Biology 446-1
Laboratory Exercise 7
RT-PCR

Objective

To gain experience in total RNA extraction from cultured cells and RT-PCR.

Background

Reverse transcription-polymerase chain reaction (RT-PCR) is a technique used by biologists to amplify a large amount of DNA from RNA template. Since an important step in gene expression is the transcription of DNA into RNA, the expression of a certain gene can be monitored if one can detect the presence of the transcribed RNA in the cell. Furthermore, pre-mRNA splicing is essential in modulating gene functions. Thus, RT-PCR can be used to detect the splicing variants of a gene for studying the control of alternative splicing.

Build on your experience with PCR, you will carried an *in vitro* reverse transcription experiment which uses RNA-directed DNA polymerase (reverse transcriptase) to synthesize single strand DNA from RNA templates. Reverse transcriptase was discovered independently by Howard Temin and David Baltimore in 1970s and for this discovery they received the 1975 Nobel Prize in Physiology and Medicine. This type of enzyme was first found in viruses, but later reverse transcriptase was also found in both prokaryotes and eukaryotes. In research laboratories, commonly used reverse transcriptases include HIV-1 reverse transcriptase from human immunodeficiency virus type 1, AMV reverse transcriptase from avian myeloblastosis virus, and M-MLV reverse transcriptase from Moloney murine leukemia virus. The main function of reverse transcriptase that is utilized in RT-PCR is the enzyme's ability to synthesize DNA single strand from 5' end to 3' end complementary to the RNA template. However, the enzyme also contains an N-terminal domain with DNA-dependant DNA polymerase activity and a C-terminal RNase H domain that may be (or may be not) active when separated form the N-terminal domain.

The normal optimal reaction temperature of the reverse transcription is 37 °C. The amount of the starting RNA template required depends on both the activity of enzyme and the property of the RNA template. For typical reverse transcription, if mRNA with poly(A) is used as template, the amount of template should be 1 pg to 100 ng. If total RNA is used as template, the amount should be about 1 μg. The product of the reverse transcription is single strand DNA which is to be used as template in a normal PCR reaction to generate double strand DNA. In the PCR step, the amount of the single strand DNA template may need to be optimized, but usually, less than 10% of the RT product is enough in a subsequent PCR amplification.

The reverse transcription and the PCR can also be carried out in one reaction mixture. Either way, the inactivation of the reverse transcriptase is necessary because the enzyme may inhibit the PCR reaction. However, heating the PCR mixture to 95 °C and keeping it at 95 °C for 5 minutes at the beginning of the PCR amplification is enough to denature the reverse transcriptase. If the RT step and the PCR step are carried out separately, the single strand DNA can also be purified first.

Compared with DNA polymerase, the error rate of reverse transcription is much higher because of reverse transcriptase lacks proofreading activity. Like with DNA polymerase, protein engineering has been used to improve the property of reverse transcriptase, such as fidelity and activity. In this lab exercise, the enzyme we are going used to catalyze the RT reaction is a recombinant heterodimeric enzyme expressed in *E. coli*.

Like DNA polymerase, reverse transcriptase does not work without a primer. Unlike in PCR, only one primer is needed in a reverse transcription reaction since only single strand DNA needs to be made. For synthesizing the anti-sense strand of DNA complementary to an mRNA, three types of deoxyribonucleic acid polymer can be used as primers, that is, gene specific, poly-T, and random hexamers primers. Gene specific primers are usually 20 to 30 nucleotides in length and complementary to the 3' end of the coding strand. It can be used to amplify the coding sequence of a particular gene from an mRNA pool. If this type of primer is used, the amount of primer in one reaction should be about 5 – 20 pico mole. Oligo(dT) is normally 12 to 18 nucleotides long and complementary to the polyA tails in mature mRNAs. A poly-T primer can start the reverse transcription of all mRNAs in the pool and the amount of primer used in the reaction should be higher, about 0.1 – 0.5 µg. A random hexamer-primer is a mixture of "all possible combinations" of A, T, C and G in all six positions in the oligo nucleotide. Thus, a random of primer can initiate the reverse transcription in any place along any mRNA. Hence, the highest amount primer is required in a RT reaction with a random primer, about 1 - 5 µg.

A specific primer is the best choice when the transcription of a particular gene is in question. A poly-T primer is generally used in a reverse transcription reaction to construct a cDNA library. In this lab exercise, gene specific primers will be used to amplify cDNA of the gene bcl-x. A primer complementary to the 3' end of the gene will be used in RT reaction and primers complementary to the 5' and 3' end of the gene will be used in the subsequent PCR reaction. Bcl-x is alternatively spliced to long of short forms that promote cell survive and apoptosis, respectively. In this exercise, the RT-PCR is expected to result in a long form product of 435 bp and a short form product of 246 bp.

In numerous research projects, RT-PCR technique is used to detect the transcription of genes and upstream RNA extraction from cells is required. These are the major steps in RNA extraction: first, lyse the cells by either physical or chemical method; second, purify the DNA/RNA from the lysate; third, get rid of the DNA by DNase digestion and/or RNA purification with columns. The final RNA purity and concentration can be measured by UV spectrophotometer and gel electrophoresis if the quality of the RNA is in question. In this lab, an RNA purification kit will be used to extract total RNA from cell lysate. The kit come with gDNA spin columns to bind and clean up the genomic DNA of the cells which is faster than DNase digestion. A second column from the kit contains a silica-gel-membrane that will bind RNA in hydrophobic condition and elute RNA in hydrophilic environment.

You need to be very careful when working with RNA because RNase contamination from your hand and breath can easily destroy the RNA and you

experiment. RNase is a large family of enzymes including RNase A, RNase B, RNase C, RNase 1, RNase T1, RNase T2, RNase H, S1 nuclease, etc. Different inhibitors are available to inhibit certain types of RNase. DEPC (diethyl pyrocarbonate) is one of the most commonly used chemicals in cleaning RNase in glassware and lab plastics. It is a highly active alkylating agent that can destroys the enzymatic activity of RNase mainly by ethoxyformylation of histidyl groups. The working concentration of DEPC solution is 0.1% in H_2O. Glassware and plastics are soaked in the solution for 1 hour at 37℃ or overnight at room temperature. Then the items are rinsed with RNase free water. DEPC may influence the activity of other enzymes. It can also carboxymethylate unpaired adenine in RNA. Thus, it must be eliminated before the lab-wares are used. This can be done by autoclaving because DEPC will hydrolyze at high temperature into CH_3CH_2OH and CO_2. Guanidinium salts, such as guanidinium isothiocyanate and guanidinium chloride, are effective RNase inhibitors at high concentration because they will destroy the three-dimensional structure of proteins. In this lab exercise, guanidinium isothiocyanate is present in the lysis buffer. It will immediately inactivate RNase after cells are lysed. However, even with the usage of RNase inhibitors, it is very important to avoid contaminating the samples with RNase. You will carry out most of the procedures in a clean biological safety cabinet. You will need to cover your skin and ware masks.

Protocol

Before the Lab day, HeLa cells should be cultured in 100×20 mm cell culture dish to 60% - 90% confluence with DMEM containing FBS, penicillin and streptomycin.

Lab day 1

Most of the manipulations are done in biological safety cabinet.

1. Remove all the DMEM from the cell culture dish by pipet or vacuum.
2. Mix 6μl β-mercaptoethanol and 600μl Buffer RLT in a 1.5 ml RNase-Free microcentrifuge tube and then apply the mixture to the culture dish with the cells.
3. Scrape off the cells from the dish in the buffer with a cell lifter.
4. Slightly lift one side of the dish and collect all of buffer with the cells from the opposite side of the dish. Lyse cells by passing them 10 to 20 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
5. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30 s at ≥ 8000 g (10000 rpm). Discard the column and save the flow-through.
6. Add 1 volume (600μl) of 70% ethanol to the flow-through and mix well by pipetting.
7. Transfer up to 700 μl of the sample including any precipitate that may have formed to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 s at ≥ 8000 g (10000 rpm). Discard the flow-through. As the sample volume from step 6 exceeds 700μl, centrifuge the

remaining sample in the same RNeasy spin column and discard the flow-through after each centrifugation.

8. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15 s at ≥ 8000 g (10000 rpm) to wash the spin column membrane. Discard the flow-through.
9. Mix 800 µl of 100% ethanol and 200 µl of 5× concentrate Buffer RPE. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 15 s at ≥ 8000 g (10000 rpm) to wash the spin column membrane. Discard the flow-through.
10. Wash the spin column membrane a second time with 500 µl Buffer RPE.
11. Optional: Place the RNeasy spin column in a new 2 ml collection tube and centrifuge at full speed for 1 min.
12. Place the RNeasy spin column in a new RNase-free 1.5 ml collection tube. Add 20 µl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at ≥ 8000 g (10000 rpm) to elute the RNA. Again add 15 µl RNase-free water directly to the spin column membrane and centrifuge in the same 1.5 ml collection tube for 1 min at ≥ 8000 g (10000 rpm) to elute any RNA that are still on the membrane after the first elution.
13. Transfer all of the 35 µl RNA solution to a new 1.5 ml RNase-free microcentrifuge tube and add 1 µl RNase inhibitor. RNA sample from this step can be safely stored at -80 °C for future use.
14. Reverse transcription. This step needs to be carried out on ice. Add the following components in the exact sequence in an RNase-free PCR tube.
    2 µl 10× Buffer RT
    9.7 µl RNase-free water
    2 µl dNTP Mix (5 mM each dNTP)
    2 µl primer YZ241 (10 µM)
    0.3 µl (about 10 units) RNase inhibitor
    3 µl template RNA from last step
    1 µl (4 units) Omniscript Reverse Transcriptase
15. Close the lid of the PCR tube. Mix the contents of the reaction by gently tap the bottom of the tube. Centrifuge the tube briefly to collect the reaction solution to the bottom.
16. Incubate the PCR tube containing the reaction mixture at 37 °C for 60 min.
17. Store the reverse transcription product at -20 °C.

Lab day 2

1. PCR, add the following components in that sequence in a PCR tube.
    2 µl 10× PCR Buffer
    9 µl ddH_2O
    2 µl dNTP Mix (5 mM each dNTP)
    2 µl primer YZ 241 (10 µM)
    2 µl primer YZ 237 (10 µM)
    2 µl template single stand DNA (the RT-PCR product from lab day 1)

1 µl DNA polymerase

2. Close the lid of the PCR tube. Mix the contents of the reaction by vertex. Centrifuge the tube briefly to collect the reaction solution to the bottom.
3. Set up the PCR in a PCR thermal cycler with the following steps with hot lid.
   Step 1 5 minutes at 95 °C
   Step 2 30 seconds at 95 °C
   Step 3 30 seconds at 62 °C
   Step 4 1 minutes at 72 °C
   Step 5 repeat step 2 – 4 for 44 more times
   Step 6 2 minutes at 72 °C
   Step 7 hold at 4 °C
   Start step 5 while the PCR cycle is in progress.
4. Prepare 60 ml of 1% agarose solution with TBE Buffer with EB (Ethidium Bromide). Heat the solution in a microwave oven until the agarose powder is dissolved. Pour the solution in the gel apparatus tray with comb.
5. Mix the PCR product with 4 µl of 6× loading dye. Load the sample into the assigned well of the agarose gel. Load a DNA ladder marker into one well of each gel.
6. Set the power supply at about 80 volts and run the electrophoresis for 40 to 70 minutes.
7. Take an image of you gel under UV light and document your results.

Materials for cell culture:

HeLa cell
Cellgro brand, DMEM (Dulbecco's Modification of Eagle's Medium), 1× with 10% Nova-Tech brand FBS (Fetal Bovine Serum) and 1% Cellgro brand antibiotic penicillin - Streptomycin Solution (10000 I.U. penicillin and 10000 µg/ml streptomycin)
GIBCO brand Hank's Balanced Salt Solution
GIBCO brand Hank's Balanced Salt Solution with 10% GIBCO brand Trypsin – EDTA (0.5% Trypsin and 5.3mM EDTA.4Na)
100×20 mm Sterile Cell Culture Dish

Materials for total RNA extraction

β-mercaptoethanol
96-100% ethanol
Qiagen brand RNeasy Plus Mini RNA Purification Kit which contains:
   gDNA Eliminator Mini Spin Column
   RNeasy Mini Spin Column
   Buffer RLT Plus
   Buffer RW1
   Buffer RPE (5× concentrated)
   RNase-Free Water
   1.5 ml Collection Tube

2 ml Collection Tube
RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/μl) from Invitrogen
Sterile Ployehtylene Cell Lifter
RNase-Free Syringe
20-gauge Needle
RNase-Free Pippet Barrier Tips (20μl, 200μl and 1000μl)
RNase-Free 1.5 ml Microcentrifuge Tube

For RT-PCR
Qiagen brand Omniscript Reverse Transcription Kit including
Omniscript Reverse Transcriptase
Buffer RT, 10×
dNTP Mix, 5 mM each
RNase-Free Water
RNase-Free PCR Tube
Primer YZ241 5' tca ttt ccg act gaa gag tga gc 3'

For PCR
Taq Polymerase
New England BioLabs brand ThermoPol 10× PCR Buffer with $MgSO_4$
dNTP Mix
ddH_2O
Primer YZ241 5' tca ttt ccg act gaa gag tga gc 3'
Primer YZ237 5' atg agg gag gca ggc gac gag 3'

For Agarose Gel Electrophoresis
Agarose
TBE Buffer with EB (Ethidium Bromide)
Sample Buffer 6×
DNA Ladder Marker
Power Supply
Electrophoresis Apparatus including Tank, Gel Tray, Rubber Spacers, Comb and Tank Lid with Power Plugs