User Manual

RNA/cDNA Quality Assay User Manual

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I. Introduction & Protocol Overview

Determination of RNA quality is essential to any application that utilizes RNA. One of the problems with conventional RNA quality assays is that they don't give any information about the quality of first-strand or amplified cDNA produced by a given sample. While RNA can look intact on a formaldehyde gel or in a chip-based assay (28S:18S ratio >1), RNA can be degraded during cDNA synthesis. The result: first-strand cDNA containing a mix of truncated and full-length cDNAs. Poor quality RNA or cDNA can lead to high background or inaccurate expression results due to the absence of intact, full-length RNA.

The Clontech **RNA/cDNA Quality Assay** assesses your human or mouse RNA or cDNA sample for its ability to produce full-length cDNA for demanding applications such as cDNA library production, RACE-ready cDNAs, microarray cDNA probes, or cDNAs for Clontech PCR-Select™ Subtractive Hybridization. This assay is especially useful for researchers who have limited starting material, such as RNA derived from laser-capture microscopy samples, cells sorted by flow cytometry, or other techniques. The Quality Assay requires as little as 10 ng of the original RNA as starting material.

The Quality Assay uses two sets of primers in a multiplex RT-PCR to amplify the 5'-end and 3'-end fragments of a long mRNA (~3 kb) for a carefully selected housekeeping gene. Since in most cases RNA degradation starts in the 5'-end region of an RNA molecule, the ratio of the 3' to 5' amplified fragments provides a direct indication of RNA integrity (Figure 1).

Because the Quality Assay uses RT-PCR, it provides a direct functional test of your sample for its ability to produce full-length cDNA for your application. It can also save you time and materials by identifying RNA samples that may contain impurities that inhibit RT. In addition, the Quality Assay doesn't involve handling inconvenient and toxic formaldehyde gels, and provides a fast, easy-to-use, and sensitive test for RNA and cDNA quality.

This User Manual provides complete protocols for using the Clontech RNA/cDNA Quality Assay, as well as guidelines for the analysis of results and determination of sample quality.

I. Introduction & Protocol Overview *continued*

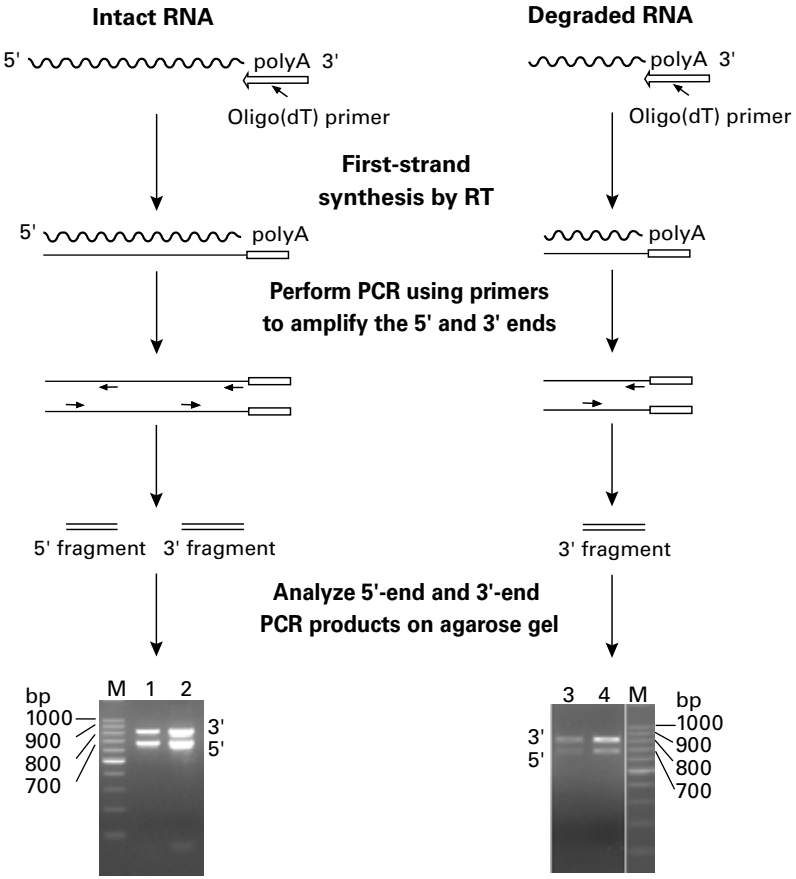

Figure 1. Method overview of the Clontech RNA/cDNA Quality Assay. Two sets of primers amplify the 5'-end and 3'-end fragments of a long housekeeping mRNA. The ratio of the 3' to 5' amplified fragments provides a direct indication of RNA integrity. An underrepresentation of the 5' fragment indicates degraded RNA or cDNA. Each lane contains 5 µl of PCR reaction using 10 ng mouse liver total RNA as starting material. To assess cDNA samples, proceed directly with PCR amplification. Lanes M: 100-bp DNA marker. Lanes 1 & 2: Intact RNA samples with a 3':5' ratio of 1 (Excellent quality rating). Lanes 3 & 4: Degraded RNA samples with 3':5' ratios >3 (Unacceptable quality rating). For more information about analyzing Quality Assay results, see Section VI.

II. List of Components

Store the Control Total RNA at –70°C.

Store all other components at –20°C.

The following reagents are suitable for 40 assays.

- **50 µl 5' Fragment Primer Mix (10 µM)**
- **50 µl 3' Fragment Primer Mix (10 µM)**
- **20 µl SMARTScribe™ MMLV Reverse Transcriptase (100 units/µl)**
- **200 µl 10X Advantage 2™ PCR Buffer**
- **30 µl 50X Advantage 2™ Polymerase Mix**
- **80 µl dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP)**
- **5 µl Control Mouse Liver Total RNA (1 µg/µl)**
- **40 µl Oligo(dT) Primer (20 µM)**
- **80 µl 5X First Strand Buffer**
- **40 µl DTT (20 mM)**

For a complete listing of all Clontech products,
please visit **www.clontech.com**

III. Additional Materials Required

The following reagents are not supplied.

- **Thermal cycler**
- **Air incubator**
- **Pipettors,** one set dedicated for PCR set-up and one set dedicated for post-PCR use.
- **PCR pipette tips** suitable for use with the above pipettors and preferably equipped with hydrophobic filters.
- **Deionized water**
  Avoid using autoclaved H_2O because recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.
- **TE buffer**

  10 mM Tris (pH 7.6)
  1 mM EDTA (pH 7.6)
- **0.5-ml PCR reaction tubes** (We recommend Perkin-Elmer GeneAmp 0.5-ml reaction tubes, Cat. No. N801-0737 or N801-0180.)
- **DNA size markers** (See Section V.B.9)
- **5X Stop/loading buffer** (Sambrook *et al.* [2001] provides several recipes)
- **Ethidium bromide** (10 mg/ml)**Advantage®-GC Polymerase mixes are covered by U.S. Patent No. 5,436,149. Foreign patents pending.**
- [Optional] **Mineral oil** (We recommend Sigma Cat. No. M3516.)
- [Optional] **Densitometer** for analysis of results. Alternatively, Quality Assay samples can be analyzed using a chip-based assay (Agilent 2100 Bioanalyzer) to determine the 3':5' ratio.

IV. General Considerations

- Prepare all solutions using deionized H_2O (Milli-Q-filtered or equivalent) that has **not** been treated with DEPC.
- Wear disposable gloves during preparation of materials and solutions that will be used for analysis of RNA, and during manipulations involving RNA. Change gloves frequently.
- Due to the tremendous amplification power of PCR, minute amounts of contaminants can produce nonspecific amplification, even in the absence of added template DNA. We recommend that you use small aliquots of starting material to avoid contaminating your stocks. When performing PCR, you should wear gloves and set up your reactions in a dedicated lab area or noncirculating containment hood using dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. We also recommend setting up a negative control reaction that does not contain any template. Finally, perform post-PCR analysis in a separate area using a separate set of pipettors.
- The first time you use this kit, you should perform cDNA synthesis with the ControlTotal RNA provided in the kit, in parallel with your experimental sample. Performing this control at least once will verify that all components are working properly, including the reverse transcriptase activity, and will also help you troubleshoot any problems that may arise.
- The cycling parameters in this protocol have been optimized using a hot-lid thermal cycler. Optimal parameters may vary with different thermal cyclers.
- To mix reactions, gently pipet them up and down and centrifuge the tube briefly to deposit contents at the bottom.
- Add enzymes to reaction mixtures last, and thoroughly mix the enzyme by gently pipetting the reaction mixture up and down.
- Do not increase the amount of enzyme added or concentration of cDNA in the reactions. The amounts and concentrations have been carefully optimized.
- The data generated by the Quality Assay can usually be analyzed by eye. For more accurate analyses, you can use densitometry to analyze your results, or run your samples on a chip-based system (Agilent 2100 Bioanalyzer).

V. RNA/cDNA Quality Assay

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

For analyzing cDNA samples, skip Section V.A and proceed with Section V.B.

A. First-Strand cDNA Synthesis

1. For each sample and control, combine the following reagents in a sterile 0.5-ml reaction tube:

1–3	µl	RNA sample* (0.025–0.5 µg of poly A^+ or 0.1–1 µg of total RNA)
1	µl	Oligo(dT) Primer (20 µM)
x	µl	Deionized H_2O
5.5	µl	Total volume

   * For the control synthesis, add 1 µl (1 µg/µl) of Control Total RNA.

2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 70°C for 3 min.
4. Cool the tube on ice for 2 min.
5. Centrifuge the tube briefly in a microcentrifuge to collect contents at the bottom.
6. Add the following to each reaction tube:

2	µl	5X First-Strand Buffer
1	µl	DTT (20 mM)
1	µl	dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP)
0.5	µl	SMARTScribe™ MMLV Reverse Transcriptase

7. Mix by gently pipetting and spin the tubes briefly in a microcentrifuge.
8. Incubate the tubes at 42°C for 1 hr 15 min in an air incubator.

   Note: If you use a water bath or thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.
9. Place the tube on ice to terminate first-strand synthesis.
10. To proceed directly to the PCR step (Section V.B), dilute an aliquot of the first-strand cDNA reaction by following the guidelines for PCR outlined in Table I.

    Note: If you used mineral oil in your first-strand reaction tube, be careful to take the aliquot from the **bottom** of the tube to avoid the oil.

11. Store the remaining first-strand reaction mixture at –20°C. First-strand cDNA can be stored at –20°C for up to three months. After determining sample quality using this assay, the stored cDNA can be used for downstream applications.

V. RNA/cDNA Quality Assay *continued*

TABLE I: GUIDELINES FOR DILUTING FIRST-STRAND cDNA

Amount of RNA (ng)	Volume of ss cDNA[a] (µl)	Volume of TE (µl)	Dilution
~25–100[b]	1 µl	–	–
~250	2 µl	3 µl	1:2.5
~500	1 µl	4 µl	1:5
~1,000	1 µl	9 µl	1:10

[a] From Step V.A.10.

[b] For first-strand reactions starting with 25–100 ng of RNA, use 1 µl directly in the PCR.

B. cDNA Amplification

These guidelines were determined using the Control Total RNA and a hot-lid thermal cycler; optimal parameters may vary with different templates and thermal cyclers. To best determine the quality of your sample, you must optimize the PCR cycling parameters by performing a range of cycles, as described in this section (Figure 2). Choosing the optimal number of PCR cycles ensures that the ds cDNA will remain in the exponential (i.e., linear) phase of amplification. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA can generate an inaccurate representation of sample quality. Undercycling, on the other hand, can yield an uninformative result.

1. Preheat a thermal cycler to 94°C.
2. Prepare a Master Mix for all reaction tubes, plus one additional tube. Combine the following components in the order shown:

per rxn	
40.5 µl	Deionized H_2O
5 µl	10X Advantage 2 PCR Buffer
1 µl	dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP)
1 µl	5' Fragment Primer Mix (10 µM)
1 µl	3' Fragment Primer Mix (10 µM)
0.5 µl	50X Advantage 2 Polymerase Mix
49 µl	Total volume

3. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
4. Aliquot 49 µl of the PCR Master Mix into each labeled 0.5-ml reaction tube.

V. RNA/cDNA Quality Assay *continued*

5. Aliquot 1 µl of each diluted cDNA (from Section V.A.10 and Table I) into each reaction tube. To test cDNA samples generated by alternative methods, use 1 µl of 10 ng/µl cDNA.

   Note: For first-strand reactions starting with 25–100 ng RNA, aliquot 1 µl directly into the reaction tube (see Table I).

6. Cap the tube, and place it in the preheated thermal cycler. If necessary, overlay the reaction mixture with two drops of mineral oil.

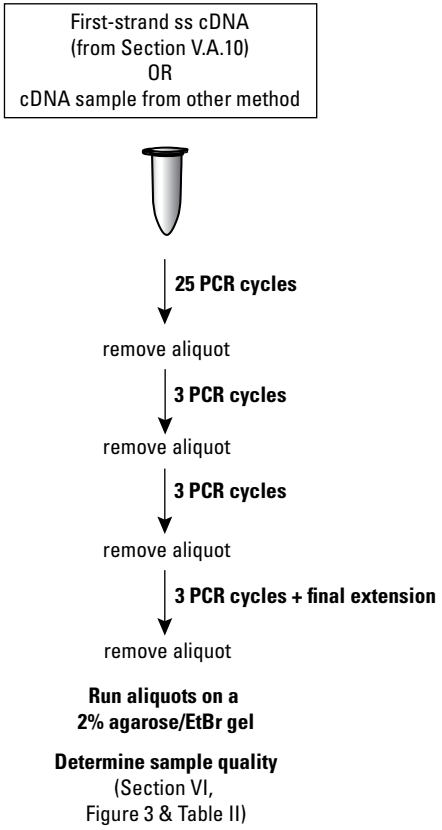

Figure 2. Optimizing PCR cycle number for the RNA/cDNA Quality Assay.

V. RNA/cDNA Quality Assay *continued*

7. Commence thermal cycling[a] using the following program:
   - 94°C 4 min 1 cycle
   - 94°C 30 sec
     68°C 2 min } x cycles[b]
   - 68°C 5 min 1 cycle[c]

   [a] Optimal parameters may vary with different thermal cyclers and different templates.

   [b] **Subject *all* tubes to 25 cycles**. Then subject all tubes to additional PCR cycles, as described in Step 8 (below), to best determine sample quality.

   [c] A final extension is performed after completion of the additional PCR cycles (see Step 8, next page).

8. For each PCR tube, determine the optimal number of PCR cycles (see Figure 2):
   a. Transfer 5 µl from the 25-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   b. Run three additional cycles (for a total of 28) with the remaining 45 µl of the PCR mixture.
   c. Transfer 5 µl from the 28-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   d. Run three additional cycles (for a total of 31) with the remaining 40 µl of PCR mixture.
   e. Transfer 5 µl from the 31-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
   f. Run three additional cycles (for a total of 34) with the remaining 35 µl of PCR mixture.

      Note: These last three cycles are especially necessary for samples starting with less than 5 ng of RNA.

   g. Perform a final extension for 5 min at 68°C (see the PCR cycling parameters from Step 7, previous page).
9. Electrophorese 5 µl of each PCR reaction alongside 0.1 µg of 1-kb DNA size marker on a 2% agarose/EtBr gel in 1X TAE buffer.

VI. Interpretation of Quality Assay Results

A. Analysis of cDNA Amplification Results

Figure 3 shows a typical gel profile of ds cDNA synthesized using mouse liver total RNA and the Quality Assay protocol outlined in Section V. As indicated by the arrows, you should observe distinct bands at 835 bp and 671 bp. These bands correspond to the 3' and 5' fragments of the housekeeping gene, respectively.

The optimal number of PCR cycles is the minimum number of cycles necessary to achieve an informative result. In this experiment, the PCR has almost reached its plateau (that is, the yield of PCR products is no longer increasing at an exponential rate) after 31 cycles. In some cases, you may also see a smear in the high-molecular-weight region of the gel, indicating that the reaction was overcycled. In contrast, the 25-cycle PCR produced very faint bands, indicating that the reaction was undercycled. The bands in over- and undercycled reactions are insufficient for determining an accurate 3':5' ratio. As indicated by the relative band intensity, the 28-cycle reaction was halted during the linear phase of amplification and is thus the optimal sample for this experiment.

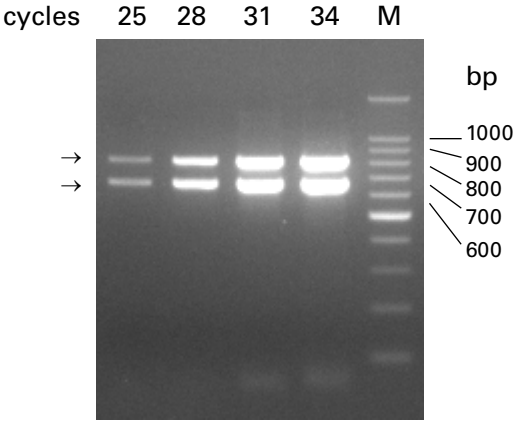

Figure 3. Analysis of Quality Assay results. 1 µg of mouse liver total RNA was used as starting material for analysis using the Quality Assay. 5 µl of each PCR product was electrophoresed on a 2% agarose/EtBr gel in 1X TAE buffer following the indicated number of PCR cycles. Lane M: 100-bp DNA ladder size marker, 650 ng loaded. The arrows indicate the bands at 835 bp and 671 bp representing the 3' and 5' fragments of the housekeeping gene, respectively. The optimal number of PCR cycles for this experiment was 28.

VI. Interpretation of Quality Assay Results *continued*

B. Determination of Sample Quality

In general, as the 3':5' fragment ratio approaches 1, the percentage of full-length transcripts in your sample increases. Additionally, the needs of your application should dictate whether your sample is suitable. Thus, if your experiment requires absolutely intact non-degraded RNA, your total RNA sample should generate a 28S:18S ratio of 2 on a formaldehyde gel and a 3':5' fragment ratio of 1±0.5 using the RNA/cDNA Quality Assay (Table II). Total RNA samples that meet these criteria are rated "Excellent" for RNA integrity. Poly A^+ RNA samples yielding a 3':5' fragment ratio of 2±0.5 (~50% full-length transcripts) are also considered good for your most demanding applications.

If you do not need high quality cDNA (e.g., you are generating cDNA for dot blots), then a 3':5' fragment ratio of 2±0.5 should suffice for your application.

Generally, samples producing a 3':5' ratio of 3–5 are rated "Unacceptable" in quality. Amplified cDNA samples are an exception. If you can only detect the 3' fragment within the linear phase of PCR, but the 5' fragment appears after 3 additional cycles, amplified cDNA samples are rated "Acceptable." The 3':5' fragment ratio should not exceed 5:1 (~20% full-length transcripts). Depending on your application, this cDNA can still be used.

TABLE II. QUALITY ASSAY CRITERIA FOR RNA & cDNA SAMPLES

		Rating based on 3':5' fragment ratio		
Sample	**28S:18S ratio**	**1±0.5**	**2±0.5**	**3–5**
Total RNA	2	Excellent	Good	Unacceptable
Total RNA	1	N/A	Good	Unacceptable
Poly A^+ RNA	N/A	Excellent	Good	Unacceptable
First-strand cDNA	N/A	Excellent	Good	Unacceptable
Amplified cDNA	N/A	Excellent	Good	Acceptable

VII. Analysis of Results and Troubleshooting Guide

If the reaction with the Control Total RNA was successful, but your experiment failed, your experimental RNA sample may be too dilute or degraded, or may contain impurities that inhibit first-strand synthesis.

- RNAs may have degraded during storage and/or first-strand synthesis. RNA must be stored at –70°C. Your working area, equipment, and solutions must be free of contamination by RNase. Check the stability of your RNA by incubating a small sample in water for 2 hr at 42°C. Then, electrophorese it on a formaldehyde/agarose/EtBr gel alongside an unincubated sample. If the RNA is degraded during incubation, it will not yield good results in the first-strand synthesis. In this case, re-isolate the RNA using a different technique, such as our NucleoSpin RNA II Kit (see Related Products for ordering information). Repeat the experiment using a fresh lot or preparation of RNA.
- You may have made an error during the procedure, such as using a suboptimal incubation temperature or omitting an essential component. Carefully check the protocol and repeat the first-strand synthesis and PCR with your sample and the control RNA.
- The conditions and parameters for PCR may have been suboptimal. The optimal number of PCR cycles may vary with different thermal cyclers or RNA samples. Check the protocol and repeat the first-strand synthesis and PCR.
- The concentration of your experimental RNA may be low. Repeat the experiment using more RNA and/or more PCR cycles.
- Your experimental RNA sample may contain impurities that inhibit cDNA synthesis. In some cases, ethanol precipitation of your existing total RNA, followed by washing twice in 80% EtOH, may remove impurities. If this fails, reisolate the RNA using a different technique, such as our NucleoSpin RNA II Kits.

VIII. Reference

Sambrook, J., & Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)

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