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HHS Public Access
Author manuscript
Methods Mol Biol. Author manuscript; available in PMC 2016 October 31.

Published in final edited form as:
Methods Mol Biol. 2012 ; 926: 99–112. doi:10.1007/978-1-62703-002-1_7.

Application of Reverse Transcription-PCR and Real-Time PCR in Nanotoxicity Research

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Abstract

Reverse transcription-polymerase chain reaction (RT-PCR) is a relatively simple and inexpensive technique to determine the expression level of target genes and is widely used in biomedical science research including nanotoxicology studies for semiquantitative analysis. Real-time PCR allows for the detection of PCR amplification in the exponential growth phase of the reaction and is much more quantitative than traditional RT-PCR. Although a number of kits and reagents for RT-PCR and real-time PCR are commercially available, the basic principles are the same. Here, we describe the procedures for total RNA isolation by using TRI Reagent, for reverse transcription (RT) by M-MLV reverse transcriptase, and for PCR by GoTaq® DNA Polymerase. And real-time PCR will be performed on an iQ5 multicolor real-time PCR detection system by using iQ™ SYBR Green Supermix.

Keywords

RNA isolation; Reverse transcription (RT); Polymerase chain reaction (PCR); Agarose gel electrophoresis; Real-time fluorescent quantitative PCR

1. Introduction

The study of gene expression in a cell or tissue at a particular moment gives an insight into the capacity of the cell for protein synthesis. Gene expression assays, for example, gene profiling, are an important tool and are widely used in nanotoxicity studies. There are several methods available to determine gene expression, such as northern blot analysis, ribonuclease protection assay (RPA), serial analysis of gene expression (SAGE), reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time polymerase chain reaction (qRT-PCR), PCR arrays, and microarrays. Among these techniques, Northern blot analysis remains a standard method for detection and quantitation of mRNA levels despite the advent of more robust techniques. Northern blotting involves the use of electrophoresis to separate RNA samples by size, then detect the mRNA with a hybridization probe complementary to part of the target sequence. RPA is an extremely sensitive technique for the quantitation of specific RNAs in solution. It can be performed on total cellular RNA or poly(A)-selected mRNA as a target. SAGE method, as well as PCR arrays and microarrays, is used to study partial or global gene expression in cells or tissues in various experimental conditions. In this chapter, we will describe the methods to determine gene expression by using RT-PCR and real-time PCR. RT-PCR as a relatively simple, inexpensive, extremely sensitive and specific tool to determine the expression level of target genes. Real-time PCR is a quantitative method for determining copy number of PCR templates, such as DNA or

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cDNA, and consists of two types: probe-based and intercalator-based. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers and an additional fluorogenic oligonucleotide probe with both a reporter fluorescent dye and a quencher dye attached. The intercalator-based (SYBR Green) method requires a double-stranded DNA dye in the PCR which binds to newly synthesized double-stranded DNA and generates fluorescence. Both methods require a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each sample at frequent intervals during the PCR. The principle techniques underlying both RT-PCR and real-time PCR are total RNA isolation, reverse transcription (RT), and PCR. Reverse transcription involves the synthesis of DNA from RNA by using an RNA-dependent DNA polymerase. PCR can amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Here we will introduce detailed procedures for RT-PCR and real-time PCR.

2. Materials

2.1. Total RNA Isolation

1. TRI Reagent. Store at 2–8°C.
2. Chloroform. Store at room temperature.
3. Isopropanol. Store at room temperature.
4. 75% Ethanol (40 ml): in a sterile and RNase-free 50 ml tube, add 10 ml of molecular grade and nuclease-free water, and 30 ml of 100% ethanol to make 40 ml of 75% ethanol. Store at −20°C.
5. Molecular grade and nuclease-free water. Store at 2–8°C.

2.2. Reverse Transcription

1. M-MLV reverse transcriptase with 5× M-MLV reaction buffer. Store at −20°C.
2. Recombinant RNasin® Ribonuclease Inhibitor. Store at −20°C.
3. dNTP mixture, 10 mM. Store at −20°C.
4. Oligo $(dT)_{18}$ primer: synthesized by SIGMA (The Woodlands, TX) and prepared at a concentration of 0.5 μg/μl with molecular grade and nuclease-free water. Aliquot and store at −20°C.
5. Molecular grade and nuclease-free water. Store at 2–8°C.
6. Thermal cycler (Mastercycler, Eppendorf, Westbury, NY).

2.3. Polymerase Chain Reaction

A basic PCR setup requires several components and reagents include the DNA template, two primers, Taq polymerase or another DNA polymerase, buffer solution, dNTPs, and divalent cations such as Mg^{2+}. The following reagents are used routinely for RT-PCR in our laboratory.

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1. Template cDNA from the above RT. Store at −20°C.
2. Upstream and downstream primers: synthesized by SIGMA (The Woodlands, TX). Diluted to 5 μM with molecular grade and nuclease-free water. Store at −20°C.
3. GoTaq® DNA Polymerase with 5× reaction buffer. Store at −20°C.
4. dNTP mixture, 10 mM. Store at −20°C.
5. Molecular grade and nuclease-free water. Store at 2–8°C.
6. Agarose. Store at room temperature.
7. Ethidium bromide solution (10 mg/ml): dilute to 0.5 mg/ml with molecular grade and nuclease-free water. Store at room temperature.
8. 50× TAE: to make 1 l of 50× TAE buffer, need 242 g Tris base, 57.1 ml glacial acetic acid, and 100 ml of 0.5 M EDTA (pH 8.0). Add enough molecular grade H_2O to dissolve solids to a final volume of 1,000 ml. Store at room temperature.

2.4. Real-Time PCR

1. iQ™ SYBR Green Supermix. Store at −20°C.
2. 96-Well PCR plate. Store at room temperature.
3. Microseal® “B” Film. Store at room temperature.
4. Molecular grade and nuclease-free water. Store at 2–8°C.

3. Methods

3.1. Isolation of Total RNA

Obtaining high quality and intact RNA is the first and often the most critical step in performing RT-PCR and real-time PCR. RNA is easily degraded since RNase is very hard to inactivate. Several precautions need to be taken to prevent RNA from degradation. People should always wear a clean lab coat, disposable gloves, and change gloves frequently. The bench should be clean. Any aqueous solutions, tubes, and pipettes used for the procedure should be sterile and RNAse-free. To avoid contaminating your sample with RNases, do not talk while processing RNA extraction.

Currently, there are a number of RNA isolation kits commercially available. Although it is convenient, time-saving, and avoids contact with phenol/chloroform using commercially available kits, those kits using silica-membrane spin columns may not be ideal for studies of insoluble nanoparticle since the nanoparticles may clog the membrane pore of the spin column. Therefore, it is important to choose the right reagents or kits for total RNA isolation according to different experiments and specific characteristics of different nanoparticles. In our laboratory, we use TRI Reagent to isolate total RNA for nanoparticle studies. TRI Reagent is a mixture of guanidine thiocyanate and phenol in a monophase solution, which can effectively dissolve DNA, RNA, and protein after homogenization or lysis of tissue

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samples. It performs well with large or small amounts of tissue or cells. Here, we describe the procedures for isolating total RNA using TRI Reagent according to the manufacturer's instructions (1).

3.1.1. Sample Preparation

1. Lyse or homogenize the sample (see Notes 1–3).

   (a) Tissue (see Note 4): homogenize tissue samples in TRI Reagent (1 ml per 50–100 mg of tissue) in an appropriate homogenizer (see Notes 5 and 6). The volume of the tissue should not exceed 10% of the volume of the TRI Reagent.

   (b) Monolayer cells: lyse cells directly on the culture dish or plate (see Notes 7 and 8). Use 1 ml of the TRI Reagent per 10 cm^2 of glass culture plate surface area. After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogenous lysate (see Note 9).

   (c) Suspension cells: isolate cells by centrifugation at 1,000 rpm for 5 min and then lyse in TRI Reagent by repeated pipetting. One milliliter of the reagent is sufficient to lyse 5–10 × 10^6 animal, plant, or yeast cells, or 10^7 bacterial cells (see Notes 10 and 11).

2. In order to minimize the possibility of DNA contamination in the RNA extracted by TRI Reagent, after homogenization, centrifuge the homogenate at 12,000 × *g* for 10 min at 2–8°C to remove the insoluble material (extracellular membranes, polysaccharides, and high molecular mass DNA). The supernatant contains RNA and protein. If the sample had a high fat content, there will be a layer of fatty material on the surface of the aqueous phase that should be removed. Transfer the clear supernatant to a fresh tube.

3. To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 min at room temperature.

4. Add 0.2 ml of chloroform (see Note 12) per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 s, and allow to stand for 2–15 min at room temperature.

5. Centrifuge the resulting mixture at 12,000 × *g* for 15 min at 2–8°C.

3.1.2. RNA Isolation

1. Transfer the colorless upper aqueous phase to a fresh tube and add 0.5 ml of isopropanol per ml of TRI Reagent used in Subheading 3.1.1, step 1 and mix. Allow the sample to stand for 5–10 min at room temperature (see Note 13).

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2. Centrifuge at 12,000 × *g* for 10 min at 2–8°C. The RNA precipitate will form a pellet on the side and bottom of the tube.
3. Remove the supernatant and wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in sample preparation (see step 1 of Subheading 3.1.1). Vortex the sample and then centrifuge at 7,500 × *g* for 5 min at 2–8°C (see Notes 14 and 15).
4. Briefly dry the RNA pellet for 5–10 min by air-drying or under a vacuum (see Note 16). Add an appropriate volume of molecular grade water to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60°C for 10–15 min (see Note 17).
5. Measure the concentration of total RNA: in a sterile and RNase-free tube, add 48 μl of molecular grade and nuclease-free water and 2 μl of total RNA from step 3 to make a total volume of 50 μl. Mix well.
6. Measure the absorbance at 260 and 280 nm with a Spectrophotometer (DU 730 Spectrophotometer, Beckman Coulter, Fullerton, CA) (see Notes 18–21). 1A_{260} unit/ml = 40 μg/ml.

3.2. Reverse Transcription

Reverse transcription involves the synthesis of DNA from RNA by using an RNA-dependent DNA polymerase, the *reverse* of normal transcription, which is from RNA to DNA. Although there are many kits commercially available for RT, the reverse transcriptase used in those kits usually is M-MLV reverse transcriptase from the Moloney murine leukemia virus or AMV reverse transcriptase from the avian myeloblastosis virus. M-MLV reverse transcriptase is the preferred reverse transcriptase in cDNA synthesis for long messenger RNA (mRNA) templates (>5 kb) because the RNase H activity of M-MLV reverse transcriptase is weaker than the commonly used AMV reverse transcriptase (2). Since M-MLV reverse transcriptase is less processive than AMV reverse transcriptase, therefore, more units of the M-MLV enzyme are required to generate the same amount of cDNA as in the AMV reaction (2). The following are the basic procedures for RT using M-MLV reverse transcriptase according to the manufacturer's instruction (2).

1. Preheat a water bath to 70°C.
2. In a sterile RNase-free 0.2 ml PCR tube, add 2 μl of Oligo $(dT)_{18}$ primer (0.5 μg/μl) and 2 μg of total RNA in a total volume of 15 μl with molecular grade and nuclease-free water (total volume is 17 μl).
3. Put the PCR tubes which contain the primer and total RNA in a plastic PCR rack (see Note 22), then put the rack in the 70°C water bath for 5 min to melt secondary structures within the template.
4. Cool the samples immediately by putting the tubes on ice to prevent secondary structures from reforming.
5. In a new sterile RNase-free 0.5 ml tube, mix the following reagents according to the number of samples. Each reaction should contain: 1 μl M-

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MLV reverse transcriptase, 1.25 μl of 10 mM dNTP, 0.75 μl Recombinant RNasin Ribonuclease Inhibitor, and 5 μl of 5× M-MLV reaction buffer (see Notes 23 and 24). Mix gently by flicking the tube, then spin briefly.

6. Spin the PCR tubes from step 4 briefly to collect the solution at the bottom of the tube and put the tubes back onto the PCR rack.
7. Add 8 μl of the above mixture (step 5) into each PCR tube, mix gently by flicking the tube, then spin briefly. The total volume in the PCR tube should now be 25 μl.
8. Put the PCR tubes on to a thermal cycler and incubate at 42°C for 60 min (see Note 25), at 94°C for 5 min, then keep at 4°C (see Note 26).
9. When finished, the samples can be stored at −20°C for later PCR experiments.

3.3. Polymerase Chain Reaction

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase. There are many DNA polymerases commercially available. Although their efficiency may be different, they are suitable for regular RT-PCR to determine the expression level of mRNA. Some experiments which will use PCR products for cloning purposes, especially those for cloning of promoter region with high G-C content, need to use high fidelity DNA polymerase. The PCR is commonly carried out in a reaction volume of 10–200 μl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The following is an example of a PCR performed in our laboratory.

1. In a sterile nuclease-free microcentrifuge tube, mix the following reagents on ice. Each reaction contains: 1 μl of 5 μM each primer, 0.5 μl of 10 mM dNTP, 5 μl of 5× Green GoTaq Buffer, 0.25 μl GoTaq DNA polymerase (5 U/μl), and 16.25 μl of nuclease-free water (total 24 μl) (see Notes 27–29). Mix gently by flicking the tube, then spin briefly.
2. In each PCR tube, add 24 μl of the above mixture to the bottom of the tube.
3. Add 1 μl of cDNA sample in each PCR tube. Mix gently by flicking the tube, then spin briefly.
4. Put the PCR tube onto a thermal cycler.
5. According to the primers and the length of PCR product, set up parameters for PCR (see Notes 30–34), then run.
6. Separate the PCR products by agarose gel electrophoresis and visualize with ethidium bromide (see Notes 35–37).
7. After separation on an agarose gel, PCR products are visualized by Gel Doc XR (Fig. 1) and analyzed by Quantity One.

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8. To semi-quantify the expression level of mRNA, intensities of target gene products are normalized to that of housekeeping gene to obtain the relative densities.

3.4. Real-Time PCR

Traditional PCR uses agarose gel for detection of PCR amplification at the final phase or endpoint of the PCR (plateau). However, real-time PCR allows for the detection of PCR amplification in the exponential growth phase of the reaction. Theoretically, there is a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number. Real-time PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR, which makes quantitation of DNA and RNA easier and more precise than traditional methods. There are two methods, which are often used in the laboratory. One is the 5′ nuclease assay in which an oligonucleotide called a TaqMan® Probe is added to the PCR reagent master mix. This probe is designed to anneal to a specific sequence of template between the forward and reverse primers and is also designed with a high-energy dye termed a Reporter at the 5′ end, and a low-energy molecule termed a Quencher at the 3′ end. When this probe is intact and excited by a light source, the Reporter dye's emission is suppressed by the Quencher dye as a result of the close proximity of the dyes. When the probe is cleaved by the 5′ nuclease activity of the enzyme, the distance between the Reporter and the Quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease. An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated. Another real-time PCR method is by using SYBR Green Dye, which can bind the minor groove of any double-stranded DNA molecule. When SYBR Green dye binds to double-stranded DNA, the intensity of the fluorescent emissions increases. As more double-stranded amplicons are produced, SYBR Green dye signal will increase. The following is an example of real-time PCR by using iQ™ SYBR Green Supermix and performed on an iQ5 multicolor real-time PCR detection system.

1. Thaw all components used in step 2 at room temperature. Mix vigorously, and centrifuge to collect contents to the bottom of the tubes, then put the tubes on ice.
2. In a sterile nuclease-free microcentrifuge tube, mix the following reagents on ice. Each reaction contains: 1 μl of 5 μM of each primer, 10 μl of 2× iQ™ SYBR Green Supermix, and 7 μl of nuclease-free water (total 19 μl) (see Notes 38 and 39). Mix gently by flicking the tube, then spin briefly.
3. In each well of 96-well PCR plate, add 19 μl of the above mixture to the bottom of the tube.
4. Add 1 μl of cDNA sample in each well (see Note 40). Cover the plate with Microseal® “B” Film (see Note 41).
5. Mix gently by flicking the tube, then spin the plate briefly.
6. Put the PCR plate in an iQ5 multicolor real-time PCR detection system.

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7. According to the primers and the length of PCR product, set up parameters for real-time PCR, then run (see Notes 42–44).
8. Analyze the data. If using SYBR Green, there should be only one peak in the melting curve (Fig. 2) (see Note 45).
9. In our laboratory, the relative expression level of each gene is calculated as fold dilution (see Note 46) by using a standard curve for each gene. Standard curves are obtained by real-time PCR using 3, 1, and 1 μl of 10-, 100-, and 1,000-fold dilution, respectively, of cDNA obtained from sample without any treatments (see Note 47). The expression level of each gene is then normalized to the relative expression level of housekeeping gene in the same sample.

4. Notes

1. Use enough TRI Reagent for the sample homogenization. Too small volume of TRI Reagent may result in DNA contamination.
2. If samples used for the isolation contain organic solvents (ethanol, DMSO), strong buffers, or alkaline solution, DNA contamination may occur.
3. Incomplete homogenization or lysis of samples may result in low yield of RNA.
4. The tissues need to be processed or frozen in liquid N_2 immediately after removing from the animal to prevent RNA from degradation. If not used immediately, the samples need to be stored at −70°C.
5. We usually put the tube which contains tissues and TRI Reagent on ice for 2–5 min after homogenizing for 10 s each time. Repeat homogenization for several times or until no tissues are visible.
6. The homogenizer we used is from Glas-Col, Terre Haute, IN.
7. TRI Reagent is *not* compatible with plastic culture plates.
8. Trypsin digestion of cells may result in RNA degradation.
9. After addition of the TRI Reagent, we usually put the dish or plate on a shaker for 5–10 min to let cells lyse totally.
10. Some yeast and bacterial cells may require a homogenizer.
11. After the cells have been homogenized or lysed in TRI Reagent, samples can be stored at −70°C for up to 1 month.
12. The chloroform used for phase separation should not contain isoamyl alcohol or other additives.
13. The mixture also can be put in −4 or −20°C for 1 h.

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14. If the RNA pellets float, after vortexing, centrifuge at 12,000 × *g* for 5 min at 2–8°C.

15. Samples can be stored in ethanol at 2–8°C for at least 1 week and up to 1 year at −20°C.

16. A more complete evaporation of ethanol is required when RNA samples are to be used in RT-PCR. This is especially critical for small volume samples, which may contain a relatively high level of ethanol if not adequately dried. However, do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Do not dry the RNA pellet by centrifugation under vacuum (Speed-Vac).

17. Incompletely dissolving the final RNA pellet may result in low RNA yield.

18. Final preparation of RNA is free of DNA and proteins. It should have a A260/A280 ratio of 1.7.

19. If the A260/A280 ratio is <1.65, it may be because: (a) the amount of sample used for homogenization may have been too small; (b) samples may not have been allowed to stand at room temperature for 5 min after homogenization; (c) there may have been contamination of the aqueous phase with the phenol phase; (d) the final RNA pellet may not have been completely dissolved; or (e) the water used for dilution of RNA may be acidic. Acidic pH can affect the A280 reading and lowers absorbance ratios. Try to dilute the RNA samples in TE buffer and measure again.

20. Typical yields from tissues (μg RNA/mg tissue): liver and spleen, 6–10 μg; kidney, 3–4 μg; skeletal muscle and brain, 1–1.5 μg; placenta, 1–4 μg.

21. Typical yields from cultured cells (μg RNA/10^6 cells): epithelial cells, 8–15 μg; fibroblasts, 5–7 μg.

22. The rack should be floated on the water surface. If a plastic PCR rack cannot be found, the plastic racks for 200 μl tips or other racks can be used.

23. Completely thaw and thoroughly vortex the buffer prior to use.

24. Spin the tubes containing reagents briefly before opening them.

25. The extension temperature may be optimized between 37 and 42°C.

26. Water bath can also be used in stand of a thermal cycler.

27. It is better to add water first and add DNA polymerase last. Put the DNA polymerase back in the −20°C freezer as soon as possible after using.

28. Completely thaw and thoroughly vortex the buffer prior to use.

29. Spin the tubes containing reagents briefly before opening them.

30. Initial denaturation of longer than 2 min at 95°C is unnecessary and may reduce the yield.

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31. Annealing temperature should be optimized for each primer set based on the primer Tm.

32. The extension time should be at least 1 min/kb target length.

33. Housekeeping gene such as β-actin, GAPDH, and so on is also run to demonstrate equal loading.

34. An example profile of PCR parameters is given as follows.

    (a) Initial denaturation: at 95°C for 2 min for 1 cycle

    (b) Amplification (25–35 cycles)

    Denaturation: at 95°C for 0.5–1 min

    Annealing: at 42–65°C for 0.5–1 min

    Extension: at 72°C for 1 min/kb

    (c) Final extension: at 72°C for 5 min for 1 cycle

    (d) Soak at 4°C indefinite for 1 cycle

35. Make 1% (w/v) agarose gel: weigh 1 g of agarose and pour it into a beaker with 100 ml of 1× TAE or 1× TBE. Put the beaker into a microwave oven. Heat 30 s, take it out to mix, then put it back to the microwave oven. Repeat this step several times until all agarose is melted down. Let it stand at room temperature for several minutes to cool it down until your hand can hold the beaker. Then add ethidium bromide (EtBr) into the gel. EtBr is a potent mutagen that must be handled carefully to avoid skin contact and contamination of the lab. The amount of EtBr to add is as follows: of a 0.5 mg/ml stock solution, add 1/1,000 to your gel. For example, if we go back to our 100 ml gel, then you would add 100 μl of EtBr. Mix gently, then pour the gel onto the casting trays. Let the gel harden at room temperature before using.

36. The percentage of gel you run mainly depends on the size fragment of PCR product. See Table 1 for reference (3).

37. For reactions containing the 5X Green GoTaq® Reaction Buffer, load amplification reaction onto the gel directly after amplification. Do not need to add any more DNA loading buffer.

38. 2× iQ™ SYBR Green Supermix contains 2× reaction buffer with dNTPs, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers.

39. We use 20 μl as a total volume of one reaction to reduce the cost of experiments. It works well.

40. Directly add 1 μl of cDNA into the mixture in the wells and make sure no liquid clings to the tip when taking the tip out of the well.

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41. Make sure that the edges of the plate are sealed securely. The film on the four corners is very easily detached.
42. The parameters for traditional PCR can also be used for real-time PCR.
43. For each gene to be determined, a test using a few samples is needed before performing many samples to make sure that the parameters are suitable and only one peak is observed in the melting curve.
44. Usually the experimental protocol consists of four programs:
    - **(a)** Initial denaturation: at 95°C for 3 min for 1 cycle
    - **(b)** Amplification (40 cycles)

      Denaturation: at 95°C for 10 s

      Annealing: at 42–65°C for 0.5–1 min

      Extension: at 72°C for 1 min/kb
    - **(c)** Analysis of the melting curve to confirm the single product amplification during the PCR assay.
    - **(d)** Cooling the rotor and thermal chamber at 25°C.
45. If there is more than one peak in the melting curve, you need to either optimize the PCR parameters or redesign the primers.
46. The expression level of each gene can also be calculated as copy number or others such as nanomoles, nanograms, and so on. However, a standard curve is necessary for either method.
47. It is better to have 4–5 points in a standard curve if possible.

Acknowledgments

This work was partly supported by American Lung Association (RG-872-N), American Heart Association (086576D), KSEF-1686-RED-11, Health Effects Institute (4751-RFA-05-2/06-12), CTSPGP 20018 from University of Louisville, T32-ES011564, and ES01443.

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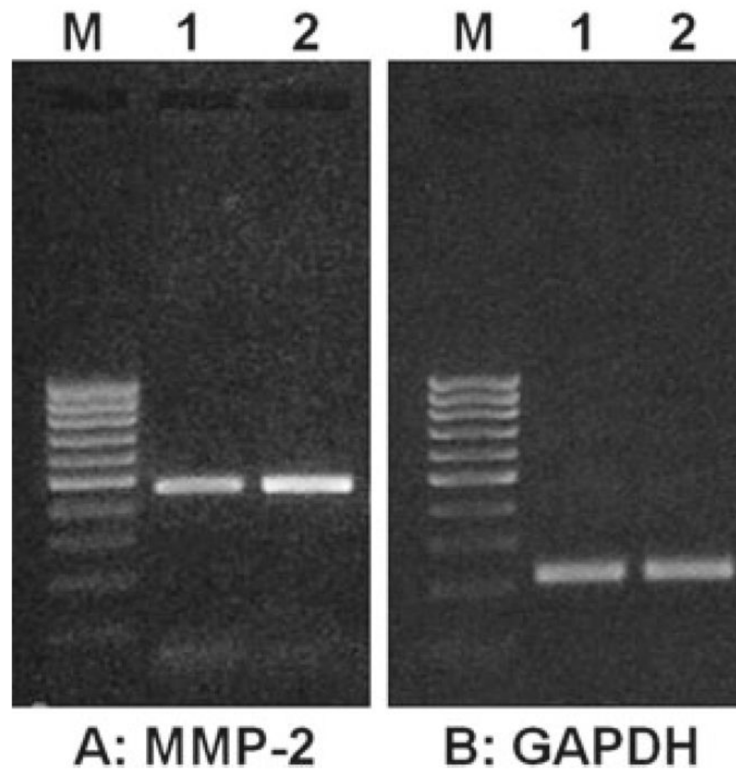

Fig. 1.
mRNA expression level of matrix metalloproteinase-2 (MMP-2) by RT-PCR. Total RNA was extracted from human monocytes U937 by using TRI Reagent and reverse transcripted to cDNA by using M-MLV reverse transcriptase (Promega). PCR was performed on a thermal cycler (Mastercycler, Eppendorf) by using GoTaq DNA polymerase (Promega). The PCR products were separated on 1% agarose gel. Housekeeping gene GAPDH was used as internal control. M, 100 bp DNA Ladder (Fisher); (*1*) cells without any treatment were used as control; (*2*) cells were treated with 5.0 μg/ml of cobalt nanoparticles for 24 h.

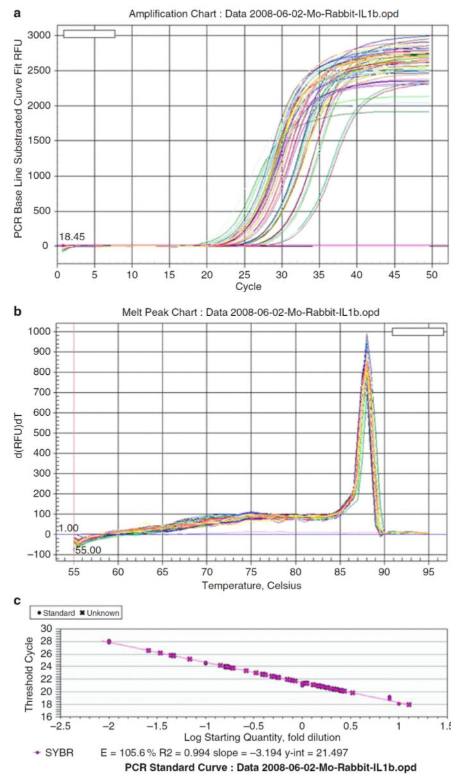

Fig. 2.

Real-time PCR for rabbit IL-1β. Rabbit ear skin wound tissues were used for total RNA isolation by TRI Reagent (Sigma) and reverse transcripted to cDNA by using M-MLV reverse transcriptase (Promega). Real-time PCR was performed on an iQ5 multicolor real-time PCR detection system (Bio-Rad) by using 2× iQ™ SYBR Green Supermix (Bio-Rad). (**a**) The amplification curves; (**b**) the melting curves. Single peak in the melting curve represents that only the real target gene is amplified; (**c**) the PCR standard curve (Color figure online).

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Table 1

The optimal concentrations of agarose gel for dsDNA separation

% Agarose (w/v)	Range of resolution Linear dsDNA (kbp)
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2