cRT-PCR PROTOCOL (Barkan lab, 2009)

All solutions (other than commercial enzymes/buffers) should be treated to make them "RNAse free"

This protocol requires that the 5' end of the RNA be monophosphorylated and the 3' end have a hydroxyl. So- primary transcripts with multiple 5' phosphates won't be detected. (A phosphatase step can be incorporated, in this case). In retrospect, it seems that not all processed RNAs will have the correct ends either (some RNAses leave 5'-OHs, for example). Nonetheless, it seems to work for processed chloroplast RNAs.

Primer Design

Design primers that are ~20-25 nts long, and that will amplify across the anticipated termini after RNA circularization. Aim to make the GC content of these two primers as similar as possible (and close to 50%). Design a third primer that can be used for nested PCR, which maps within the anticipated primary PCR product, and adjacent to one of the two primary PCR primers.

RNA ligation

Total RNA is isolated with TriReagent (Molecular Research Center) according to the manufacturer's instructions.

Total RNA (10 μg)	
T4 RNA Ligase 1 (20,000 U/ml, New England Biolabls)	1.5 μl
10 X T4 RNA Ligase 1 buffer	5 μl
rRNasin (40 U/μl) (Promega)	1 μl
filtered, deionized water	to bring total volume to 50 μl

Mix and incubate at 37° C, 1 h; then at 65° C, 15 min.
Add 5 μl 3M sodium acetate (pH 5.2) .
Add 100 μl ice-cold 100% ethanol and mix.
Store on ice for at least 15 minutes.
Microcentrifuge at maximum speed, at 4° C for 10 min.
Carefully pour off the supernatant.

Add ~0.4 ml 70% ethanol to rinse the pellet.
Centrifuge at maximum speed for 5 min.
Pour off the ethanol carefully and invert the tube on to a clean Kimwipe.
Let the pellet dry at room temperature until no drops are visible.
Dissolve the pellet in 70 μl TE buffer. (TE: 10 mM Tris-HCl pH 7.5, 1 mM EDTA)
Store the RNA at -70° C until used.
(This is the circularized RNA, cRNA)

Reverse transcription.

cRNA	7 µl
Oligo RP1 (5 pmole/µl)	0.5 µl (mapping within ~100 nt of the anticipated 5' end)
Filtered, deionized H_2O	4.5 µl
Total volume	12 µl

Mix and incubate at 98° C, 2 min.
Add 3 µl of 5X Annealing Buffer (250 mM KCl, 25 mM Tris-HCl, pH 8).
Transfer to 46-48˚C for 15 min to anneal the primer.

Initiate primer extension by adding 10 µl AMV mix:

AMV mix:

5X AMV RT buffer	4 µl*
dNTPs mix (2.5 mM each)	5 µl
rRNasin (40 U/µl)	1 µl
AMV Reverse Transcriptase (Promega) (10U/µl)	1 µl

(* Although 5 µl is one fifth of the final reaction, this volume might bring the [KCl] up too high, when added to the KCl from the annealing buffer.)

Incubate at 46-48° C, 1 h.
Keep the synthesized cDNA at 4° C while setting up the PCR reaction.

PCR (1^{st} round)

cDNA	1 µl
ExTaq 10X buffer	2 µl
dNTPs mix	2 µl
Oligo RP1 (5 pm/µl)	2 µl
Oligo FP1 (5 pm/µl)	2 µl
Filtered, deionized H_2O	11 µl
ExTaq	0.1 µl

30 cycle amplification.

If sharp, strong bands are obtained- simply clone and sequence them. You should sequence 20 or so clones in order to be confident that the ends detected are the major ones.

If the bands are weak or fuzzy, do a nested PCR:
cut out the bands, purify the DNA from the gel with the QIAquick Gel extraction kit and do a nested amplification..

Nested PCR (2nd round)

DNA from PCR round 1	0.5 μl*
ExTaq 10X buffer	2 μl
dNTPs mix (Takara)	2 μl
Oligo RP2 (5 pm/μl)	2 μl
Oligo FP1 (5 pm/μl)	2 μl
Filtered, deionized H_2O	11.4 μl
ExTaq (Takara)	0.1 μl

*This volume is taken from a 1:50 dilution prepared from the 50 μl eluted band recovered from gel of 1st PCR (higher dilutions up to 1:10,000 might be required).

Abundant products are cut out from the gel, purified with the QIAquick Gel extraction kit, cloned into pGEM-T and sequenced.