GE Healthcare

illustra™ Ready-to-Go RT-PCR Beads

Product Booklet

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27-9267-01

Page finder

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2. Handling

2.1. Safety warnings and precautions

Warning: ***For research use only.*** Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store at ambient room temperature in an airtight foil pouch with the dessicant. Once opened, completely reseal the pouch, fold the sealed edge over several times and seal with a clip. Store unopened and resealed pouches in a dessicator to maximize product lifetime.

Store reconstituted $pd(N)_6$ and $pd(T)_{12–18}$ at -20°C.

3. Components of the kit

The following components are included in this product:

RT-PCR Beads	Individual RT-PCR reactions. When brought to a final volume of 50 µl, each reaction will contain ~2.0 units of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 60 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (FPLC*pure*™), RNAguard™ Ribonuclease Inhibitor (porcine) and stabilizers, including RNase/DNase-Free BSA.
Control mix beads (red tubes)	Five beads, rabbit globin mRNA (1 ng) and 8 pmol each of 5′-specific globin primer (5′-d[ACACTTCTGGTCCAGTCCGACTGAG]-3′) and 3′-specific globin primer (5′-d[GCCACTCACTCAGACTTTATTCAAA]-3′).
Collection tubes	2 ml capless microcentrifuge tubes.
$pd(N)_6$	Lyophilized; 275 µg. Sufficient for 100 reactions using up to 2.5 µg/reaction
$pd(T)_{12–18}$	Lyophilized; 55 µg. Sufficient for 100 reactions using up to 0.5 µg/reaction.
Strip bubble caps	36 strips of eight caps. Provided only with the 0.2 ml tubes/plate format.

4. Quality control

Each batch of Ready-To-Go™ RT-PCR Beads is tested to ensure its ability to generate an RT-PCR product using a control mix bead and an RT-PCR Bead. In addition, a second test is performed using *Drosophila melanogaster* mRNA (7.6 kb), an RT-PCR Bead and primers specific for a 425 bp region of the *fsh* gene.

5. Materials not supplied

Reagents

- **Water**—Use only deionized or distilled water that is sterile and free of contaminating nucleic acid.
- **Mineral oil**—If required for the thermal cycler being used.

Equipment

- **Supplies for liquid handling**—Gloves, vials, and pipette tips should be sterile; pipettor and microcentrifuge. Perform all reactions in the plastic microcentrifuge tubes provided in the kit; these tubes are suitably sterile for thermal cycling.
- **Ice bucket or cold block**—For maintaining RT-PCR beads at 4°C during rehydration and prior to thermal cycling.
- **Thermal cycler**—For cycling according to the specified conditions.

6. Introduction

Ready-To-Go RT-PCR Beads utilize Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and *Taq* DNA polymerase to generate PCR product from an RNA template. Each bead is optimized to allow the first-strand cDNA synthesis and PCR reactions to proceed sequentially as a single-tube, single-step reaction. Simply add RNA, first-strand primer and PCR primers to the reaction, incubate at 42°C for 15 minutes, and cycle. The first-strand cDNA synthesis reaction may be primed with an oligo(dT) primer, a random primer such as $pd(N)_6$ or a custom (gene-specific) primer complementary to a specific mRNA sequence. The PCR reaction may be primed with two gene-specific primers or with $pd(T)_{12–18}$ and a single gene-specific primer.

First-Strand cDNA generated with the RT-PCR Beads is designed to be used directly as a template for PCR (i.e., RT-PCR, ref. 1–4). In this procedure, the double-stranded RNA:cDNA heteroduplex made during first-strand synthesis is heat-denatured to allow the cDNA strand to be used as a template for polymerization in PCR (5–7).

The specificity of the PCR amplification is based on two amplification primers which flank the cDNA segment to be amplified and hybridize to complementary strands. Repeated cycles of denaturation, primer annealing, and primer extension by *Taq* DNA polymerase can result in exponential amplification of a target cDNA. Even cDNA made from relatively rare transcripts can be successfully amplified using this technique.

Ready-To-Go RT-PCR Beads provide the reagents for RT-PCR reactions in a convenient ambient-temperature-stable bead. The beads are manufactured using a proprietary technology licensed to GE Healthcare. Ready-To-Go RT-PCR Beads have been optimized for first-strand cDNA synthesis and PCR reactions and contain buffer, $MgCl_2$, nucleotides, M-MuLV reverse

transcriptase, RNAguard and *Taq* DNA polymerase. The only reagents that must be added to the reaction are template RNA, a first-strand primer and PCR primers. The Ready-To-Go Bead format significantly reduces the number of pipetting steps, thereby increasing the reproducibility of the RT-PCR technique and minimizing the risk of contamination.

Handling

Bead potency and shelf-life is maximized when handled as per the instructions on page 5.

Requirements/Conditions for First-Strand cDNA Synthesis

The use of intact RNA is of primary importance to the success of first-strand cDNA synthesis. The amount of RNA needed in the first-strand reaction will vary depending on the relative abundance of the message of interest. We recommend using 100 pg to 1 µg of mRNA or 20 ng to 2 µg of total RNA. DEPC-treated water is preferred over TE buffer for preparing RNA. In some cases, the EDTA in TE buffer may inhibit the PCR.

Choices for primers include 0.2–2.5 µg of $pd(N)_6$, 0.1–0.5 µg of $pd(T)_{12-18}$ or 5–25 pmol of gene-specific primers. In a limited number of systems it may be beneficial to use up to 5 µg of $pd(N)_6$ to minimize nonspecific bands. This may require the purchase of additional $pd(N)_6$ (see page 27).

Assemble reactions on ice to preserve M-MuLV activity and to minimize production of nonspecific first-strand products.

The standard reaction temperature for first-strand cDNA synthesis using RT-PCR Beads is 42°C. The reaction temperature can be increased to 48°C if nonspecific priming is a problem; however, signal intensity may decrease.

Requirements/Conditions for PCR

In general, PCR primers should be 15–30 bp long with a GC content of approximately 50%. In order to minimize the production of primer-dimers, avoid complementarity between primer pairs and within each primer.

Each RT-PCR Bead reaction is formulated so that the $MgCl_2$ concentration for PCR is 1.5 mM. See Appendix if a higher $MgCl_2$ concentration is desired or if more than 10 µl of RNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA) is used.

Standard PCR consists of multiple cycles of denaturation (95°C), annealing (40–60°C) and elongation (72°C). An initial denaturation step (95°C for 5 minutes) is recommended to ensure complete denaturation of the template DNA and inactivation of the reverse transcriptase. The optimal annealing temperature varies depending on the sequence of the primers and their homology to the template DNA. In RT-PCR Bead reactions, a slightly higher annealing temperature, compared to a standard "wet reaction", may be used if products other than the desired one are present following amplification.

For most standard, three-step PCR reactions, 35 cycles results in a 10^5 to 10^9-fold amplification of the target sequence. The yield of PCR product may be increased by increasing the number of cycles to 45. However, an increased number of cycles may also produce spurious bands and increased background. The time of each step when using a "rapid cycler" such as a Perkin-Elmer 9600 thermal cycler (or equivalent) should be approximately half the time as when using a Perkin-Elmer 480 thermal cycler (or equivalent)

7. Protocols

Overview

Procedure 7.1 details the recommended handling of the 0.2 ml tubes/plate. Therefore, it may be skipped if the 0.5 ml tube format has been purchased.

Procedure 7.2 (One-Step Protocol for RT-PCR) is the most convenient protocol for use since primers for first-strand cDNA synthesis and PCR are added along with the template to an RT-PCR Bead. Most templates will amplify using the One-Step Protocol. Procedure 7.3 (Two-Step Protocol for RT-PCR) is slightly less convenient because only $pd(N)_6$ or $pd(T)_{12–18}$ is added with the template, and each tube must be opened prior to PCR amplification to add the gene-specific PCR primers. In certain cases, the Two-Step Protocol will give higher yield and greater specificity than that achieved with the One-Step Protocol. For example, if nonspecific priming is a problem or if an extremely low amount of template RNA is being used, Procedure 7.3 may give better results.

The reaction conditions described in Procedures 7.2 and 7.3 are general recommendations. Optimal conditions for each system must be determined empirically.

The use of intact, undegraded RNA is of primary importance to the success of first-strand cDNA synthesis. We strongly recommend the use of our mRNA purification kits for preparing high-quality mRNA. For rapid purification of mRNA directly from cells or tissues, we recommend either QuickPrep™ *Micro* mRNA Purification Kit or QuickPrep mRNA Purification Kit (see Ordering Information on page 27).

For purification of total RNA we recommend the use of the QuickPrep Total RNA Extraction Kit. With this kit, total RNA from cells and tissue can be extracted and purified in approximately 1 hour. The purified total RNA is of sufficient quantity and purity for use in RT-PCR.

Cautions

- Wear gloves when preparing RNA and when setting up RT-PCR reactions to avoid contamination with ribonucleases from the skin.
- Each tube of Ready-To-Go RT-PCR Beads (white tube) contains one bead sufficient for an RT-PCR reaction of 50 µl. Do not resuspend a single bead in more or less volume.
- When performing PCR, exercise extreme care to prevent contamination by nucleic acids. Always use sterile filter pipette tips and microcentrifuge tubes, and avoid carry-over contamination of stock solutions.

$pd(N)_6$ and $pd(T)_{12–18}$

The tube of $pd(N)_6$ contains 275 µg of lyophilized product. Reconstitute the $pd(N)_6$ with 550 µl of DEPC-treated water to give a final concentration of 0.5 µg/µl. The tube of $pd(T)_{12–18}$ contains 55 µg of lyophilized product. Reconstitute the $pd(T)_{12–18}$ with 110 µl of DEPC-treated water to give a final concentration of 0.5 µg/µl. Store reconstituted $pd(N)_6$ and $pd(T)_{12–18}$ at -20°C.

Control mix

The five control beads included in the kit are packaged in red 0.5 ml microcentrifuge tubes. Each contains one room-temperature-stable bead containing 1 ng of rabbit globin mRNA and 8 pmol each of two globin-specific PCR primers. A control mix bead can be used to evaluate the performance of the RT-PCR Beads by adding the rehydrated control mix to a tube of RT-PCR Beads and performing RT-PCR.

The globin primer specific for the 3′ end of the globin message will anneal to the mRNA and serve as a primer for the reverse transcriptase to produce first-strand globin cDNA. Subsequent PCR amplification of the first-strand product, utilizes the 5′ globin-specific primer and the remaining 3′ globin-specific primer as PCR primers, resulting in a 550 bp PCR product.

7.1. Handling of 0.2 ml tubes/plate

For RT-PCR Beads in 0.2 ml tubes, you have the option of using all 96 tubes at once, a strip of 8 tubes or a single tube. The seal covering the plate is composed of a top paper layer and a lower foil layer. To access the complete plate, both layers can be removed simultaneously. To access individual strips of tubes while keeping the remaining strips sealed, the top paper layer must first be removed keeping the lower foil seal intact.

Option A. Use all 96 tubes at once

1. Remove the plate from the pouch

2. Check that the bead in each tube is visible at the bottom of the tube. If necessary, gently tap the plate against a hard surface to force each bead to the bottom of the tube.

3. Place the plate, paper side up, on the bench top or other hard surface. Following the instructions printed on the paper layer, grasp the edge of the paper layer along with the edge of the foil layer. Carefully pull off together the paper and foil and discard.

Option B. Use less than 96 tubes

The 96 well plate is designed to allow a single strip of 8 tubes to be pushed out from the holding tray. To access individual strips of tubes while keeping the remaining strips sealed, the top paper layer must first be removed keeping the lower foil seal intact. Follow the directions below to remove a single strip of 8 tubes from the tray.

1. Remove the plate from the pouch.

2. Locate the side that allows access to the individual strips indicated by the arrow and instructions printed on the paper layer.

3. Grasp the edge of the seal (paper *and* foil) and carefully pull it back to separate the paper layer from the bottom foil layer at the first cut closest to the holes.

4. Hold down the edge of the bottom foil seal and carefully remove and discard the top paper layer.

Note: If the foil layer is accidentally removed, reseal the unused tubes with the strip bubble caps that are provided. If the reactions will not be used immediately, store in the pouch with desiccant.

5. Gently remove the outer perforated edge of foil that connects the individual strips and discard.
6. Gently apply pressure to the bottoms of all of the tubes in the strip that will be used. Gently rock the strip of tubes while continuing to apply gentle pressure until the strip of tubes come free from the plate tray. Be careful not to remove the foil seal from the tubes when pushing out the strip.
7. Place the tubes that you have removed into an appropriate holder. If you want to use less than 8 reactions, you may cut off the desired number of reactions with scissors. Snip the plastic strip connecting the individual tube from the adjacent tube then, carefully cut the foil seal between the tubes.
8. Place the plate and any other unused tubes into the resealable pouch with desiccant.

7.2. One-step protocol for RT-PCR

When performing RT-PCR, exercise extreme care. Wear gloves when setting up reactions to prevent the introduction of exogenous RNases from the skin. Always use sterile, filter pipette tips and avoid carry-over contamination of stock solutions. RT-PCR Beads are designed for a 50 µl reaction volume (one RT-PCR Bead/tube). When brought to a final volume of 50 µl, each reaction will contain 1.5 mM $MgCl_2$. If a higher concentration of $MgCl_2$ is desired for PCR or if more than 10 µl of RNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA) is used, refer to Appendix.

1. Check that the bead in each tube is visible at the bottom of the tube. If necessary, tap the tubes against a hard surface to force each bead to the bottom of the tube. Remove the caps from 0.5 ml tubes or remove the foil from the 0.2 ml tubes to be used.

2. Place the tubes or plate that you plan to use on ice. If desired, one or more tubes may be used for a negative control to test for DNA contamination (see step 2.6).

3. Determine the total volume of RNA and primers that will be used in an RT-PCR Bead reaction. It is necessary to calculate this volume in order to determine the volume of water in which to dissolve the bead.

first-strand primer	V µl
PCR primer 1	W µl
PCR primer 2 (if necessary)	X µl
template RNA*	Y µl
DEPC-treated water	Z µl
	(to a total of 50 µl)

*See Appendix, if the RNA is in a solution containing EDTA.

4. To the side of each tube, add Z µl of room temperature DEPC-treated water. If necessary, tap the tube to mix the water with the bead. Incubate on ice until the bead dissolves (approximately 5 minutes).

5. Flick each tube with a finger to mix or pipet gently up and down and replace on ice. Do NOT vortex since this may cause foaming.

6. (Optional) To prepare a negative control reaction to test for DNA contamination, incubate the rehydrated bead without template and primers at 95°C for 10 minutes to inactivate the M-MuLV reverse transcriptase.

7. Add template and primers individually to each dissolved bead, including the bead that was dissolved and incubated in Step 6.

Note: Some systems are sensitive to the order of addition of template and primers.

8. For the positive control reaction, add 50 µl of DEPC-treated water to the control mix bead. Transfer the entire contents of the red tube to a tube containing an RT-PCR Bead.

9. Overlay the reaction with 50 µl of mineral oil only if the addition of mineral oil is a requirement for your thermal cycler.

10. Close caps on 0.5 ml tubes or apply bubble caps to the 0.2 ml tubes/plate. Push down firmly to ensure that the caps fit tightly on the tubes.

11. Transfer reactions to a prewarmed incubator, heat block or thermal cycler and incubate at 42°C for 15–30 minutes

Note: Bubble caps may become deformed if this incubation is carried out in a thermal cycler. If this occurs, use one of the additional bubble caps provided.

12. Incubate the reactions at 95°C for 5 minutes to inactivate the reverse transcriptase and to completely denature the template. Cycle 20–45 times depending on the abundance of the target.

Cycling conditions for the control beads are listed in Protocol 7.3. below

Note: Bubble caps may be deformed after cycling. If this occurs, use one of the additional bubble caps provided.

7.3. Thermal cycling parameters for the control reaction beads

Perkin-Elmer 480 (or equivalent)	Perkin-Elmer 9600 (or equivalent)
95°C, 1 minute	95˚C, 30 seconds
55°C, 1 minute	55°C, 30 seconds
72°C, 2 minutes	72°C, 1 minute
Repeat for a total of 32 cycles	Repeat for a total of 32 cycles

7.4. Two-step protocol for RT-PCR

When performing RT-PCR, exercise extreme care. Wear gloves when setting up reactions to prevent the introduction of exogenous RNases from the skin. Always use sterile, filter pipette tips and avoid carry-over contamination of stock solutions. RT-PCR Beads are designed for a 50 µl reaction volume (one RT-PCR Bead/tube). When brought to a final volume of 50 µl, each reaction will contain 1.5 mM $MgCl_2$. If a higher concentration of $MgCl_2$ is desired for PCR, refer to Appendix 7.1.

1. Check that the bead in each tube is visible at the bottom of the tube. If necessary, tap the tubes against a hard surface to force each bead to the bottom of the tube. Remove the caps from 0.5 ml tubes or remove the foil from the 0.2 ml tubes to be used.

2. Place the tubes or plate to be used on ice. If desired, one or more tubes may be used for control to test for DNA contamination.

3. Determine the total volume of RNA and primers that will be used in an RT-PCR Bead reaction. It is necessary to calculate this volume in order to determine the volume of water in which to dissolve the bead.

first-strand primer*	V µl
PCR primer 1**	W µl
PCR primer 2**	X µl

template RNA***	Y µl
DEPC-treated water	Z µl
	(to a total of 50 µl)

*For increased sensitivity, use pd(N)$_6$ or pd(T)$_{12–18}$ as the first-strand primer. The success of this approach will be system-dependent.

**Add these primers after the first-strand cDNA synthesis reaction is completed (see Step 13). The combined total volume of the two PCR primers should be ≤ 10 µl.

***See Appendix, if the RNA is in a solution containing EDTA.

4. To the side of each tube, add Z µl of room temperature DEPC-treated water. If necessary, tap the tube to mix the water with the bead. Incubate on ice until the bead dissolves (approximately 5 minutes).
5. Flick each tube with a finger to mix or pipet gently up and down and replace on ice. Do not vortex since this may cause foaming.
6. (Optional) To prepare a negative control reaction to test for DNA contamination, incubate the rehydrated bead without template and primers at 95°C for 10 minutes to inactivate the M-MuLV reverse transcriptase.
7. Add template and only the primer for first-strand cDNA synthesis individually to the dissolved bead, including the bead that was dissolved and incubated in Step 6.
8. For the positive control reaction, add 50 µl of DEPC-treated water to the control mix bead. Transfer the entire contents of the red tube to a tube containing an RT-PCR Bead.
9. Overlay the reaction with 50 µl of mineral oil only if the addition of mineral oil is a requirement for your thermal cycler.

10. Close caps on 0.5 ml tubes or apply bubble caps (provided) to the 0.2 ml tubes/plate. Push down firmly to ensure that the caps fit tightly on the tubes.

11. Transfer reactions to a prewarmed incubator, heat block or thermal cycler and incubate at 42°C for 15–30 minutes.
Note: Bubble caps may become deformed if this incubation is carried out in a thermal cycler. If this occurs, use one of the additional bubble caps provided.

12. Incubate the reactions at 95°C for 5 minutes to inactivate the reverse transcriptase and to completely denature the template.
Note: Bubble caps may become deformed. If this occurs, use one of the additional bubble caps provided.

13. Add gene-specific primers for PCR. (Do not add to the positive control reaction.)

14. Cycle 20–45 times depending on the abundance of the target.
Note: Bubble caps may be deformed after cycling. If this occurs, use one of the additional bubble caps provided.

15. We recommend the following thermal cycle profiles as starting points:

Perkin-Elmer 480 (or equivalent)
95°C, 1 minute
55°C, 1 minute
72°C, 2 minutes
Repeat for a total of 32 cycles

Perkin-Elmer 9600 (or equivalent)
95°C, 30 seconds
55°C, 30 seconds
72°C, 1 minute
Repeat for a total of 32 cycles

8. Appendix: Adding $MgCl_2$ (optional)

EDTA present in the RNA solution will chelate Mg^{2+} required for the RT-PCR and may lead to suboptimal results. To compensate, additional Mg^{2+} may be added when assembling the reactions. As a starting point, add 0.4 µl of 25 mM $MgCl_2$ to the dissolved bead for each 10 µl of RNA solution. The final volume of the assembled reaction should be kept to 50 µl.

RT-PCR Bead reaction will contain 1.5 mM $MgCl_2$ in a final volume of 50 µl. Extra $MgCl_2$ can be added to the reaction if a higher concentration of Mg^{2+} is desired. **Note:** *$MgCl_2$ concentrations above 4.0 mM will inhibit the PCR reaction.* Use the data in Table 1 to determine the volume of a sterile 25 mM $MgCl_2$ solution that should be added to increase the Mg^{2+} concentration of the reaction. If extra $MgCl_2$ is added, decrease the amount of water added to the reaction so that the final volume equals 50 µl.

Final [$MgCl_2$]	Volume of 25 mM $MgCl_2$
2.0 mM	1.0 µl
2.5 mM	2.0 µl
3.0 mM	3.0 µl
3.5 mM	4.0 µl
4.0 mM	5.0 µl

Table 1. Adjusting the reacton concentration of Mg^{2+}

9. Troubleshooting

Problem: No bands are visible on the gel.

Possible causes	Solutions
1. *The thermal cycler did not function properly.*	**1.** Improper cycling conditions can result in poor amplification. Your thermal cycler may not be functioning properly if the RT-PCR Bead control reaction failed to produce a specific product at ~550 bp. Switch to a different thermal cycler.
2. *No primer was added to the reaction.*	**2.** RT-PCR Beads contain no primer. Primers for both first-strand cDNA synthesis and PCR must be added by the researcher. In Procedure C, at least one PCR primer must be added after first-strand synthesis.
3. *Insufficient RNA was used in the reaction.*	**3.** The amount of RNA needed to give a good signal in different RT-PCR systems can vary. Titrate the amount of template required.
4. *The quality of the template RNA was poor.*	**4.** Impure or degraded RNA may fail to be primed during first-strand cDNA synthesis or may fail to amplify. Isolate and handle RNA using precautions against RNases.
5. *Template RNA is in a buffer containing EDTA.*	**5.** EDTA present in the RNA solution will chelate Mg^{2+} required for the RT-PCR and may lead to suboptimal results. To compensate, add additional Mg^{2+} when assembling the reactions. Refer to Appendix for details.
6. *The dissolved bead remained at room temperature*	**6.** M-MuLV reverse transcriptase will lose significant activity if kept at room temperature for 30 minutes. Maintain

Problem: No bands are visible on the gel *continued*.

Possible causes	Solutions
6. *Continued before initiating the RT-PCR.*	**6.** *Continued* dissolved beads on ice until the tube is moved to the 42°C incubation.
7. *RNases were introduced into the reaction.*	**7.** Use only DEPC-treated water and wear gloves when setting up reactions.
8. *The wrong reaction volume was used.*	**8.** Each RT-PCR Bead should only be used in a final reaction volume of 50 µl.

Problem: There are extra, nonspecific bands on the gel

Possible causes	Solutions
1. *In Procedure 7.2, the primers are binding nonspecifically to the RNA during first-strand cDNA synthesis.*	**1.** Decrease the amount of template and/or primers. Determine if your system is sensitive to the order of addition of template and primers. Increase the amount of $pd(N)_6$ in the reaction and/or use Procedure C.
2. *The PCR primers are hybridizing to a secondary site on the template.*	**2.** Design new primers that are less specific for the secondary site. Decrease the amount of template and/or primers. Increase the annealing temperature during PCR by 5°C and/or decrease the number of cycles.
3. *DNA contamination is present in the RNA template.*	**3.** Try using other purification methods which will reduce/eliminate DNA in the RNA template (e.g. QuickPrep *Micro* mRNA Purification Kit)

Problem: There is excessive smearing on the gel.

Possible causes	Solutions
1. *Too much template RNA was added to the reaction.*	**1.** Reduce the amount of template RNA in the reaction until the smearing is eliminated.
2. *The quality of the template RNA was poor.*	**2.** Impurities or degraded RNA may serve as a template for priming during first-strand cDNA synthesis. Isolate and handle RNA using precautions against RNases.
3. *RNases were introduced into the reaction.*	**3.** Use only DEPC-treated water and wear gloves when setting up reactions.
4. *Too much primer was added to the reaction.*	**4.** Reduce the amount of primer in the reaction.
5. *$MgCl_2$ concentration was too low.*	**5.** Titrate the amount of $MgCl_2$ in the reaction (Appendix) until the smearing is eliminated.
6. *The annealing temperature during PCR was too low.*	**6.** The optimal annealing temperature varies depending upon the sequence of the primers and their homology to the template cDNA. In RT-PCR Bead reactions, a slightly higher annealing temperature, compared to standard "wet" reaction, may be required. Try increasing the annealing temperature 5°C.
7. *The reaction was cycled for more than 35 cycles.*	**7.** The yield of PCR product may be increased by increasing the number of cycles to 45. However, an increased

Problem: There excessive smearing on the gel *continued*.

Possible causes	Solutions
	7. *Continued.* number of cycles may also produce spurious bands and increased background. Reduce the number of cycles until the smearing is eliminated.
8. *Cycling conditions suitable for a Perkin-Elmer 480 thermal cycler (or equivalent) were used with a "rapid cycling" thermal cycler.*	**8.** The length of each step when using a "rapid cycler" such as a Perkin-Elmer 9600 thermal cycler (or equivalent) should be approximately half the length as when using a Perkin-Elmer 480 thermal cycler (or equivalent).

Problem: There is excessive primer-dimer on the gel.

Possible causes	Solutions
1. *Too much primer was added to the reaction.*	**1.** Excessive primer to template ratios can cause an abundance of low molecular weight bands and smearing. Titrate the amount of primers until the primer-dimer band is eliminated.
2. *Primers were not properly designed.*	**2.** In general, PCR primers should be 15–30 bp long with a GC content of approximately 50%. Complementarity between primer pairs and within each primer should be avoided.

10. References

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11. Companion products

Product	Product number
QuickPrep™ Total RNA Extraction Kit	27-9271-01
QuickPrep *Micro* mRNA Purification Kit	27-9255-01
$pd(N)_6$	27-2166-01
$pd(T)_{12–18}$	27-7858-02 27-7858-03

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