Extraction of ds-RNA

1. Weigh out plant tissue (minimum of 20 grams).

2. Place the tissue in a mortar and freeze using liquid nitrogen.

3. Grind the tissue to a fine powder.

4. Transfer the powder to a homogeniser and add the appropriate amount of extraction buffer (for 20 grams add 60 ml 1XSTE, 20 ml phenol, 20 ml chloroform, 7.5 ml 10% SDS and 750 μl 2-mercaptoethanol).
NB. This is a minimum volume to use as for some tissue, that may contain a lot of papain and phenolics, you may need twice this amount of buffer.
(For Ockra, 4% (w/v) ρ-aminosalicylic acid can be added)

5. Blend the tissue and buffer at high speed for at least 1 minute making sure that the buffer doesn't overheat.

6. Pour the blended tissue into a beaker, flask or centrifuge tube.

7. Stir for at least 1 hour at room temperature (or 4°C).

8. Separate the phases by centrifuging at 8000 rpm for 15 minutes.

9. Carefully collect the upper aqueous phase.

10. Measure the volume.

11. Add ethanol (100%, AR grade) while slowly stirring the extract to give a final concentration of 15%.
(To calculate the amount of ethanol - 0.176 X volume = amount of ethanol to add)

12. Add CF-11 (use 0.15 grams per gram of tissue weighed out in procedure 1 - for 20 grams of tissue use 3 grams of CF-11).

13. Stir the solution for 15 - 30 minutes at room temperature.

14. Pour the suspension into the column.

15. Wash the CF-11 with STE buffer containing 15% ethanol until the absorbance of the eluate at 254 nm is the approximately the same as that of the unused buffer. (This is approximately 500 ml per 20 grams of tissue but can be more of less depending on the tissue used. The best thing to do is to use more buffer rather than less as the dsRNA will also remain bound to the CF-11).

16. Once the buffer has completely passed through the column, elute the dsRNA using 1.5 ml of STE per gram of tissue weighed out in procedure 1 (for 20 grams of tissue, use 30 ml of STE).

17. Measure the amount of eluate (should be the same amount as used in procedure 16) and add at least 2 volumes of ethanol (100%, AR grade) and 0.1 volumes of sodium acetate (3 M, pH 5.4) - for 30 ml of eluate add at least 60 ml of ethanol and 3 ml of sodium acetate.

18. Leave the solution at -20°C overnight.

19. Precipitate the dsRNA by centrifuging at 15 000 rpm for 20 minutes in the ultra-centrifuge swing bucket 27 - 3 rotor (4°C). (May need to use 2 tubes for 20 grams of tissue.)

20. Carefully pour off the supernatant into the original flask and invert the tube to drain off as much ethanol as possible.

21. Resuspend the pellet (may not be visible) at the base of the tube using 400 μl of 70% ethanol. If there are more than 2 tubes, transfer the 400 μl of 70% from one tube and use it to resuspend the pellet in other tubes.

22. Transfer the contents to one eppendorf tube per sample. Make the volume up to 1.5 ml, vortex and pellet the dsRNA by centrifuging in the microfuge at 4 °C.

23. Carefully pour off the supernatant and remove as much of the liquid as possible. Dry the inside of the tube with clean tissue.

24. Dry the pellet in the incubator at 55°C for 30 minutes.

25. Resuspend the pellet in 20 μl of TE (pH 8.0). Pipette lightly to resuspend the pellet.

26. Store the sample at 4°C for short periods or at -20°C for extended periods.

Confirmation of dsRNA by digestion with DNase and RNase

The nature of the dsRNA can be confirmed by testing with DNase (will digest only single- and double-stranded DNA) and RNase (will digest single- and double-stranded RNA under both low salt conditions and only single stranded RNA under high salt conditions). For routine dsRNA extractions only the DNase reaction need be performed ie as part of the routine screening of samples.

Digestion with DNase (this is the most important of the digestions):
Treat all samples with 1 unit of DNase per 10 µl of reaction mix (stock 10 u/µl) and incubate at room temperature (best at 37°C) for at least 30 minutes (best for 60 minutes).

Always check the DNase and mung bean nuclease (MBN) by digesting plasmid DNA in a controlled experiment first.

For example:

Sample	Plasmid (µl)	DNase (1 U/µl)	MBN	10 X MBN buffer	dH_20 (µl)	TOTAL (µl)
MBN control	1 (100 ng)	0	1	1	7	10
DNase control	1 (100 ng)	1	0	0	8	10
DNA	1 (100 ng)	-	0	0	9	10

NB. 1 µl of DNase (stock of 10 U/µl) was diluted in 10 µl of dH_2O. MBN was also diluted from a stock of 75 U/µl to 10 U/µl ie. 1 µl of stock and 6.5 µl of dH_2O - the stock of MBN (or S1 nuclease) often varies. Some are 100 U/µl but the rule is 1 U/10µl of digest.

Incubate at 37°C for 30 minutes.

MBN 10X buffer:

	Stock	make 1 ml
300 mM sodium acetate	3 M	100 µl
500 mM NaCl	5 M	100 µl
10 mM $ZnCl_2$	0.5 M	20 µl
50% glycerol	100 %	500 µl

Example reaction: (Makes a final volume of 200 µl)

Sample	DNase (10 U/µl)	MBN (75 U/µl)	10 X MBN buffer	dH_20 (µl)	TOTAL (µl)
10	20	2.7	20	147.3	200

Incubate at 37°C for 30 minutes.

Extract with an equal volume of phenol/chloroform (1:1). Vortex and centrifuge for 2 - 3 minutes at room temperature. Transfer the supernatant to a sterile eppendorf and add 0.1 volumes of sodium acetate (3 M, pH 5.4) and 2.5 volumes of ethanol (AR). Lightly vortex and store at -20°C for 2 hours (or overnight) and pellet the dsRNA by centrifuging at 4°C for 15 minutes. Remove and discard the supernatant and add 500 µl of 70% ethanol. Vortex and pellet by centrifuging at 4°C for 5 minutes. Remove the supernatant and dry the pellet. Resuspend the pellet in 10 µl of TE (pH 8.0). Store at 4°C until use (or -20°C for long-term).

Digestion with RNase under high salt conditions (digests ss- and dsRNA):
Treat samples with 5 u of RNase (stock 10 u/μl) per reaction having a final concentration of 2 X SSC and incubate at 37°C for at least 30 minutes (best for 60 minutes). Electrophorese samples immediately or store at 4 °C.

Example reaction:

Sample	Extract (μl)	X 20 SSC (μl)	RNase (10 u/μl)	dH_20 (μl)	TOTAL (μl)
1	10	1.5	1.0	2.5	15

Incubate at 37°C for at least 30 minutes.

Make the volume up to 200 μl with dH_2O and extract with an equal volume of phenol/chloroform (1:1). Vortex and centrifuge for 2 - 3 minutes at room temperature. Transfer the supernatant to a sterile eppendorf and add 0.1 volumes of sodium acetate (3 M, pH 5.4) and 2.5 volumes of ethanol (AR). Lightly vortex and store at -20°C for 2 hours (or overnight) and pellet the dsRNA by centrifuging at 4°C for 15 minutes. Remove and discard the supernatant and add 500 μl of 70% ethanol. Vortex and pellet by centrifuging at 4°C for 5 minutes. Remove the supernatant and dry the pellet. Resuspend the pellet in 10 μl of TE (pH 8.0). Store at 4°C until use (or -20°C for long-term).

Digestion with RNase under low salt conditions (digests ssRNA):
Treat samples with 5 u of RNase (stock 10 u/μl) per reaction having a final concentration of 0.1 X SSC and incubate at 37°C for at least 30 minutes (best for 60 minutes). Electrophorese samples immediately or store at 4 °C.

Example reaction:

Sample	Extract (μl)	X 1 SSC (μl)	RNase (10 u/μl)	dH_20 (μl)	TOTAL (μl)
1	10	1.5	1.0	2.5	15

Incubate at 37 ° for at least 30 minutes.

Make the volume up to 200 μl with dH_2O and extract with an equal volume of phenol/chloroform (1:1). Vortex and centrifuge for 2 - 3 minutes at room temperature. Transfer the supernatant to a sterile eppendorf and add 0.1 volumes of sodium acetate (3 M, pH 5.4) and 2.5 volumes of ethanol (AR). Lightly vortex and store at -20°C for 2 hours (or overnight) and pellet the dsRNA by centrifuging at 4°C for 15 minutes. Remove and discard the supernatant and add 500 μl of 70% ethanol. Vortex and pellet by centrifuging at 4°C for 5 minutes. Remove the supernatant and dry the pellet. Resuspend the pellet in 10 μl of TE (pH 8.0). Store at 4°C until use (or -20°C for long-term).

Polyacrylamide Gel Electrophoresis

Thoroughly wash the glass plates firstly with detergent rinsing with distilled water (dH_2O) followed by ethanol (use AR grade as denatured ethanol has methanol added which can be harmful when used frequently). Dry the plates with clean soft tissue or cloth and assemble them using the apparatus provided.

There are two recipes for the PAGE. The first one (and the preferred one) uses TBE buffer and the second one uses a Tris/glycine buffer which is also used for proteins.

Prepare the resolving gel - 12% polyacrylamide (lower fraction) as described below. Using a pasteur pipette or a pipetter set on 1 ml gently add approximately 3 ml of the solution to the gel apparatus (this should take the level of gel to approximately 2/3 rds the total height of the glass). Overlay the solution by gently adding dH_2O - drop by drop - across the top of the glass at intervals of approximately 10 mm. Gently move the apparatus to ensure that the gel surface is flat. Allow the gel to polymerase - this can be seen by checking whether the left-over gel solution has set and then the poured gel can be checked - and then pour off the water and carefully insert filter paper to dry between the glass plates.

Now prepare the stacking gel - 5% polyacrylamide - and using a pasteur pipette or a pipetter set on 1 ml gently add approximately 1.5 ml of the solution to the gel apparatus (this should take the level of gel to the top of the glass). Now place the comb between the glass plates by gently pushing it into the solution at a slight angle to ensure that no air is trapped under the teeth. Add a little more of the stacking solution so the space between the two plates is totally filled. Allow the gel to set.

Using TBE running buffer:

Solution	12%	5%
dH_2O	4795 μl	3565 μl
30% acrylamide	4000 μl	830 μl
10X TBE	1000 μl	500 μl
Temed	5 μl	5 μl
10% APS	200 μl	100 μl
TOTAL	10000 μl	5000 μl

Using Tris/glycine based running buffer:

Solution	12%	5%
dH_2O	3295 μl	2805 μl
30% acrylamide	4000 μl	830 μl
1.5 M Tris (pH 8.8)	2500 μl	-
0.5 M Tris (pH 6.8)	-	1260 μl
Temed	5 μl	5 μl
10% APS	200 μl	100 μl
TOTAL	10000 μl	5000 μl

Now that the gel has set, assemble the apparatus and place it into the gel chamber. Fill the central cavity with X1 Tris-glycine buffer (diluted from X5 stock solution) and gently remove the comb. Wash out the wells by pipetting buffer into the wells using a pipetter or pasteur pipette. Now load the samples avoiding well number 1 and 10 (often samples run in these wells will be at a slight angle often referred to as “smiling”). Molecular weight markers should be run in the first lane and if the volume of samples are large then a space of one lane should be left between the samples (this latter suggestion is just that, a suggestion and is not necessary after you have had a little experience with running the samples - remember that having a space between the samples limits the number of samples that can be run and also increases the cost if you need to run more than 5 samples!!!!).

After the samples have been loaded ensure that the central chamber is full of buffer and the bottom of the plates is covered by at least 1 cm of buffer (if buffer leaks from the central cavity then the outer chamber must be filled to the top of the plates). Connect the electrodes with the red positive cable attached to the red terminal on the apparatus. Switch on the power supply and electrophorese the samples for - 4 hours to see bands in the range of 1.5 kb and greater, 6 hours to see bands only in the range of 3 kb and above and 8 hours to see in the range of 4 kb and above - at 150 V. If you know that your dsRNA is greater or equal 7 kb then consider running a resolving gel or either 6 or 8%. Remember, if you do this run some markers on a trial gel first to gauge the correct running time.

Switch off the power supply, disassemble the apparatus splitting the glass plates using a spacer. With the gel on one plate stain in ethidium bromide for at least 10 minutes before observing under UV light..

BUFFERS

1 M Tris (pH 8.0)
- 121.1 g Tris
- add 900 ml dH_2O
- pH to 8.0
- make volume up to 1000 ml

0.5 M EDTA (pH8.0)
- 186.15 g of EDTA
- add 900 ml dH_2O
- pH to 8.0
- make volume up to 1000 ml

5 M NaCl
- 292 g NaCl
- add 800 ml dH_2O
- dissolve crystals
- make volume up to 1000 ml

1 X STE buffer

10 mM Tris.Cl (pH 8.0)	10 ml of 1M Tris.Cl (pH 8.0)
100 mM NaCl	20 ml of 5 M NaCl
1 mM EDTA (pH 8.0)	2 ml of 0.5 M EDTA (pH 8.0)
	Make up to 1 litre

1 X STE/15% buffer

10 mM Tris.Cl (pH 8.0)	10 ml of 1M Tris.Cl (pH 8.0)
100 mM NaCl	20 ml of 5 M NaCl
1 mM EDTA (pH 8.0)	2 ml of 0.5 M EDTA (pH 8.0)
15% ethanol	150 ml ethanol (100%, AR grade)
	Make up to 1 litre

Phenol
1. Weigh out 500 g (**USE GLOVES - PHENOL IS CORROSIVE AND TOXIC**)
2. In the fume hood, add 200 ml dH_2O
   If the phenol crystals are hard then estimate the weight and add water directly to the solid phenol and continue.
3. Add 0.7 g hydroxyquinoline (not necessary if you don't have this - just to give a yellow colour)
4. Leave to stir in a fume hood at room temperature overnight (or at least 30 minutes)
5. Store at 4°C in a brown glass bottle or in a clear glass bottle covered with aluminium foil

Optional:

5. pH the phenol using 0.1 M Tris.Cl, pH 8.0
   (a) add 100 ml and stir for 5 minutes
   (b) remove the supernatant
   (c) add an additional 100 ml of 0.1 M Tris.Cl, pH 8.0 and repeat (a) to (c) until pH of the supernatant is about 8.0 (usually need 500 ml of buffer)
   NB. pH the supernatant using the pH test strips NOT the pH meter.

TE (pH 8.0)	1000 ml
10 mM Tris.Cl (pH 8.0)	10 ml
1 mM EDTA (pH 8.0)	2 ml

TAE BUFFER (50X)
For 1000 ml:

Tris	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

(NB. MAY NEED TO ADJUST pH TO 8.0 BUT SHOULDN'T HAVE TO)

Tris Glycine Buffer (5X)
For 1000 ml:

Tris	15.1 g
Glycine	94 g

Make volume up to 1000 ml (pH should be around 8.3 but it shouldn't be necessary to pH the solution). Use this for polyacrylamide gel electrophoresis at X1 concentration.

For making up:

RNase
Using equipment only for making up RNase, measure out 0.01 g RNase (in an eppendorf tube) and add 990 µl of dH_2O. Heat in a boiling water bath at 100 °C for 15 minutes before allowing to cool at room temperature. Aliquot into 200 µl lots and store at -20°C.

If the RNase is bought in small 10 mg amounts add 990 µl of dH_2O and continue as above.

DNase
This is often purchased in buffer and stored at -20°C. If it comes as a powder it should be made up according to the manufacturers recommendations. The reaction buffer is as per Sambrook et al. but it is not always necessary to use if just for this use.

Silver Staining Procedure

(This procedure is taken from Blum, H., Beier, H. and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99.)

STEP	SOLUTION (100 ml final in dH_2O unless stated otherwise)	TIME
1. Fix	50 ml methanol, 12 ml acetic acid, 50 μl of 37% formaldehyde (fixing is best done overnight but also can be for as little as 30 min if placed on a shaker)	60 min
2. Wash	50 ml Ethanol	20 min
3. Wash	50 ml Ethanol	20 min
4. Wash	30 ml Ethanol	20 min
5. Pretreat	ml sodium thiosulphate ($Na_2S_2O_3.5H_2O$) 0.1 g / 500 ml dH_2O	1 min
6. Rinse	dH_2O	20 s
7. Rinse	dH_2O	20 s
8. Rinse	dH_2O	20 s
9. Impregnate	0.2 g $AgNO_3$,, 75 μl of 37% formaldehyde	20 min
10. Rinse	dH_2O	20 s
11. Rinse	dH_2O	20 s
12. Rinse	dH_2O	20 s
13. Develop	6 g sodium carbonate (Na_2CO_3), 50 μl of 37% formaldehyde, 0.4 mg sodium thiosulphate (or 2 ml of solution from step 5) Develop until bands develop to the required intensity.	10 min
14. Rinse	dH_2O	2 min
15. Rinse	dH_2O	2 min
16. Stop	50 ml methanol, 12 ml acetic acid	10 min
17. Storage	10 ml ethanol, 10 ml acetic acid	

CRUDE EXTRACTION OF NUCLEIC ACID BY THE PHENOL:GLYCINE METHOD

Crude extraction:

1. Grind approximately 0.05 - 0.1 g of fresh/frozen leaf sample (or as little as 0.02 of freeze dried) after freezing with liquid nitrogen in an eppendorf tube. Grinding in a mortar can also be done but using a larger amount of sample - approximately 0.5 g.

2. To no more than 0.1 g of ground sample add 500 µl of buffer G (0.375 g glycine, 1 ml 5M NaCl, 1 ml 0.5 M EDTA, make up to 50 ml, pH 9.5, filter sterilise with 0.2µ filter) and grind until the sample has thawed.

3. Add 500 µl of phenol and vortex for approximately 1 minute.

4. Separate phases by centrifuging at 4 °C for 5 minutes.

5. Transfer the upper aqueous phase to a fresh tube.

6. Add 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% absolute ethanol (or 1 volume of isopropanol as the total volume using absolute ethanol may exceed the capacity of the eppendorf tube).

7. Pellet the nucleic acid by centrifuging at 4 °C for 15 minutes.

8. Pour off the supernatant being careful not to loose the pellet.

9. Add 500 µl of 70% ethanol and lightly pipette the pellet paying particular attention to release the pelleted material on the side of the tube.

10. Pellet the nucleic acid by centrifuging at 4 °C for 5 minutes.

11. Pour off the supernatant again being careful not to loose the pellet.

12. Dry the pellet in the incubator for 10 - 15 minutes.

13. Resuspend the pellet in 50 µl of dH_2O.

14. Store for short periods at 4 °C or for more than a day at -20 °C.

REVERSE TRANSCRIPTION REACTION

A 20 μl reaction should be set up in the following way.

To a sterile eppendorf tube add:

crude extract -	5 μl	
primer A -	1 μl	
primer B -	1 μl	
dH_2O -	5 μl	*(if using formamide of random primers use 4 μl)
*(Formamide -	1 μl)	
*(Random Primers @ 200 ng/μl)	1 μl)	

(Caution: Formamide is moderately toxic)

Heat the mixture to 75 °C for 3 minutes and immediately quench on ice.

Spot spin the tube and add:

5X buffer (supplied with enzyme) -	4μl
0.1 M DTT (supplied with enzyme) -	2 μl
10 mM dNTP mix -	1 μl

Mix the contents gently by pipetting up and down and incubate at 37 °C for 10 minutes. Remove The tube from the 37 °C block and add 1 μl (200 units) of Superscript II. Mix the contents by gently pipetting up and down.

Incubate at 42 °C for 50 minutes.

Store on ice until used or at -20 °C for longer term.

ADDITIONAL PROCEDURE (BUT OPTIONAL): RNA HYDROLYSIS PROCEDURE

During the RT reaction the DNA is synthesised using the RNA as template. The final product after the RT reaction is therefore an RNA:DNA hybrid ie one strand is DNA and the complementary strand is RNA; normally dsRNA (RNA:RNA), ssRNA or dsDNA (DNA:DNA). The "strength" of this molecule (ie. tolerance to denaturing to ssRNA and ssDNA molecules by boiling) often makes it difficult to denature. Occasionally this can cause problems in any subsequent PCR reactions. Should this be apparent (and may only be apparent if the PCR doesn't work very well or not at all) this RNA hydrolysis procedure should be used. Alternatively, RNase H could be used which destroys any RNA which has a complementary DNA strand. But, RNase H costs more money and often is not as efficient as RNA hydrolysis. A drawback of the RNA hydrolysis procedure is that the resulting ss cDNA usually needs to be precipitated often resulting in some loss of the little DNA that may be resulting from the RT reaction. Best advice, do the PCR without the RNA hydrolysis procedure first. If it doesn't work (and you think that it should) then try the RNA hydrolysis procedure.

1. Stop the RT reaction by adding 1 μl of 0.5 M EDTA (pH 8.0) (final now approx. 20 mM) and 31 μl of 0.5 M NaOH (final now approx. 0.3 M).

2. Incubate at 65°C for 1 hour.

3. Neutralise the solution by adding 30 μl Tris-HCl pH 8.0 and 30 μl 1 M HCl (ie 1 in 12 dilution of AR grade HCl).

4. Extract with an equal volume of Phenol:chloroform (1:1).

5. Spin for 5 minutes at RT.

6. Retain the supernatant in a sterile eppendorf tube.

7. Add 0.1 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of 100% ethanol (AR).

8. Vortex and place in -20 °C for 2 hours (or overnight) or -80 °C for 30 minutes.

9. Precipitate the DNA by centrifuging for 15 minutes at 4 °C.

10. Pour off the supernatant being careful not to loose the pellet.

11. Add 500 μl of 70% ethanol and lightly pipette the pellet paying particular attention to release the pelleted material on the side of the tube.

12. Pellet the nucleic acid by centrifuging at 4 °C for 5 minutes.

13. Pour off the supernatant again being careful not to loose the pellet.

14. Dry the pellet in the incubator for 10 - 15 minutes.

15. Resuspend the pellet in 20 μl of dH_2O (or less if you want to use a larger amount for subsequent PCR reactions).

16. Store for short periods at 4 °C or for more than a day at -20 °C.

PCR USING cDNA AS TEMPLATE

PCR should be done as previously described with the exception that varying amounts of cDNA may need to be used ie. you may need to do two PCR reactions of each cDNA reaction using 5μl (and 1 μl) of template. Typically, a single 50 μl (or 10 μl) PCR reaction would be as previously described or as below:

	50 μl	**10**
dNTP's (@ 1.25 mM)	8	1.6
X10 buffer	5	1
$MgCl_2$ (25mM)	2.5 (can use up to 4)	0.5
primer 1 (@ 30 □m/μl)	1	0.2
primer 2 (@ 30 □m/μl)	1	0.2
dH_2O	26.5 (33)	5.3
Taq **(dilute stock @ 5 u/μl to 1 u/μl)**	1	0.2
Template	5 (1)	1
TOTAL	**50**	**10**
Add oil:	**35**	**35**

Cycling conditions:

94°C	3 minutes	X1
94°C	15 seconds	
45°C	15 seconds (May need to play around with this temperature)	
72°C	30 seconds	X30
72°C	10 minutes	X1

After the PCR is complete, remove 10 μl of each sample, mix with 2 μl of loading dye and electrophorese in a 1% agarose gel at 100 volts and visualise by UV light. Remember to run molecular weight markers with each gel. If using 10 μl reactions, add 2 μl to the reaction tube and spot spin. Load all the sample which now is blue in colour.

TOTAL EXTRACTION RNA

This is another total RNA extraction method which is taken from Chang, S., Puryear, J. and Cairney, J. (1993). "A simple and efficient method for isolating RNA from Pine Trees". *Plant Molecular Biology Reporter*, **11 (2)**. This specifically extracts the RNA from the samples which may not have been possible using other methods such as that of the phenol:glycine. It will also extract the ssRNA which is not extracted using CF-11. It has been very successful for use in extracting mRNA in banana and could therefore be useful for Okra and wax gourd.

1.Warm 5 ml of extraction buffer to 65 °C in a water bath using sterile capped tubes (just before use add 100 μl of 2-mercaptoethanol).

2. Grind 1 gram of tissue in liquid nitrogen and immediately add to the buffer.

3. Mix thoroughly by inverting and vortexing.

4. Add equal volume of chloroform:isoamyl alcohol (24:1) and vortex.

5. Centrifuge at approximately 3000 g for 10 minutes (will need to use high speed sorval).

6. Transfer supernatant to a sterile tube and add an equal volume of chloroform:isoamyl alcohol (24:1) and vortex.

7. Centrifuge at approximately 3000 g for 10 minutes (will need to use high speed sorval).

8. Transfer the supernatant to a sterile tube and add 0.25 volumes of 10 M lithium chloride and shake. Allow RNA to precipitate by storing at 4 °C overnight.

9. Pellet RNA by centrifuging at approximately 8000 g for 20 minutes.

10. Carefully pour off supernatant. Immediately resuspend the pellet in 500 μl of STE.

11. Transfer the contents into a sterile eppendorf tube and add an equal volume of chloroform:isoamyl alcohol (24:1) and vortex.

12. Separate the phases by centrifuging at room temperature for 5 minutes.

13. Transfer the supernatant to a sterile eppendorf tube. Add 2 volumes of 100% ethanol (AR) and mix by shaking.

14. Store at -70 °C for 30 minutes or -20 °C for 2 hours (or overnight).

15. Pellet the RNA by centrifuging at 4 °C for 20 minutes.

16. Carefully remove the supernatant using a pipetter and dry the pellet in the incubator for 15 minutes.

17. Resuspend the pellet in approximately 20 - 100 μl of dH_2O. Allow to resuspend on ice for 30 minutes before mixing by gently pipetting.

18. At this point the RNA concentration using 2 - 5 μl can be done. However, this may not be possible if a large cuvette volume is required (ie greater than 100 μl).

19. Store RNA at -20 °C.

Extraction buffer

2 % CTAB	
100 mM Tris-HCl (pH 8.0)	(use stock solution of 1 M, pH 8.0)
25 mM EDTA	(use stock solution of 0.5 M, pH 8.0)
2 M NaCl	(use stock solution of 5 M)
0.5 g/l spermidine	

Mix and autoclave. Store at room temperature.

SSTE

1 M NaCl	(use stock solution of 5 M)
0.5% SDS	
10 mM Tris-HCl (pH 8.0)	(use stock solution of 1 M, pH 8.0)
1 mM EDTA (pH 8.0)	(use stock solution of 0.5 M, pH 8.0)