16% SDS-PAGE followed by Western blot analysis.

3.4 In Vitro Assembly of Empty and RNA Containing Virions

Materials

1. RNA transcripts
2. RNA assembly buffer: 50 mM NaCl; 50 mM Tris-HCl, pH 7.2; 10 mM KCl; 5.0 mM $MgCl_2$; 1.0 mM DTT
3. Re-assembly buffer: 1.0 M Nacl; 50 mM NaAc, pH 4.8; 1.0 mM EDTA; and 1.0 mM DTT
4. Dialysis buffer B: 1.0 M NaCl; 20 mM Tris-HCl, pH 7.5, 1.0 mM EDTA; 1.0 mM DTT and 1.0 mM PMSF

3.5 In vitro assembly of empty virions

1. Empty virions of BMV can be assembled in vitro by dialyzing only coat protein subunits of about 200–500 g against reassembly buffer at 4 °C for 24 h.
2. Dispense the reaction mixture to Centricon-100 microconcentrators (Amicon, Beverley, MA; Follow instructions supplied by the manufacturer for using microconcentrators)
3. Centrifuge at 2,000 *g* at 4 °C for 30 min
4. Wash the reaction mixture by adding 1.5 ml of reassembly buffer
5. Repeat the above step two more times
6. Finally elute the virions by centrifugation at 4 °C for 5 min
7. Assess virion assembly under an electron microscope (see later)

3.6 In Vitro Assembly of RNA Containing Virions

1. Prepare RNA transcripts to be assembled and calculate the concentration
2. Mix the coat protein subunits and RNA transcript at a ratio of 1:5 (wt/wt)

3. Dispense above mixture to a dialysis bag/cassette and properly secure to avoid any leaks
4. Prepare 1,000 ml of RNA 1x assembly buffer
5. Place the dialysis bag into beaker and stir
6. Dialyze the assembly reaction at 4 °C for 24 h
7. After 24 h, collect the mixture containing assembled virions and add 1.5 ml of RNA assembly buffer
8. Pass this mixture through Centricon-100 column and centrifuge at low speed (2,000 *g*) for 30 min
9. Wash the column once with 1.5 ml of RNA assembly buffer at 4 °C for 30 min
10. Repeat the above washing step
11. Elute the virions by centrifugation at 4 °C for 5 min
12. Verify the concentration at OD 260 nm

3.7 Electron Microscopy

1. Adjust the concentration of virus particles to 10–25 g ml^{-1}
2. Place a drop of solution containing virus particles in a glow discharged carbon coated copper grid
3. Add 10 l of 1% uranyl acetate and leave it for a minute
4. Wash once with sterile distilled water and allow drying for 1–5 min
5. Examine grids under an electron microscope

3.8 Extraction Viral RNA from Encapsidated Virions

Materials

Eppendorf tubes (1.5 ml), bentonite (2.5 mg ml^{-1}), 20% SDS, phenol/chloroform/isoamyl alcohol-PCI (25:24:1, v/v) and ethanol.

1. Collect in vitro assembled virions and transfer them to 1.5 ml eppendorf tube
2. Add 1/10 vol bentonite and 20% SDS; Mix well
3. Add equal volume of phenol/chloroform/isoamyl alcohol and vortex for 5 min
4. Centrifuge at 12,000 *g* for 20 min at 4 °C
5. Collect supernatant and precipitate RNA by adding 1/10th of 3 M NaOAc (pH 5.2) and 2.5 volume of cold 100% ethanol
6. Keep at −20 °C for overnight or at −80 °C for 3 h
7. Centrifuge at 12,000 *g* for 20 min at 4 °C
8. Wash pellet with 70% ethanol and dry
9. Dissolve pellet in RNase-free water and estimate concentration of the RNA by spectrophotometer

4 Assaying RNA Encapsidation in Plant Protoplasts

Materials

Barley or Chenopodium quinoa or Nicotiana benthamiana leaves; 0.55 M mannitol in water pH 5.9; 0.55 M sucrose in water; Sterilize the solution by filtration. Polyethylene glycol-40%, (weigh 40 g PEG-2000, add 50 ml sterile distilled water, 10 ml of 5% MES (morpholineethanesulfonic acid) buffer (pH 5.9), and 1 ml of 0.3 M $CaCl_2$. Briefly warm the mixture in microwave oven and stir the mixture until it dissolved. Adjust the pH 5.9 with 0.1 M KOH. Finally, filter the solution using 0.45- m pore size filter. Store at room temperature. Use immediately or store upto 3 weeks).

Enzymes: Cellulose, Macerozyme, Driselase, and Macerozyme, BSA

4.1 Protoplast Culture Medium

Solution A (1,000×): Dissolve 0.00249 g copper sulphate, 0.0166 g potassium iodide (KI), and 26.648 g magnesium sulphate in 100 ml of 0.055 M mannitol, pH 5.9. Check the pH (5.8) of the solution and sterilize by autoclaving.

Solution B (1,000×): Dissolve 2.712 g of potassium phospahte (KH_2PO4) and 10.111 g potassium nitrate (KNO_3) in 100 ml sterile distilled water and adjust pH to 6.5 with 1N KOH. Sterilize by autoclaving.

Solution C (1,000×): Dissolve 14.70 g of calcium chloride in 100 ml of 0.55 M mannitol pH 5.9. Adjust the pH to about 6.2 and sterilize the solution.

Gentamycin: Prepare 10 mg ml^{-1} stock solution in sterile water. Store at −20 °C.

Cephaloridine: Prepare 30 mg ml^{-1} stock solution in sterile water. Keep at −20 °C.

4.2 Preparation of Protoplasts

1. Prepare the enzyme solution: For 0.5 g leaf tissue, use 0.25 g cellulose, 12.5 mg BSA, and 12.5 mg macerozyme. Add 12.5 ml 0.55 M mannitol, pH 5.9 and stir at room temperature. Filter the solution through 0.45- mpore size filter.
2. Collect 0.5 g leaves of barley plants (5–6 days old). Slice the leaves lengthwise and then crosswise with a razor blade. Incubate the sliced (1 mm^2) leaf materials in the enzyme solution for 3–4 h at 28 °C.
3. After 3–4 h, decant the enzyme solution containing the protoplasts into beaker. Filter through gauze (300–350 m) into another beaker. Transfer the solution to a sterile 50 ml polypropylene tube underlaid with 10 ml of 0.55 M sucrose. Use 12.5 ml protoplast solution per tube.
4. Centrifuge in a Beckmann centrifuge at 400 rpm for 5 min at 20 °C.

5. Remove the protoplasts from the interface of mannitol and sucrose. (They will constitute a dark green band at the interface.) Transfer them into another 50 ml tube containing 10–15 ml 0.55 M mannitol, pH 5.9. Mix gently and centrifuge at 600 rpm for 4 min at 20 °C. Remove most of the supernatant and gently resuspend the cells in the remaining liquid. Add 10 ml 0.55 M mannitol, pH 5.9 and repeat the wash as described earlier.
6. Resuspend the protoplasts in a known volume of 0.55 M mannitol, pH 5.9 and determine the number of viable protoplasts with a hemacytometer. [Stain the protoplasts with fluorescein diacetate by combining 1–2 drops of protoplasts suspension solution with 1–2 l of fluorescein diacetate (5 mg ml^{-1} in acetone). Count the bright fluorescent cells].
7. Centrifuge at 600 rpm for 3 min at 20 °C. Remove most of the supernatant and keep the volume as quite small and add either virus or RNA transcript.

4.3 Transfect the Protoplasts with Viral RNA or Transcripts

1. Gently shake the pellet to resuspend the protoplasts
2. Add RNA transcript or/viral RNA and then add 150 l of 40% PEG
3. Gently shake for 10 min, and add two drops of 0.55 M mannitol, pH 5.9. Gently mix the suspension
4. Continue to add mannitol dropwise over the next 5–10 min until the volume has reached 1.5 ml. Incubate on ice for 15 min
5. Pellet the protoplasts at 600 rpm for 3 min at 20 °C
6. Wash once with 1 ml 0.55 M mannitol, pH 5.9
7. Resuspend 1×10^5 protoplasts in 1 ml culture medium containing 0.55 M mannitol (pH 5.9), a 1x concentration each of solutions A, B, and C, 10 g of Gentamycin and 0.3 mg Cephaloridine.
8. Place the transfected protoplasts in a culture plate and keep under fluorescent lamp for 20–24 h.

4.4 Isolation of Assembled Virions and Extraction of Encapsidated RNA

Materials

Protoplast lysis buffer: 100 mM glycine, 10 mM EDTA, 100 mM NaCl (pH 9.5), 2% SDS, bentonite 2.5 mg ml^{-1}; phenol/chloroform (25:24, v/v); 100% ethanol.

1. Collect the transfected protoplasts by centrifugation at 600 rpm for 3 min at 20 °C.
2. Discard the supernatant and add 250 l protoplasts lysis buffer and add 250 l phenol/chloroform.

3. Vortex for 5 min at room temperature
4. Centrifuge at 12,000 rpm for 10 min at 4 °C.
5. Collect the supernatant and repeat the phenol/chloroform extraction.
6. Collect the supernatant and add 1/10th of 3 M NaoAc (pH 5.6) and add 2.5 volumes of 100% cold ethanol.
7. Mix the contents and keep at −80 °C for 15–30 min.
8. Pellet the RNA by centrifugation (12,000 rpm) for 20 min at 4 °C. Wash the pellet with 70% ethanol, dry the pellet, and dissolve the RNA in 25 l of water.

5 RNA Encapsidation Assay in Planta (Agroinfiltration)

First, to facilitate agroinfiltration, the genome of RNA virus was placed in the expression vector (binary vector) under the control of the CaMV 35S promoter and transformed into suitable agrobacterium strains. Transformed agrobacterial cultures are grown to log phase, collected by low speed centrifugation, and resuspended in an infiltration solution (10 mM $MgCl_2$, 10 mM MES). The suspension is used further for leaf infiltration. After 2–6 days, the infiltrated leaves can be used for the analysis of gene of interest and its encapsidation. The agroinfiltration system has several advantageous over the conventional methods. Using agroinfiltration, the expression and encapsidation of two or more genes can be analyzed by different combinations of agrobacteria containing genes of interest (6, 7). For example, in the case of bipartite or tripartite viruses, coexpression of individual viral RNAs can be achieved by simply mixing agrobacterium strains each containing one of the RNAs prior to inoculation.

Materials: Binary expression vector, agrobacterium strain, LB medium, antibiotics (50 g ml^{-1} Kanamycin, 10 g ml^{-1} Rifampicin), 10 mM $MgCl_2$, 10 mM MES pH 5.6; 100 mM Acetosyringone in methanol.

5.1 Construction of T-DNA Based Plasmids

1. Select the appropriate T-DNA vector (for example pCASS vector).
2. Amplify the desired gene of interest by PCR having unique restriction sites at both ends. This will facilitate to clone the gene of interest in to T-DNA vector.
3. Digest the T-DNA vector and the PCR product.
4. Purify the digested product by Gel elution
5. Check the concentration of eluted producs for ligation.
6. Ligate the PCR produt with linearized T-DNA vector, transform it to *E. coli*, and plate on LB agar plates amended with Kanamycin or appropriate selection marker.

7. Incubate the plates for overnight at 37 °C.
8. Pick up few colonies and grow on LB liquid medium with appropriate antibiotics for overnight at 37 °C.
9. Extract the plasmid DNA, and screen by restriction analysis for the presence of gene of interest.
10. Once the positive clone has been identified (the plasmid DNA can be used to transform into agrobacterium).

5.2 Transformation into Agrobacterium

1. Take 4–6 g plasmid DNA (T-DNA construct having the gene of interest) and add to the agrobacterium competent cells (EHA105 or).
2. Keep on ice for 45 min.
3. Freeze the competent cells containing plasmid DNA on liquid nitrogen for 1 min.
4. Thaw the cells at 37 °C for 3 min.
5. Add growth medium (LB medium) and incubate in a shaker for 3–4 h at 28 °C.
6. Centrifuge the mixture (at low speed 2,000 rpm for 5 min).
7. Discard most of the supernatant and suspend the cells with little less than 0.1 ml medium.
8. Plate the mixture on LB agar plate supplemented with appropriate antibiotics.
9. Incubate the plates at 28 °C for 2–3 days.
10. Several colonies will appear on plates after 2–3 days.
11. Screen the colonies for presence of T-DNA plasmid (by mini preparation and restriction analysis).
12. Select the positive transformant for further studies.
13. Alternatively, store the positive transformant of agrobacterium by making glycerol stock and store at −80 °C.

5.3 Agroinfiltration

1 Steak appropriate transformed *A. tumefaciens* on LB agar plates supplemented with appropriate antibiotics and incubate at 28 °C for 2 days.
2. A single colony to be inoculated into 5 ml LB broth with 50 g ml^{-1} of kanamycin and 10 g ml^{-1} of rifampicin (or use an antibiotic of the helper Ti plasmid encoded resistance) and keep the tubes at 28 °C in an orbital shaker at 250–300 rpm for 2 days.
3. Inoculate 1 ml of fresh culture into 50 ml LB broth supplemented with kanamycin 50 g ml^{-1} as well as rifampicin 10 g ml^{-1}, 10 mM MES pH 5.6, and 100 M acetosyringone.
4. Keep the flask at 28 °C for 16 h in an orbital shaker, which can rotate at 250–300 rpm.

5. Check the absorbance of the fresh culture at OD_{600}. The OD_{600} of the culture must have reached to 1.0.
6. Transfer the fresh culture to screw cap oak ridge tube or sterile Falcon tube and centrifuge at 5,000 rpm for 10 min at room temperature in a Beckman or Table top centrifuge.
7. Decant the supernatant and resuspend the bacterial pellet with 50 ml of 10 mM $MgCl_2$.
8. Centrifuge at 5,000 rpm for 10 min at room temperature and decant the supernatant.
9. Resuspend the cells with 50 ml of 10 mM $MgCl_2$ and add 100 M acetosyringone (from 100 mM acetosyringone stock) and gently mix well.
10. Check the final optical density at 600 nm (OD_{600}). The final OD_{600} must be of 1.0. When two or more *A. tumifaciens* strains needed to infiltrate together, grow the each strain independently as mentioned above and mix equal amount prior to infiltration.
11. The bacterial culture should be kept at room temperature for at least 3 h without shaking.
12. After 3 h, the culture is ready for infiltration. Infiltration should be performed with a 1 ml syringe without needle.
13. Two or three expanded leaves of young seedlings of *N. benthamiana* (5 leaves stage, 2- to 3-week-old plants) should be ideal for infiltration.
14. Perform the infiltration by gently punching the tip of the syringe on the backside of the leaf with blocking by finger from the other side. Gently and slowly push the syringe barrel. Note the bacterial suspension spreads into the intracellular space of the leaf and further spreads up to tip of leaf. Once this has been done, infiltrate into the other leaf. After agroinfiltration, transfer plants to greenhouse.
15. Collect the leaf samples from 2 days and analyze the expression and encapsidation of gene of interest by extracting total RNA and virion RNA.
16. Analyze RNAs by Northern blot hybridization using specific probes.

6 Analysis of Encapsidated RNA by Northern Hybridization

6.1 Materials

The materials include agarose, gel casting tray, gel tank, 10× MOPS buffer (0.2 M MOPS, 40 mM NaOAc, 5 mM EDTA, adjust pH 7.0 with solid NaoH, filter the solution), 37% formaldehyde; 20× SSC (3 M sodium chloride, 0.3 M trisodium citrate), nylon membrane, blotting unit, sample buffer: 10× MOPS buffer/formaldehyde/formamide/H_2O (0.5 ml Formamide; 0.18 ml formaldehyde, 0.1 ml 10x MOPS buffer, 0.22 ml H_2O).

Hybridization solution: For 10 ml: 4.0 ml 5 M NaCl; 1.0 ml 20% SDS; 2.0 ml Denhardt's solution; 0.3 ml salmon sperm DNA; 0.2 ml yeast tRNA and 2.5 ml water.

6.2 Preparation of Formaldehyde-Denatured RNA Gel

1. Prepare 1.2% agarose gel by melting 2.4 g agarose in 174 ml RNAse free distilled water.
2. Use microwave until it completely dissolved.
3. Allow the mixture cool for 5–10 min.
4. Add 20 ml, 10× MOPS, and 6 ml of 37% formaldehyde and throughly mix and pour into the gel tray.

6.3 Sample Preparation and Electrophoresis

1. Take known amount of viral RNA (0.5–1.0 g), add 10–15 l sample buffer, and mix thoroughly.
2. Heat the reaction mixture containing viral RNA at 65 °C for 10 min and cool the reaction mixture for 5 min on ice.
3. Add 2 l of 6× loading dye.
4. Load the sample into the well.
5. Electrophorese the sample for 2–3 h at 100 V or until the dye front reached 2/3 of the way to the bottom of gel.

6.4 RNA Transfer from Gel to Nylon Membrane

1. Rinse the gel in 7× SSC for 10 min. RNA transfer from gel to a nylon membrane to be performed with transVac vaccum blotting unit (Amersham). Alternatively, transfer can be done by conventional methods.
2. After transfer, wash the membrane briefly with 7× SSC and dry at room temperature.
3. Place the membrane in UV cross linker (Strategene) for optimal cross link.
4. To certify equal loading of RNA samples, stain the membrane with methylene blue solution (0.04% methylene blue in 0.5 M sodium acetate, pH 5.2).
5. Estimate the equal amount of RNA by visualization.

6.5 Preparation of Radiolabeled RNA Probes

1. Use sterile Eppendorf tube to mix the following components:

   Transcription buffer (5×) 4.0 l
   100 mM Tris-HCl (pH 8.3), 250 mM KCl, 25 mM MgCl2, BSA (1 mg ml^{-1})
   DTT (0.1 M) 2.0 l

RNA guard (40 U ml^{-1}) 1.0 l
ATP, CTP, GTP (2.5 mM each) 4.0 l
UTP (100 M) 2.4 l
[32P]UTP (~3,000 Ci $mmol^{-1}$) 4.0 l
DNA template linearized (1 g l^{-1}) 1.0 l
T7 or T3 RNA polymerase (25 U l^{-1}) 1.0 l
Water 0.6 l

2. Mix the contents by vortexing and briefly centrifuge.
3. Incubate the reaction mixture at 37 °C for 1 h.
4. Terminate the reaction by adding 20 l TE buffer.
5. Extract once with an equal volume of phenol/chloroform.
6. Collect the supernatant, add 1.0 l carrier Yeast RNA (1 g ml^{-1}) ½ volume of 7.5 ammonium acetate and 2.0 volume of cold ethanol.
7. Incubate at −80 °C for 1 h.
8. Centrifuge the tubes at 4 °C for 20 min.
9. Wash the pellet with 70% ethanol, dry, and suspend in 50 l water.
10. Use the probes immediately or store at −80 °C for latter use.

6.6 Prehybridization and Hybridization

Materials: Prehybridization solution, Hybridization oven, 2× SSC, SDS.

Prehybridization Solution: Deionized Formamide 10.0 ml, SDS 10% 2.0 ml, Denhardt's solution (50×) 2.0 ml, Denatured Salmon Sperm DNA (10 mg ml^{-1}) 0.3 ml, NaCl (5.0 M) 4.0 ml, Sterile distilled water 1.7 ml

1. Place the membrane into hybridization bottle.
2. Add 5–10 ml prehybridization solution and keep the tubes at 55 °C or 65 °C for 3–6 h.
3. Label the RNA probe using p^{32}-UTP.
4. Add the probe directly to prehybridization mixture and hybridize for overnight or at least 12–16 h at 65 °C.
5. After hybridization, wash the membrane once with 2× SSC; 02% SDS at room temperature for 30 min.
6. Wash the membrane twice with 0.2× SSC and 0.2% SDS at 65 °C for 30 min.
7. Remove the membrane from the hybridization bottle and air dry for 5–10 min.
8. Wrap with saran wrap and expose to PhosphorImager Casette or expose to X-ray film.
9. Analyze the results.

Acknowledgments This study was supported by a grant GM 064465–01A2 from the National Institutes of Health to A.L.N. Rao.

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