Chapter 3

The Use of Real-Time Reverse Transcription-PCR for Assessing Estrogen Receptor and Estrogen-Responsive Gene Expression

Michelle L. Booze and Kathleen M. Eyster

Abstract

Real-time reverse transcription-polymerase chain reaction (RT-PCR), also known as quantitative RT-PCR (qRT-PCR), is a powerful tool for assessing gene transcription levels. The technique is especially useful for measuring estrogen receptor transcript levels as well as gene expression changes in response to estrogen stimulation as it is quick, accurate, robust, and allows the measurement of gene expression in a variety of tissues and cells. This chapter describes the protocols used for the real-time RT-PCR assay using hydrolysis (TaqMan-type) probes.

Key words Estrogenreceptors, Gene expression, Primer, Fluorogenic probe, Real-time RT-PCR, Master mix, Custom design, Normalization

1 Introduction

The purpose of real-time reverse transcription-polymerase chain reaction (RT-PCR) is the measurement of the presence of a given messenger RNA (mRNA). The procedure involves the conversion of mRNA to complementary DNA (cDNA) in an initial step, and then amplification of a specific region of the target gene, the amplicon, from the cDNA by polymerase chain reaction (PCR). Fluorescent dyes are used to visualize the amplification in "real time." This technique is more powerful than its predecessors because of its quantitative nature and because additional steps after the reaction (e.g., separation of PCR products on a gel) are not required.

A variety of choices in machines and detection methods are available; most measure the level of fluorescence as an indicator of mRNA transcript levels. The increased detection of the fluorophore directly correlates to a greater quantity of the target mRNA transcript in the starting sample [1]. The fluorophore is attached to an oligomer probe. Several oligomer probe options are available

Kathleen M. Eyster (ed.), *Estrogen Receptors: Methods and Protocols*, Methods in Molecular Biology, vol. 1366,
DOI 10.1007/978-1-4939-3127-9_3,

for this assay. These include agents that intercalate into double stranded DNA (e.g., SYBR Green) [2, 3], hydrolysis probes (e.g., Taqman probes) [4, 5], dual-hybridization probes, molecular beacons [6], and scorpions [7]. Each different probe type has its own advantages and disadvantages [8].

A real-time RT-PCR experiment using hydrolysis fluorogenic probes requires forward and reverse primers that flank the amplicon, as well as the hydrolysis probe which is an oligonucleotide that is complementary to a sequence within the amplicon. The probes are typically designed to span exon-exon junctions so as to decrease the likelihood of amplification from contaminating genomic DNA (*see* **Note 1**). The oligomer probes carry a fluorescent reporter dye at one end and a quencher dye at the other end. As long as the reporter dye and the quencher are held in close proximity by their attachment to the oligomer probe, no fluorescence is released from the reporter dye. The release of the fluorophore from the oligomer probe relies on the 5′ nuclease activity of the DNA polymerase [9]. (Taq polymerase is the most commonly used enzyme in this assay, but others are also available [10, 11].) As the DNA polymerase synthesizes the new DNA strand of the amplicon during each PCR cycle, it encounters and hydrolyzes the probe, thereby releasing the reporter dye from the quencher. This release allows the fluorophore to fluoresce which is registered by the real-time PCR machine. The quantity of increasing fluorescence that is detected with each cycle is directly proportional to the quantity of starting material (mRNA) in the initial sample [1].

The accuracy of real-time RT-PCR requires diligent care by the investigator [12, 13]. Careful experimental design from the beginning, whether working with cells or tissues, will provide the foundation needed for reproducible results. This chapter describes the use of hydrolysis probes for detecting gene expression by real-time RT-PCR (Fig. 1).

2 Materials

It is extremely important to work cleanly and mitigate the presence of RNases (*see* **Note 2**).

1. Nuclease-free water (*see* **Note 3**).
2. Primer/Probe unique to the target experimental gene (e.g., Assays on Demand, Life Technologies) or investigator-designed primers and probes (*see* **Note 4**).
3. One-step real-time RT-PCR master mix containing the appropriate polymerase (e.g., TaqMan One-Step Master Mix, Life Technologies) (*see* **Note 5**).
4. Samples of total RNA for analysis with known concentration.

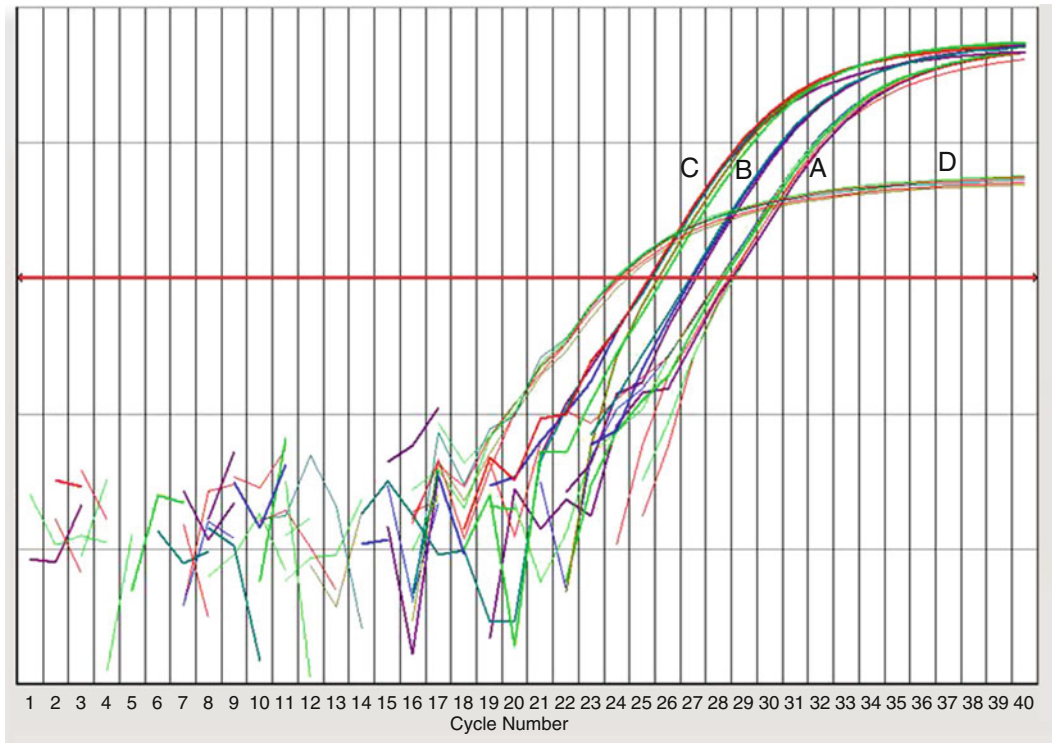

Fig. 1 Amplification curve for a one-step real-time RT-PCR reaction illustrating amplification of an experimental gene, fatty acid synthase (FAS), and a reference gene, cyclophilin. Vehicle, estradiol benzoate (EB), and ethinyl estradiol (EE) were administered by gavage for 3 weeks. Total RNA was extracted from liver and a one-step real time RT-PCR reaction was carried out on samples from control (*curve A*), EB-treated (*curve B*), and EE-treated (*curve C*) samples. EB increased FAS expression by 2.2-fold and EE increased FAS by 8.0-fold compared to control in this group of rats. *D* = housekeeping gene, cyclophilin. *N* = 5/group

5. RNase inhibitor.
6. RNA dilution pool: The same amount of RNA will be needed for each reaction and the easiest way to ensure this is to prepare an RNA dilution pool for each biological replicate and/or treatment from the concentrated mRNA samples after isolation (*see* **Note 6**). Calculate the necessary volumes of the RNA dilution pool (amount of RNA sample plus nuclease-free water) by multiplying the quantity of RNA needed per well (calculated using the validation curves described in **Note 7**) by the total volume required (6 µL/well times the number of wells. Always include the volume of one extra well for pipetting overage.). Divide this value by the concentration of RNA in the sample. For example, if you want 50 ng/well for nine wells at 6 µL/well and the concentration of RNA in the sample is 500 ng/µL, the calculation will be ((50 ng/well × 54 µL) divided by 6 µL) divided by 500 ng/µL = 0.90 µL of total RNA. Mix 0.90 µL total RNA with 53.1 µL nuclease-free

water for the first RNA dilution pool. Perform this calculation for each of the biological replicates, as the concentration of the total RNA will be different for each sample, and make an RNA pool for each (*see* **Notes 8** and **9**).

7. Master Mix pool: Make a separate Master Mix pool for each experimental and reference gene; the only difference among the Master Mix pools will be the primer-probe mix specific for a given gene. The Master Mix pool contains the following per-well volume of reagents times the number of wells: 1.25 μL primer-probe mix, 4.625 μL nuclease-free water, 0.625 μL 40× reverse transcriptase + RNase inhibitor, and 12.5 μL 2× Master Mix (*see* **Note 10**). Multiply these amounts by the number of wells needed for the assay. If less than 50 wells are needed, add one more well volume for overage. If more than 50 wells are needed, add two well volumes.
8. No-RT Master Mix pool: Make separate Master Mix pools for each experimental and reference gene for the no-RT controls. The No-RT Master Mix pools contain the following per-well volume of reagents: 1.25 μL primer-probe mix, 4.625 μL nuclease-free water, 0.625 μL RNase inhibitor, and 12.5 μL 2× Master Mix. As before, multiply these amounts by the number of wells needed for the assay. If less than 50 wells are needed, add one more well volume for overage. If more than 50 wells are needed, add two well volumes.
9. 96-Well microtiter plates and plate cover film compatible with the appropriate real time PCR machine (*see* **Note 11**).
10. Real-time PCR machine.
11. A pipette set that can dispense liquid amounts ranging from 0.1 μL to 1 mL.
12. Filter pipette tips that can dispense liquid amounts from 0.1 μL to 1 mL.

3 Methods

The instructions given here describe a one-step real-time RT-PCR reaction in which the reverse transcription reaction and the PCR cycles are carried out in the same well of the PCR plate (*see* **Note 12**). Choose the appropriate reference genes for your experiment (*see* **Note 13**). Perform a concentration-response curve (also called a validation curve) for the experimental gene and for the reference genes (*see* **Note 7**). To reduce the number of pipetting steps (and thereby reduce pipetting errors), prepare reagent pools of RNA and Master Mix (Subheading 2, **items 6–8**).

1. Design a 96-well plate map (Fig. 2) to guide experimental design. Include technical replicates of each RNA sample

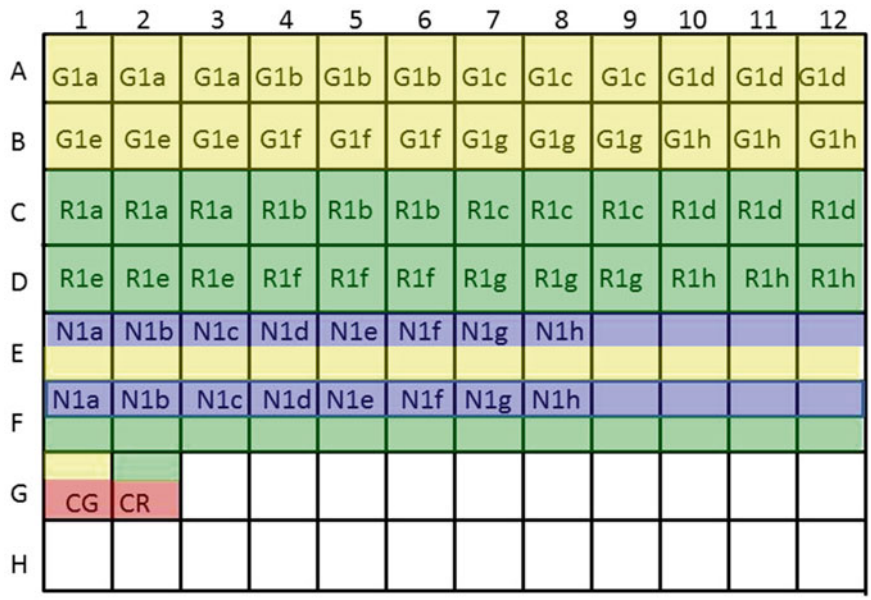

Fig. 2 96-Well plate map for a real-time RT-PCR experiment. Prepare a color-coded plate map to make it easier to find your place on the plate while pipetting. In this experimental design, *rows A* and *B*, labeled G1a–G1i, represent the experimental gene. *Rows C* and *D*, labeled R1a–R1i, represent the reference (housekeeping) gene. *Row A* illustrates four biological replicates for the vehicle control-treated sample and *row B* illustrates four biological replicates of estrogen-treated samples. Similarly, *rows C* and *D* illustrate the same four biological replicates for the vehicle control-treated and estrogen-treated samples. Triplicate wells are shown for each biological replicate. *Rows E* and *F* show a single well for each biological replicate in which the reverse transcriptase enzyme is left out of the reaction. *Row G* shows a single well for each gene (CG for the experimental gene and CR for the reference gene); no RNA is added to these wells. The reactions in *rows A–D* should yield amplification curves as in Fig. 1. No amplification should be observed in the no RT wells or in the no RNA wells. If amplification is observed in the no-RT wells, this suggests contamination of the RNA sample with genomic DNA. If amplification is observed in the no-RNA wells, it suggests that the reagents are contaminated

(preferably triplicates). Also include biological replicates from different animals or different passages of a cell line for each treatment group for the purposes of statistical analysis.

2. Control reactions: For a one-step RT-PCR reaction, each biological replicate must have a no-reverse transcription (NRT) control. Each experimental gene and each reference gene requires the inclusion of a no-RNA control (NRC) (Fig. 2).
3. Load 19 μL of the appropriate Master Mix pool or No-RT Master Mix pool into the assigned wells on the plate following the plate map.
4. Load 6 μL of the appropriate RNA pool into the assigned wells on the plate following the plate map.
5. Carefully seal the plate with the appropriate 96-well plate film (*see* **Note 14**).

6. Centrifuge the plate at 1200 × *g* for 2 min.
7. Place the plate in the real-time PCR machine and program the run as instructed by the manufacturer (*see* **Note 15**).
8. Analyze the data (*see* **Note 16**).

4 Notes

1. The precaution of designing hydrolysis probes to cross exon-exon boundaries does not provide absolute protection against contaminating genomic DNA since DNA can loop out and allow the juxtaposition of those boundaries. Therefore, it is important to eliminate genomic DNA from the RNA samples for real-time RT-PCR even when using this probe design.
2. RNases are ubiquitous and contamination with RNases will destroy your experiment. Cleanliness is key and gloves should be worn at all times. Detergents that denature RNases such as RNaseZap and RNase Away are very useful for cleaning the surfaces of the laboratory bench, pipettes, pipette tip boxes, gloved hands, and any other surfaces that may come in contact with the samples. It is recommended that a dedicated PCR hood is used; however, plates can be loaded on a bench that is dedicated to RNA work if appropriately cleaned. Use dedicated filtered pipette tips for best results.
3. Aliquot nuclease-free water into volumes of 1 mL or less to keep the stock solution clean and free of contamination. Do not use diethylpyrocarbonate-treated (DEPC) water for the real time RT-PCR reactions.
4. Several companies provide pre-designed primer-probe sets that contain forward and reverse primer as well as the oligomer probe in one solution. Often these reagents are available for human, rat, and mouse genes and some other species as well. Primer and probe oligonucleotide design programs (e.g., Primer Express, Life Technologies or Primer3, Massachusetts Institute of Technology) are available if pre-designed reagents are not available for the species or gene of interest. Primers, probes, and amplicons for real-time RT-PCR have different requirements from primers and amplicons for conventional RT-PCR, so be sure to pay attention to these specifications when designing these reagents. While the commercially designed primer/probes have a high success rate, it is important to be aware that some do fail on occasion depending on the sample being tested. Failure of amplification of any given primer/probe set could be due to an issue with the primers/probes or because a given tissue may not express the gene target. The addition of a positive control along with the mRNA samples is useful for determining the efficacy of the reaction.

5. Master mixes can be created in-house but commercially available mixes are often a better choice because of the rigorous validation of each lot. Make sure to carefully investigate which master mix is the correct fit for your experimental design and is compatible with the primer/probes sets that you plan to use.
6. The reaction volume calculations included here are for a total volume of 25 μL, a commonly used reaction volume for real-time RT-PCR. Total volume can be reduced to as low as 10 μL to conserve precious samples or to reduce reagent costs.
7. Efficiency is a term that is used to describe how well the primer and probe oligonucleotides bind with the target sequence [14]. The quality of this attachment is especially important when choosing the correct reference gene; the experimental gene and the reference genes should have comparable efficiencies. PCR efficiency is determined by performing a concentration-response curve with a range of dilutions of the starting sample [15]; for example, set up the reaction with 25, 50, 100, 150, and 200 ng of total RNA. The target gene and the reference gene should have similar concentration-response curves and efficiency should be close to 100 % [16, 17]. The best sample to use for this test is a pool that includes a representative population from the experiment, whether it is tissue or cell derived. This concentration-response curve is also known as a validation curve and it serves the additional purpose of identifying the RNA concentration that will produce a valid curve in the real time PCR reaction.
8. The RNA for real-time RT-PCR should be pure and of high quality. The sample should have been treated with DNase during the purification process to reduce the likelihood of DNA contamination. Analysis of the RNA sample on an Agilent Bioanalyzer is an excellent method of determining the quality of the RNA [18]. We have observed that RNA that has been purified on a spin column such as the RNeasy column (Qiagen) is of higher and more reproducible quality than that purified by the phenol:chloroform:isoamyl alcohol method.
9. If self-designed primer/probe sets are being used, the forward, reverse, and probe oligonucleotides will be separate. As such, the volume of water added to each reaction will have to be changed to accommodate this method.
10. Genes can be multiplexed; that is, the primer-probe sets for both the experimental gene and the reference gene can be amplified in the same well. In a multiplexed assay, adjust the volume of water in the Master Mix pool. Be aware that if one or both of the amplifications in a multiplexed reaction is especially robust, the reaction may exhaust the substrate and produce an artifactual amplification curve.

11. The 96-well microtiter plates may be machine specific. Always check to make sure that the plates and the transparent film covers are compatible with the real time PCR machine available.
12. When the sample of RNA is limited or when it is necessary to maintain long-term storage of a sample of cDNA, it may be preferable to perform a two-step reaction in which the reverse transcription reaction is carried out in a conventional PCR machine, and then the real time PCR reaction is performed using cDNA from the first step. Convenient kits are available for the reverse transcription reaction (e.g., High Capacity cDNA Archive Kit, Life Technologies); the cDNA should be purified using a spin column (e.g., QIAquick PCR Purification Kit, Qiagen) as the residual nucleotides, salts, and buffers from the RT reaction may interfere with the subsequent real-time PCR reaction. Another caveat to the two step reaction is that any evidence of potential contamination of the sample with genomic DNA will be lost.
13. Proper normalization is critical for a successful real-time RT-PCR experiment. This requires identification of a reference/housekeeping gene that does not change expression in the sample that is being tested in response to the experimental paradigm [14]. While it is common to choose a single gene such as glyceraldehyde-3-phosphate as the reference, it is important to be aware that no reference gene displays exactly the same expression across all cell types. Therefore, more than one reference gene should be validated for each new cell type or treatment [1, 13]. This is especially important in the context of estrogen treatment as many of the commonly used reference genes show a response to estrogen in a tissue-specific manner [15, 16].
14. When scheduling is an issue, it is possible to freeze a plate overnight and run the plate the next day. Pipette the plate as usual and seal with the transparent film. Wrap the plate in aluminum foil and freeze at –20 °C overnight. Allow the plate to come to room temperature and centrifuge at $1200 \times g$ for 2 min to make sure that any condensate is returned to the bottom of the well. Run the plate as usual.
15. Most machines take readings from all of the wells, whether they contain sample or not. This is useful when setting up the analysis, especially if wells are mistakenly mislabeled.
16. Several data analysis programs are available for real-time RT-PCR data (e.g., qBase, DataAssist™, Real-Time StatMiner®) which are easy to use and allow quick evaluation of the data. These programs can incorporate the use of several reference genes to normalize real time RT-PCR data. Most analysis programs do have freeware versions or free trials. Real time RT-PCR data can be manually analyzed using two methods: the

standard curve/absolute quantification method or the relative quantification method. The standard curve method is often used when assessing a small number of genes or when quantifying viral load [17, 18]. This method can also be used if an adequate reference gene cannot be found. The relative quantification method is the most common method and relies on a reference gene for normalization. The equation for this comparative threshold method is $2^{-\Delta\Delta^{Ct}} = \Delta Ct(\text{sample}) - \Delta Ct(\text{reference gene})$ [19, 20]. Perform statistical analyses on the data.

Acknowledgements

This work was supported, in part, by NIH P20GM103443. The authors would like to thank Dr. Rozzy Finn for reviewing this chapter.

References

1. Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6(10):995–1001
2. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22(1):130–131, 134–138
3. Morrison TB, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24(6):954–958, 960, 962
4. Lee LG, Connell CR, Bloch W (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res 21(16):3761–3766
5. Tyagi S (1996) Taking DNA probes into a protein world. Nat Biotechnol 14(8):947–948
6. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14(3):303–308
7. Whitcombe D, Theaker J, Guy SP, Brown T, Little S (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 17(8):804–807
8. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR (2005) Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 5(2):209–219
9. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6(10):986–994
10. Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A 88(16):7276–7280
11. Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H (1999) One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. J Virol Methods 77(1):37–46
12. Derveaux S, Vandesompele J, Hellemans J (2010) How to do successful gene expression analysis using real-time PCR. Methods 50(4):227–230
13. Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 15(3):155–166
14. Boda E, Pini A, Hoxha E, Parolisi R, Tempia F (2009) Selection of reference genes for quantitative real-time RT-PCR studies in mouse brain. J Mol Neurosci 37(3):238–253
15. Schroder AL, Pelch KE, Nagel SC (2009) Estrogen modulates expression of putative housekeeping genes in the mouse uterus. Endocrine 35(2):211–219
16. Zou K, Ing NH (1998) Oestradiol up-regulates oestrogen receptor, cyclophilin, and glyceraldehyde phosphate dehydrogenase mRNA concentrations in endometrium, but down-regulates them in liver. J Steroid Biochem Mol Biol 64(5–6):231–237
17. Jurado J, Prieto-Alamo MJ, Madrid-Risquez J, Pueyo C (2003) Absolute gene expression patterns of thioredoxin and glutaredoxin redox systems in mouse. J Biol Chem 278(46): 45546–45554

18. Castelain S, Descamps V, Thibault V et al (2004) TaqMan amplification system with an internal positive control for HCV RNA quantitation. J Clin Virol 31(3):227–234
19. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402–408
20. Pfaffl MW (2004) Quantification strategies in real-time PCR. In: Bustin SA (ed) A-Z of quantitative PCR. International University Line, La Jolla, CA, pp 87–112