PT410
PCR protocols for the detection of chrysanthemum stunt and potato spindle tuber viroids

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Know-how for Horticulture™

PT410

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INDUSTRY SUMMARY.

New tests have been developed for the detection of chrysanthemum stunt (CSV) and potato spindle tuber viroids (PSTV). These new tests are more cost effective, sensitive and reliable than existing tests, and the turn around time for diagnosis has been reduced from seven days to one day.

CSV and PSTV are both highly contagious and cause two of the most devastating diseases of horticultural crops. They are spread by the handling and propagation of infected material, and by contaminated machinery and tools. The only way to control these diseases is the use of pathogen tested propagating material combined with good hygiene. The key to controlling CSV and PSTV is the ability to detect them in plant tissue prior to their introduction into a Pathogen-Tested scheme or during an epidemic.

CSV causes a wide variety of symptoms that reduce the quality and yields of chrysanthemums. Outbreaks of CSV can cause devastating crop losses: in 1987 a major outbreak occurred in Victoria that was estimated to have cost the chrysanthemum industry in excess of $ 3 million. CSV is controlled in Australia by the use of pathogen tested planting material.

PSTV is a quarantinable disease of potatoes that is not known to occur in Australia. PSTV causes a wide variety of symptoms that affect the yield and quality of potatoes. An outbreak of PSTV in breeding material in Victoria and NSW in 1982, resulted in the destruction of all but 100 single tested tubers, in the National Breeding Program. All seed potatoes that enter Australia are tested for PSTV during post entry quarantine.

In the past the test used for the detection of CSV and PSTV was a commercially available cDNA probe kit manufactured by Bresatec. However, due to limited demand and developments in the more sensitive polymerase chain reaction (PCR) technology, Bresatec has ceased production of these kits. The aim of this project was to develop PCR tests for the detection of CSV and PSTV and compare them to the cDNA kits currently used for detection.

As a result of this development and comparative study the PCR tests have been adopted by Crop Health Services and the Australian Quarantine Inspection Service (AQIS) for the diagnosis of both CSV and PSTV.

TECHNICAL SUMMARY.

The aim of this study was to develop reverse transcriptase polymerase chain reaction (RT-PCR) tests for the detection of chrysanthemum stunt viroid (CSV) in chrysanthemums and potato spindle tuber viroid (PSTV) in potatoes. The sensitivity and the cost effectiveness of the RT-PCR was compared to the cDNA probe in the laboratory for both viroids.

Sequences for both CSV and PSTV were obtained through the Australian National Genetic Information Service (ANGIS) and compared through computer analysis for regions of homology between strains. Primers were developed in highly conserved regions for the two known strains of CSV and the 13 known strains of PSTV. Where possible ANGIS was used to compare these primers with other DNA sequences from possible contamination sources during template preparation.

RT-PCR was found to be more sensitive, far less time consuming and more cost effective than cDNA probes for both CSV and PSTV. It was also found that RT-PCR could be carried out on sap extracts of expanding leaf tissue from new, emerging potato sprouts or chrysanthemum tips. However, where plant material is older, a more rigorous RNA purification is recommended. Dilution experiments showed that PCR could detect less than 0.0148 ng of viroid RNA and at least 1 infected plant in 200 for CSV. PSTV could be detected in 16.8 ng of total RNA from PSTV infected plants. One infected PSTV plant in 20 could be detected. Turn around time for a viroid test could be reduced from seven days using the cDNA probe to one day using the RT-PCR.

2. RECOMMENDATIONS.

Extension/ adoption by industry of research findings.

PCR was compared to a cDNA probe for the detection of chrysanthemum stunt (CSV) and potato spindle tuber viroids (PSTV), and the following recommendations are made.

- The PCR for CSV was found to be cost effective, reliable, and very sensitive. It is recommended that the PCR test be used for the routine detection of CSV in chrysanthemums.

- The PCR for PSTV was found to be cost effective, reliable, and very sensitive. It is recommended that the PCR test be used for the routine detection of PSTV in potatoes.

- The PCR tests for both viroids have been adopted by Crop Health Services at the Institute for Horticultural Development, and staff from this team have been trained in this technology. The Australian Quarantine Inspection Service has adopted these tests for post entry quarantine testing. These new services are currently being advertised to all potential clients.

Directions for future research/extension.

Improvements in crop monitoring and hygiene have reduced losses due to CSV. Industry needs to continue the development of an awareness program which in combination with regular diagnostic checks can be used to minimise outbreaks of CSV. Through vigilant quarantine, PSTV has been prevented from entering the country. To maintain Australia's PSTV free status AQIS should to continue with regular PSTV checks on all imported potato cultivars.

As only one strain of each viroid was available for assessment due to quarantine regulations, it is recommended that further strains be assessed if they become available.

3. TECHNICAL REPORT.

Development of RT-PCR for the detection of chrysanthemum stunt and potato spindle tuber viroids.

Jane Moran and Fiona Constable.

Introduction.

Chrysanthemum stunt viroid (CSV) and potato spindle tuber viroid (PSTV) are highly contagious diseases of chrysanthemums and potatoes respectively. They spread quickly throughout a crop by the handling and cultivation of infected plants. PSTV is not known to occur in Australia and is a quarantinable disease, however, there was an outbreak in the breeding material in Victoria and NSW in 1982. As a result all breeding material in the National Breeding Program was destroyed except for 100 single tested tubers. During 1987 a major outbreak of CSV in Victoria caused a $3 million loss to the chrysanthemum industry.

The only way to deal with a CSV or PSTV outbreak is to destroy infected plants. This means economic loss to the grower, especially where whole crops are involved. The best way to control these diseases is to use high health propagating material combined with good hygiene. In Australia these two diseases are controlled by pathogen-tested schemes that are managed by the relevant industry, and quarantine regulations. It is a part of the current Australian Quarantine Inspection Service (AQIS) post entry quarantine protocols that all potatoes are tested for PSTV and other diseases before they are released to the industry. High health chrysanthemum cuttings are tested for CSV and other diseases prior to release to industry.

Potato spindle tuber was first recognised as a disease of potatoes by Martin (1922) in the United States and the causal agent was originally thought to be a virus. Diener (1971) eventually showed the causal agent be a low molecular weight RNA and named the agent a "viroid". PSTV causes a wide variety of symptoms that affect the yield and quality of potatoes. Although symptoms on the plant are often not obvious, tubers are severely affected. Infected

tubers are smaller, may be elongated with tapered ends and they have more cracking than usual.

Chrysanthemum stunt was first recognised as a disease by Dimock (1947) in the United States. Again the causal agent was originally thought to be a virus but, in 1972, evidence showed that chrysanthemum stunt disease is also caused by a viroid (Diener and Lawson, 1972). CSV causes a wide variety of symptoms that reduce the quality and yields of chrysanthemums. Flowering can occur up to 10 days earlier, and flowers may be bleached and distorted. Infected plants are often stunted, attaining only half their normal size.

Viroids are the smallest disease agents of plants known to man. They exist as single stranded, covalently closed circular RNA molecules (Gross, 1985) that are unencapsidated and range from 250 bases to almost 600 bases long (Singh and Singh, 1995). CSV and PSTV are approximately 354 bases and 359 bases respectively.

At the Institute for Horticultural Development a virus diagnostic service is offered to chrysanthemum and potato growers for the detection of viroids. In the past the test used for the detection of PSTV and CSV was a commercially available cDNA probe kit. This test is time consuming and costly and the kit is no longer being manufactured. With the development of the polymerase chain reaction (PCR) for the detection of small amounts of DNA and RNA, much more rapid and cost effective tests for all types of pathogens, including viroids can be developed. Reverse transcriptase (RT)-PCR has been developed for the detection of CSV by Kusunoki *et al.* (1993), showing that this disease agent can be detected by a routine RT-PCR method. There are no known RT-PCR tests available for PSTV.

The aim of this study was to develop PCR techniques for the detection of CSV in chrysanthemums and PSTV in potatoes, and then compare these tests with the cDNA probe with respect to sensitivity, reliability and cost effectiveness.

Materials and Methods.

1. Samples.

One isolate of CSV and one isolate of PSTV were imported with approval from AQIS. The infected plants were maintained in the AQIS post entry quarantine nursery, at IHD Knoxfield, under strict quarantine conditions, isolated in a secure glasshouse, operating on normal day length and a temperature range of 25-30°C. Healthy plants were maintained in a separate glasshouse operating on normal day length and a temperature range of 18-25°C. Fresh young growing tips from chrysanthemums and new emerging sprouts from potatoes were used for testing.

A pure preparation of the circular form of CSV (3.7 ng/µl) was produced by Brendan Rodoni (1989) at the Plant Research Institute, Department of Agriculture, Burnley, Victoria and is held at IHD.

2. RNA extraction methods

a. Total RNA extraction procedure

Total RNA was purified using a modification of the method from Rowhani *et al.* (1993), (Yan Wan Chow Wah, pers. comm.). Where possible, RNase, DNase and protease free chemicals were used. All buffers were sterile filtered prior to use. Mortars and pestles were bleach, acid and sterile distilled water washed prior to use. Glassware, stirring beads and spatulas were baked at 180°C for 3 hours. Re-useable plastic-ware was soaked with 3% hydrogen peroxide then sterile distilled water rinsed.

One gram of plant material was chopped and placed in a cold mortar and pestle containing 5 ml of cold extraction buffer (95 mM $K_2HPO_4.3H_2O$, 30 mM KH_2PO_4, 10% sucrose, 2% polyvinyl pyrrolidine (PVP-10), 0.15% BSA fraction V, and 0.1mM ascorbic acid, bovine serum albumin (BSA, fraction V) and ascorbic acid were added just prior to use then the pH was adjusted to 7.6). The sample was incubated for 10 minutes to plasmolyse the cells and

then ground to a slurry. A further 5 ml of extraction buffer was added and the sample ground again. The slurry was centrifuged at 750 g for 5 minutes to remove larger organelles and cell debris. The supernatant was transferred into a clean centrifuge tube and centrifuged at 16,800 g for 20 minutes to pellet the nucleic acids. The supernatant was discarded and the tube allowed to drain briefly then placed on ice. The pellet was resuspended in 2 ml of TE-1 buffer (50 mM Tris, 10 mM EDTA, pH 8.0 containing 0.1% β-mercaptoethanol added just prior to use). After resuspension, 250 µl 10% sodium dodecyl sulphate (SDS) was added and the sample incubated at 60°C for 10 minutes. The sample was cooled on ice for one minute then centrifuged at 16,800 g at 4°C for 5 minutes. The supernatant was transferred to sterile tubes, 400 µl of 5M potassium acetate was added and the sample was incubated on ice for 30 minutes and then centrifuged at 16,800 g for 15 minutes. The supernatant was transferred to a sterile tube, 300 µl 3M sodium acetate pH 5.2 was added followed by 3 ml of ice cold isopropanol, incubated for one hour at -20°C and then centrifuged at 14,500 g for 20 minutes at 4°C. The pellet was resuspended in 500 µl TE-2 (10 mM Tris, 1 mM EDTA, pH 7.4), then 250 µL 4M NaCl and 370 µl ice cold isopropanol were added. The sample was incubated at -20°C for one hour. The sample was centrifuged at 16,800 g for 20 minutes at 4°C. The pellet was rinsed with 70% ethanol and vacuum dried, then resuspended in 200-300 µl of TE-2. RNA concentration was determined spectrophotometrically.

b. Rapid extraction procedure

Sap extracts were produced using a modification of the method of Thomson and Dietzgen (1995). A 1 mm square of expanding leaf tissue from new emerging potato sprouts or chrysanthemum tips, were squashed in 30 µl of 100 mM Tris, pH 7.6, containing 1 M KCl and 10 mM EDTA. Samples were boiled for 10 minutes and then placed immediately on an ice slurry. A 1µl aliquot of this extract was diluted in 9 µl of distilled water and 2 µl of this diluent was used as template for RT-PCR.

3. Sequences and primers.

Sequences for two isolates of CSV and 13 isolates of PSTV were obtained through the Australian National Genetic Information Service (ANGIS). All sequences were aligned against each other to determine conserved regions. Primers were designed for CSV and PSTV in the areas where the sequences for each viroid showed the greatest homology. The primers were compared where possible against likely contaminants, such as plant RNA, other plant pathogens and human contamination, using ANGIS.

4. PCR.

Sap extracts, total RNA or pure viroid RNA, were denatured by boiling for 10 minutes, placed onto an ice slurry to prevent renaturation. Sap extracts were diluted as above, and 2µl used as template for the RT-PCR reaction. The GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT), or Access RT-PCR System (Promega Corporation), were used according to the manufacturers' directions for cDNA synthesis and PCR amplification (Appendix 1).

The GeneAmp RNA PCR kit has a two step protocol where the RNA is copied to DNA, the reverse transcription (RT) step, and with the addition of further ingredients PCR is performed on the copy DNA (cDNA). For the RT reaction 1µl of a 50µM solution of the downstream primer was used. The RT reaction was carried out in a thermocycler at 23°C for 5 minutes, 42°C for 20 minutes, 99°C for 5 minutes and a 4°C hold. For the PCR reaction, 1µl of a 50 µM solution of each primer pair was used. The PCR mixture was added to the RT reaction and an initial denaturing step was carried out at 94°C for 1 minute followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds at 57°C for CSV or 55°C for PSTV and extension at 72°C for 1 minutes. A final extension at 72°C for 5 minutes was then done finishing with a 4°C holding step.

The Access RT-PCR System is a one step reaction where all components are added to the tube at the same time. For the RT-PCR reaction, 1µl of a 50 µM solution of each primer pair

was used. The RT-PCR was carried out in a thermocycler using the following cycling method. RT was carried out at 48°C for 45 minutes followed by a 94°C denaturation for 2 minutes. PCR was then carried out for 40 cycles, DNA was denatured at 94°C for 30 seconds, annealed for 1 minute at 57°C for CSV or 55°C for PSTV and extended at 68°C for 2 minutes. A final extension at 68°C for 7 minutes was then performed finishing with a 4°C hold.

Amplification products were analysed by electrophoresis in 1.5% agarose gels in TBE (0.045 M Tris borate, 0.001 M EDTA pH 8.0), visualised by staining with ethidium bromide (5 µg/ml) and photographed with UV transillumination.

4. cDNA probe

The CSV and PSTV non-radioactive kit (Bresatec) using a photobiotin labelled cDNA probe for the detection of viroids in plant extracts was used according to the manufacturer's specifications (see Appendix 2).

5. cDNA/RT-PCR comparison.

In order to test the sensitivity of the RT-PCR and the cDNA probe, crude and purified extracts of CSV and PSTV were diluted and tested as above. Pure CSV RNA or total RNA from PSTV infected plants were diluted in water to 1/1000 for both the cDNA probe and RT-PCR. Sap extracts of CSV or PSTV infected material were diluted in healthy chrysanthemum or potato sap extracts respectively to 1 plant in 200 for CSV and 1 plant in 100 for PSTV. Diluted sap extracts from CSV infected plants were tested to 1 plant in 100 for the cDNA probe.

Results.

1. Extraction

The modified Rowhani *et al.* (1993) method proved to be reliable for extracting total RNA from old and young PSTV infected potato plants. The rapid extraction method was unreliable on older tissue for both CSV and PSTV and should only be used on expanding leaf tissue from new emerging potato sprouts or chrysanthemum tips. Neither method was useful where chrysanthemum plants were kept in a cold environment for greater than one month (results not shown).

2. Primers.

One primer set was designed for CSV and four sets of primers were designed for PSTV (Table 1). All primer sets were tested against sap extracts and pure viroid RNA for CSV or total RNA for PSTV.

The primers developed for CSV RT-PCR gave consistent results using both RT-PCR kits with sap and pure CSV RNA. A single band of 354 bp was produced from both pure CSV RNA and infected sap extracts and no bands were seen from healthy sap extracts.

The primer pair TG21/CT20 was selected. They gave consistent results for PSTV using both RT-PCR kits with sap and total RNA extracts. A single band of 258 bp was produced from infected sap extracts and total RNA from infected material. No bands were seen from sap extracts or total RNA from healthy material. The primer pairs PSTV-F/PSTV-R, CG20/CT18 and CG19/CA19 were unreliable (results not shown).

Table 1. Primers developed for CSV and PSTV

Viroid	Primer pairs	Primer sequences	Upstream or Downstream	Position*	PCR product size (bp)
CSV	CSV-F	5'-CGG GGA AAC CTG GAG GAA G-3'	upstream	93-111	354
	CSV-R	5'-GAT CCC TGA AGG ACT TCT TCG-3'	downstream	90-70	
PSTV	PSTV-F	5'-TGT GGT TCA CAC CTG ACC TCC-3'	upstream	22-42	359
	PSTV-R	5'-GGA ACC ACG AGT TTA GTT CC-3'	downstream	21 - 2	
PSTV	CG19	5'-CGG CCG ACA GGA GTA ATT C-3'	upstream	144-162	330
	CA19	5'-CAG TTC GCT CCA GGT TTC C-3'	downstream	115-97	
PSTV	TG21	5'-TGT GGT TCA CAC CTG ACC TCC-3'	upstream	22-42	258
	CT20	5'-CTT CAG TTG TTT CCA CCG GG-3'	downstream	280-261	
PSTV	CG20	5'-GGA AAC CTG GAG ACT GG-3'	upstream	97-113	349
	CT18	5'-CTG AAG CGC TCC TCC GAG-3'	downstream	86-69	

*Position numbers of primers for CSV are the location from the first nucleotide base in the first published sequence (Haseloff and Symons 1981). Similarly position numbers of primers for PSTV are the location from the first nucleotide base in the first published sequence (Gross *et al.* 1978).

3. Comparison of sensitivity of the GeneAmp RNA PCR kit and the Access RT-PCR System for CSV and PSTV in sap and RNA extracts.

a. CSV

Results from dilution series showed that the RT-PCR kits had similar sensitivity using pure CSV RNA. CSV could be detected to levels as low as 0.0148 ng by the Access RT-PCR System (Fig. 1) and the GeneAmp RNA PCR kit (Fig. 2).

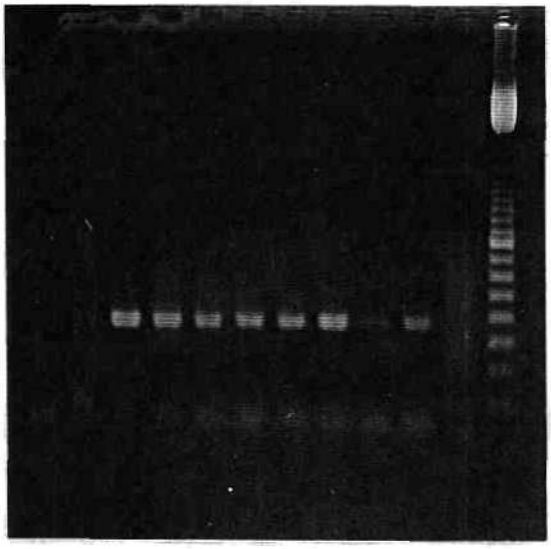

Figure 1. Agarose gel showing dilutions of pure CSV RNA amplified by RT-PCR using the Access RT-PCR System. Lane 1, water control; Lane 2, total RNA from healthy chrysanthemum; Lane 3-11 pure CSV RNA (ng): 7.4, 1.48 0.74, 0.0.37, 0.148, 0.074, 0.037, 0.0148; Lane 12, 100 bp ladder.

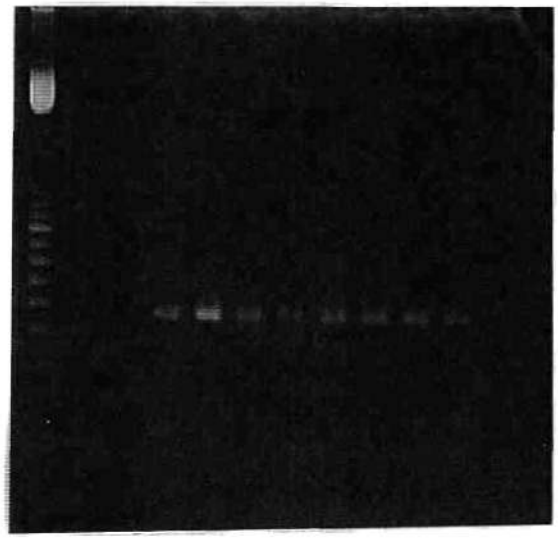

Figure 2. Agarose gel showing dilutions of pure CSV RNA amplified by RT-PCR using the GeneAmp RNA PCR kit. Lane 1, 100 bp ladder, Lane 2, water control; Lane 3, total RNA from healthy chrysanthemum; Lane 4-12 pure CSV RNA (ng): 7.4, 1.48 0.74, 0.0.37, 0.148, 0.074, 0.037, 0.0148.

Using the sap extracts both the Access RT-PCR System (Fig. 3) and the GeneAmp RNA PCR kit (Fig. 4) could detect one CSV infected plant in 200 while still giving a bright signal.

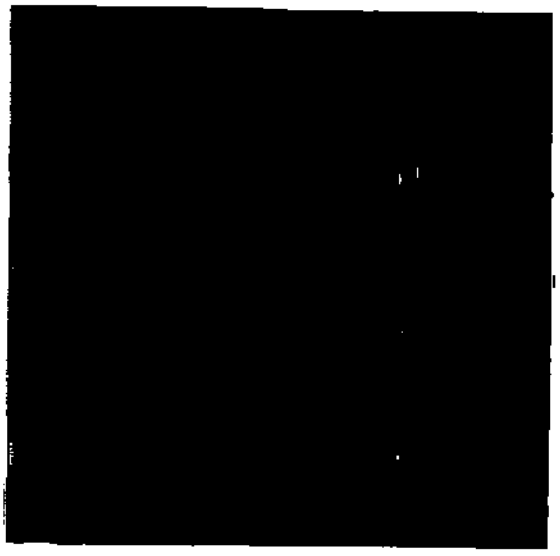

Figure 3. Agarose gel showing CSV infected sap extracts diluted with healthy sap extracts and amplified by RT-PCR using the Access RT-PCR System. Lane 1, water control; Lane 2, healthy chrysanthemums. Lane 3, CSV infected chrysanthemum; Lane 4, 1:5 (infected : healthy); Lane 5, 1:10; Lane 6, 1:20; Lane 7, 1:50; Lane 8, 1:100; Lane 9, 1:200; Lane 10, 100 bp ladder.

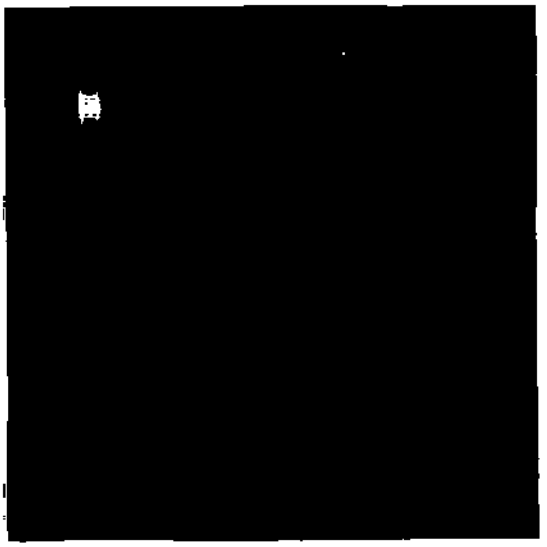

Figure 4. Agarose gel showing CSV infected sap extracts diluted with healthy sap extracts and amplified by RT-PCR using the GeneAmp RNA PCR kit. Lane 1, 100 bp ladder, Lane 2, water control; Lane 3, healthy chrysanthemums. Lane 4, CSV infected chrysanthemum; Lane 5, 1:5 (infected : healthy); Lane 6, 1:10; Lane 7, 1:20; Lane 8, 1:50; Lane 9, 1:100; Lane 10, 1:200.

b. PSTV

PSTV could be detected in 16.8 ng of total RNA purified from PSTV infected plants with the TG21/ CT20 primers using the Access RT-PCR System (Fig. 5) and the GeneAmp RNA PCR kit (Fig 6).

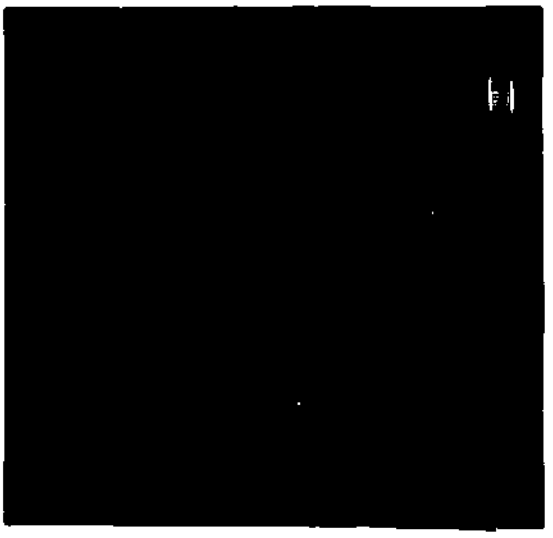

Figure 5. Agarose gel showing dilutions of total RNA from PSTV positive plants amplified by RT-PCR using the Access RT-PCR System. Lane 1, water control; Lane 2, total RNA from healthy potatoes; Lane 3-11, dilutions of total RNA from PSTV infected plants (ng): 1680, 336, 168, 84, 33.6, 16.8, 8.4, 3.36, 1.68, ; Lane 12, 100 bp ladder.

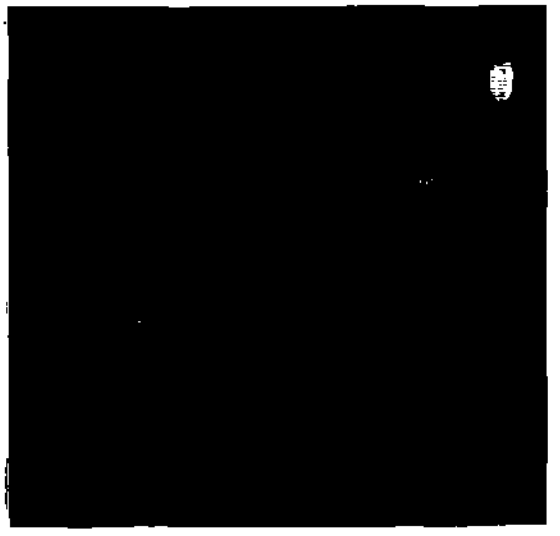

Figure 6. Agarose gel showing dilutions of total RNA from PSTV positive plants amplified by RT-PCR using the GeneAmp RNA PCR kit. Lane 1, 100 bp ladder; Lane 2, water control; Lane 3, RNA from healthy potatoes; Lane 4-12, dilutions of total RNA from PSTV infected plants (ng): 1680, 336, 168, 84, 33.6, 16.8, 8.4, 3.36, 1.68.

The dilution series on sap extracts of PSTV infected potatoes showed that one plant in 20 could be detected by both the Access RT-PCR System (Fig. 7) and the GeneAmp RNA PCR kit (Fig. 8).

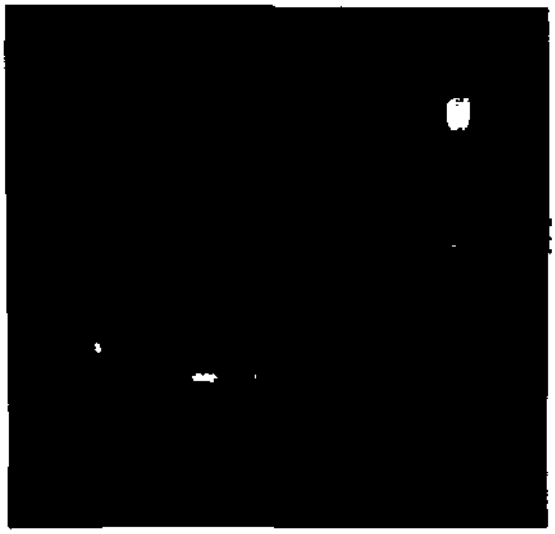

Figure 7. Agarose gel showing dilutions of PSTV sap extracts amplified by RT-PCR using the Access RT-PCR System. Lane 1, water control; Lane 2, healthy potato; Lane 3, PSTV infected potato; Lane 4, 1:5 (infected : healthy), Lane 5, 1:10; Lane 6, 1:20; Lane 7, 1:50; Lane 8, 1:100; Lane 9, 100 bp ladder.

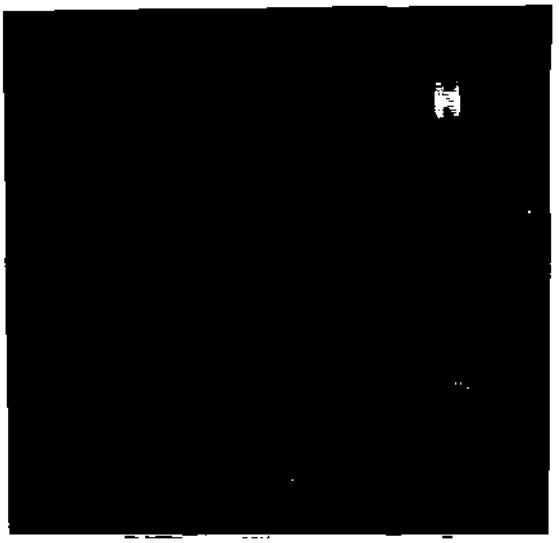

Figure 8. Agarose gel showing dilutions of PSTV sap extracts amplified by RT-PCR using the GeneAmp RNA PCR kit. Lane 1, 100 bp ladder; Lane 2, water control; Lane 3, healthy potato; Lane 4, PSTV infected potato; Lane 5, 1:5 (infected : healthy); Lane 6, 1:10; Lane 7, 1:20; Lane 8, 1:50; Lane 9, 1:100.

The GeneAmp RNA PCR kit occasionally gave inconsistent results with both the sap extracts and total RNA from PSTV infected material using all primer sets (results not shown).

4. cDNA probe

Results from the cDNA probe show that it is not as sensitive as PCR. Reasonably strong signals could be obtained from 1.11 ng of pure CSV and very weak signals were obtained down to 0.222 ng (Fig. 9). Total RNA extracts of PSTV infected material had reasonable signals down to 504 ng and very weak signals were obtained down to 42 ng (Fig. 10).

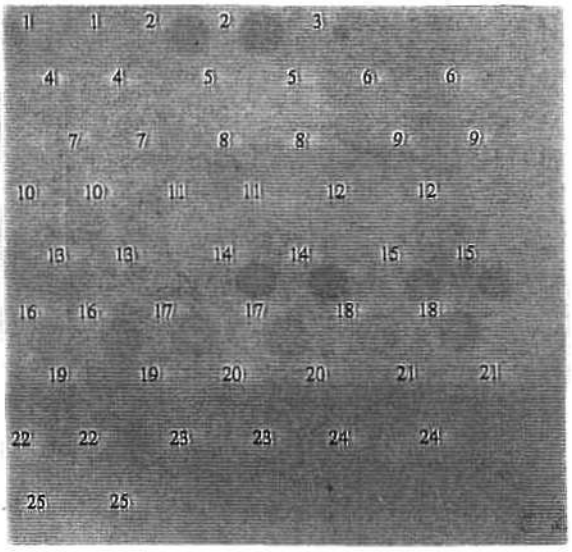

Figure 9. Detection by cDNA probe of CSV infected material. 1, negative control; 2, positive control; 3, kit positive control DNA; 4-9, sap extract dilutions as for RT-PCR to 1:100; 10, sap extract negative control; 11-19, pure CSV RNA dilutions as for RT-PCR; 20, chrysanthemum total RNA.

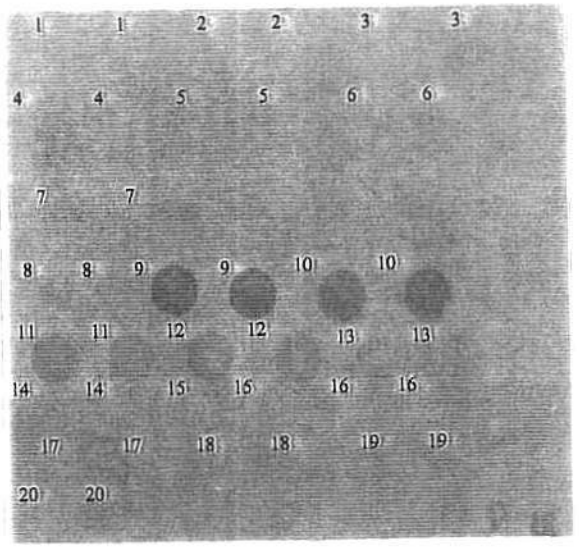

Figure 10. Detection by cDNA probe of PSTV infected material. 1-6, sap extract dilutions as for RT-PCR to 1:100; 7. sap extract negative control; 8. negative control; 9, positive control; 10, kit positive control DNA; 11-19, total RNA from PSTV infected plants dilutions as for RT-PCR; 20, total RNA from healthy potato plants.

When sap extracts were used neither viroid could be detected.

5. cDNA/RT-PCR comparison.

The RT-PCR, using either kit, was found to be at least 75 times more sensitive than the cDNA probe for the detection of CSV and 30 times more sensitive for the detection of PSTV. (Table 2.)

Table 2. Levels of detection (ng) of pure CSV RNA and total RNA from PSTV infecetd potatoes using the Access RT-PCR System, the GeneAmp RNA PCR kit or the cDNA probe.

	Access RT-PCR System	GeneAmp RNA PCR kit	cDNA probe
CSV	0.0148	0.0148	1.11
PSTV	16.8	16.8	504

RT-PCR, using either kit, was also able to detect viroids in sap extracts while the cDNA probe could not.

Discussion.

In order for a grower to implement disease control strategies a correct diagnosis must be made. Both CSV and PSTV can be devastating to their respective host crops and rapid identification of these disease agents can help to prevent further spread of the disease in the field. This is especially important as the symptoms of both viroids in their hosts may not be immediately obvious to the grower, especially for PSTV where the above ground symptoms are minor.

Any test offered by a commercial diagnostic service must be accurate, reliable and have a rapid turn around time. It is extremely important that the test is reliable and easy to use, growers cannot afford to wait a week for a commercial laboratory to trouble-shoot a test that has failed to work. A rapid turn around time is extremely important for a disease that can spread easily and rapidly.

The RT-PCR for both viroids has a rapid turn around time and results can be obtained within six hours after the receipt of the samples. The ability of the RT-PCR to detect both viroids in sap extracts decreases the turn around time since lengthy purification procedures for RNA are not required. This results in cost savings in labour that can be passed onto growers. Also, the results from sap extracts indicate that it is possible to bulk samples when many are being tested, again resulting in cost savings to growers.

The rapid extraction procedure, since it is simple and rapid, enables many samples to be processed simultaneously. This extraction procedure is most reliable when expanding leaf tissue from new emerging potato sprouts or chrysanthemum tips are used. Plants for testing should be maintained at 25°C as this is the optimum temperature for viroid replication. This increases viroid titre (Singh and Singh, 1995). Older chrysanthemum and potato material may give unreliable results with the rapid extraction procedure. The modified method of Rowhani *et al.* (1993) is more likely to produce a reliable result with older material but this method takes one day to produce total RNA and only a small number of samples can be processed at a time.

The RT-PCR test for CSV had been developed by Kusunoki *et al.* (1993) where a full length product was produced. However this method required a rigorous extraction procedure that slowed down the turn around time for testing and was therefore not used.

While both kits were equally sensitive the one step PCR is most likely to be adopted as the routine diagnostic test. The one step PCR is far easier to use since all ingredients for the RT reaction and the PCR reaction are added to the tube at the one time, limiting the chance of contamination. The two step RT-PCR requires the tube to be opened after the RT reaction, increasing the chance of contamination and thus false positives. Also, it was found that the one step kit was more reliable than the two step kit for PSTV testing, making it the preferred option for routine viroid diagnosis by RT-PCR. Whether this is true of other one step kits has yet to be determined.

The cDNA probe, while reasonably sensitive, is tedious and time consuming and takes one week before results can be obtained. The RNA must be purified before use, and the number of extracts that can be produced at any one time is limited. The test itself takes three days to run and many hours of labour adding greatly to the cost. The one advantage of the test is that a large number of samples can be processed at once. However, it is unlikely that this number would exceed that done comfortably via PCR within the time it would take to carry out a cDNA test.

The RT-PCR test will replace the cDNA probe, especially as the commercial cDNA probe kit is no longer available. New technology is often more sensitive, rapid and cost effective than older methods and can improve our ability to test for disease organisms. Indeed, the RT-PCR is more sensitive, rapid and cost effective than the cDNA probe for CSV and PSTV detection. Also, in our hands the RT-PCR was reliable with crude and purified extracts of both viroids. The RT-PCR is the diagnostic test that we recommend for the detection of CSV and PSTV.

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Appendix 1.

Manufacturers' instructions for the use of the GeneAmp RNA PCR kit (Perkin-Elmer-Cetus) and the Access RT-PCR System (Promega Corporation).

GeneAmp®

RNA PCR Kit
Part No. N808-0017

RNA PCR Core Kit
Part No. N808-0143

PERKIN ELMER

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES
See notice to purchaser

The Perkin-Elmer *GeneAmp*® RNA PCR Kit and RNA PCR Core Kit are designed for use in the detection and analysis of gene expression at the RNA level. Included are reagents to perform the reverse transcription of RNA to cDNA (using cloned Murine Leukemia Virus [MuLV] Reverse Transcriptase) and subsequent amplification (using the *GeneAmp* Polymerase Chain Reaction ["PCR"] process and *AmpliTaq*® DNA Polymerase) all in a single reaction tube.[1,3,20,21] A recombinant RNase Inhibitor, originally isolated from human placenta, is included for inhibition of certain mammalian RNases.[4] Sufficient reagents are provided for 100 reverse transcription reactions (20 µL each) and 100 PCR amplifications (100 µL each).

The complete GeneAmp RNA PCR Kit also contains a positive control RNA template transcribed from the plasmid pAW109. Plasmid pAW109 contains an insert of a synthetic linear array of primer sequences for multiple target genes constructed such that "upstream" primer sites are followed by complementary sequences to their "downstream" primer sites in the same order. The insert in pAW109 is identical to the insert previously described for plasmid pAW106 (Figure 1).[1] The primers included in this kit flank an IL-1α site and can be used to amplify a 308 bp sequence within the site. Sufficient pAW109 RNA is provided for 50 reverse transcription/amplification reactions at 10^6 copies per reaction.

The *GeneAmp* PCR process is a simple and powerful method invented by K. Mullis, which allows *in vitro* amplification of DNA segments (including cDNA)[1-3] through a succession of incubation steps at different temperatures.[5-9] The *GeneAmp* PCR process is based on the repetition of this cycle and can amplify DNA segments by at least 10^5 fold and potentially as high as 10^9 fold under the conditions described below.[9]

NOTICE TO PURCHASER: LIMITED LICENSE

The purchase price of this product includes a limited, non-transferable license under U.S. Patents 4,683,202, 4,683,195 and 4,965,188 or their foreign counterparts, owned by Hoffmann-La Roche Inc. and F. Hoffmann-La Roche Ltd. to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with an authorized thermal cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, Applied Biosystems Division, 850 Lincoln Centre Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

LIST OF KIT COMPONENTS

Store the *GeneAmp* RNA PCR Kit or the RNA PCR Core Kit at -20°C in a constant temperature freezer. If stored under the proper conditions, the product will maintain performance through the control date printed on the label. ***Important Note:* Reverse Transcriptase and RNase Inhibitor are sensitive to air oxidation. Keep these reagents at -20°C until just prior to use.** All water is autoclaved, deionized, ultrafiltered water, referred to as "DI water". Before use, thaw frozen components and mix well by vortexing.

GeneAmp RNA PCR Core Kit Part No. N808-0143

Reagent	Part No.	Volume	Description
MuLV Reverse Transcriptase	N808-0018	100 µL	50 U/µL. Reverse transcribes RNA to cDNA
RNase Inhibitor	N808-0119	100 µL	20 U/µL. Inhibits RNase activity
AmpliTaq® DNA Polymerase	N801-0060	50 µL	5 U/µL. Extends primers during PCR amplification
dATP	N808-0007	320 µL	10 mM — Deoxyribonucleoside triphosphates dissolved in DI water, titrated with NaOH to pH 7.0
dCTP		320 µL	10 mM
dGTP		320 µL	10 mM
dTTP		320 µL	10 mM
10X PCR Buffer II	N808-0010	1.5 mL	500 mM KCl, 100 mM Tris-HCl, pH 8.3. Provides preferred pH and ionic strength for reverse transcription and PCR amplification
25 mM $MgCl_2$ Solution		1.5 mL	25 mM $MgCl_2$. Required for enzyme activity
Random Hexamers	N808-0127	100 µL	50 µM* Random primers for reverse transcription of RNA
Oligo dT$_{16}$	N808-0128	100 µL	50 µM* of Poly dT tailed primer for reverse transcription of RNA. Sequence: 5'-TTTTTTTTTTTTTTT-3'
** Primer DM152		50 µL	15 µM* of "Downstream" IL-1α primer for reverse transcription and amplification of pAW109 RNA. Sequence: 5'-CATGTCAAATTTCACTGCTTCATCC-3'
** Primer DM151		50 µL	15 µM* of "Upstream" IL-1α primer for amplification of pAW109. Sequence: 5'-GTCTCTGAATCAGAAATCCTTCTATC-3'
pAW109 RNA	N808-0037	50 µL	1 x 10^6 copies/µL. RNA transcribed from pAW109; in 30 µg/mL *E.coli* rRNA; in 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0

* Supplied in 10 mM Tris-HCl, pH 8.3

** Can also be purchased separately as a set of 200 µL each of 25 µM Solutions (**Part No. N808-0078**).

ENZYME CHARACTERISTICS

***AmpliTaq*® DNA Polymerase**

(Deoxyribonucleoside triphosphate: DNA Deoxynucleotidyltransferase, EC 2.7.7.7.)

The key component in the *GeneAmp* RNA PCR Kit or RNA PCR Core Kit is *AmpliTaq* DNA Polymerase (**Part No. N801-0060**). Perkin-Elmer *AmpliTaq* DNA Polymerase is a recombinant, thermostable, 94 kDa DNA polymerase encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene which has been inserted into an *Escherichia coli* host.[10-16] Many of the features of *AmpliTaq*® DNA Polymerase also make it excellent for DNA sequencing[15] (**Part Nos. N808-0001, N808-0035, N808-0110**). *AmpliTaq*® DNA Polymerase and Native *Taq* DNA Polymerase are covered by U.S. Patents 4,889,818 and 5,079,352 owned by Hoffmann-La Roche Inc. Additional patent applications on genes encoding ther-

Concentration:	5 Units/µL
Unit Definition:	One unit of enzyme is defined as the amount that will incorporate 10 nmoles of dNTPs into acid insoluble material i 30 minutes in a 10 minute incubation at 74°C under the analysis conditions below.
Analysis Conditions[10]	25 mM TAPS (*tris*-(hydroxymethyl)-methyl-amino-propanesulfonic acid, sodium salt), pH 9.3 (at room temperature), 50 mM KCl; 2 mM $MgCl_2$; 1 mM β-mercaptoethanol; 200 µM *each of* dATP, dGTP, dTTP; 100 µM [α-^{32}P]-dCTP (0.05-0.1 Ci/mmole); salmon sperm DNA, activated by a modification of methods in ref. 19; mixed in a final volume 50 µL and incubated at 74°C for 10 minutes.
Storage Buffer:	20 mM Tris-HCl, pH 8.0 (at room temperature), 100 mM KCl, 0.1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM (dithiothreitol), 50% (v/v) Glycerol, 0.5% (w/v) Tween 20®, 0.5% (w/v) Nonidet P40®.
Storage Temperature:	Store *AmpliTaq*® DNA Polymerase at -20°C, in a constant temperature freezer. If stored under proper conditions, t enzyme will remain active through the control date printed on the label.
Associated Activities:	Endonuclease and exonuclease activities were not detectable after one hour incubation of 600 ng of supercoiled pBR322 (dam$^-$, dcm$^-$) or 600 ng of *MspI*-digested pBR322 DNA, respectively, at 74°C, in the presence of 8 units *AmpliTaq*® DNA Polymerase. The enzyme has a fork-like structure dependent, polymerization enhanced 5' to 3' n ase activity but lacks a 3' to 5' exonuclease activity.[13,21]

MuLV Reverse Transcriptase

(DNA Nucleotidyltransferase [RNA Directed] EC 2.7.7.49)

MuLV Reverse Transcriptase (Murine Leukemia Virus Reverse Transcriptase) is an RNA-dependent DNA polymerase that uses single strande RNA as a template in the presence of a primer to synthesize a complementary DNA strand. This enzyme is suitable for synthesis of first strand

Concentration:	50 Units/µL
Source:	This enzyme is purified from *Escherichia coli* expressing the *pol* gene of MuLV on a plasmid.
Unit Definition:	One unit is the amount of enzyme that incorporates 1 nmole of dTTP into acid precipitable material in 10 minutes a 37°C using poly rA • oligo $d(T)_{12-18}$ as template primer. The ratio of oligo d(T) to poly rA is 3:1.
Storage Buffer:	20 mM Tris-HCl, pH 7.5, 0.1 mM Na_2EDTA, 1 mM DTT, 0.01% (w/v) Nonidet P40®, 50% (v/v) Glycerol, in DEPC (diethylpyrocarbonate) treated DI Water.

The schematic diagram in Figure 1 on next page (not drawn to scale) shows the general arrangement of the RNA transcribed from plasmid pAW109. The DNA sequences for the other GeneAmplimer® primers in the control cDNA sequences are provided in Table 1.[1] The DM151 and DM152 primer set amplify a 421-bp sequence from authentic IL-1α mRNA. Based on the positioning of the DM151/DM152 primer pair, a 308-product is amplified from pAW109-derived cDNA. See following table for correct sizes of the products produced with these primers, from eit pAW109 or mRNA.

Table 1. GeneAmplimer® Primers for Lymphokine and Other Amplicons in pAW109 RNA.

GENE NAME	PRIMER INFORMATION		PCR PRODUCT SIZE FROM		PE PART NUMBE
	NAME	SEQUENCE	pAW109	mRNA	
TNF	AW112 AW113	Sequence: 5'-CAGAGGGAAGAGTTCCCCAG-3' Sequence: 5'-CCTTGGTCTGGTAGGAGACG-3'	301bp	325bp	N808-0
M-CSF	AW111 GM20	Sequence: 5'-GAACAGTTGAAAGATCCAGTG-3' Sequence: 5'-TCGGACGCAGGCCTTGTCATG-3'	302bp	192bp	N808-0
PDGF-A	AW116 AW117	Sequence: 5'-CCTGCCCATTCGGAGGAAGAG-3' Sequence: 5'-TTGGCCACCTTGACGCTGCG-3'	301bp	225bp	N808-0
PDGF-B	AW118 AW119	Sequence: 5'-GAAGGAGCCTGGGTTCCCTG-3' Sequence: 5'-TTTCTCACCTGGACAGGTCG-3'	300bp	226bp	N808-0
LDL-R	AW125 AW126	Sequence: 5'-CAATGTCTCACCAAGCTCTG-3' Sequence: 5'-TGTGTCTCGAGGGGTAGCTG-3'	301bp	258bp	N808-0
HMG	AW102 AW104	Sequence: 5'-TACCATGTCAGGGGTACGTC-3' Sequence: 5'-CAAGCCTAGAGACATAATCATC-3'	303bp	247bp	N808-0
IL-1α	DM151 DM152	Sequence: 5'-GTCTCTGAATCAGAAATCCTTCTATC-3' Sequence: 5'-CATGTCAAATTTCACTGCTTCATCC-3'	308bp	421bp	N808-0
IL-1β	DM155 DM156	Sequence: 5'-AAACAGATGAAGTGCTCCTTCAGG-3' Sequence: 5'-TGGAGAACACCACTTGTTGCTCCA-3'	306bp	391bp	N808-0
IL-2	DM135 DM136	Sequence: 5'-GAATGGAATTAATAATTACAAGAATCCC-3' Sequence: 5'-TGTTTCAGATCCCTTTAGTTCCAG-3'	305bp	229bp	N808-0
PDGF-R	AW158 AW159	Sequence: 5'-TGACCACCCAGCCATCCTTC-3' Sequence: 5'-GAGGAGGTGTTGACTTCATTC-3'	300bp	228bp	N808-0
LPL	AW155 AW157	Sequence: 5'-GAGATTTCTCTGTATGGCACC-3' Sequence: 5'-CTGCAAATGAGACACTTTCTC-3'	301bp	276bp	N808-0

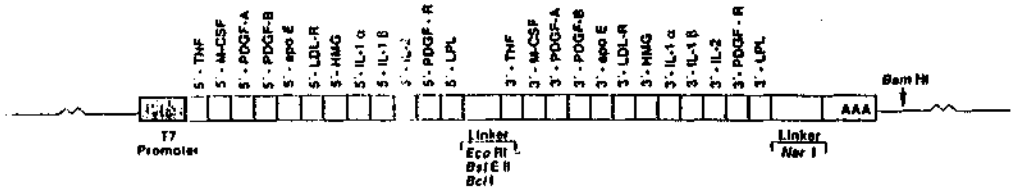

Figure 1. pAW109 Insert.

PROTOCOLS FOR REVERSE TRANSCRIPTION OF RNA AND PCR AMPLIFICATION OF cDNA

.0 Reverse Transcription Protocol. This protocol for the control in the complete *GeneAmp*® RNA PCR Kit will provide an example for users of ther kit. Control components can be purchased separately.

.1 Prepare a Master Mix for reverse transcription by adding the reagents in the order and proportions shown below (See Sections 4.1 and 4.2).

	Component	Volume	Final Concentration
Master Mix	25 mM $MgCl_2$ Solution	4 µL	5 mM
	10X PCR Buffer II	2 µL	1X
	DEPC Treated DI Water	2 µL*	—
	dGTP	2 µL	1 mM
	dATP	2 µL	1 mM
	dTTP (or dUTP, Section 6.4)	2 µL	1 mM
	dCTP	2 µL	1 mM
	RNase Inhibitor	1 µL	1 U/µL
	MuLV Reverse Transcriptase	1 µL	2.5 U/µL
	Random Hexamers -or- Oligo $d(T)_{16}$ -or- DM152: "Downstream" Primer	1 µL	2.5 µM -or- 2.5 µM -or- 0.75 µM
	** pAW109 RNA -or- User provided experimental sample	1 µL*	10^5 copies (or more) -or- ≤ 1 µg total RNA
	Total reverse transcription volume, including sample	20 µL	

* Any combination of water and sample volumes can be used as long as the final volume equals 20 µL.
** See Section 5.1 for instructions on diluting the pAW109 RNA.

using random hexamers or Oligo $d(T)_{16}$ as reverse transcriptase primer, allow all tubes to incubate at room temperature for 10 minutes. The room temperature incubation allows for the extension of the hexameric primers or Oligo $d(T)_{16}$ by reverse transcriptase. The extended primers will then remain annealed to the RNA template upon raising the reaction temperature to 42°C. Incubate all tubes in a Perkin-Elmer *GeneAmp* PCR Instrument System as follows.

Table 2. Perkin-Elmer *GeneAmp* PCR Instrument Systems' Reverse Transcriptase Profile Times and Temperatures.

Perkin Elmer *GeneAmp*™ PCR Instrument System	Tube (for reverse transcription)		Times & Temperatures for Reverse Transcription		
			1 Cycle Only		
	Tube Type	Volume in mL/tube (Oil to µL)	Reverse Transcribe	Denature	Cool
DNA Thermal Cycler or *DNA Thermal Cycler 480*	*GeneAmp PCR Reaction (N801-0180)*	*20 µL* (Oil: 50-100)	STEP CYCLE		
			15 min 42°C	*5 min* 99°C	*5 min* 5°C
GeneAmp PCR System 9600	*MicroAmp*™ Reaction (N801-0533) (N801-0540) (N801-0612)	20 µL (no oil needed)	CYCL		
			15 min 42°C	5 min 99°C	5 min 5°C

.2 Except for amplifications in the Perkin-Elmer *GeneAmp*™ PCR System 9600, control evaporation or refluxing by overlaying the mix with 50 to 00 µL of mineral oil (Sigma Chemical Co., St. Louis, MO., Cat. No. M5904). The oil should not interfere when withdrawing samples. If the entire volume is to be recovered, 100 µL of high purity chloroform can be added after amplification. The aqueous phase containing the DNA will then float on he chloroform-oil mixture, allowing easy collection. The use of *AmpliWax*™ PCR Gem 100 (Part No. N808-0100) as an alternative to oil may also ncrease reproducibility in amplification when used for Hot Starts at low target number or with high background DNA concentrations (Section 6.5).[17]

2.0 PCR Protocol

2.1 For each sample prepare a minimum of 78 µL of PCR Master Mix as shown below. (See Sections 4.1 and 4.3)

Component	Volume	Final Concentration
25 mM $MgCl_2$ Solution	4 µL	2 mM
10X PCR Buffer II	8 µL	1X
DI Water	65.5 µL	--
AmpliTaq® DNA Polymerase	0.5 µL	2.5 U/100 µL
Total PCR Master Mix volume per sample	78 µL	

2.2 Dispense 78 µL of the PCR Master Mix into each reverse transcription reaction tube. Change tips between additions to avoid sample carr

2.3 Dispense the primers into each tube. Change tips between primer additions to avoid sample carryover. For the pAW109 RNA supplied w this kit, use the DM151/DM152 primer set. If a "downstream" primer was used for the reverse transcription reaction (e.g., DM152), then it sho not be added again. Instead, substitute 1 µL DI water.

Component	Volume	Final Concentration	
DM152 "Downstream" Primer	1 µL	0.15 µM	Can be mixed first, then add 2 µL per tube.
DM151 "Upstream" Primer	1 µL	0.15 µM	Higher concentrations may be used.

The final volume of each tube should now be 100 µL (including 20 µL from the reverse transcription reaction).

2.4 Spin the tubes for approximately 30 seconds in a microcentrifuge.

3.0 Temperature Cycling for the Control Reagents. Optimum performance of the *GeneAmp* PCR Process is achieved using a *GeneAmp* ® PCR Instrument System.

Store samples at 6°C or less until ready to use (with the *GeneAmp*® PCR Carry-over Prevention Kit [Part No. N808-0066] use 72°C).

Table 3. Perkin-Elmer *GeneAmp* PCR Instrument Systems' PCR Profile Times and Temperatures.

Perkin-Elmer *GeneAmp* ™ PCR Instrument System	Tube (for PCR)		Times & Temperatures for Amplification with this Kit			
	Tube Type	Volume µL/tube (vapor barrier)	Initial Step	Each of 35 Cycles		Final Step
				Melt	Anneal-Extend	
DNA Thermal Cycler or *DNA Thermal Cycler 480*	*GeneAmp PCR Reaction (N801-0180)*	*100* (oil:50-100 µL)	STEP CYCLE	STEP CYCLE		TIME DELAY
			120 sec. 95°C 1 cycle	*60 sec. 95°C*	*60 sec. 60°C*	*7 min. 72°C*
DNA Thermal Cycler 480	*GeneAmp* Thin-Walled Reaction (N801-0537) (N801-0611) (N801-0737)	100 (oil:50-100 µL)	STEP CYCLE	STEP CYCLE		TIME DELAY
			120 sec 95°C 1 cycle	45 sec 95°C	45 sec. 60°C	7 min. 72°C
GeneAmp ™ PCR System 9600	*MicroAmp*™ Reaction (N801-0533) (N801-0540) (N801-0612)	100 (no vapor barrier needed)	HOLD	CYCL		HOLD
			105 sec. 95°C	15 sec 95°C	30 sec. 60°C	7 min. 72°C

4.0 General Notes

4.1 Because of the enormous amplification possible with the *GeneAmp* PCR process, small levels of DNA contamination, especially from pre PCR amplification reactions, samples with high DNA levels and positive control templates, can result in product formation even in the absence purposefully added template DNA.[12] If possible, all reactions should be set up in an area separate from PCR product analysis. The use of ded ed or disposable vessels, solutions and pipettes (preferably positive displacement pipettes or tips with hydrophobic filters) for DNA preparatio reaction mixing, and sample analysis will minimize cross contamination.[15]

4.2 Mix gently (avoid generating bubbles) the reverse transcriptase, RNase Inhibitor, *AmpliTaq* ® DNA Polymerase or other recently thawed reagents, then spin down in a microcentrifuge before pipetting. Pipette the enzyme carefully and slowly: the viscosity of the 50% glycerol in the buffer can lead to pipetting errors. Using Master Mixes (see above) will increase accuracy, reduce enzyme loss on tips, and reduce tube-to-tube ability.

4.3. For both reverse transcription and PCR amplification, Master Mixes of reagents (water, buffers, dNTPs, magnesium chloride, and enzym all samples can be prepared first, then aliquotted to individual tubes when needed. Using such mixes will minimize reagent pipetting losses, increase accuracy, and reduce the number of reagent transfers. Any combination of water, primer, and experimental sample volumes can be as long as the final total combined volume equals 20 µL for the reverse transcription reaction (See Section 1.1) and 100 µL for the PCR amplif tion (See Sections 2.1 and 2.3). However, care should be taken not to add additional EDTA, citrate or other chelators with the primers or exp mental sample. Final chelator concentration should not exceed 0.11 mM.

Reverse transcription and PCR amplifications are performed in polypropylene reaction tubes. Perform amplifications in the Perkin-Elmer PCR action tube appropriate for your Perkin-Elmer *GeneAmp*™ PCR Instrument System (see **Section 5** and the instrument manual). Perkin-Elmer R reaction tubes provide the best heat transfer because of their uniform fit in the wells of the corresponding instrument. Since DNA may stick to stic and since nucleases are often found on surfaces, it may be preferable to use sterile tubes and pipette tips.

The reagent concentration ranges in the reaction mix recipe described in **Sections 1.0** and **2.0** are a useful starting place for reverse transcription and PCR amplification of different RNA targets using primers designed by the user. Optimization of reactions for each primer-template pair may necessary and can be achieved by varying magnesium chloride concentration, primer concentration and anneal-extend temperature (**Section**). The effect of these variations can be monitored by examining the intensity and distribution of product samples electrophoresed on Agarose Separation of *GeneAmp* PCR Products (**Part No. N930-2774**) followed by visualization with ethidium bromide staining of the gel.[9]

The optimal magnesium chloride concentration for both reverse transcription and PCR amplification needs to be determined empirically, by ting concentrations of magnesium chloride in the range of 0.8 to 5 mM in 0.5 mM increments for each primer set. Too little or too much magnesium chloride could reduce reverse transcription and/or amplification efficiency or result in non-specific products. If the samples contain EDTA, citrate or other chelators, raise the magnesium chloride concentration in the reaction mix proportionately. Magnesium chloride concentrations should to be adjusted in parallel with significant changes in the concentrations (higher or lower) of sample RNA, cDNA, DNA and dNTPs, to keep free agnesium ion constant.

Keep concentrations of dNTPs in the reaction mix balanced; if the concentration of any one dNTP is significantly different from the rest, the *mpliTaq*® DNA Polymerase may tend to misincorporate, slow down and terminate prematurely.[13]

The sequence-specific primers for RNA and cDNA should be 15 to 30 bases in length. The %G+C of primers should be near 50%, to maximize specificity. To avoid potential problems, primers should be purified by gel electrophoresis or HPLC ion-exchange chromatography. The optimal primer concentrations need to be determined empirically, by testing concentrations in the range of 0.1 to 1.0 µM. Primer concentrations that too low will result in little or no PCR product, while concentrations that are too high may result in amplification of non-target sequences. Primer ncentrations in the range of 0.2 to 0.5 µM will work for most PCR amplifications. Primer sequences should not complement within themselves to each other, particularly at the 3' ends. The primers in *GeneAmp*® PCR Reagent Kit have been designed with GG/CC overlap at the 3' end, to ow one consequence of complementary sequences. These use results in production of 46 to 50 base pair products called primer dimer, which n lead to other, larger primer artifacts. This product may occur to some extent even without such an overlap. Reducing each primer concentration (up to 5 fold, to 0.2 µM) will greatly reduce such products, as will use of *AmpliWax*™ PCR Gem 100 in a *Hot Start*.[17]

RNA Reverse Transcription

The pAW109 RNA used as a positive control RNA is recommended to be used as supplied (**See Section 1.1**) or can be diluted (in autoclaved PC treated DI water) to as little as 5×10^3 copies per µl before adding 2 µL to the reaction and is stable for up to one hour diluted. In order to ess user sensitivity in RNA PCR, working dilutions of the pAW109 RNA should be made from the stock concentration. RNAs are very labile, d therefore precautions must be taken to minimize ribonuclease activity during sample preparation and subsequent dilution.[22]

The primer of cDNA synthesis can be Random Hexamers, Oligo $d(T)_{16}$ (if poly rA tailed mRNA is used) or a "downstream" primer of your own sign (DM152 is used in the example protocol). The choice of primer for reverse transcription depends on many factors and the choice is best de on the basis of experimentation to evaluate all three priming systems. For short RNAs containing no hairpins, any of the three priming systems usually work equally well. For longer transcripts or sequences containing hairpins, the following guidelines should be considered.

5.2.1 Random hexamers. Try first, especially if efficiency of downstream priming is low, for long reverse transcripts, or for reverse transcripts containing hairpins. Use to reverse transcribe all RNA (rRNA, mRNA, and tRNA). The concentration of random hexamers provided in this kit will allow efficient reverse transcription of at least 1 µg RNA per reaction.

5.2.2 "Downstream" Primer. Try second. Use to reverse transcribe only RNA that contains a complementary sequence. You will need to have prior knowledge of the target sequence and have to synthesize the primers.

5.2.3 Oligo $d(T)_{16}$. Avoid for long mRNA or large transcripts if target is several Kb upstream. Use to increase specificity by reverse transcribing only eukaryotic mRNAs and retroviruses with poly rA tails (Prokaryotic RNA, rRNA, tRNA, and some eukaryotic mRNA do not have poly rA tails.). Use to transcribe entire eukaryotic mRNA message.

The RNA segment to be transcribed and later amplified can be at least 3 kb long, although 100 to 1000 bases are more typical and easier to ptify. Start with enough copies of the template to be sure of obtaining a signal after 35 cycles: preferably $>10^4$ copies but less than 1 µg total mple RNA. The pAW109 RNA is provided at a high enough concentration to allow first users of the kit to add much higher levels of this template a control. Low concentrations of target cDNA may require up to 45-50 cycles to produce sufficient product for analysis.

Sample RNA should be extracted with phenol/chloroform, precipitated with ethanol, and redissolved in RNase-free 1 mM EDTA, 10 mM NaCl, mM Tris-HCl, pH 8.0.[11]

Note: Reverse transcriptase binds to cDNA and is thereby inhibitory to PCR amplification. Under the conditions provided (2.5 U/µL), the 99°C ubation for 5 minutes inactivates the reverse transcriptase and removes the inhibitory effect. If the concentration of reverse transcriptase is reased, inactivation of reverse transcriptase becomes more difficult. At 10 U/µL of reverse transcriptase, PCR yields may be variable or spotic. Therefore, for synthesis of longer RNA transcripts, it is advisable to increase the incubation time during reverse transcription up to 60 minutes rather than increase the amount of reverse transcriptase added.

PCR Amplification

The selection of 60°C for a combined anneal-extend temperature is optimal for amplification of the pAW109 cDNA. It may be necessary to wer or raise (in the range of 37° to 65°C) the anneal-extend temperature for other primer-template pairs. Higher anneal-extend temperatures generally result in a much more specific product.[9,16] The optimal anneal-extend temperature can be determined empirically by testing increments of C or less until the maximum in specificity and product yield is reached. At these temperatures (37° to 65°C) *AmpliTaq*® DNA Polymerase has significant activity and extension of primed templates can occur. For some primer-template pairs, two temperature rather than three temperature cles may work where the anneal temperature is above 60°C. The extension is completed at the anneal temperature. The slower extension rate lower temperatures may require optimization of the time for such combined anneal-extend steps.

6.2 The length of the target sequence will affect the required extension time. Typically, *AmpliTaq* DNA Polymerase has an extension rate of 2 to 4,000 bases per minute at 70° to 80°C. Polymerization rates are significant even below 55°C and with some templates up to 85°C.[13] As the amount of DNA increases in later cycles, the number of *AmpliTaq* DNA Polymerase molecules may become limiting for the extension time alk Increasing the extend times in later cycles may be necessary to maintain efficiency of amplification. Use the Auto-Segment Extension feature Perkin-Elmer DNA Thermal Cycler or DNA Thermal Cycler 480 or the AUTO program for the *GeneAmp*™ PCR System 9600.

6.3 The half life of *AmpliTaq*® DNA Polymerase (<35 minutes at >95°C)[14] suggests 95°C as the maximum practical melting temperature. It is important in the early cycles to be sure to completely melt the template DNA. With high G+C content DNA, melting at 97°C for the first few c will help produce single stranded template for the PCR amplification. The melting temperature *should be reduced* for subsequent cycles bec the smaller PCR product usually melts completely at a lower temperature (unless the PCR product is excessively G+C rich) than the starting

6.4 Because *AmpErase*™ UNG (UNG), a component in the *GeneAmp*® PCR Carry-Over Prevention Kit (**Part No. N808-0068**), is active at 42 would remove any incorporated Uracil from cDNA during reverse transcription. Users of the *GeneAmp*® PCR Carry-over Prevention Kit shoul UNG only to the PCR Master Mix and should keep mixes above 60°C while adding UNG if dUTP is used during reverse transcription.

6.5 *AmpliWax*™ PCR Gem 100 (**Part No. N808-0100**) might also facilitate use of these Kits. After first pipetting the PCR Master Mix into the R Reaction tube, put in one *AmpliWax* PCR Gem 100. The PCR Master Mix preferably would have only one primer, to reduce the formation of p dimer. Next, melt and solidify the wax before pipetting the reverse transcriptase mix on top of the wax layer. After incubating at 42°C for the reverse transcriptase step, the temperature should be raised to 95°C for 10 minutes, to inactivate the reverse transcriptase without inactivatir *AmpliTaq* DNA Polymerase. The PCR cycling can then proceed normally. Because of the relatively small volume in the upper layer, this melt might work best using the maximum volume for the tube (**Section 3.0**).

PERFORMANCE CHARACTERISTICS

Each lot of the complete GeneAmp® RNA PCR Kit (**Part No. N808-0017**) has been shown, using the reagent conditions in **Sections 1.0** and **2** and the cycling conditions in bold italics of **Table 2** in **Section 3.0**, to yield a visible 308-bp band on Agarose for Separation of *GeneAmp* PCR Products (**Part No. N930-2774**) gels or equivalent stained with ethidium bromide, starting with 10^4 copies of pAW109 RNA.

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N801-0101 DNA Thermal Cycler 480, 240 V, 50 Hz
N801-0102 DNA Thermal Cycler 480, 100 V, 50/60 Hz

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Technical Bulletin

Access RT-PCR* System and Access RT-PCR Introductory System

I. Description

Numerous techniques have been developed to measure gene expression in tissues and cells. These include Northern blots, coupled reverse transcription and PCR amplification (RT-PCR), RNase protection assays, *in situ* hybridization, dot blots and S1 nuclease assays. Of these methods, RT-PCR is the most sensitive and versatile. This technique can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.

The Promega Access RT-PCR* System is designed for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from either total RNA or mRNA (1). This one tube, two-enzyme system provides sensitive, quick, and reproducible analysis of even rare RNAs. The system uses AMV Reverse Transcriptase (AMV RT) from Avian Myeloblastosis Virus for first strand DNA synthesis, and the thermostable *Tfl* DNA Polymerase from *Thermus flavus* (2) for second strand cDNA synthesis and DNA amplification. The Access RT-PCR System includes an optimized single-buffer system that permits extremely sensitive detection of RNA transcripts, without a requirement for buffer additions between the reverse transcription and PCR amplification steps (Figure 1). This simplifies the procedure and reduces the potential for contaminating the samples. In addition, the improved performance of AMV Reverse Transcriptase at elevated temperatures (48°C) in the AMV/*Tfl* 5X Reaction Buffer minimizes problems encountered with secondary structures in RNA.

II. Ordering Information

Product	Cat.#
Access RT-PCR System	A1250

Contains sufficient reagents for 100 reactions, including 25 reactions containing Positive Control RNA and primers. Includes:

- 500u AMV Reverse Transcriptase, 5u/µl
- 500u *Tfl* DNA Polymerase, 5u/µl
- 1ml AMV/*Tfl* 5X Reaction Buffer
- 1.2ml $MgSO_4$, 25mM
- 100µl dNTP Mixture, 10mM each of dATP, dCTP, dGTP and dTTP
- 50µl Positive Control RNA with Carrier (1.25 attomole/µl)
- 100µl Upstream Control Primer, 15µM
- 100µl Downstream Control Primer, 15µM
- 13ml Nuclease-Free Water

*PCR is a patented process. Purchase of this product does not include a license to perform PCR. Use of this product is recommended for persons that either have a license to perform PCR, or are not required to obtain a license.

Product	Cat.#
Access RT-PCR Introductory System	A1260

Contains sufficient reagents for 20 reactions and includes reagents for positive control reactions (Positive Control RNA and primers). Includes:

- 100u AMV Reverse Transcriptase, 5u/µl
- 100u *Tfl* DNA Polymerase, 5u/µl
- 1ml AMV/*Tfl* 5X Reaction Buffer
- 1.2ml $MgSO_4$, 25mM
- 20µl dNTP Mixture, 10mM each of dATP, dCTP, dGTP and dTTP
- 50µl Positive Control RNA with Carrier (1.25 attomole/µl)
- 100µl Upstream Control Primer, 15µM
- 100µl Downstream Control Primer, 15µM
- 13ml Nuclease-Free Water

Storage Conditions: Store all system components at –20°C. For long-term storage, the Positive Control RNA with Carrier may be stored at –70°C. The Access RT-PCR Systems are stable for six months from the date of purchase when stored and handled properly.

III. General Considerations

A. Factors to Consider When Optimizing the Reverse Transcription-PCR Reaction

RNA Template

Successful reverse transcription is dependent on the integrity and purity of the mRNA used as the template. Procedures for creating and maintaining an RNase-free environment are described in reference 3. Use sterile tubes, pipette tips, gloves and diethyl pyrocarbonate (DEPC)-treated water. When isolating RNA from samples high in ribonuclease activity, use of a ribonuclease inhibitor such as Promega's RNasin® Ribonuclease Inhibitor is recommended. For the routine and rapid purification of total RNA from eukaryotic sources, we suggest the use of Promega's RNAgents® Total RNA Isolation System followed by digestion with RQ1-RNase-Free DNase (Cat.# M6101), extraction and ethanol precipitation. Poly(A)+ RNA can be efficiently isolated from total RNA using the PolyATtract® mRNA Isolation Systems, or directly from eukaryotic sources with the PolyATtract® System 1000. These systems enable the isolation of total or poly(A)+ RNA from crude cell or tissue lysates, and result in RNA preparations sufficiently pure for use in the Access RT-PCR System.

For optimal results using this system, the RNA template, whether a total RNA preparation, an mRNA population or a synthesized RNA transcript, should be DNA-free. The system's *Tfl* DNA Polymerase has no reverse transcriptase activity under the standard reaction conditions (1), but amplification product will be generated out of these reactions if trace amounts of DNA with similar sequences are present in the template preparation.

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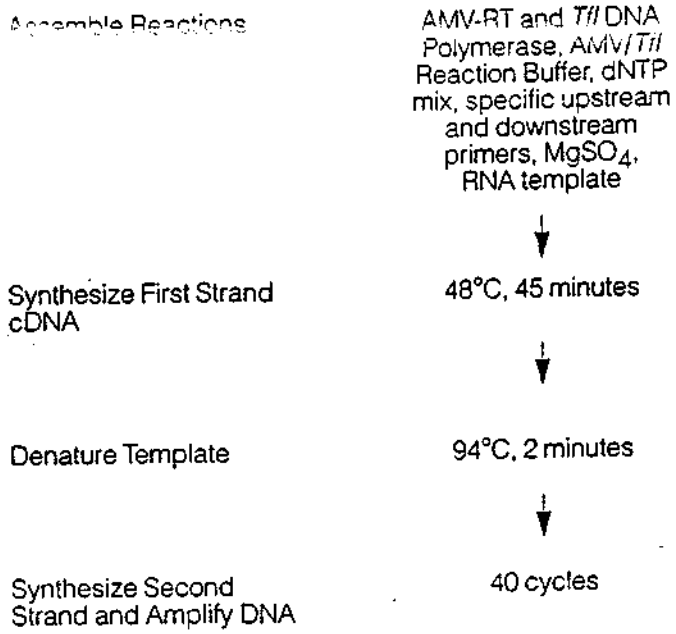

Figure 1. Outline of the Access RT-PCR System protocol.

The minimum amount of RNA that can be amplified using RT-PCR is both template and primer dependent. For the Positive Control RNA provided, the minimum amount of RNA required is 10^3 molecules (2.5 zeptomoles, 2.5×10^{-21} moles) (Figure 2A). Excellent amplification results can be obtained with the Access RT-PCR System using total RNA template levels in the range of 10pg–1µg per reaction (Figure 2B), or poly(A)+ RNA template levels in the range of 1pg–100ng.

Control Reactions

To facilitate optimization and troubleshooting of the RT-PCR reactions, perform both positive and negative control reactions. Use the supplied Positive Control RNA with Carrier, the Upstream Control Primer and the Downstream Control Primer for positive control reactions (see Figure 2A). For a negative control, substitute sterile nuclease-free water for the RNA template in the reaction.

Avoiding Contamination of Nucleic Acids

Take great care to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acid (RNA and DNA) from one experiment to the next. Use a separate work area and pipettor for pre- and post-amplification steps. Use positive displacement pipets or aerosol resistant tips to reduce cross-contamination during pipetting. Wear gloves and change them often. Use UNG (4) or another sterilization technique to prevent DNA carryover to subsequent reactions. **Note:** It is critical to vortex the magnesium stock prior to use.

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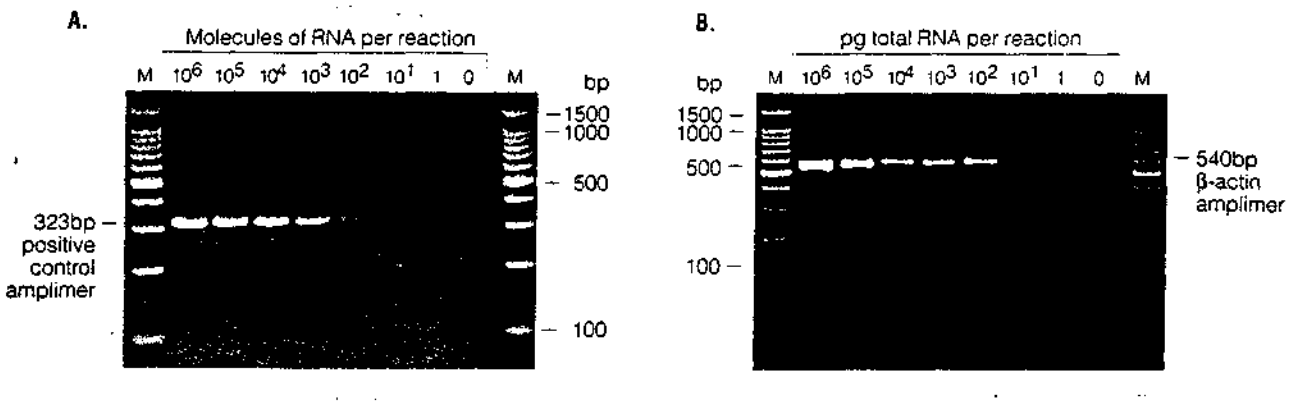

Figure 2. Amplification of specific RNAs using the Access RT-PCR System. Panel A: Serial 10-fold dilutions of the Positive Control RNA supplied with the system were prepared in nuclease-free water. RT-PCR reactions containing the indicated amounts of RNA were performed as described in the Section IV using the control oligonucleotide primers provided with the Access RT-PCR System. Equivalent aliquots of each amplification reaction were separated on a 3% NuSieve®/1% agarose gel in 1X TAE buffer containing 0.5µg/ml ethidium bromide The specific 323bp amplimer is indicated. Lanes M, Promega's 100bp DNA Ladder (Cat.# G2101). **Panel B:** RT-PCR reactions containing the indicated amounts of mouse liver total RNA were performed as described in Section IV, using oligonucleotide primers specific to the mouse β-actin transcript. The specific 540bp amplimer is indicated. Equivalent aliquots of each amplification reaction were separated on a 3% NuSieve®/1% agarose gel in 1X TAE buffer containing 0.5µg/ml ethidium bromide. Lanes M, Promega's 100bp DNA Ladder (Cat.# G2101).

Magnesium Concentration

The magnesium requirement of both the AMV Reverse Transcriptase and the *Tfl* DNA Polymerase in the Access RT-PCR System reactions is affected by the final concentration of nucleotides, oligonucleotide primers and template. The magnesium sulfate concentration should be optimized for each experimental target/primer combination. Although 1.0–2.5mM magnesium sulfate is suitable for most applications, titration of the magnesium sulfate concentration can significantly improve the sensitivity, specificity and quality of the reverse transcription and amplification products. To determine the optimal magnesium concentration for a specific template/primer combination, prepare a reaction series containing 0.5–3.0mM magnesium sulfate in 0.5mM increments by adding 1, 2, 3, 4, 5 or 6µl of the 25mM magnesium sulfate stock to 50µl reactions.

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Primer Design

A specific primer should be used for first strand synthesis. Specific primers anneal only to defined sequences and can be used to synthesize cDNA from particular mRNAs, rather than from the entire mRNA population in the sample. To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, primers may be designed to anneal to sequences in exons on opposite sides of an intron. An amplification product derived from genomic DNA will be much larger than the product of the RT-PCR reaction. This size difference not only makes it possible to differentiate the two products by gel electrophoresis, it also favors the synthesis of the smaller cDNA derived product (PCR favors the amplification of smaller fragments). Regardless of primer choice, the final concentration of the primer in the reaction must be optimized. We recommend adding 50pmol of primer (1µM final concentration in reaction) as a starting point for optimization.

Temperatures

The Access RT-PCR System does not require a template denaturation step prior to initiation of the reverse transcription reaction. If desired, however, a denaturation step may be incorporated by incubating a separate tube containing the primers and RNA template at 94°C for 2 minutes. (**Do not** incubate the AMV Reverse Transcriptase at 94°C; it will be inactivated.) The template/primer mixture can then be added to the RT-PCR reaction mix for the standard reverse transcription incubation at 48°C.

AMV Reverse Transcriptase is active in the AMV/*Tfl* 5X Reaction Buffer at temperatures between 37° and 48°C. We recommend that the reverse transcription reaction be performed at 48°C to minimize the effects of RNA secondary structure and to encourage full-length cDNA synthesis. Following the reverse transcription incubation, we recommend a two minute incubation at 94°C to denature the RNA/cDNA hybrid and inactivate the AMV Reverse Transcriptase. It has been reported that the AMV Reverse Transcriptase enzyme must be inactivated to obtain high yields of amplification product using thermophilic DNA polymerases such as *Tfl* DNA Polymerase (5,6).

The sequences of the primers are a major consideration in determining the temperature of the PCR amplification cycles. An amplification cycle typically consists of a denaturation step (94°C), a template/primer annealing step (42°–60°C) and an extension step (68°C). For primers with a high T_m, it may be advantageous to increase the suggested annealing and extension temperatures. The higher temperature minimizes nonspecific primer annealing, thus increasing the amount of specific product produced. For upstream primers with a low T_m, it may be necessary to decrease the annealing temperature to allow the primer to anneal to the target template.

Incubation Times and Number of Cycles

Efficient first-strand cDNA synthesis can be accomplished in a 20–60 minute incubation at 37°–48°C. We recommend a 45 minute incubation at 48°C as a general starting point.

Following the first-strand cDNA synthesis, the AMV Reverse Transcriptase is inactivated and the RNA/cDNA hybrid denatured using a 2 minute incubation at 94°C. This step leads directly into the second-strand cDNA synthesis and PCR amplification phase of the procedure. Most

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RNA samples can be detected using 40 cycles of amplification. If the target RNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles to 45 or 50. During the extension step, allow approximately 1 minute for every 1kb of amplimer (minimum extension time = 1 minute). A final 7 minute extension at 68°C improves the quality of the final product by extending truncated product to full-length.

IV. Synthesis and Analysis of RT-PCR Products Using the Access RT-PCR System

The reverse transcription and PCR cycling profile provided below should serve as a guideline for initial experiments. These conditions work well for the detection of the 323bp PCR product generated from the Positive Control RNA using the Upstream and Downstream Control Primers provided with the Access RT-PCR System. We recommend optimizing the parameters discussed in Section III for each target RNA.

First Strand cDNA Synthesis

1 cycle	48°C for 45 minutes	reverse transcription
	↓	
1 cycle	94°C for 2 minutes	AMV RT inactivation and RNA/cDNA/primer denaturation
	↓	

Second Strand cDNA Synthesis and PCR Amplification

40 cycles	94°C for 30 seconds	denaturation
	60°C for 1 minute	annealing
	68°C for 2 minutes	extension
	↓	
1 cycle (Optional)	68°C for 7 minutes	final extension
	↓	
1 cycle	4°C	soak

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A. Protocol

Reagents to Be Supplied by the User

- template RNA
- downstream oligonucleotide primer
- upstream oligonucleotide primer
- nuclease-free light mineral oil (e.g., SIGMA Cat.# M5904)

1. Prepare the reaction mix by combining the indicated volumes of Nuclease-Free Water, AMV/*Tfl* 5X Reaction Buffer, dNTP Mix, the specific upstream and downstream primers, and 25mM $MgSO_4$ in a thin-walled 0.5ml reaction tube on ice. Mix the components by pipetting. Add the AMV Reverse Transcriptase and *Tfl* DNA Polymerase to the reaction. Gently vortex the tube for 10 seconds to mix the components. If working with multiple samples, a Master Mix may be assembled on ice by combining appropriate multiples of each of the indicated components and transferring 48µl of the master mix to each reaction tube. Initiate the reaction by adding the template. Use individual pipet tips for all additions, being careful not to cross-contaminate the samples.

	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50 µl)	Xµl	
AMV/*Tfl* 5X Reaction Buffer (See Note 1)	10µl	1X
dNTP Mix (10mM each dNTP) (See Note 2)	1µl	0.2mM
Downstream primer	*50pmol	1µM
Upstream primer	*50pmol	1µM
25mM $MgSO_4$ (See Note 2)	2µl	1mM
AMV Reverse Transcriptase (5u/µl)	1µl	0.1u/µl
Tfl DNA Polymerase (5u/µl)	1µl	0.1u/µl
RNA sample (See Section III.A)	**Yµl	
final volume	50 µl	

*A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng x b; where b is the number of bases in the primer. For the positive control reaction, use 3.3µl of both the Downstream and Upstream Control Primers (50pmoles).
**10^3–10^6 copies of a specific target template or 1pg–1µg total RNA. Use 2µl of the Positive Control RNA with Carrier (2.5 attomoles or 1 x 10^6 copies).

2. Overlay the reaction with one or two drops (20–40µl) of nuclease-free mineral oil to prevent condensation and evaporation.

3. Place the tubes in a controlled temperature heat block equilibrated at 48°C and incubate for 45 minutes.

4. Proceed directly to thermal cycling the reactions for second-strand cDNA synthesis and amplification (refer to the thermal cycling profile outlined above).

Notes:

1. If a precipitate forms in the buffer, re-solubilize by incubating at 65°C for 15 minutes.
2. Vortex prior to use.

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B. Analysis

1. Analyze the PCR reaction products by agarose gel electrophoresis of 5% of the total reaction. The products will be readily visible by UV transillumination of an ethidium bromide stained gel. The amplification product obtained using the Positive Control RNA with the Upstream and Downstream Control Primers is 323bp long (Figure 3).

   Note: An approximately 220bp amplification product is occasionally observed with the Positive Control RNA. This product arises from the amplification of a sequence in the carrier RNA added to the Control RNA.

2. Store the reaction products at –20°C until needed. The reaction products may be purified using the Wizard™ PCR Preps DNA Purification System (7).

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```
   1  GAATACAAGC TTGGGCGTGT CTCAAAATCT CTGATGTTAC ATTGCACAAG
  51  ATAAAAATAT ATCATCATGA ACAATAAAAC TGTCTGCTTA CATAAACAGT
 101  AATACAAGGG GTGTTATGAG CCATATTCAA CGGGAAACGT CTTGCTCGAG
 151  GCCGCGATTA AATTCCAACA TGGATGCTGA TTTATATGGG TATAAATGGG
 201  CTCGCGATAA TGTCGGGCAA TCAGGTGCGA CAATCTATCG ATTGTATGGG
 251  AAGCCCGATG CGCCAGAGTT GTTTCTGAAA CATGGCAAAG GTAGCGTTGC
 301  CAATGATGTT ACAGATGAGA TGGTCAGACT AAACTGGCTG ACGGAATTTA
 351  TGCCTCTTCC GACCATCAAG CATTTTATCC GTACTCCTGA TGATGCATGG
 401  TTACTCACCA CTGCGATCCC CGGGAAAACA GCATTCCAGG TATTAGAAGA
 451  ATATCCTGAG TCAGGTGAAA ATATTGTTGA TGCGCTGGCA GTGTTCCTGC
 501  GCCGGTTGCA TTCGATTCCT GTTTGTAATT GTCCTTTTAA CAGCGATCGC
 551  GTATTTCGTC TCGCTCAGGC GCAATCACGA ATGAATAACG GTTTGGTTGA
 601  TGCGAGTGAT TTTGATGACG AGCGTAATGG CTGGCCTGTT GAACAAGTCT
 651  GGAAAGAAAT GCATAAGCTT TTGCCATTCT CACCGGATTC AGTCGTCACT
                Upstream Control Primer 5'-GCCATTCT CACCGGATTC AGTCGTC-3'
 701  CATGGTGATT TCTCACTTGA TAACCTTATT TTTGACGAGG GGAAATTAAT
 751  AGGTTGTATT GATGTTGGAC GAGTCGGAAT CGCAGACCGA TACCAGGATC
 801  TTGCCATCCT ATGGAACTGC CTCGGTGAGT TTTCTCCTTC ATTACAGAAA
 851  CGGCTTTTTC AAAAATATGG TATTGATAAT CCTGATATGA ATAAATTGCA
 901  GTTTCATTTG ATGCTCGATG AGTTTTTCTA ATCAGAATTG GTTAATTGGT
 951  TGTAACACTG GCAGAGCATT ACGCTGACTT GACGGGACGG CGGCTTTGTT
                Downstream Control Primer 3'-GACTGAA CTGCCCTGCC GCCGA-5'
1001  GAATAAATCG AACTTTTGCT GAGTTGAAGG ATCAGATCAC GCATCTTCCC
1051  GACAACGCAG ACCGTTCCGT GGCAAAGCAA AAGTTCAAAA TCACCAACTG
1101  GTCCACCTAC AACAAAGCTC TCATCAACCG TGGCGACTCT AGAGGATCCC
1151  CGGGCGAGCT CCCAAAAAAA AAAAAAAAAA AAAAAAAAAA AAACCGAATT
```

Figure 3. Sequence of the Positive Control RNA, Upstream Control Primer and Downstream Control Primer. The expected cDNA product is 323bp long.

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V. Troubleshooting

Symptoms	Possible Causes	Comments
Low yield or no first strand product	RNA degraded	Verify the integrity of the RNA by denaturing agarose gel electrophoresis.
		Use Promega's RNAgents® System, PolyATtract® mRNA Isolation Systems or PolyATtract® System 1000 to rapidly isolate intact RNA and mRNA.
		Ensure that reagents, tips and tubes are RNase-free. Isolate the RNA in the presence of a ribonuclease inhibitor (e.g., Promega's RNasin® Ribonuclease Inhibitor)
	AMV Reverse Transcriptase thermally inactivated	If an initial denaturation/annealing step is introduced into the protocol, be certain to add the enzyme mix containing AMV Reverse Transcriptase **after** the denaturation step and subsequent 48°C equilibration.
	Primer specificity	Verify that the "downstream" primer was designed to be complementary to the downstream sequence of the RNA.
	Primer annealing	If oligo(dT) was used as a "downstream" primer, verify that the annealing incubation was carried out at an appropriate temperature prior to reverse transcription.
	RNA purification problem	Carryover of reagents (e.g., SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with RT-PCR. Reduce volume of target RNA, perform additional purification steps or change purification method.
		Use Promega's RNAgents® System, PolyATtract® mRNA Isolation Systems or PolyATtract® System 1000 to isolate highly pure RNA and mRNA.
Amplification product has a higher than expected molecular weight	Genomic DNA sequences related to the RNA template contaminate the RNA preparation	Digest the RNA using RQ1 RNase-Free DNase.
Low yield or no amplification product	Insufficient number of cycles	Return reactions to thermocycler for 5 more cycles.
	Thermocycler programmed incorrectly	Verify that times and temperatures are correct.
	Temperature too low in some positions of thermocycler	Perform a set of control reactions to determine if certain positions in the thermocycler give low yields.
	Top of thermocycler open	The top must be closed for correct heating and cooling.
	Improper reaction conditions	Reduce the annealing temperature and/or allow longer extension times for longer amplimers.

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Symptoms	Possible Causes	Comments
Low yield or no amplification product (continued)	Missing reaction component	Check the reaction components and repeat the reaction.
	Mineral oil problem	The reaction must be overlaid with high quality, nuclease-free light mineral oil. Do NOT use autoclaved mineral oil.
	Reaction tubes not autoclaved	Autoclaving tubes eliminates contaminants that inhibit amplification.
	Insufficient first strand product	See discussion above under "No first strand product".
	Poor primer design	Make sure primers are not self-complementary or complementary to each other. Try a longer primer.
	Incorrect primer specificity	Verify that the primers were designed to be complementary to the appropriate strands.
	Suboptimal reaction conditions	Optimize $MgSO_4$ concentration, annealing temperature and extension time. Verify that primers are present in equal concentration. Vortex the $MgSO_4$ prior to use.
	Nucleotides degraded	Keep nucleotides frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze/thaw cycles.
	Target sequence genuinely not present in target RNA	Redesign experiment or try other sources of target RNA.
Multiple, nonspecific amplification products	Suboptimal reaction conditions	Optimize $MgSO_4$ concentration and annealing temperature. Vortex the $MgSO_4$ prior to use.
	Poor primer design	Make sure primers are not self-complementary or complementary to each other, especially near the 3′ ends. Try a longer primer. Avoid using three G or C nucleotides in a row at the 3′ end of a primer.
	Contamination by another target RNA/DNA	Use positive displacement pipets or aerosol resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettor for pre- and post-amplification. Wear gloves and change them often. Use UNG (4) or another sterilization technique to prevent DNA carryover to subsequent reactions.
	Multiple target sequences genuinely exist in target RNA	Design new primers.

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VI. Appendix

A. Composition of Buffers and Solutions

10mM dNTP Mixture

10mM each of dATP, dCTP, dGTP and dTTP in water.

Positive Control RNA with Carrier

1.25amol/µl 1.2kb RNA (prepared by *in vitro* transcription)
3µg/ml *E. coli* rRNA
10mM Tris-HCl, pH 8.0
0.1mM EDTA

$MgSO_4$ Solution

25mM $MgSO_4$ in water.

TAE 50X Buffer

242g Tris Base
57.1ml glacial acetic acid
100ml 0.5M EDTA, pH 8.0

Add deionized water to 1 liter.

B. Control Primer Sequences

Upstream Control Primer
5' GCCATTCTCACCGGATTCAGTCGTC 3'

Downstream Control Primer
5' AGCCGCCGTCCCGTCAAGTCAG 3'

C. Related Products

Enzymes

Product	Size	Conc. (u/µl)	Cat.#
Tfl DNA Polymerase*	100u	2-6	M1941
	500u (5 x 100u)	2-6	M1942
Tli DNA Polymerase*	50u	1	M7101
	250u (5 x 50u)	1	M7102
Tth DNA Polymerase*	100u	4-6	M2101
	500u (5 x 100u)	4-6	M2102
AMV Reverse Transcriptase	300u	5-10	M5101
	1,500u (5 x 300u)	5-10	M5102
	600u	20-25	M9004

*Some applications in which this product may be used are covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application, users of the product may be required to obtain a patent license depending upon the particular application and country in which the product is used. For more specific information, please contact Promega.

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PCR-Related Reagents

Product	Size	Conc. (u/µl)	Cat.#
Recombinant RNasin® Ribonuclease Inhibitor	2,500u	20-40	N2511
	10,000u (4 x 2,500u)	20-40	N2512
	20,000u (2 x 10,000u)	20-40	N2513
	60,000u (6 x 10,000u)	20-40	N2514

This product is a recombinant protein isolated from *E. coli*.

Product	Size	Conc. (u/µl)	Cat.#
RNasin® Ribonuclease Inhibitor	2,500u	20-40	N2111
	10,000u (4 x 2,500u)	20-40	N2112
	20,000u (2 x 10,000u)	20-40	N2113
	60,000u (6 x 10,000u)	20-40	N2114

This product is a protein isolated from human placenta.

Product	Size	Conc. (u/µl)	Cat.#
RQ1 RNase-Free DNase	1,000u	1	M6101
	5,000 (5 x 1,000)	1	M6102

Product	Size	Cat.#
RNA Markers, 0.36-9.49kb	50µg	G3191
pGEM® DNA Markers	50µg	G1741
100bp DNA Ladder	250µl	G2101
Random Primers	20µg	C1181
Nuclease-Free Water	50ml	P1193
	(2 x 25ml)	
PCR: A Practical Approach book	1 book	A1000
dATP, 100mM	40µmoles	U1201
dCTP, 100mM	40µmoles	U1221
dGTP, 100mM	40µmoles	U1211
dTTP, 100mM	40µmoles	U1231
dATP, dCTP, dGTP, dTTP, each at 100mM	40µmoles of each	U1240
	10µmoles of each	U1330
rATP, 10mM	0.5ml	P1132
rCTP, 10mM	0.5ml	P1142
rGTP, 10mM	0.5ml	P1152
rUTP, 10mM	0.5ml	P1162
rATP, rCTP, rGTP, rUTP, each at 10mM	0.5ml of each	P1221

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PCR-Related Systems

Product	Cat.#
Wizard™ PCR Preps DNA Purification System	A7170

Each system provides sufficient reagents for the purification of 50 DNA samples, either with or without prior gel purification.

Product	Cat.#
pGEM®-T Vector System I	A3600
pGEM®-T Vector System II	A3610

The pGEM®-T Vector System II includes JM109 Competent Cells (High Efficiency).

Product	Cat.#
PinPoint®*-Xa1 T-Vector System I	V2610
PinPoint®*-Xa1 T-Vector System II plus competent cells	V2850

PinPoint®-Xa1 T-Vector System II includes JM109 Competent Cells (High Efficiency).

Product	Cat.#
E. coli S30 Extract System for Linear Templates	L1030

Sufficient reagents are provided for 30 x 50µl coupled reactions.

Product	Cat.#
PolyATtract® System 1000 (includes Magnetic Separation Stand)	Z5420
PolyATtract® System 1000 (excludes Magnetic Separation Stand)	Z5400

Product	Cat.#
PolyATtract® mRNA Isolation System I	Z5210
PolyATtract® mRNA Isolation System II	Z5200
PolyATtract® mRNA Isolation System III	Z5300
PolyATtract® mRNA Isolation System IV	Z5310

Product	Cat.#
RNAgents® Total RNA Isolation System	Z5110

This system contains sufficient reagents for six RNA isolations, each from 1 gram of tissue or 10^8 cultured cells.

Product	Cat.#
Reverse Transcription System	A3500

Each system contains sufficient reagents for 100 reactions, processing up to 1µg of RNA per reaction.

Product	Cat.#
SILVER SEQUENCE™ DNA Sequencing System	Q4130

Each system provides sufficient reagents for 100 sets of sequencing reactions and staining reagents for 10 gels.

*For research purposes only. Not for diagnostic or therapeutic use. For non-research uses of the portion of the vector encoding the biotinylation sequence, please contact Promega Corporation for licensing information. For non-research uses of the SoftLink™ Resin, contact Toso Haas, 156 Keystone Drive, Montgomeryville PA 18936, 1-800-366-4875 or (215) 283-5000.

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Product	Cat.#
fmol® DNA Sequencing System	Q4100

Each system provides sufficient reagents for 100 sets of sequencing reactions.

VII. References

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Appendix 2.

Manufacturer's instructions for the use of the non-radioactive kit (Bresatec) using a cDNA probe and photobiotin for the detection of viroids in plant extracts.*

*Only the protocol for CSV is included since the cDNA method for both viroids is the same with the exception of the treatment of RNA extracts from potatoes. RNA extracts from potatoes are incubated at 60°C for 10 minutes and immediately placed on ice before spotting onto nitrocellulose membranes.

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CHRYSANTHEMUM STUNT VIROID (CSV)

NON-RADIOACTIVE KIT FOR DETECTION IN PLANT EXTRACTS. USE OF NON-RADIOACTIVE, PHOTOBIOTIN™-LABELLED DNA PROBES.

© Bresatec Limited, 1988.

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* Photobiotin™ is a trademark of Bresatec Limited. Patent pending on the preparation and use of Photobiotin and related compounds.

November, 1988

I. INTRODUCTION

The causative agent of chrysanthemum stunt disease, chrysanthemum stunt viroid (CSV), is a member of the unique group of plant pathogens known as viroids. Viroids differ from conventional viruses by several properties; (a) they consist of single-stranded covalently closed circular RNA species which exist as highly base-paired rod-like structures, (b) they are not encapsidated, (c) they do not seem to code for any proteins, and (d) they appear to depend completely and absolutely on host enzymes for their replication and circularisation. Several sequence variants of CSV have been reported, with sizes ranging from 353-356 nucleotides (1,2).

Since viroids lack the antigenic coat protein characteristic of viruses, they cannot be detected by immunological approaches such as the enzyme-linked immunosorbent assay (ELISA). An alternative approach is to employ nucleic acid hybridization techniques using recombinant DNA probes (3).

Biotin-labelled probes have advantages over radioactively-labelled probes in terms of stability, safety and time of detection. This protocol describes the use of a biotinylated recombinant DNA probe, containing a full-length monomer insert of CSV, for the routine indexing of CSV in plant extracts by dot-blot nucleic acid hybridization. This photobiotin-labelled probe has a comparable sensitivity to a ^{32}P-labelled probe with no detectable non-specific binding to healthy plant tissue (4).

II. PRINCIPLE OF METHOD

A photoactivatable analogue of biotin, PHOTOBIOTIN™, is used for the rapid and reliable preparation of stable, non-radioactive, biotin-labelled DNA probes for use in hybridization studies. When a mixture of Photobiotin and nucleic acid is exposed to strong visible light for 15-20 minutes, one biotin is coupled per 100-150 residues of nucleic acid (5). After hybridization to target nucleic acid, detection involves addition of an avidin-enzyme conjugate followed by a substrate; a positive signal is identified by the formation of an insoluble blue pigment at the site of hybrid formation.

III. COMPONENTS OF THE KIT

Tube 1.	POSITIVE CONTROL DNA (VECTOR PLASMID), 1 µg, lyophilized. Resuspend in 100 µl sterile 0.1 mM EDTA. Prior to spotting, denature the DNA by heating at 100°C for 5 minutes followed by snap cooling. Store at -20°C.
Tube 2.	SONICATED SALMON SPERM DNA, 50 mg in 10 ml 0.1 mM EDTA. Store at -20°C.
Tube 3.	150 x DENHARDT'S SOLUTION, 1.5 ml, sterilized. Store at -20°C and keep free of bacterial contamination. [1 x Denhardt's solution: 0.02% bovine serum albumin, 0.02% Ficoll 400, and 0.02% polyvinyl pyrollidone.]
Tube 4.	PHOTOBIOTIN-LABELLED double-stranded DNA probe. 5 µg, lyophilized. Resuspend in 250 µl sterile 0.1 mM EDTA. Store at -20°C in 50 µl batches.
Tube 5.	AVIDIN-ALKALINE PHOSPHATASE, 100 µg, lyophilized. Resuspend in 200 µl sterile water to give a 500x stock solution. Store at -20°C in small batches (e.g. 20-40 µl).
Tube 6.	NITRO BLUE TETRAZOLIUM (NBT), 400 µl of a stock solution of 75 mg/ml in 70% dimethylformamide. Store at -20°C in the dark.
Tube 7.	5-BROMO-4-CHLORO-3-INDOLYL PHOSPHATE (BCIP), 400 µl of a stock solution of 50 mg/ml in dimethylformamide. Store at -20°C in the dark.

IV. EQUIPMENT AND SOLUTIONS REQUIRED

A. EQUIPMENT

Microcentrifuge
Micropipettors and tips (sterile)
Roller leaf press (e.g. Erich Pollahne, West Germany)
[or: glass rod filed at one end to fit 1.5 ml Eppendorf tubes]
Sterile forceps, scissors
Wash trays (e.g. lunch boxes 10 cm x 20 cm x 20 cm)
Heat sealable polythene rolls or polythene bags
Polythene bag heat sealer
Shaking water bath
Oven (preferably with vacuum)
Nitrocellulose membranes (Schleicher and Schuell 0.45 μM)
Whatman filter papers

B. SOLUTIONS

(a) PREPARATION OF PLANT EXTRACTS

1. **Extraction buffer:** 0.5 M sodium acetate, pH 6.0, 10 mM $MgCl_2$, 3.0% ($^w/_v$) SDS, 20% ($^v/_v$) ethanol, 1% ($^v/_v$) 2-mercaptoethanol (100 ml).
2. **Water-saturated phenol, 0.1% 8-hydroxyquinoline** (100 ml).
3. **Chloroform.**
4. **Re-distilled** or **A.R. ethanol** and **70% ethanol.** Store at -20°C.
5. ***E. coli* tRNA (Sigma) solution:** 10 mg/ml in 0.1 mM EDTA (1 ml). Store at -20°C.

(b) DOT-BLOT HYBRIDIZATION

1. **20 x SSC:** 3 M sodium chloride, 0.3 M tri-sodium citrate (1 litre).
2. **Deionized formamide** (100 ml).
   Stir 100 ml formamide with 2 g Amberlite resin (Sigma MB-1) for 1 hr, filter and store at -20°C. Discard the resin.
3. **Sodium phosphate solution:** 1 M, pH 6.5 (100 ml).
4. **Sodium EDTA solution:** 0.2 M, pH 8.0 (100 ml).
5. **Dextran Sulphate (Pharmacia) solution:** 50% ($^w/_v$) in water (100 ml).
6. **Sodium dodecyl sulphate (SDS) solution:** 10% ($^w/_v$) in water (200 ml).
7. **Pre-hybridization buffer:** To prepare 20 ml, add the following:

Reagent	Volume (ml)
20 x SSC	5.0
150 x Denhardt's solution (Tube 3)	0.14
10% SDS solution	0.4
Sodium phosphate solution, 1 M, pH 6.5	1.0
Sonicated salmon sperm DNA (5 mg/ml, Tube 2)	1.0
Deionized formamide	10.0
Sodium EDTA solution, 0.2 M, pH 8.0	0.5
Water	2.0
	~ 20 ml

8. **Hybridization buffer:** To prepare 10 ml, mix the following:
   Pre-hybridization buffer 8 ml
   50% ($^w/_v$) Dextran sulphate solution 2 ml

9. **Post-hybridization Washing Solution A:** 2 x SSC, 0.1% SDS (1 litre).
10. **Post-hybridization Washing Solution B:** 0.1 x SSC, 0.1% SDS (1 litre).

Sterile stock solutions of 20 x SSC, 1 M sodium phosphate (pH 6.5), 0.2 M sodium EDTA (pH 8.0) and 10% ($^{w}/_{v}$) SDS can be stored at room temperature.
Stock solutions of deionized formamide, 150 x Denhardt's solution, sonicated denatured salmon sperm DNA and dextran sulphate should be stored at -20°C.
Pre-hybridization and Hybridization buffers should be prepared fresh as required.

(c) COLORIMETRIC DETECTION

1. **Washing Solution I:** 0.1 M Tris-HCl, pH 7.5, 1 M NaCl, 2 mM $MgCl_2$, 0.05% ($^{v}/_{v}$) Triton X-100 (1 litre).
2. **Blocking Solution:** 3% ($^{w}/_{v}$) BSA (Fraction V, Sigma, Cat. No. A-4503) in Washing Solution I (100 ml).
   Untreated BSA can give coloured backgrounds when colour development is allowed to proceed for more than 1-2 hours. For optimum results, it is strongly recommended that the BSA solution be prepared as follows:
   Dissolve 3 g BSA in 70 ml sterile water in a screw-capped glass bottle and adjust the resulting solution to pH 3.0 with conc. HCl. Heat in a boiling water bath for 20 minutes, cool to room temperature and adjust to pH 7.5 with 10 M NaOH. During this pH adjustment, the solution briefly turns turbid but readily clarifies as the pH nears neutrality. Add 10 ml Solution B (see below) and 5.8 g solid NaCl to give a final molarity of 1.0. Make to 100 ml with sterile water. This solution can be stored for several months at 4°C.
   Solution B: 1.0 M Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.5% (v/v) Triton X-100.
3. **Avidin-Alkaline Phosphatase Solution:** Prepared by 500x dilution of the stock solution (tube 5) in Blocking Solution (e.g. 2 µl per ml).
4. **Washing Solution II:** 0.1 M Tris-HCl, pH 9.5, 1 M NaCl, 5 mM $MgCl_2$ (1 litre).
5. **Solution S:** 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$ (100 ml).
6. **Termination Solution:** 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA (500 ml).

V. PREPARATION OF PLANT EXTRACTS

Healthy and CSV-infected chrysanthemum leaves for diagnosis should be freshly harvested. Leaf material should be transported cooled and stored at 4°C prior to extraction. Storage at 4°C (or -20°C) for several days results in negligible loss of viroid. Long term storage of CSV-infected chrysanthemum tissue at -20°C should be avoided due to considerable loss of viroid (our unpublished observations). The following two methods of extraction are recommended:

A. SAP EXTRACTION BY ROLLER LEAF PRESS

1. To each gram leaf tissue inserted between the rollers, add 5 ml Extraction buffer in small amounts and collect the slurry in a 50 ml centrifuge tube.
2. Add 2.5 ml water-saturated phenol and 2.5 ml chloroform, shake for 1 min and leave on ice for 10 min.
3. Spin at 10,000 x g at 4°C for 20 min and collect the aqueous phase.
4. Add 2.5 volume chilled ethanol to all or part of the aqueous phase (50 ml Corex or 1.5 ml Eppendorf tubes) and leave at -20°C for 1 hour. Centrifuge at 10,000 x g at 4°C for 20 min to obtain the nucleic acid pellet.
5. Wash the pellet in cold 70% ethanol, recentrifuge as above for 10 min, dry *in vacuo* and resuspend in 0.1 mM EDTA (100-200 µl/g starting material) and store at -20°C.

B. LEAF DISC (SMALL SCALE) METHOD

1. Remove a leaf disc of 1 cm. diameter (approx. 15 mg chrysanthemum tissue) and place it in a 1.5 ml Eppendorf tube. The leaf disc is best obtained by placing the leaf between the cap and the top of the Eppendorf tube and then closing the cap firmly. Alternatively, the end of a disposable pipette tip (blue, 1 ml) can substitute for the Eppendorf cap.
2. Add 50 µl Extraction buffer to the leaf disc, followed by 50 µl water-saturated phenol and 100 µg *E. coli* tRNA (10 µl of 10 mg/ml stock). Homogenize with a *clean* filed glass rod for 2 min.
3. Add 50 µl chloroform, vortex for 30 sec and centrifuge at 12,000 x g for 10 min at room temperature. Collect the supernatant with a drawn-out Pasteur pipette.
4. Add 150 µl cold ethanol and leave at -20°C for 1 hour.
5. Collect the nucleic acid pellet by spinning at 12,000 x g for 30 min at 4°C.
6. Rinse the pellet in cold 70% ethanol, dry *in vacuo*, resuspend in 10 µl 0.1 mM EDTA and store at -20°C.

VI. DOT-BLOT HYBRIDIZATION PROCEDURE

A. SPOTTING

1. Soak a pre-stamped (6 x 6 mm squares) sheet of nitrocellulose in distilled water for 5 minutes followed by 20 x SSC for 10 minutes or until use. Dry the filter under a lamp for 5 minutes.
2. Spot the samples (3 or 4 µl) on the nitrocellulose sheet and dry.
3. Place the filter between two sheets of Whatman filter paper, clip and bake at 80°C for 2 hours (preferably in a vacuum oven). Place the filter in a heat sealable polythene bag.

B. HYBRIDIZATION PROTOCOL

1. Add Pre-hybridization buffer to the bag (0.1 ml/cm^2 filter). Remove all air bubbles and carefully seal the bag. Incubate at 42°C for 15 hr (overnight, see Notes) in a water bath which allows slight agitation.
2. To denature the probe, add an equal volume of 0.1 M NaOH and incubate at room temperature for 10 minutes.
3. Replace the Pre-hybridization buffer with an equal volume of Hybridization buffer containing 50-100 ng/ml denatured probe. Re-seal the bag after carefully removing the air bubbles.
4. Hybridize in the water bath with slight agitation at 55°C for up to 23 hr (see Notes).
5. Transfer the filter to a wash tray. Use 200-300 ml solution for each 50 cm^2 of the filter. Washing steps are performed as follows:
   (a) Two washes in Post-hybridization Washing Solution A for 15 minutes each at room temperature.
   (b) Three washes in Post-hybridization Washing Solution B for 20 minutes each at 55°C.

C. COLORIMETRIC DETECTION

1. Place the filter in a new polythene bag, add the Blocking Solution (IVB(c), 0.1 ml/cm^2). Remove air bubbles, seal the bag and incubate in a water bath at 42°C for 1 hour, with occasional agitation.
2. Remove the Blocking Solution and replace with 1 µg/ml Avidin-Alkaline Phosphatase Solution. Seal the bag and incubate at room temperature for 15-20 minutes.

3. Remove the filter, place in a plastic tray and wash as follows:
   (a) Three washes in Washing Solution I for 20 minutes each at room temperature.
   (b) Two washes in Washing Solution II for 10 minutes each at room temperature.
4. Transfer the filter to a new polythene bag, and incubate in the dark with Substrate Solution (0.1 ml/cm^2 filter).

   PREPARATION OF SUBSTRATE SOLUTION: Add 20 µl NBT Solution (Tube 6) to 5 ml Solution S and gently mix by inverting the tube. To this mixture add 20 µl BCIP Solution (Tube 7) and gently mix. The Substrate Solution can be freshly prepared just before use or it can be stored in aliquots at -20°C for two weeks.

5. Terminate the colour development when required by rinsing the filter in Termination Solution.
6. Store the filter in a sealed plastic bag in the dark at 4°C. Photograph while moist.

VII. NOTES

1. Aqueous solutions should be prepared with Distilled Water or water of equivalent quality and autoclaved wherever possible.
2. Chrysanthemum extracts, once spotted onto nitrocellulose, may give rise to darkened spots. These will disappear during the pre-hybridization and hybridization steps, thus allowing colorimetric detection.
3. The use of a vacuum manifold apparatus for the application of extracts is not recommended; direct spotting of extracts results in a greater sensitivity of the method (4).
4. A different hybridization protocol involving a four hour pre-hybridization and a sixteen hour (overnight) hybridization may be used. However, a probe concentration of 100 ng/ml is recommended for this alternative method.
5. Wash trays should be cleaned thoroughly. Nitrocellulose filters should always be removed carefully by forceps prior to changing washing solutions.
6. Stock solutions of 1 M Tris-HCl (pH 7.5), 1 M Tris-HCl (pH 9.5), 2 M $MgCl_2$ and 5 M NaCl are used for the preparation of the solutions required for colorimetric detection.
7. Maximum colour development normally is obtained within 1-2 hours. The optimal period of colour development will vary, however, depending upon the amount of biotin-labelled probe annealed to the target nucleic acid and it may be necessary to continue for a longer period. Overnight incubations may result in background problems.

VIII. REFERENCES

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