



Promega

Technical Manual

Plexor[®] One-Step qRT-PCR System

INSTRUCTIONS FOR USE OF PRODUCTS A4021 AND A4041.



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PRINTED IN USA.
Revised 11/09

Part# TM263

Plexor[®] One-Step qRT-PCR System

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this system. E-mail techserv@promega.com.

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1. Description

The **Plexor[®]** One-Step qRT-PCR System^(a,b) is a novel real-time RT-PCR system for the quantitation of specific sequences within an RNA sample. This one-tube, two-enzyme system uses ImProm-II[™] Reverse Transcriptase and the **Plexor[®]** qPCR technology for quantitation of any type of RNA sample, including total cellular RNA, poly(A)⁺ RNA or viral RNA. This Technical Manual describes the **Plexor[®]** One-Step qRT-PCR System and provides instructions for reaction setup. Instructions for the use of the **Plexor[®]** System with various real-time PCR instruments and information on data analysis using the **Plexor[®]** Analysis Software are provided in separate, instrument-specific Instrument Setup and Data Analysis Technical Manuals.

Before using the **Plexor®** One-Step qRT-PCR System, be sure you have the following:

- **Plexor® System primer pair(s).** The Plexor® One-Step qRT-PCR System is designed to work with pairs of PCR primers where one of the primers contains a fluorescent label adjacent to an iso-dC residue. The other primer is unlabeled. We recommend using the free, web-based Plexor® Primer Design Software to design primers and match fluorescent reporter choices to your specific real-time instrument and your preferred oligonucleotide manufacturer. For more information, refer to Section 3.B.
- **Instrument-specific manual for instrument setup and data analysis.** These manuals provide details for programming thermal cycling conditions, exporting raw data from the instrument software and data analysis. A complete list of Instrument Setup and Data Analysis Technical Manuals is available at: www.promega.com/plexorresources/. These manuals are available by request from your local Promega Branch Office or Distributor or at: www.promega.com/plexorresources/
- **Plexor® Analysis Software.** The key to the Plexor® technology is the quenching of a fluorescent reporter due to the site-specific incorporation of dabcyI-iso-dGTP. As a result, the fluorescent signal from a Plexor® System reaction decreases as PCR product accumulates. Real-time instrument software can record the quenching data but cannot convert the data into cycle thresholds or melt temperatures. Data analysis is accomplished by exporting the data to the Plexor® Analysis Software. The Plexor® Analysis Software is compatible with data from all supported instruments and is available via free download at: www.promega.com/plexorresources/. The software is also available on CD-ROM; contact your local Promega Branch Office or Distributor, or e-mail: techserv@promega.com. The software is compatible with Windows® 98 and later operating systems.

The Plexor® One-Step qRT-PCR System takes advantage of the specific interaction between two modified nucleotides to achieve quantitative RT-PCR analysis (1-3). One of the PCR primers contains a modified nucleotide (iso-dC) linked to a fluorescent label at the 5' end. The second PCR primer is unlabeled. The reaction mix includes deoxynucleotides and iso-dGTP modified with the quencher dabcyI. DabcyI-iso-dGTP is incorporated opposite the iso-dC residue in the primer. The incorporation of the dabcyI-iso-dGTP at this position results in quenching of the fluorescent dye on the complementary strand and a reduction in fluorescence, which allows quantitation during amplification (Figure 1).

With the Plexor® One-Step qRT-PCR System, the accumulation of product is accompanied by a decrease in fluorescence as shown in Figure 2.

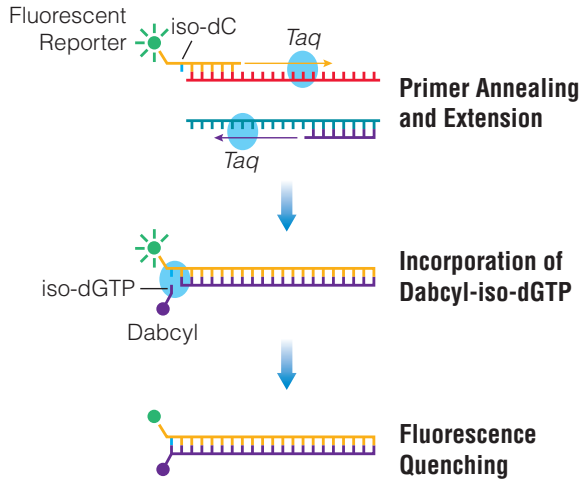


Figure 1. Schematic diagram illustrating the Plexor® System real-time PCR process.

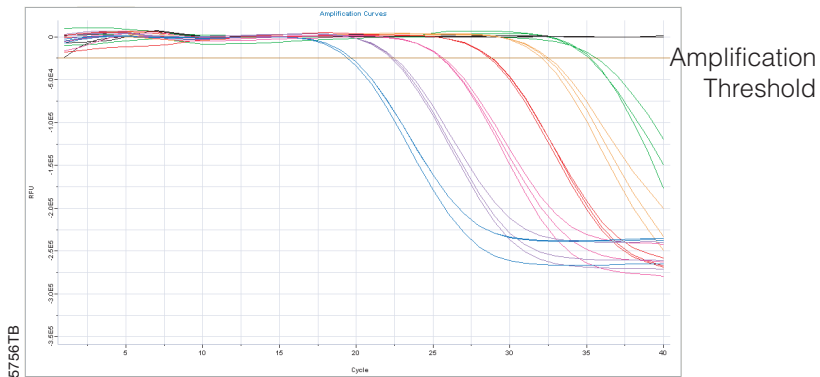


Figure 2. Representative Plexor® System amplification curve. The amplification curves show the fluorescence (in relative fluorescence units, RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This line is used to determine the cycle threshold (C_t) for the samples.

The Plexor® One-Step qRT-PCR System allows the use of a melt or dissociation curve to determine the melting temperature (T_m) of the products following amplification (Figure 3). This is useful in assessing the specificity of the reaction.

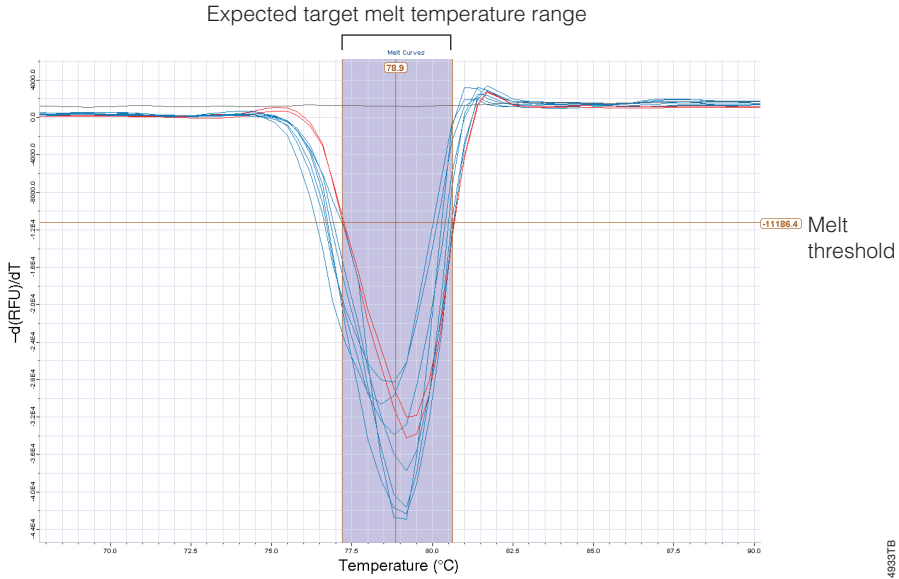


Figure 3. Representative Plexor® System melt curve. Melting temperature is empirically determined by plotting the change in fluorescence with temperature ($-dRFU/dT$) versus temperature and determining the temperature at which the greatest rate of change in fluorescence occurs.

2. Product Components and Storage Conditions

Product	Size	Cat. #
Plexor® One-Step qRT-PCR System	200 reactions	A4021
	200 reactions	A4041*

For Research Use Only. Not for use in diagnostic procedures. *This catalog number only available in Europe or through Distributors supported by Promega European Branch Offices. Includes:

- 2 × 1.25ml Plexor® Master Mix, 2X
- 2 × 10µl ImProm-II™ Reverse Transcriptase
- 50µl ImProm-II™ Enzyme Diluent
- 5µg 1.2kb Kanamycin Positive Control RNA (0.5mg/ml)
- 10µl Plexor® Kanamycin Control Primer Pair, 25X
- 3 × 10ml MOPS/EDTA Buffer
- 2 × 1.25ml Nuclease-Free Water
- 2 × 2,500u RNasin® Plus RNase Inhibitor

Storage Conditions: Store all components at -20°C.

Items Available Separately

Product	Cat.#
Plexor® Analysis Software	A4071

Not for Medical Diagnostic Use. This CD-ROM contains the Plexor® Analysis Software and instructions for use of the software. This software can also be downloaded at: www.promega.com/plexorresources/

Product	Size	Cat.#
MOPS/EDTA Buffer	3 × 10ml	Y5101

3. General Considerations

1. We recommend using designated work areas and pipettes for pre- and post-amplification steps to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acid from one experiment to the next.
2. Wear gloves and change them often.
3. Do not open the reactions after amplification is complete. Opening increases the risk of contaminating subsequent reactions with the amplified product.
4. Prevent contamination by using aerosol-resistant pipette tips.
5. Always include a no-template control (NTC) reaction to detect contamination.
6. Always dilute primers in MOPS/EDTA Buffer to maintain the integrity of the iso-dC-containing primers. **Do not** dilute the primers in water.
7. The ImProm-II™ Reverse Transcriptase and Control RNA should be kept chilled

before use. Thaw on ice; do not use a heat block.

8. Total RNA, poly(A)+ mRNA or synthetic RNA transcript may be used. For optimal results, the RNA should be free of DNA contamination and should be diluted in MOPS/EDTA Buffer.

3.A. Assay Design

The Plexor® One-Step qRT-PCR System reactions are similar to standard single-tube RT-PCR reactions except that one of the two amplification primers contains the modified nucleotide, iso-dC, adjacent to the 5' fluorescent label, and the Plexor® Master Mix contains dabcyI-iso-dGTP. Plexor® One-Step qRT-PCR System reactions can be performed on a variety of real-time PCR instruments. A complete list of instrument-specific Technical Manuals is available at: www.promega.com/plexorresources/



It is possible to design assays to quantify multiple targets within the same reaction. In these multiplex reactions, use a different fluorophore for each target. When analyzing the results from multiplex reactions, you may observe a small deflection in the Melt Curve plot in addition to the melt curve from the expected product. Color-to-color cross-talk is the most likely source of this nonspecific signal if this deflection has approximately the same T_m as the product observed in another channel. The ability to detect fluorescent dyes varies with instrument and instrument filter type.

3.B. Primer-Design Software

Promega has developed a web-based, primer-design software for use with the Plexor® One-Step qRT-PCR System. The Plexor® Primer Design Software designs primers for monoplex and multiplex qRT-PCR reactions using the parameters described in Section 5.B. We strongly recommend using this software, which can be found at: www.promega.com/plexorresources/

The software assists in selecting the appropriate fluorescent labels for primers used in single and multiplex qRT-PCR assays and will match fluorescent reporter choices to your specific real-time instrument and your preferred oligonucleotide manufacturer. Fluorescently labeled oligonucleotides for use as primers with the Plexor® System assays must be ordered with an iso-dC residue adjacent to the 5' fluorescent label. A number of oligonucleotide suppliers have been licensed to provide these iso-dC-containing primers. Convenient links to these suppliers are included within the Plexor® Primer Design Software.

Plexor® primers have been designed and tested for several different genes that may be used to normalize gene expression data. These “housekeeping” gene primers have been incorporated into the Plexor® Primer Design Software. The sequences for these primers can be included in a multiplex primer design to minimize primer interactions. They can be ordered from the licensed oligonucleotide suppliers.

3.C. RNA Reference Standards

Relative Quantitation

Relative quantitation requires a reference or calibrator sample. A reference sample provides the basis for comparison in a relative quantitation assay. Examples of such reference samples are untreated samples or a zero-time-point control in a time-course experiment. The reference RNA should be prepared in the same manner as the experimental sample RNA (4).

Absolute Quantitation

For absolute quantitation, use an RNA sample of known concentration as the RNA reference standard. In general, standard curves are generated based on copy number or mass. However, other units, such as plaque forming units or dilution factors from a known stock, can also be used.

Serial dilutions of the RNA reference standard are amplified, and the results are used to generate a standard curve and determine the concentrations of unknown samples. **The same primers that are used to amplify your samples must be used to amplify the RNA reference standard.** We recommend performing duplicate or triplicate amplification reactions with each dilution of the RNA reference standard. Instructions for preparing serial dilutions of the reference standard are provided in Section 5.C. An example of a plate layout for the standard curve is shown in Figure 4.

Dilute the RNA reference standard in MOPS/EDTA Buffer. Prepare the dilution series, so 5µl of RNA is added to each reaction. For example, to add 1,000 copies per reaction, dilute the RNA reference standard to 1,000 copies/5µl or 200 copies/µl.

Note: The use of fewer than ten copies of target may produce inconsistent results due to the random distribution inherent in dilutions of samples with low copy numbers.

	1	2	3	4	5	6	7	8	9	10	11	12
A	buffer	buffer	buffer									
B	10 ⁰	10 ⁰	10 ⁰									
C	10 ¹	10 ¹	10 ¹									
D	10 ²	10 ²	10 ²									
E	10 ³	10 ³	10 ³									
F	10 ⁴	10 ⁴	10 ⁴									
G	10 ⁶	10 ⁶	10 ⁶									
H	10 ⁸	10 ⁸	10 ⁸									

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Figure 4. Schematic diagram showing distribution of diluted RNA reference standard in a 96-well PCR plate. Row A. No-template control. **Row B.** 1 copy of target sequence. **Row C.** 10 copies. **Row D.** 100 copies. **Row E.** 1,000 copies. **Row F.** 10,000 copies. **Row G.** 1,000,000 copies. **Row H.** 100,000,000 copies.

3.D. Control Reactions

No-Template Control: Include no-template control (NTC) reactions for each set of reactions. Add 5µl of MOPS/EDTA Buffer to these reactions in place of the template RNA. There should be no amplification product detected in the NTC reaction. Amplification in the NTC reaction indicates nonspecific amplification or the introduction of contaminants. Nonspecific amplification products can be differentiated by T_m in the melt curve analysis.

Positive Control: Include a positive control reaction to verify that reagents and instrumentation perform consistently. The positive control reactions contain the Plexor® Kanamycin Control Primer Pair, 25X, and the 1.2kb Kanamycin Positive Control RNA (diluted to 7.5×10^3 copies/µl). One of the primers in the control primer pair has an iso-dC residue adjacent to a 5' fluorescein (FAM) label. Product should be detected in the FAM channel during amplification of the positive control reaction. The absence of product in the positive control reaction indicates a problem with the Plexor® System reagents, reaction assembly or the real-time PCR instrument.

An additional positive control reaction can also be used to detect amplification inhibitors in the experimental RNA sample. To test for inhibitors, add the RNA sample in question to the positive control reaction. Decrease the amount of Nuclease-Free Water included in the reaction mix, so the final reaction volume is 25µl (20µl final volume if using LightCycler® 20µl capillaries). A decrease in product formation or a large increase in the C_t value (>5 cycles) for the mixed sample positive control reaction indicates the presence of amplification inhibitors in the RNA sample.

No Reverse Transcriptase Control: Include control reactions that do not contain reverse transcriptase (no-RT) to test each experimental RNA sample for the presence of DNA or amplicon contamination. Amplification of such contaminating DNA will produce product with the expected T_m in the melt curve analysis.

Normalization: Normalization of relative quantitation assays is recommended to minimize the variability between different samples. This is usually accomplished by amplification of a gene with an expression level that does not vary with the experimental treatment. The normalization reaction can be performed in a multiplex reaction along with amplification of the gene of interest or can be performed in a separate reaction. Examples of genes commonly used for normalization include GAPDH and β -actin. Total RNA or cell number can also be used to normalize results.

4. Protocol

Prior to reaction assembly, review the appropriate Technical Manual for the specific real-time instrument to be used. A complete list of Instrument Setup and Data Analysis Technical Manuals can be found at:

www.promega.com/plexorresources/

When using the Plexor® One-Step qRT-PCR System for the first time, we recommend programming the thermal cycling conditions and checking that the instrument is compatible with the dyes used and is configured for those dyes before assembling the reactions, so that the assembled reactions are not kept on ice for prolonged periods of time before thermal cycling. Once you are familiar with the programming process, the instrument can be programmed after reaction assembly.

Materials to Be Supplied By the User


(Solution compositions are provided in Section 5.E.)

- RNA reference standard
- sterile, aerosol-resistant pipette tips
- pipettes dedicated to pre-amplification work
- iso-dC-containing, fluorescently labeled primer and unlabeled primer
- crushed ice
- real-time PCR instrument and related consumables (e.g., capillaries, cuvettes or PCR plate and plate covers)
- the appropriate Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used
- Plexor® Analysis Software

4.A. ImProm-II™ Reverse Transcriptase Dilution

One microliter of the supplied ImProm-II™ Reverse Transcriptase is sufficient to perform 16 Plexor® One-Step qRT-PCR reactions (0.0625µl per reaction). If you are performing a small number of reactions (less than 16), we recommend diluting the enzyme by adding 5µl of the supplied ImProm-II™ Reverse Transcriptase to 11µl of ImProm-II™ Enzyme Diluent and using 0.2µl of diluted enzyme per reaction. Once diluted, the enzyme can be stored at -20°C for up to 1 month.

4.B. Primer Preparation

 Iso-dC-containing primers are sensitive to pH and must be resuspended and diluted in MOPS/EDTA Buffer to maintain primer integrity. **Do not use water to resuspend or dilute primers or prepare primer mixes.** The fluorescently labeled primer is light-sensitive and must be stored in the dark.

Primers can be prepared and stored individually or as pairs. Prepare 25X primer stocks using MOPS/EDTA Buffer. For most assays, the 25X concentration will be 5µM (giving a final concentration of 200nM for each primer). We have successfully used 1X primer concentrations in the range of 50–400nM. Multiplex assays may require optimization of primer concentrations.

4.C. RNA Preparation

Experimental RNA Templates

Thaw RNA on ice, vortex at low speed and return any unused portion to the freezer as soon as aliquots are taken. If the RNA target needs to be diluted, dispense the required dilution volumes of MOPS/EDTA Buffer beforehand and chill on ice.

Note: The amount of RNA required per reaction depends upon the abundance of the RNA of interest. A high-copy-number RNA can be detected in as little as 0.01pg while low-copy-number RNA may require >1ng. Up to 1µg RNA can be used in each reaction. A good starting point for a standard mass of RNA to add for an expression level of unknown abundance would be 100ng of total RNA.

Dilute the RNA in MOPS/EDTA Buffer to an appropriate concentration, so that 5µl of template is added per reaction.

Positive Control RNA

Thaw the 1.2kb Kanamycin Positive Control RNA on ice, vortex at low speed and return any unused portion to the freezer as soon as aliquots are taken. The 1.2kb Kanamycin Positive Control RNA is provided at 7.5×10^{11} copies/µl. One or more concentrations of the Positive Control RNA can be used. If using a single concentration, we recommend using 3.8×10^4 copies/reaction. Four serial 1/100 dilutions of the Positive Control RNA in MOPS/EDTA Buffer will provide a solution with 7.5×10^3 copies/µl. Use 5µl of that final dilution for a concentration of 3.8×10^4 copies/reaction.

4.D. Reaction Setup

Note: The specificity of some assays can be improved by combining and denaturing the template and primers prior to adding them to the reaction. For a description of this alternate reaction setup, see Section 5.A.

1. Thaw the Plexor® Master Mix and primers on ice.
2. Briefly vortex the Plexor® Master Mix and primers to mix. Store on ice.
3. Prepare the reaction mix as indicated in Table 1. The reaction mix will be used to amplify the experimental and RNA reference standard samples, and to perform the NTC reaction.

Prepare sufficient reaction mix for the desired number of reactions on ice.

Note: Multiplex assays can be performed by adding additional primer pairs specific for each target.

Table 1. Preparation of 25µl Reaction Mix. Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix , 2X	12.5µl
RNasin® Plus RNase Inhibitor	0.5µl
25X (5µM) primer pair ^{1,2}	1µl
ImProm-II™ Reverse Transcriptase (see Section 4.A)	0.2µl (diluted) or 0.0625µl (undiluted)
Nuclease-Free Water	to a final volume of 20µl

¹Individual primers can be used. Add 1.0µl of each primer at 5µM.

²For assays where more than one pair of primers will be used, add 1µl of each primer pair.

- Vortex the reaction mix briefly to mix.
- Assays with sample RNA or RNA reference standards:** Add 20µl of the reaction mix prepared in Step 3 (Table 1) to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the sample or reference RNA to the appropriate well, cuvette or capillary.

No-template control (NTC) reactions: Add 20µl of the reaction mix prepared in Step 3 (Table 1) to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of MOPS/EDTA Buffer to each well, cuvette or capillary.

- Assemble the positive control reaction mix as indicated in Table 2.

Table 2. Assembling the Positive Control Reaction Mix (25µl). Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix , 2X	12.5µl
RNasin® Plus RNase Inhibitor	0.5µl
Plexor® Kanamycin Control Primer Pair, 25X	1µl
ImProm-II™ Reverse Transcriptase (see Section 4.A)	0.2µl (diluted) or 0.0625µl (undiluted)
Nuclease-Free Water	to a final volume of 20µl

- Add 20µl of the positive control reaction mix to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of 1.2kb Kanamycin Positive Control RNA diluted to 7.5×10^3 copies/µl (Section 4.C) to each well, cuvette or capillary designated as a positive control. Vortex briefly to mix.
- Assemble the no-RT control reaction mix as indicated in Table 3.

Table 3. Assembling the no-RT Control Reaction Mix. Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix , 2X	12.5µl
RNasin® Plus RNase Inhibitor	0.5µl
25X (5µM) primer pair ^{1,2}	1µl
Nuclease-Free Water	to a final volume of 20µl

¹Individual primers can be used. Add 1.0µl of each primer at 5µM.

²For assays where more than one pair of primers will be used, add 1µl of each primer pair.

9. Add 20µl of the no-RT reaction mix to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the sample RNA to the appropriate well, cuvette or capillary designated as a no-RT control. Vortex briefly to mix.

4.E. Real-Time Analysis


After the amplification reactions have been assembled, prepare the capillaries, cuvettes or plates as described in the appropriate Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used (these manuals are available at: www.promega.com/plexorresources/).

More information about cycling conditions for specific real-time instruments and instructions on data export for the Plexor® Analysis Software are included in the specific Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used.

5. Appendix

5.A. Alternate Protocol: RNA and Primer Combination and Denaturation

RNA and primer denaturation as an initial step can increase the specificity of some RNA sequence quantitations using the Plexor® One-Step qRT-PCR System. In this procedure, the RNA and primers are combined and denatured prior to adding the reaction mix.

 Iso-dC-containing primers are sensitive to pH and must be resuspended and diluted in MOPS/EDTA Buffer to maintain primer integrity. **Do not use water to resuspend or dilute primers or prepare primer mixes.** The fluorescently labeled primer is light-sensitive and must be stored in the dark.

1. Place sterile, thin-walled dilution tubes and reaction tubes on ice. Thaw RNA on ice, vortex at low speed and return any unused portion to the freezer as soon as aliquots are taken. If the RNA target needs to be diluted, dispense the required dilution volumes of MOPS/EDTA Buffer beforehand and chill on ice. Use 3µl of RNA for each reaction. For example, for 1ng of RNA per reaction, prepare RNA at 1ng/3µl or 0.33ng/µl.

Note: The amount of RNA required per reaction is dependent upon the abundance of the RNA of interest. A high-copy-number RNA can be detected in as little as 0.01pg while low-copy-number RNA may require >1ng. A good starting point for a standard mass of RNA to add for an unknown expression level would be 100ng of total RNA.

- On ice, combine the components listed in Table 4 for the experimental RNA, RNA reference standard, NTC and positive controls reactions. The final volume should be 5µl per RT reaction. Multiply the volumes to accommodate multiple reactions if more than one reaction is planned using a single RNA:primer combination.

Table 4. Target RNA and Primer Denaturation.

Component	Volume Per Reaction		
	RNA Samples	Positive Control	NTC
RNA (up to 1µg/reaction)	Xµl	—	—
1.2kb Kanamycin Positive Control RNA (diluted to 7.5×10^3 copies/µl. See Section 4.C)	—	4µl	—
25X primer pair ^{1,2}	1µl	—	1µl
Plexor® Kanamycin Control Primer Pair, 25X	—	1µl	—
MOPS/EDTA Buffer	to 5µl	—	to 5µl

¹Individual primers can be used. Add 1.0µl of each primer at 5µM.

²For assays where more than one pair of primers will be used (i.e., multiplex assays), add 1.0µl of each primer pair.

- Close each tube of RNA tightly. Place the tubes into a preheated 70°C heat block for 5 minutes. Immediately chill in ice-water for at least 5 minutes. Centrifuge each tube for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume.

Reaction Setup After Target RNA and Primer Denaturation

- Thaw the Plexor® Master Mix on ice and vortex briefly.
- Prepare the qRT-PCR reaction mix in a sterile 1.5ml microcentrifuge tube on ice as indicated in Table 5. The reaction mix will be used to amplify experimental RNA, RNA reference standards, NTC and positive control reactions. Prepare sufficient reaction mix for the desired number of reactions on ice.

Table 5. Reaction Mix Setup After Target RNA and Primer Denaturation (25µl). Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix, 2X	12.5µl
RNasin® Plus RNase Inhibitor	0.5µl
ImProm-II™ Reverse Transcriptase (see Section 4.A)	0.2µl (diluted) or 0.0625µl (undiluted)
Nuclease-Free Water	to a final volume of 20µl

- Vortex the reaction mix briefly.
- Add 20µl of the reaction mix (Table 5) to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the experimental sample, reference standard, positive control or NTC (prepared as described in Table 4) to the appropriate well, cuvette or capillary.
- Assemble the no-RT control reaction mix as indicated in Table 6.

Table 6. No-RT Control Reaction Mix (25µl). Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix , 2X	12.5µl
RNasin® Plus RNase Inhibitor	0.5µl
Nuclease-Free Water	to a final volume of 20µl

- Vortex briefly to mix.
- Add 20µl of the No-RT Reaction Mix prepared in Table 6 to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the denatured RNA and Primers (Table 4) to the appropriate well, cuvette or capillary.

5.B. Primer-Design Parameters for Quantitative RT-PCR

The web-based Plexor® Primer Design Software, which is described in Section 3.B, uses the following parameters to design primers for use with the Plexor® One-Step qRT-PCR System.

- The primers amplify a region of target sequence that is a minimum of 40bp in size and a maximum of 150bp. The optimal size is 100bp.
- The primers have a T_m of 60–70°C. The optimal T_m is 65°C.

Note: The software uses T_m -calculating algorithms that are not available elsewhere, so the melting temperature calculated with this software may not be identical to melting temperatures calculated by other means.

3. The primers are typically 20–35 bases in length.
4. The primers must not contain more than 4 consecutive G residues or more than 4 consecutive C residues.
5. The primers must not contain more than 10 consecutive A residues or more than 10 consecutive T residues.
6. The primers do not contain a GC clamp. A GC clamp is defined as 3 G or C residues at the 3' end of the primer.
7. One of the primers must contain a 5' fluorescent label attached to an iso-dC residue. The chosen fluorescent label depends upon the detection capabilities of the real-time PCR instrument. More information on the compatibility of fluorescent labels and real-time PCR instruments is available at: www.promega.com/plexorresources/

A number of oligonucleotide suppliers have been licensed to provide these iso-dC-containing primers. Convenient links to these suppliers' web sites are available at: www.promega.com/plexorresources/

5.C. Serial Dilution of an RNA Reference Standard

An RNA reference standard with any units of concentration or amount can be used to generate the standard curve. In general, copy number or mass is used, but other units that are appropriate for your experiment can be used, such as plaque forming units or dilution factors from a known stock.

1. Thaw and vortex an RNA reference standard of known concentration.
2. Prepare serial dilutions of the RNA reference standard. Be sure to change pipette tips between dilutions.

An example of a tenfold dilution series starting with an RNA reference standard at a concentration of 10^7 copies/ μ l is given below.

- a. For a tenfold dilution series, label microcentrifuge tubes as 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 10^0 .
- b. Add 90 μ l of MOPS/EDTA Buffer to each tube.
- c. Add 10 μ l of the RNA reference standard to the first tube (10^6) and vortex to mix.
- d. Transfer 10 μ l from the 10^6 tube to the next tube in the series (10^5) and vortex to mix.
- e. Transfer 10 μ l from the 10^5 tube to the next tube in the series (10^4) and vortex to mix.
- f. Continue this dilution series with the tubes labeled 10^3 , 10^2 , 10^1 and 10^0 .

5.D. References

1. Sherrill, C.B. *et al.* (2004) Nucleic acid analysis using an expanded genetic alphabet to quench fluorescence. *J. Am. Chem. Soc.* **126**, 4550-6.
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3. Moser, M.J. and Prudent, J.R. (2003) Enzymatic repair of an expanded genetic information system. *Nucl. Acids Res.* **31**, 5048-53.
4. Bustin, S.A. (2004) A-Z of quantitative PCR. International University Line, La Jolla, 86-120.

5.E. Composition of Buffers and Solutions

1M MOPS (pH 7.5)

209.3g MOPS free acid (Sigma
Cat.# M1254)
100ml 5M sodium hydroxide

Dissolve MOPS free acid in 750ml of autoclaved, deionized water. Adjust to pH 7.5 with sodium hydroxide. Bring the volume to 1 liter with autoclaved deionized water.

MOPS/EDTA Buffer (1mM MOPS, 0.1mM EDTA)

1.0ml 1M MOPS (pH 7.5)
0.2ml 0.5M EDTA (pH 8.0)
998.8ml autoclaved, deionized water

5.F. Related Products

RNA Purification, Manual Systems

Product	Size	Cat. #
SV Total RNA Isolation System	10 preps	Z3101
	50 preps	Z3100
	250 preps	Z3105
RNAagents® Total RNA Isolation System	Scalable	Z5110

For Laboratory Use.

Manual or Automated RNA Purification

Product	Size	Cat. #
SV 96 Total RNA Isolation System*	1 x 96 preps	Z3500
	5 x 96 preps	Z3505
Vac-Man® 96 Vacuum Manifold	1 each	A2291

*For Laboratory Use.

Automated RNA Purification

Product	Size	Cat. #
MagneSil® Total RNA mini-Isolation System	4 plate	Z3351

For Laboratory Use.

cDNA Synthesis

Product	Size	Cat. #
ImProm-II™ Reverse Transcription System	100 reactions	A3800

For Laboratory Use.

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^(b)The PCR process, which is the subject of European Pat. Nos. 201,184 and 200,362 owned by Hoffmann-LaRoche*, is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

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