CHAPTER 8

RT-PCR of Human Genes

Introduction

The goal of this laboratory is to isolate and purify total human RNA and learn how to analyze gene expression. The source of RNA will be your cheek cells obtained from a saline mouthwash (a bloodless and non-invasive procedure). You will also learn how to work with RNA and determine its concentration and purity. The isolated RNA will be used in a RT-PCR (polymerase chain reaction) experiment for determining gene expression.

The exercise will be carried out in two laboratory periods. In the first period you will isolate and purify total RNA. The RNA will be analyzed using RNA gel electrophoresis for identifying major RNA species present in the cells. In the second laboratory period you will run an RT-PCR to determine expression of the β-actin gene in cheek cells. The product of this reaction will be analyzed by agarose gel electrophoresis.

Figure 8.1 presents an overall timetable for these experiments.

Background

Obtaining pure RNA is an essential step in the analysis of patterns of gene expression and understanding the mechanism of gene expression. Isolation of pure, intact RNA is one of the central techniques in today's molecular biology.

Two strategies of RNA isolation are usually employed: isolation of total RNA and isolation of mRNA. A typical eucaryotic cell contains approximately 10–20 pg of RNA, most of which is localized in the cytoplasm. Approximately 80–85 percent of eucaryotic RNA is ribosomal RNA, while 15–20 percent is composed of a variety of stable low molecular weight species such as transfer RNA and small nuclear RNA. Usually approximately 1–3 percent of the cell RNA is messenger RNA (mRNA) that is heterogeneous in size and base composition. Almost all eucaryotic mRNAs

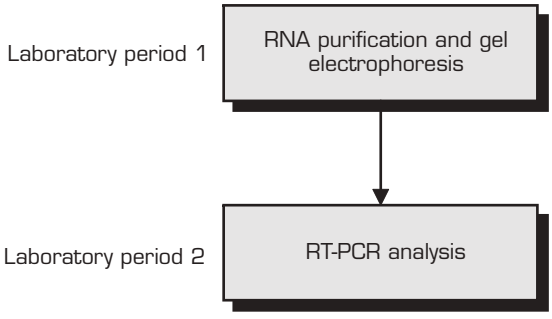

Figure 8.1 Schematic outline of the procedures used in the RT-PCR laboratory.

are monocystronic and contain a post-transcriptionally added poly-adenylic acid (poly A) tract at their 3′-terminal. This poly-A 3′-tail permits separation and isolation of mRNA from all other classes of RNA present in the cell.

FIRST LABORATORY PERIOD

Experiment 1: purification of total RNA

Introduction

The goal of this experiment is to isolate total RNA from cheek cells. We will use a simple and quick procedure for obtaining a sufficient quantity of cellular RNA for analyzing its purity, carrying out RNA gel electrophoresis, and studying gene expression using an RT-PCR.

Background

Isolation of total RNA is most frequently used when pure RNA is required for experiments. This is because the techniques are less laborious and require less time to perform than isolation of mRNA. Various techniques for purification of total RNA are now in use and their application depends on the nature of the RNA required. For example, if RNA is going to be used for a quantitative RT-PCR, the intactness of the purified RNA is not critical, whereas intact RNA is required for cDNA library preparation or Northern blot analysis. Complete removal of DNA contamination is critical if RNA is used in an RT-PCR, but is not important in *in vitro* translation.

The physical and chemical properties of RNA and DNA are very similar. Thus, the basic procedures used in RNA purification are similar to those of DNA. All of the RNA purification methods incorporate the following steps.

1. Disruption of cells or tissue.
2. Effective denaturation of nucleoprotein complexes and removal of proteins.
3. Concentration of RNA molecules.
4. Determination of the purity and integrity of isolated RNA.

In addition, methods must include procedures that remove co-purified DNA from the preparation. In contrast to DNA purification, guarding against physical shearing of RNA molecules is not necessary because RNA molecules are much smaller and much more flexible than DNA molecules. In RNA protocols, strong physical forces during cell and tissue breakage are frequently used and the use of wide-mouth pipettes is not required. However, RNA isolation is much more difficult than DNA purification largely due to the sensitivity of RNA to degradation by internal and external ribonucleases. These enzymes are omnipresent and are very stable molecules that do not require any co-factors for their function. A crucial aspect of any procedure for RNA purification is fast and irreversible inactivation of endogenous RNases and protection against contamination with exogenous RNase during the isolation procedure. To these ends, all extraction buffers

include powerful RNase inhibitors and all solutions and equipment used are treated to remove exogenous RNases.

Elimination of RNases

The most commonly used inhibitors included in extraction buffers for inhibiting endogenous RNase are as follows.

1. Strong protein denaturation agents. These include guanidinium hydrochloride and guanidinium isothiocyanate used at a concentration of 4 M. These chaotropic agents can quickly inactivate endogenous RNases and contribute to denaturation of nucleoprotein complexes. In order to denature RNase irreversibly by these compounds, a high concentration of 2-mercapthoethanol is also included. This type inhibitor will be used in our RNA preparation procedure.

2. Vanadyl–Ribonucleoside complexes. Oxovanadium IV ions form complexes with any ribonucleoside and bind to most RNases, inhibiting their activity (Berger and Birkenmeier, 1979). A 10 mM solution is used during cell breakage and is added to other buffers used in RNA isolation. Complexes can be used with deproteinizing agents (phenol or chloroform : isoamyl alcohol or CIA) and with chaotrophic agents. The compound is difficult to remove from purified RNA. Any residual amount of vanadyl will inhibit many enzymes used in subsequent RNA manipulations.

3. Aurintricarboxylic acid (ATA). This compound binds selectively to RNase and inhibits its activity. ATA is usually incorporated into extraction buffers used for bacterial RNA preparations (Hallick et al., 1977). The inhibitor can affect certain enzymes and is not used if RNA will be used in primer extension or S1 nuclease experiments.

4. Macaloid. This is a naturally occurring clay (sodium magnesium lithofluorosilicate). Being negatively charged, it strongly absorbs all RNase. The macaloid and bound RNase are removed from the preparation by centrifugation (Marcus and Halvorson, 1967).

5. Protein RNase inhibitors such as RNasin. A protein originally isolated from human placenta, it inhibits RNase by non-competitive binding. It cannot be used in extraction buffers containing a strong denaturant. It is usually included in solutions used in the later stages of purification or in buffers used in storage or subsequent RNA manipulations. We will be using this inhibitor in the RT-PCR experiment.

The most frequent sources of exogenous RNase contamination are one's hands and bacteria and fungi present on airborne dust particles. The most frequently used inhibitors for removing exogenous RNase contamination are as follows.

1. Diethyl pyrocarbonate (DEPC). DEPC causes enzyme inactivation by denaturing proteins. Inactivation of RNase is irreversible. The compound is used for removing RNase from solutions and glassware used in

RNA preparation. DEPC should be used with care because it is highly flammable and a suspected strong carcinogen.

2. RNaseZap™ or RNaseOff solutions. These commercially available reagents destroy RNases on contact very effectively. The decontamination solutions are not toxic and can be used for removing RNase from all surfaces and equipment. The compositions of these reagents are trade secrets.

Methods of RNA isolation

Three methods of RNA isolation or their modification are most frequently used: a guanidinium hot-phenol method, a high-salt lithium chloride method, and a TRI-Reagent™ method.

The guanidinium hot-phenol method is a modification of the procedure first described by Chirgwin et al. (1979) and Chomczynski and Sacchi (1987). This single-step extraction procedure takes advantage of the characteristic of RNA under acidic conditions to remain in the aqueous phase containing 4 M guanidine thiocyanate, while DNA and proteins are distributed into the phenol–chloroform organic phase. Distribution of DNA into the organic phase is particularly efficient if the DNA molecules are small. The method therefore uses a procedure for fragmenting DNA into molecules not larger than 10 kb. This method is used for isolating total RNA from a variety of procaryotic and eucaryotic cells. The efficiency of this method is very high (80–90 percent), affording purification of a large quantity of high-quality RNA.

The high-salt lithium chloride method is frequently used for isolating RNA from plant tissues that are particularly rich in various secondary products such as anthocyanins, phenolic compounds, polysaccharides, and latex. It has been shown that it is very difficult to isolate pure RNA from such plants using chaotropic agents (Schultz et al., 1994; Bugos et al., 1995). The procedure involves cell breakage in low pH, high salt buffer in the presence of RNase inhibitors. Protein and DNA are removed by acidic phenol–CIA extraction and RNA is recovered by lithium chloride precipitation.

The TRI-Reagent™ method is a single-step method of RNA isolation using a monophasic solution of phenol and guanidine isothiocyanate combined with precipitation of RNA by isopropanol in the presence of high salt (Chomczynski and Mackey, 1995). The method is particularly useful for fast isolation of RNA from numerous small samples and can be used with all types of cells and tissues. We will use a modification of this method (RNAwiz™).

Safety precautions

Each student should work only with his or her own cells. However, this restriction does not apply to purified RNA. Students that do not wish

to work with their own cells can work with RNA isolated by anybody in the class.

RNAwiz™ contains phenol and guanidinium isothiocyanate. Both reagents are harmful to the skin. The reagents can be rapidly absorbed by and are highly corrosive to the skin. It initially produces a white softened area, followed by severe burns. Because of the local anesthetic properties of phenol, skin burns may not be felt until there has been serious damage. Gloves should be worn when working with this reagent. Because some brands of gloves are soluble or permeable to phenol, they should be tested before use. If TRI-Reagent™ is spilled on the skin flush off immediately with a large amount of water and treat with a 70 percent solution of PEG (polyethylene glycol) 4000 in water. Used reagent should be collected into a tightly closed glass receptacle and stored in a chemical hood until proper disposal.

Technical tips

The success of this experiment critically depends on rigorous control of RNase contamination. In order to prevent contamination of equipment and solutions with RNase, the following precautions should be taken.

1. Students should wear gloves at all time. Because gloves can be easily contaminated with RNase they should be changed frequently.

2. All tubes should be kept closed at all times.

3. Whenever possible disposable, certified RNase-free tubes, pipette tips, and plasticware should be used. Regular microfuge tubes and tips usually are not contaminated with RNase and they do not require special treatment if they are used from unopened bags.

4. All glassware should be treated with 0.1 percent DEPC water solution and autoclaved to remove DEPC. It is also possible to inactivate RNase by baking glassware at 180°C for at least 2 hours or overnight. Alternatively, RNase can be easily and efficiently eliminated from glassware, countertops, pipettors, and plastic surfaces using RNaseZap™ solution.

5. All solutions should be made with DEPC-treated water. Deionized water from a MilliQ RG apparatus can be used directly in all applications instead of DEPC-treated water because it does not contain RNase.

6. Since RNase treatment is frequently used in DNA isolation procedures, gel electrophoresis of DNA can cause electrophoresis tray and gel box contamination. Before their use for RNA gel electrophoresis, the gel tray and box should be decontaminated. To decontaminate the gel apparatus and gel-casting trays, treat them with RNaseZap™ solution. Instead of RNaseZap™ solution, the electrophoresis equipment can be treated with 0.2 N NaOH for 15 minutes and rinsed before use with RNase-free water.

Protocol

Collecting human cheek cells

1. Pour 13 ml of PBS into a 15 ml conical centrifuge tube. Transfer the solution into a paper cup. Pour all the solution into your mouth and swish vigorously for 30–40 seconds. Expel the PBS wash back into the paper cup.
2. Transfer the solution from the paper cup into a 25 ml Corex centrifuge tube and place it on ice.
3. Collect the cells by centrifugation at 6,000 r.p.m. for 10 minutes at 4°C.
4. Pour as much supernatant as possible back into the paper cup. Be careful not to disturb the cell pellet. Discard the supernatant from the paper cup into the sink. Invert the Corex centrifuge tube with cells on a paper towel to remove the remaining PBS.

RNA purification

1. Add 700 µl of RNAwiz™. Close the tube and mix by vortexing. Incubate for 5 minutes at room temperature. **Transfer the solution into a microfuge tube.**
2. Add 140 µl (0.2 volumes) of chloroform (not CIA) and mix by vortexing for 20 seconds. Incubate at room temperature for 10–15 minutes.
3. Centrifuge for 10 minutes at room temperature. After centrifugation, the mixture will be separated to two phases: a bottom phase containing chloroform and an upper aqueous phase containing RNA. **Note**: if an aqueous phase does not appear, add 100 µl of chloroform, vortex it for 20 seconds and repeat step 3.
4. Without disturbing the interphase, transfer the top aqueous phase to a fresh RNase-free microfuge tube. Add 350 µl (0.5 of starting volume) of RNase-free water and mix well by inverting the tube several times. Divide the solution into two new tubes. **Note**: if the combined volume of aqueous phase and water is less then 800 µl it is not necessary to divide this mixture into two tubes. Instead add 700 µl of isopropanol to the tube, mix well, and let it stand at room temperature for 10 minutes. Next, follow the procedure from step 10.
5. Precipitate RNA by the addition of 350 µl of isopropanol to each tube. Mix by inverting the tubes several times and incubate at room temperature for 10 minutes.
6. Place the tubes into the centrifuge, orienting the attached end of the tube lid away from the center of rotation. Centrifuge for 15 minutes at room temperature to pellet the RNA.
7. Remove the tubes from the centrifuge. Remove the supernatant using a P200 Pipetman. Wash the pellet with 700 µl of cold 70 percent ethanol. Add ethanol to each tubes and mix by inverting several times.

8. Place the tubes into a microfuge and centrifuge for 5 minutes at room temperature. Remove ethanol with a P200 Pipetman.

9. Place the tubes into the centrifuge, making sure that the side containing the pellet faces away from the center of rotation. Start the centrifuge until it reaches 500 r.p.m. (1–2 seconds). This will collect ethanol from the sides of the tube. Remove ethanol using a P200 Pipetman equipped with capillary tip.

10. Prewarm RNAsecure solution in a 60°C water bath for 5 minutes. Add 15 µl of this solution to one tube and dissolve the pelleted RNA. Transfer the solution to the second tube and dissolve the pellet.

11. As fast as possible heat the sample to 60°C for 10 minutes to inactivate potential RNase contamination. Store the RNA sample at –70°C. **Note**: since RNAsecure only inactivates RNases at 60°C the best results are obtained using prewarmed solution and transferring the tube as quickly as possible to a 60°C water bath.

12. Determine the concentration of RNA by measuring the absorbance at 260 nm. Initially use a 1 : 100 dilution of the sample in PBS. The absorbance reading should be in the range 0.1–1.5. Calculate the concentration of RNA using the equation $N = A_{260}/\varepsilon_{260}$, where N is the RNA concentration in micrograms per milliliter, ε_{260} is the RNA extinction coefficient, and A_{260} is the absorbance reading (corrected for dilution). The absorption coefficient for total RNA is usually taken to be $0.025\,\mu g^{-1}\,cm^{-1}$ giving a solution of $40\,\mu g\,ml^{-1}$ of RNA an absorbance of 1.0 (e.g. $1/0.025 = 40\,\mu g\,ml^{-1}$).

13. To determine the purity of the RNA, measure the absorbance at 260 nm, 280 nm, and 234 nm and calculate the 260 nm : 280 nm and 260 nm : 234 nm ratios. The concentration of your RNA should be between 0.1 and $0.3\,\mu g\,\mu l^{-1}$.

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Experiment 2: RNA agarose gel electrophoresis

Introduction

In this experiment you will continue the analysis of total RNA using agarose gel electrophoresis. We will use a native agarose gel system that is easy to prepare and does not use toxic chemicals. You will run a mini-gel that is sufficient for demonstrating the presence of stable species of RNA in your preparation. Native gels can also be used as an analytical tool for assessing the efficiency of RNA purification.

Background

Gel electrophoresis of RNA molecules requires techniques that are different from those used for DNA. In order to separate RNA molecules according to their size it is necessary to maintain their complete denaturation before and during electrophoresis. Non-denatured RNA can form secondary structures such as "hairpins" that profoundly influence their electrophoretic mobility. A number of denaturants have been used. Among these are glyoxal with DMSO (McMaster and Carmicheal, 1977), formaldehyde (Lehrbach et al., 1977; Rave et al., 1979), and methylmercuric hydroxide (Bailey and Davidson, 1977; Thomas, 1980). Formaldehyde and glyoxal-DMSO are presently used more often than the highly toxic methylmercuric hydroxide.

The buffers used for RNA electrophoresis differ from those used for DNA. These buffers are of very low ionic strength, frequently resulting in the creation of a pH gradient along the length of the gel that causes overheating of the gel and distortion of RNA bands. To prevent this, RNA gels are usually run at low field strength ($<5\,V\,cm^{-1}$) using a large volume of buffer and constant stirring to prevent gradient formation.

It is also possible to run native RNA agarose gels that do not include toxic denaturants in the agarose gel. The native gel system is simple and, in general, does not affect electrophoretic separation of RNA (Liu and Chou, 1990).

The glyoxal-DMSO method does not use toxic chemicals, but it is more difficult to use than the native method. This method requires very careful

control of pH during electrophoresis to a pH below 8. This is because glyoxal denatures RNA by binding covalently to the guanine residue, forming products that are stable only at a pH below 8. At a pH above 8, glyoxal dissociates from RNA. Submarine gels require continuous recirculation and mixing of electrophoresis buffer in order to maintain the pH within an acceptable limit. In addition, commercially available glyoxal must be purified before use in order to remove glyoxylic acid, which is readily formed by oxidation and degrades RNA. The electrophoresis time is longer than for native gels.

Safety precautions

Agarose gel contains ethidium bromide, which is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Students should wear gloves when handling ethidium bromide solution and gels containing ethidium bromide. Discard the used gel into the designated container.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench. To avoid electric shock always disconnect the red (positive) lead first.

Ultraviolet (UV) light can damage the retina of the eye and cause severe sunburn. Always use safety glasses and a protective face shield to view the gel. Work in gloves and wear a long-sleeved shirt or laboratory coat when operating UV illuminators.

Technical tips

The most common problems with gel electrophoresis of RNA are inadequate denaturation of the samples and overloading the gel with RNA. Inadequate denaturation will appear either as multiple rRNA bands or rRNA bands that appear to be smeared but at a correct ratio. Overloading the gel will result in very broad rRNA bands that run on the gel with excessive "smearing." Bands could have a U-shape appearance and their mobility might be faster than expected from their base number.

Sample degradation will be indicated by an incorrect ratio between 28S and 18S rRNA or, in more severe cases, the total disappearance of these bands. Students can use even a severely degraded sample for the RT-PCR experiment.

Figure 8.2 presents an image of native gel electrophoresis of total human RNA isolated from cheek cells.

Protocol

1. Prepare a mini-gel using a casting tray not larger than 7.5 cm × 7.5 cm and a thin gel (0.2 cm). Seal the ends of the gel-casting tray with tape.

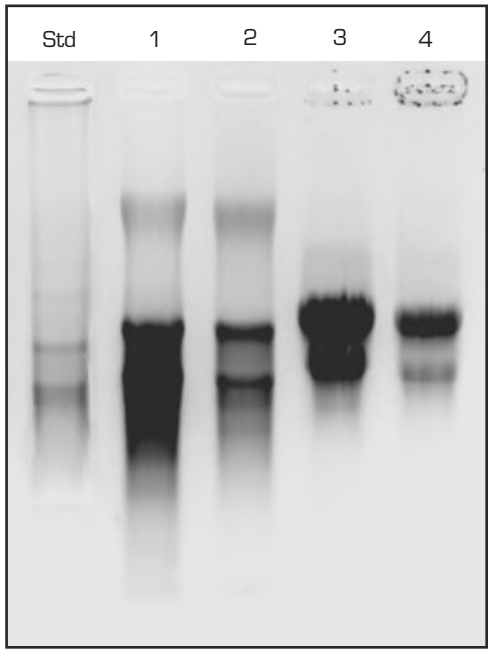

Figure 8.2 Native agarose gel electrophoresis of human total RNA. Lane 1 standard RNA and lanes 2–4 RNA isolated from cheek cells. Electrophoresis was carried out on a 1 percent agarose gel for 1 hour at 5 V cm^{-1}.

Regular labeling tape or electrical insulation tape can be used. Use a mini-gel comb with wells 0.2–0.5 cm long and 1 mm (or less) wide. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom. To adjust this height it is most convenient to place a plastic charge card (for example MasterCard) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb. Wipe the comb with RNaseZap™ immediately before use.

2. Prepare 500 ml of one times TBE (Tris–borate EDTA) buffer by adding 50 ml of a ten times TBE stock solution to 490 ml of RNase-free water.

3. Prepare a 1 percent agarose gel. Place 15 ml of the buffer into a 100 ml flask and add 150 mg of agarose powder. Melt the agarose by heating the solution in a microwave oven at full power for 1–2 minutes until the agarose is fully dissolved. If evaporation or spillage occurs during melting, adjust the volume to 15 ml with deionized water.

4. Cool the agarose solution to approximately 60°C and add 1 µl of ethidium bromide stock solution. Slowly pour the agarose into the casting tray. Remove any air bubbles by trapping them in a 10 ml pipette. Place the comb 1 cm away from one end of the gel. Allow the gel to solidify for 20–30 minutes. **Note**: native agarose gels should be as thin as possible (2–3 mm) in order to shorten the electrophoresis time.

5. Remove the tape from the ends of the gel-casting tray and place the tray on the central supporting platform of the gel box. Add electrophoresis buffer until it reaches a level approximately 2–3 mm above the surface of the gel.

6. Prepare the RNA sample for electrophoresis in a 0.2 ml thin-wall, RNase-free tube. Prepare the sample as follows. Add 2 µl of five times RNA sample buffer to a sterile microfuge tube and 1 or 2 µl of your RNA and fill it up with sterile RNase-free water to a total volume of 10 µl.

7. Place the tube into a LightCycler and incubate at 65°C for 10 minutes. Transfer it immediately to ice. Incubate on ice for at least 2 minutes. Centrifuge the tube for 20 seconds to collect condensation and place the tube back on ice until ready to load onto the gel.

8. Load the samples into the wells using a yellow, RNase-free tip. Place the tip **under** the surface of the electrophoresis buffer and **above** the well. Expel the sample slowly, allowing it to sink to the bottom of the well. Take care not to spill the sample into a neighboring well. During sample loading, it is very important not to place the end of the tip into the sample well or touch the edge of the well with the tip. This can damage the well, resulting in uneven or smeared bands.

9. Place the lid on the gel box and connect the electrodes. RNA will travel towards the positive (red) electrode. Turn on the power supply. Adjust the voltage to approximately 5 V cm^{-1}. For example, if the distance between electrodes (not the gel length) is 20 cm, in order to obtain a field strength of 5 V cm^{-1} the voltage should be set to 100 V. Continue electrophoresis until bromophenol blue moves at least two-thirds of the length of the gel. It will take the tracking dye approximately 30 minutes to reach this position.

10. Turn the power supply off and first disconnect the positive (red) and then the negative lead from the power supply. This order of disconnecting leads prevents the occurrence of accidental electrical shock. Remove the gel from the electrophoresis chamber. You can photograph the gel using a computer imaging system to record the results.

11. Two sharp bands will appear on the gel, 28S RNA (4.7 kb) and 18S RNA (1.9 kb). The 5S and 5.8S RNA bands are located on the leading edge of the gel, running together with tRNA. **Note**: the integrity of the prepared RNA samples and lack of RNA degradation can be easily judged from the appearance of rRNA bands. Degradation of the sample appears as diffused 28S and 18S bands or an incorrect ratio of stain between rRNA bands. This ratio should be approximately 2 : 1 for 28S and 18S, respectively (see Fig. 8.2).

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SECOND LABORATORY PERIOD

Experiment 3: running an RT-PCR

Introduction

During this laboratory period you will perform an RT-PCR using the RNA purified in experiment 1. You will analyze β-actin genes expression in cheek cells. There are six known actin proteins in mammalian cells: two sarcomeric muscle actins (α-skeletal and α-cardiac), two smooth muscle actins (α and γ), and two non-muscle cytoskeletal actins (β and γ). Three genes have been mapped for human actin protein: β-actin gene on chromosome 7, α-skeletal actin gene on chromosome 1, and α-cardiac antigen on chromosome 15. The β-actin gene on chromosome 7 was mapped to the 7p15 position. In addition to one functioning β-actin gene, there are approximately 20 pseudo-genes widely distributed in the human genome. Only four β-actin pseudo-genes were mapped to other chromosomes (chromosomes 5, 13, and 18).

We will be using the "One Tube RT-PCR Kit" from Roche Molecular Biochemicals Co. This kit is designed for sensitive, quick, and reproducible analysis of RNA with high fidelity. The one step reaction system uses avian myoblastosis virus (AMV) transcriptase for first-strand synthesis and Expand^{TM} High Fidelity enzyme blend, which consists of *Taq* DNA polymerase and *Pwo* DNA polymerase, for the PCR.

The expected length of the product of our RT-PCR reaction should be 557 bases (see Figure 8.3 for details). Products of different size can also appear. This will depend on the structure of your gene (a missing processing site at the intron–exon boundary) or partial expression of pseudo-genes (mRNA synthesis).

Background

Sensitive methods for the detection and analysis of RNA molecules are an important aspect of most cell/molecular biology studies. Commonly used methods include *in situ* hybridization, Northern blots, dot or slot blot analysis, S1 nuclease analysis, and RNase protection assays. *In situ* hybridization is very sensitive, but is a rather difficult technique. Other common methods lack sensitivity for detecting small amounts of RNA.

The adaptation of PCR methodology to the investigation of RNA provides a method having speed, efficiency, specificity, and sensitivity. Since RNA cannot serve as a template for a PCR, reverse transcription is combined with PCR to make RNA into a complementary DNA (cDNA) suitable for PCR. The combination of both techniques is named RT-PCR.

The process of RT-PCR has proven to be invaluable for detecting gene expression, for amplifying RNA sequences prior to subcloning and analysis, and for the diagnosis of infectious agents or genetic diseases. This technique is the most sensitive procedure for determining the presence or absence of RNA templates or quantifying the level of gene expression. Furthermore, RT-PCR allows cloning of rare messages without having to construct cDNA libraries. The use of RT-PCR for analyzing genetic diseases is particularly advantageous because it circumvents inefficient amplification of long DNA fragments caused by long introns and provides additional information about phenomena such as alternative splicing.

Two different techniques are used for RT-PCR.

1. **Two step RT-PCR**. The synthesis of cDNA is performed with reverse transcriptase from AMV, moloney murine leukemia virus (M-MuLV), or *Thermus thermophilus* (*Tth*) DNA polymerase in the first step, followed by PCR with an appropriate thermostable DNA polymerase. The two-step reaction requires that the reaction tube is opened after cDNA synthesis and reagents are added for the PCR part of the procedure. This is inconvenient and increases the risk of contamination.
2. **One-step RT-PCR**. The cDNA synthesis and the PCR are performed together in a single tube. Two techniques are in use for running one-step RT-PCRs. The first technique uses *Tth* DNA polymerase for carrying reverse transcription and PCR reactions. The second uses AMV reverse transcriptase and *Taq* DNA polymerase.

The first method relies on the ability of the *Tth* DNA polymerase to use an RNA template for DNA synthesis (reverse transcription) as well as to use a DNA template (PCR). This enzyme is thermostable allowing both reactions to be carried at elevated temperature. Carrying out the reaction at elevated temperature helps unravel secondary structures of RNAs, thereby allowing the synthesis of longer products and increasing the efficiency synthesis of the first strand of DNA. However, low processivity of the *Tth* DNA polymerase limits the RT-PCR products to less than 1.0 kb.

The second method uses AMV reverse transcriptase and *Taq* DNA polymerase. This technique allows amplification of fragments of up to 2.0 kb with lower error rates as compared to the use of *Tth* DNA polymerase. However, the reverse transcription step of the reaction has to be performed at 42°C and, therefore, is strongly affected by the secondary structure of mRNA.

Reverse transcriptases

Reverse transcriptases are RNA-dependent DNA polymerases that have predominantly been used for catalyzing first-strand cDNA (complementary DNA) synthesis. However, reverse transcriptases are also capable of synthesizing a DNA strand complementary to a primed single-stranded DNA.

Three different enzymes with reverse transcriptase activity are now

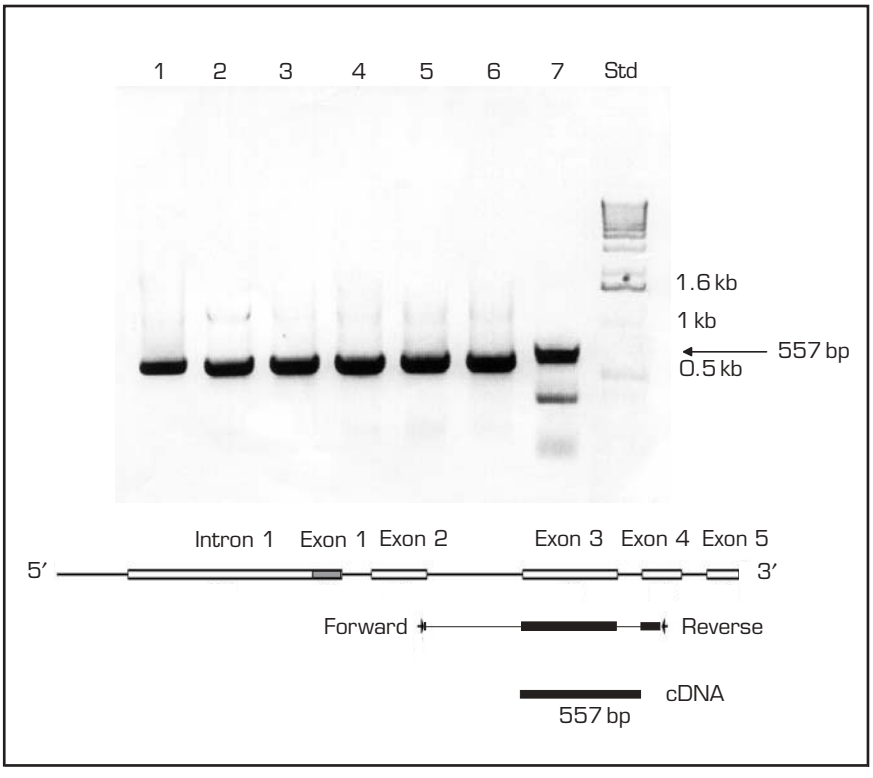

Figure 8.3 Agarose gel electrophoresis of the products of RT-PCR reactions. The structure of genomic DNA encoding the human gene for β-actin is shown. The size of the expected cDNA and the position of primers in the genomic DNA are indicated. The expected product of PCR amplification, when genomic DNA is used, is 1,200 bp.

commercially available: the viral reverse transcriptases (RTases) from avian myoblastosis virus (AMV), M-MuLV, and the heat-stable DNA polymerase derived from *T. thermophilus*. All these enzymes require different pH, salt concentration, and incubation temperatures for optimal activity.

The AMV and M-MuLV viral RTases are highly processive and are able to synthesize cDNAs of up to 10 kb. *Tth* DNA polymerase is able to synthesize cDNA in the range of 1.0–2.0 kb, which is sufficient since fragments of <1 kb are usually used for PCRs.

The unique advantage of the *Tth* DNA polymerase is its ability to perform both reverse transcription and PCR amplification in a one-step reaction.

Priming of a reverse transcriptase reaction

There are three types of primers that may be used for reverse transcription.
1. Oligo(dT)12–18 primer. This primer binds to the endogenous poly

(A)+ tail at the 3′-end of mammalian mRNA. A reaction with this primer frequently produces a full-length cDNA product.

2. **Random hexanucleotide primers**. These primers can bind to mRNA templates at any complementary site and will give partial length (short) cDNAs. These primers may be better for overcoming the difficulties caused by template secondary structure. The random primers may also transcribe more 5′-regions of the RNA.
3. **Specific oligonucleotide primers**. These primers can be used for selectively priming the RNA of interest. This approach has been used very successfully in diagnostic assays, as well as in basic research. We will be using specific primers for human β-actin genes.

Safety precautions

The agarose gel contains ethidium bromide, which is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Students should wear gloves when handling ethidium bromide solution and gels containing ethidium bromide. Discard the used gel into the designated container.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench. To avoid electric shock always disconnect the red (positive) lead first.

UV light can damage the retina of the eye and cause severe sunburn. Always use safety glasses and a protective face shield to view the gel. Work in gloves and wear a long-sleeved shirt or laboratory coat when operating UV illuminators.

Technical tips

The Titan one-tube RT-PCR Kit from Roche Molecular Biochemicals Co. is used in this experiment. The reverse transcriptase reaction is performed using AMV polymerase at elevated temperatures minimizing the influence of the secondary structure of mRNA on the synthesis of the first strand. The Expand^{TM} High Fidelity enzyme blend is used for the PCR part of the reaction. Synthesis of cDNA is much faster than in the two-step system and also faster than synthesis achieved by many other one-step kits. This makes it an ideal kit for use in a class environment when performing RT-PCR reactions and product analysis during the same laboratory period.

RT-PCR reactions should when possible make use of primers that do not amplify genomic DNA or produce fragments that are larger than fragments amplified from cDNA. This is possible if primers are positioned in different exons with very large introns located between them. This is necessary because it is very difficult to remove genomic DNA from purified RNA to the

Table 8.1 Master mix 1 (MX1)

Ingredient concentration	For one reaction	For three reactions	Final concentration
4dNTP (ten times)	2.0µl	6.0µl	200.0mM
DTT	1.0µl	3.0µl	5.0mM
RNase inhibitor	0.5µl	1.5µl	5.0 units
Primers mix	1.0µl	3.0µl	20.0µM
RNA	up to 3.0µl	–	At least 150.0ng
Water	5.0µl	15.0µl	
Total	12.5µl	28.5.0µl	

Table 8.2 Master mix 2 (MX2)

Ingredient concentration	For one reaction	For three reactions	Final concentration
Five times RT-PCR buffer	5.0µl	15.0µl	One times
Enzyme mix	0.5µl	1.5µl	One times
Water	7.0µl	21.0µl	
Total	12.5µl	37.5µl	

extent that it will not be amplified by a PCR. This principle is very well illustrated in the experiment described. The amplification of residual genomic DNA, when present in RNA, should result in a 1,200 bp DNA fragment, while amplification of cDNA results in a 557 bp fragment (see Figure 8.3). However, the presence of a larger than expected fragment is not always the result of the amplification of contaminating genomic DNA. These fragments can result from amplification cDNA obtained from unprocessed RNA or RNA originating from pseudo-genes.

Protocol

1. Label two RNase-free 1.5 ml microfuge tubes MX1 and MX2 (master mix 1 and master mix 2, respectively). Place the tubes into an ice bucket.
2. Prepare master mix 1 and master mix 2 as described in Tables 8.1 and 8.2. Remember to start assembling the reactions by the addition of water and buffer. Add the enzyme (for MX2) last. Mix all of the ingredients by pipetting up and down. Centrifuge the tubes for 30 seconds.
3. You will need to run your RNA in a single reaction. Therefore, you will have two reactions per group. Prepare three reactions per group in order to compensate for pipetting errors.
4. Label two RNase-free thin-walled tubes with your group number and

Table 8.3 RT-PCRs

Ingredient cencentration	Your reaction	Partner's reaction	Final concentration
MX1 mix	9.5 µl	9.5 µl	One times
MX2 mix	12.5 µl	12.5 µl	One times
Your RNA	3.0 µl	–	100–200 ng
Partner's RNA	–	3.0 µl	100–200 ng
Total	25.0 µl	25.0 µl	

place them on ice. Assemble RT-PCR reactions in these tubes using MX1 and MX2 as indicated in Table 8.3. Mix by pipetting up and down and centrifuge briefly.

5. Place the tubes into the thermocycler and run the RT-PCR reaction using the following program: 50°C for 30 minutes and 94°C for 30 seconds. Cycle 1 with denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and elongation at 68°C for 1 minute ($n = 10$). Cycle 2 with denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and elongation at 68°C for 2.5 minutes ($n = 25$) and end with 5 minutes at 68°C. Store tubes in a –70°C freezer.

Analysis of the results of the RT-PCR

1. Prepare a mini-gel using a casting tray not larger than 7.5 cm × 7.5 cm. Seal the ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom. To adjust this height, it is most convenient to place a plastic charge card (for example MasterCard) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb.

2. Prepare a 1.6 percent agarose mini-gel in one times TAE (Tris–acetate EDTA) buffer. Use 30 ml of agarose solution. Weigh 480 mg of agarose and add it to 30 ml of one times TAE buffer. Dissolve agarose in a microwave oven, adjust the volume to 30 ml with water, and add 1 µl of ethidium bromide. Pour the agarose into the casting tray. Allow the gel to solidify for 20–30 minutes.

3. Add 5 µl of stop solution to each tube with PCR reactions. Mix by pipetting up and down. Load prepared samples onto the gel. If only two or three samples are loaded onto the gel, use wells in the center of the gel.

4. Load 6 µl of a size standard (1 kb ladder) into the well to the left of your samples. Run gel electrophoresis for 20–30 minutes at 60–80 V.
Photograph the gel and analyze the results.

5. A typical result of an RT-PCR gel is presented in Fig. 8.3. Line 7 contains a RT-PCR using mRNA of human β-actin.