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## 1. PURPOSE

This protocol describes how to perform real-time Reverse Transcription PCR in a two-step procedure (Reverse Transcription being a separate step from the PCR) using SYBR Green as the detected fluorophore and ROX as the passive reference.

## 2. SCOPE

This procedural format is utilized by the Human Colon Cancer and Mouse, Rat, and Arabadopsis microarray projects under the supervision of John Quackenbush and Norman Lee within the Mammalian Genomics Department.

## 3. MATERIALS

3.1 TaqMan Reverse Transcription Reagents (Applied Biosystems; Cat # N808-0234)
3.2 SYBR Green PCR Master Mix (Applied Biosystems; Cat # 4309155) **OR**
3.3 QuantiTect SYBR Green PCR Kit (Qiagen; Cat # 204143) (**preferred**)
3.4 RNaseZAP (Ambion; Cat # 9780)
3.5 MicroAmp Optical 96-well Reaction Plate (Applied Biosystems; Cat # N801-0560) **OR**
3.6 Optical 384-well Reaction Plate (Applied Biosystems; Cat # 4309849)
3.7 ICycler iQ PCR Plates, 96 well (BioRad; Cat # 2239441) **OR**
3.8 Thin Wall Tubes; 200 µl (BioRad; Cat # 2239473)
3.9 Optical Caps (8 Caps/Strip) (Applied Biosystems; Cat # 4323032) **OR**
3.10 Optical Adhesive Covers (Applied Biosystems; Cat # 4311971)
3.11 RNAse and DNAse Free Water

**Note:** Reaction vessels MUST be DNAse and RNAse free.

## 4. PREPARATION

4.1 Decontamination

4.1.1 The workbench along with the tube racks and pipetmen should all be sprayed down with RNaseZAP (or any RNAse decontaminant) before use.

4.1.2 RNases are very difficult to kill and can seriously affect the run.

4.2 RNA

4.2.1 RNA should be thawed and stored on ice throughout the reverse transcription preparation and frozen immediately after using.

4.2.2 Repeated freeze/thaw of RNA samples causes significant degradation in RNA integrity.

## 5. PROCEDURE

5.1 Reverse Transcription (RT)

5.1.1 Thaw all reagents and store on ice.

5.1.2 Maintain an RNAse and DNAse free work environment.

5.1.3 Mix all individual reagents thoroughly and spin down.

**Note:** A 100-µl RT reaction efficiently converts a maximum of 2.0 µg total RNA to cDNA. Multiple RT reactions should be performed if more than 2.0 µg total RNA is used.

**Note:** The reverse transcription can be performed using 2 µg in a 100 µl reaction OR using 1 µg in a 50 µl reaction volume.

5.1.4 Master mix (for a **single 100 ml reaction**):

| | (in µl) | Final Concentration |
|---|---|---|
| 10X TaqMan RT Buffer | 10.0 | 1X |
| MgCl2 (25 mM) | 22.0 | 5.5 mM |
| dNTP Mixture | 20.0 | 500 µM/dNTP |

| Random Hexamer (50 µM) | 5.0 | 2.5 µM |
|---|---|---|
| RNase Inhibitor (20 U/µl) | 2.0 | 0.4 U/µl |
| Reverse Transcriptase (50 U/µl) | 2.5 | 1.25 U/µl |
| Total RNA | | 2.0 µg |
| RNAse free Water | **to 100 mL** | |

**Note:** As with all enzymatic reactions, mix all nonenzymatic components first and then add the enzymatic components.

**Note:** For the reverse transcription reaction, the final volume does not have to be exact, however for sample comparison, the amount of starting material (added RNA) MUST be equal.

5.1.5 Combine all reagents **except RNA** and mix thoroughly and spin down.

5.1.6 Aliquot master mix into either thin wall tubes **OR** a 96-well reaction plate.

**Note:** The BioRad iCycler iQ PCR plate is designed to be broken into four separate 24-well sections which is useful in place of individual tubes.

5.1.7 Add RNA samples to individual tubes or wells and seal.

**Note:** A weak seal of the tube/well could lead to evaporation during the reverse transcription and loss of reaction.

5.1.8 Mix tubes/plate and then spin down to remove any air bubbles.

5.1.9 Thermal cycling parameters:

| Incubation | 25°C | 10 minutes |
|---|---|---|
| Reverse Transcription | 48°C | 30 minutes |
| Inactivation | 95°C | 5 minutes |

**Note:** The incubation step is necessary to maximize primer-RNA template binding.

**Note:** Store all cDNA samples at –15 to –25°C

5.2 Real-Time PCR (for either Applied Biosystems SYBR Green Master Mix Kit or Qiagen QuantiTect SYBR Green PCR Kit)

5.2.1 Keep all reagents on ice during set up.

**Note:** It is not necessary to clean up the cDNA reactions for use in the PCR reaction.

5.2.2 Although an RNAse free environment is not necessary for the PCR of cDNA, a clean workspace is a good idea.

5.2.3 Mix 2X Master Mix thoroughly before use.

**Note:** The PCR step can be done in either a 50 µl or a 20 µl reaction volume for the 96-well plates with similar results and in a 20 µl reaction volume for the 384-well plates.

**EITHER:**

5.2.4 Master mix (for a **single 50 ml reaction**):

| | (in µl) | Final Concentration |
|---|---|---|
| 2X PCR Master Mix | 25.0 | 1X |
| Forward primer (10 µM stock) | 1.0 | 200 nM |
| Reverse primer (10 µM stock) | 1.0 | 200 nM |
| RNAse free Water | 22.0 | |
| Total | 49.0 | |

5.2.5 Add 49.0 µl of mix to the necessary wells in the 96-well plate.

**OR:**

5.2.4 Master mix (for a **single 20 ml reaction**):

| | (in µl) | Final Concentration |
|---|---|---|

| | | |
|---|---|---|
| 2X PCR Master Mix | 10 | 1X |
| Forward primer (10 µM stock) | 0.4 | 200 nM |
| Reverse primer (10 µM stock) | 0.4 | 200 nM |
| RNAse free Water | 8.2 | |
| Total | 19.0 | |

5.2.5 Add 19.0 µl of mix to the necessary wells in the 96-well or 384-well plate.

5.2.6 Add 1.0 µl of cDNA to the appropriate wells in the plate.

**Note:** Additional cDNA template may be added to the reaction if desired.

5.2.7 Seal plate and mix. Spin down to remove any bubbles in the wells.

**Note:** Samples for a standard curve should be included in EVERY real-time run for quantitation.

Note: Negative controls for every primer set used should be included to verify the absence of primer dimer amplification and contamination.

5.3 Thermal Cycling Parameters

5.3.1 For **Qiagen QuantiTect** SYBR Green PCR Kit

| | | |
|---|---|---|
| Incubation | 50.0°C | 2 minutes |
| Taq Activation | 95.0°C | 15 minutes |

40 cycles of:

| | | |
|---|---|---|
| Denaturation | 95.0°C | 15 seconds |
| Annealing | 56.0°C | 30 seconds |
| Extension (with detection) | 72.0°C | 30 seconds |

Dissociation/Melting Curve (machine specific)

5.3.2 For **Applied Biosystems** SYBR Green PCR Master Mix:

| | | |
|---|---|---|
| Incubation | 50.0°C | 2 minutes |

| Taq Activation | 95.0°C | 10 minutes |
|---|---|---|

40 cycles of:

| Denaturation | 95.0°C | 15 seconds |
|---|---|---|
| Anneal/Extension (detection) | 72.0°C | 1 minute |

Dissociation/Melting Curve (machine specific)