

# Chapter 2

## Reverse-Transcriptase Polymerase Chain Reaction to Detect Extracellular mRNAs

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### Abstract

The presence of extracellular nucleic acids has been reported in serum/plasma from cancer and diabetes patients that may help in disease diagnosis. Taking insulin-producing cells as examples here, RT-PCR was used to investigate a correlation between the presence and amounts of extracellular mRNA(s) and cell mass and/or function. RT-PCR was performed on a range of mRNAs, including *Pdx1*, *Npy*, *Egr1*, *Pld1*, *Chgb*, *InsI*, *InsII*, and *Actb* in biological triplicate analyses.

Reproducible amplification of these mRNAs from MIN6, MIN6 B1, and Vero-PPI cells and their CM suggests that beta cells transcribe and release these mRNAs into their environment. mRNAs secreted from insulin-producing cells into their extracellular environment may have potential as extracellular biomarkers for assessing beta cell mass and function.

**Key words:** Extracellular nucleic acid, RT-PCR, Insulin-producing cells, Conditioned media, MIN6, Beta cell function, Beta cell mass

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### 1. Introduction

In 1948, Mandel and Metais first reported detection of nucleic acids in plasma (1). Extracellular nucleic acid presents a good potential to be used as a tool for early diagnosis of disease. Extracellular RNA is detected in the serum and plasma of various forms of cancer and diabetes patients (2–4). DNA and mRNA from foetal origin have been discovered in the plasma of pregnant women (5–7).

There are many laboratory techniques developed to evaluate mRNA levels. The most commonly used technique to amplify nucleic acid is the reverse-transcriptase polymerase chain reaction (RT-PCR). This is a very sensitive method, and the genes expressed

in very low levels are also detectable after exponential amplification. For this study, medium conditioned by a range of insulin-producing cell types, including glucose-responsive and nonresponsive murine beta cells, MIN6 (L) and (H), respectively, a glucose-responsive clonal population of MIN6 (MIN6 B1), and monkey kidney fibroblast cells engineered to produce human insulin (Vero-PPI) were used. Vero-PPI cells were previously engineered to produce human (pro)insulin (8). MIN6 B1 was seeded at a range of densities ( $1 \times 10^6$ ,  $5 \times 10^6$ , and  $1 \times 10^7$ ) to investigate if the abundance of specific mRNAs detectable in conditioned media (CM) reflected the cell numbers conditioning the medium. A number of transcripts, including pancreatic and duodenal homeobox gene-1 (*Pdx1*), early growth response gene 1 (*Egr1*), chromogranin B (*Chgb*), insulin I (*InsI*), insulin II (*InsII*), neuropeptide Y (*Npy*), phospholipase D1 (*pld1*), and paired box transcription factor 4 (*Pax4*) were investigated.

### **1.1. Polymerase Chain Reaction**

PCR was invented by Kary Mullis, and was awarded Nobel Prize for this (9). The word “polymerase” is derived from the enzyme DNA polymerase that plays an important role in copying DNA during replication or mitosis. PCR is the molecular biology technique used to exponentially amplify a DNA template using a thermal cycler. Selective and repeated amplification of the cDNA is performed using primer (consisting of complimentary sequences to the target) and DNA polymerase enzyme. *Taq* polymerase is the DNA polymerase enzyme isolated by Thomas D. Brock in 1976 from the thermophilic bacterium *Thermus aquaticus*, hence the name “*Taq*” polymerase (10).

### **1.2. Reverse Transcription**

Howard Temin and David Baltimore shared the 1975 Nobel Prize in Physiology or Medicine with Renato Dulbecco for their discovery of an enzyme to transcribe DNA from RNA. PCR can only be used on DNA strands, but with the discovery of reverse-transcriptase, analysis of RNA molecules using PCR is possible. Synthesis of RNA from DNA is termed as “transcription.” RT-PCR is reverse of “transcription” hence the term “reverse-transcription.”

Reverse-transcriptase is a polymerase enzyme that transcribes single-stranded RNA into single-stranded DNA called complementary DNA or cDNA using oligo dT primer, gene-specific primers or random oligomers. Reverse transcription reaction could be carried out using total cellular RNA or poly(A) RNA, reverse-transcriptase enzyme, and primers, and can be converted into cDNA for further amplification using PCR. mRNA is isolated from tissues, cells, serum, CM, or any other desired samples and RT reaction is carried out using these mRNA. This chapter deals with performing RT-PCR using cell and CM RNA. CM is the medium that had been conditioned by cells for 48 h (see Note 1).

## 2. Materials

### 2.1. Cell Culture

1. MIN6 B1 cell line – DMEM media (stored at 4°C) with 150 mL/L heat-inactivated foetal calf serum (FCS) (stored at –20°C) (see Note 2), 2 mM L-Glut (stored at –20°C), and 75 µM β-mercaptoethanol (store at room temperature). β-Mercaptoethanol should be prepared in flow cabinet and filter sterilised before use. It may be stored at 4°C for a month. The medium could be prepared as required and must be used within 1 month.
2. MIN6 cell line – DMEM containing 25 mM glucose, supplemented with 200 mL/L heat-inactivated FCS (see Note 2).
3. Vero-PPI cell line – modified Eagle’s medium (MEM) (store at 4°C) with 5.6 M glucose and 10 g/L non-essential amino acids (store at –20°C).
4. Solution of trypsin (0.25%) (store at –20°C) and ethylenediaminetetraacetic acid (EDTA) (1 mMol) (prepare, autoclave, and store at room temperature).
5. Phosphate-buffered saline (PBS): sodium chloride 8 g/L, potassium chloride 0.2 g/L, di-sodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L, pH 7.3 at 25°C, prepare and store at room temperature.

### 2.2. Isolation of RNA from Cells and CM

1. 0.45-µm filters (Millipore).
2. TRI Reagent (Sigma-Aldrich) should be used in fume hood; store at 4°C.
3. Chloroform (Sigma-Aldrich) should be used in fume hood; store at room temperature.
4. Glycogen (Sigma-Aldrich): final concentration 120 µg/mL (store at –20°C for up to 1 year).
5. Isopropanol (Sigma-Aldrich): store at room temperature.
6. Ethanol: make 75% using UHP and store at room temperature (For UHP see Note 3).
7. RNase-free water (Ambion); store at room temperature.

### 2.3. DNase Treatment of RNA

1. RNase-free DNase (Promega) (store at –20°C).
2. Reaction buffer: supplied as 10× – 400 mM Tris–HCl (pH 8.0), 100 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub> (Promega) (store at –20°C).
3. DNase Stop Solution: 20 mM EGTA (pH 8.0) (Promega) (store at –20°C).

### 2.4. RNase Treatment of RNA

1. RNase ONE Ribonuclease (Promega) (store at –20°C).
2. Reaction buffer: supplied as 10× – 100 mM Tris–HCl (pH 7.5 at 25°C), 50 mM EDTA, 2 M sodium acetate (Promega) (store at –20°C).

**2.5. Reverse Transcription**

1. Oligo dT primers (MWG); store at  $-20^{\circ}\text{C}$ .
2. Human RNase inhibitor (Sigma-Aldrich); store at  $-20^{\circ}\text{C}$ .
3. Moloney murine leukaemia virus (MMLV) reverse-transcriptase (RT) enzyme (Sigma-Aldrich); store at  $-20^{\circ}\text{C}$ .
4. Reverse-transcriptase buffer: supplied as  $10\times - 500\text{ mM}$  Tris-HCl (pH 8.3),  $500\text{ mM}$  KCl,  $30\text{ mM}$   $\text{MgCl}_2$ ,  $50\text{ mM}$  DTT; store at  $-20^{\circ}\text{C}$ .

**2.6. PCR Analysis**

1.  $\text{MgCl}_2$ : supplied with *Taq* polymerase enzyme (Sigma-Aldrich; store at  $-20^{\circ}\text{C}$ ).
2. Oligonucleotide primers, see Note 4 (MWG; store at  $-20^{\circ}\text{C}$ ).
3. *Taq* DNA polymerase enzyme (Sigma-Aldrich; store at  $-20^{\circ}\text{C}$ ).
4. Deoxynucleoside triphosphate (dNTP) (Sigma-Aldrich; store at  $-20^{\circ}\text{C}$ ).

**2.7. Electrophoresis**

1. Agarose (Sigma-Aldrich); store at room temperature.
2. Tris-borate-EDTA (TBE) buffer:  $10.8\text{ g/L}$  Tris base;  $5.5\text{ g/L}$  boric acid,  $4\text{ mL/L}$   $0.5\text{ M}$  EDTA (pH 8.0). Prepare in UHP and store at room temperature.
3. Ethidium bromide, a carcinogen, should be used with caution (Sigma-Aldrich; ( $10\text{ mg/mL}$ )), prepare and store at room temperature.
4.  $\Phi\text{X174}$  DNA HaeIII digest (Sigma-Aldrich; stored at  $-20^{\circ}\text{C}$ ).
5.  $6\times$  loading buffer:  $50\%$  glycerol,  $1\text{ mg/mL}$  bromophenol blue,  $1\text{ mM}$  EDTA; prepare in UHP and store at room temperature.

**2.8. Equipments Required**

1. Thermal cycler.
2. NanoDrop.
3. LabWorks Analysis Software (version 3.0; UVP).
4. Electrophoresis unit (Bio-Rad).
5. EpiChemi II Darkroom, UVP Laboratory Products.

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**3. Methods**

RNA from cells and their CM are isolated using TRI Reagent and are quantified using NanoDrop. RNA is easily degraded by ubiquitously present RNase (ribonuclease) enzymes, so precaution should be taken prior to RNA work. All Eppendorfs, PCR tubes, Gilson pipette tips, etc., should be RNase-free, and disposable nitrile gloves should be worn.

Proper controls should be included to ensure that the amplified product is of RNA origin, and not from contaminating genomic DNA. Some of the relevant controls to include involve analysing

samples after DNase treatment; after RNase treatment; and without reverse-transcriptase enzyme – an enzyme which is necessary for the formation of cDNA on an mRNA template. Water, on its own, should also be included as a control to determine the presence of any contaminant (PCR-VE and RT-VE). Treatment with DNase enzyme would destroy any contaminating DNA leaving only RNA, with RNase enzyme would destroy all RNA leaving only genomic DNA if any (see Note 5).

### **3.1. Preparation of Conditioned Media and Cells**

1. Cells are grown to ~80% confluency, re-feeding every 3 days. To condition the medium for analysis, fresh medium (8 mL) is added for a further 48 h (by which time the cells have reached ~90% confluency).
2. CM is passed through a 0.45- $\mu$ m filter to ensure that no cells or large cell particles are present.
3. 250  $\mu$ L aliquots of filtered CM is added to 750  $\mu$ L of TRI Reagent and incubated at room temperature for 5–10 min to completely dissociate nucleoprotein complexes. Aliquots are stored at  $-80^{\circ}\text{C}$  until RNA extraction and analysis.
4. The corresponding cells from the flasks are washed with PBS, trypsinised, and centrifuged at  $7,500\times g$  for 5 min. The pellets are then washed twice with cold PBS, resuspended in 1 mL TRI Reagent, incubated as described above, and stored at  $-80^{\circ}\text{C}$ .

### **3.2. Isolation of RNA from Cells and CM**

1. The frozen TRI Reagent samples are allowed to thaw at room temperature. Upon thawing, allow these to sit for at least 5 min for complete dissociation of nucleoprotein complexes.
2. To this, add 0.2 mL of chloroform per mL of TRI Reagent. Shake samples vigorously for 15 s and allow to stand for 15 min at room temperature (see Note 7).
3. The resulting mixture is then centrifuged at  $15,700\times g$  in a microfuge for 15 min at  $4^{\circ}\text{C}$ .
4. The colourless upper aqueous phase (containing RNA) is removed into a fresh RNase-free 1.5-mL Eppendorf tube.
5. To this tube, add 1.25  $\mu$ L of glycogen (added only to the CM samples) and 0.5 mL of ice-cold isopropanol. Mix the samples, incubate at room temperature for 5–10 min, and store at  $-20^{\circ}\text{C}$  overnight to ensure maximum RNA precipitation (see Note 6).
6. To pellet the precipitated RNA, centrifuged the Eppendorf tubes at  $13,400\times g$  for 30 min at  $4^{\circ}\text{C}$ .
7. Taking care not to disturb RNA pellet, remove the supernatant. Wash the pellet by the addition of 750  $\mu$ L of 75% ethanol and vortex. Centrifuge at  $5,400\times g$  for 5 min at  $4^{\circ}\text{C}$ .
8. Repeat step 7.
9. The RNA pellet is then allowed to air-dry for 5–10 min, and is then resuspended in 15  $\mu$ L of RNase-free water. To facilitate dissolution of the RNA pellet repeated pipetting may be included.

**3.3. DNase****Treatment of RNA**

To digest any contaminating genomic DNA from RNA isolates, cell and CM samples are treated with RNase-free DNase as follows:

1. 1 µg and 4 µL of RNA isolate is taken from cell and CM samples, respectively.
2. To this, add 1 U RNase-free DNase.
3. Add 1 µL of reaction buffer to this mixture.
4. Then add RNase-free water, bringing the volume up to 10 µL.
5. This mixture is then incubated at 37°C for 30 min.
6. The DNase is then inactivated by adding 1 µL DNase Stop Solution.
7. The reaction mixture is incubated at 65°C for 10 min.

**3.4. RNase****Treatment of RNA**

To digest all RNAs present in aliquots from all cell and CM samples, treat with RNase ONE Ribonuclease as follows:

1. RNA isolates are treated with 1 U RNase ONE Ribonuclease per 0.1 µg RNA.
2. To this, add 10 µL of reaction buffer.
3. This mixture is further incubated at 37°C for 30 min to destroy all RNA present.

**3.5. Reverse****Transcription**

1. First-strand cDNA is synthesised using 1 µL of 500 ng/L oligo dT primers per 1 µg RNA from cultured cells and 4 µL of the RNA suspension from CM (see Note 8).
2. RNase-free water is added to make up the final volume to 5 µL and should be incubated at 72°C for 10 min.
3. This mixture is then cooled on ice, and the following reaction mixture is added to make up 15 µL of final volume:  
1 µL of human RNase inhibitor, 10 mM of each (dNTP, 1 µL of MMLV (200 U/µL) reverse-transcriptase, 2 µL of 10× reverse-transcriptase buffer, and RNase-free water.
4. This mixture is incubated at 37°C for 1 h.
5. RT reaction is subsequently set up for all the controls using aliquots of isolates treated with DNase enzyme; with RNase enzyme; or untreated (see Note 5).
6. In parallel, an RT reaction is set up without RT enzyme (MMLV-RT) as control (see Note 5).

**3.6. PCR Analysis**

1. cDNAs (2.5 µL) from CM and cells are amplified in a 25 µL PCR reaction solution containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 20 µM oligonucleotide primers, and 2.5 U *Taq* polymerase enzyme.
2. To detect if mRNAs detected extracellular to cells are likely to be full-length transcripts or if they are fragmented products, PCR primers are designed in such a way as to amplify regions

close to the 3' end; close to the 5' region; or stretching along most of the length of the transcript (see Note 4).

3. The mixture is then amplified using following conditions.

95°C for 3 min	(Denaturation)
Followed by	
30–45 cycles of (see Note 8):	
95°C for 30 s	(Denaturation)
52–60°C for 30 s	(Annealing)
72°C for 45 s	(Extension)
And	
72°C for 10 min	(Extension)

### 3.7. Gel Electrophoresis of PCR Products

1. The PCR-amplified products are subsequently separated by electrophoresis on a 2% agarose gel prepared in a 1× TBE buffer by melting in a laboratory microwave. Thick gloves and suitable face-protecting safety gear should be used when handling boiling agarose.
2. Upon cooling, the gel is supplemented with 5 µL ethidium bromide (10 mg/mL) to allow visualisation of the DNA upon intercalation.
3. The gel is then poured in to the electrophoresis unit and allowed to set. Sample wells are formed by placing a comb into the top of the gel prior to its setting.
4. To run the samples, 2 µL of 6× loading buffer is added to 10 µL of PCR product and the mixture is loaded to the gel with an appropriate size marker (e.g. ΦX174 DNA HaeIII digest).
5. Gels are electrophoresed at 120–150 V for 1–2 h (depending on size of the target gene, i.e. to get adequate separation).
6. Once the internal control and target bands have migrated to the required extent, the gel is taken to the gel analyzer (EpiChemi II Darkroom, UVP Laboratory Products), photographed, and densitometrically analysed using Labworks software (UVP).

### 3.8. Densitometry Analysis

1. Densitometric analysis of the PCR products may be performed using the MS Windows 3.1 compatible Molecular Analyst software/PC image analysis software available for use on the 670 Imaging Densitometer (Bio-Rad. CA) Version 1.3; or other suitable packages.
2. Developed negatives of gels are scanned using transmission light, and the image is transferred to the computer.
3. The amount of light blocked by the DNA band is in direct proportion to the intensity of the DNA present. A standard area is selected and scanned. A value is taken for the optical density (OD) of each individual pixel on the screen.

4. The average value of this OD (within a set area, usually  $\text{cm}^2$ ) is normalised for the background of an identical selected area.
5. The normalised reading is taken as the densitometric value used in analysis. These OD readings are unit less, i.e. are assigned arbitrary units.
6. The results are imported into Microsoft Excel, and bar charts may be generated from this data.

### 3.9. Results

1. While 30 cycles of PCR is found to be adequate for analysis of transcripts using RNA isolated from cell lines, often products are undetectable or very low in intensity when analysing RNA from CM after 30 cycles of PCR. Forty-five cycles of PCR, however, is generally adequate to produce a detectable band from CM RNA (11) (see Note 9).
2. Amplified products are obtained from cell and CM isolates that were untreated with a digestive enzyme or that were treated with DNase enzyme. However, no bands were detected where samples had been treated with RNase enzyme (see Note 5; Fig. 1).
3. The absence of RT enzyme (MMLV-RT) results in no amplified PCR products, regardless of whether the cell and CM isolates were untreated, RNase- or DNase treated (Fig. 1).
4. In the case of chromogranin b, the intensity of the band produced following 45 cycles of PCR is directly associated with the numbers of cells conditioning the medium. Increased

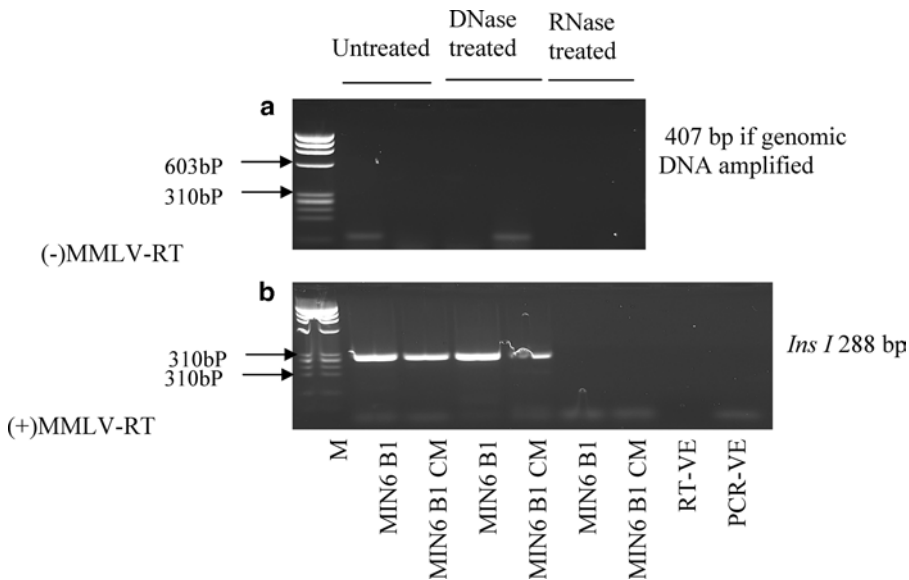


Fig. 1. Cell and CM RNA isolates treated with RNase or DNase prior to cDNA formation, using MMLV-RT enzyme, and subsequent amplification using *Ins1* primers. (a) (+) MMLV-RT = reverse-transcriptase (RT) reaction performed with necessary RT enzyme, MMLV-RT. (b) (-) MMLV = RT cycle performed in the absence of MMLV-RT enzyme, as control. RT-VE = reaction with  $\text{H}_2\text{O}$  instead of RNA as control; PCR-VE = PCR reaction with  $\text{H}_2\text{O}$  instead of cDNA as a control. M = molecular weight marker:  $\Phi$ X174 DNA HaeIII digest.



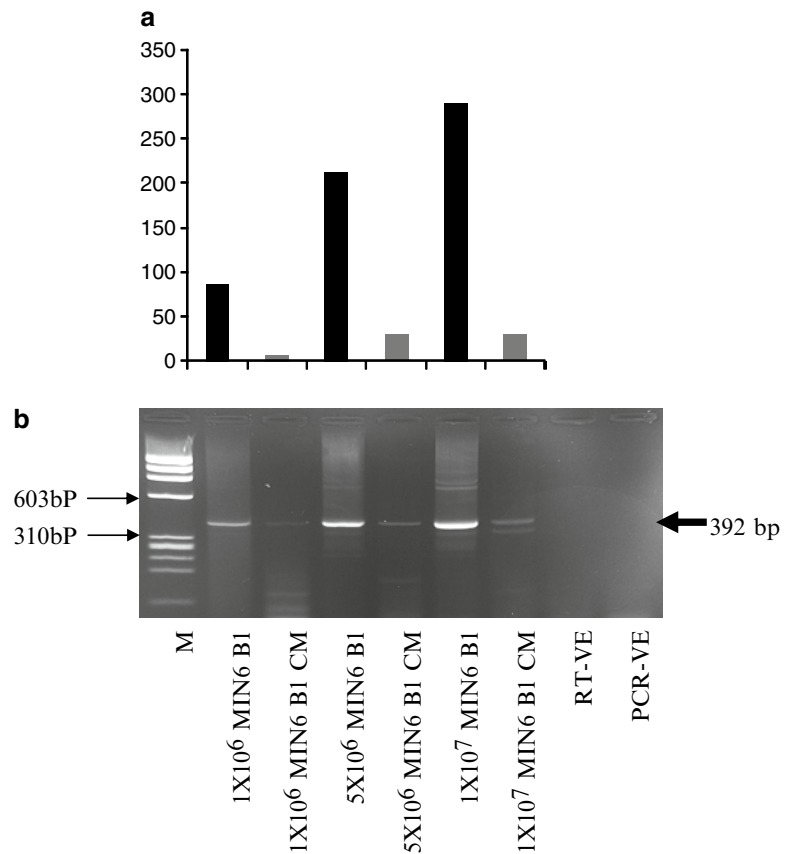


Fig. 2. (a) Densitometric analysis (b) *Glucokinase* RT-PCR on RNA isolated from MIN6 B1 cells, grown at a range of densities, and their corresponding CM. RT-VE= reaction with H<sub>2</sub>O instead of RNA as control; PCR-VE= PCR reaction with H<sub>2</sub>O instead of cDNA as control. Densitometry analysis of amplified gene products is shown above the gel image. M= molecular weight marker:  $\Phi$ X174 DNA HaeIII digest. Results are representative of  $n=3$  biological repeats.

number of cells resulted in increased levels of these transcripts detectable in a fixed volume of CM (Fig. 2).

## 4. Notes

1. To identify a suitable time-point at which mRNA could be routinely amplified, CM samples are collected at four time-points (24, 48, 72, and 96 h) after seeding cells. Beta-actin, a house keeping gene that is highly expressed gene transcript may be isolated and amplified at all time-points evaluated. However, for low levels of gene transcripts expressed by the cell populations, amplified product is undetected after 24 h, but is present when analysed after 48 h, or more, of culture.

To ensure that all CM analysed is from healthy, proliferating cells, it may be recommendable to analyse 48 h CM.

2. FCS should be thawed at 4°C overnight, or at room temperature, before heat-inactivating. Thawed serum may be heat-inactivated at 56°C for 1 h in a water bath.
3. Ultra high pure water (UHP) is the pretreatment of water involving activated carbon, pre-filtration and anti-scaling. Water is then purified by a reverse osmosis system (Millipore Milli-RO 10 Plus) and has a resistivity of 18.2 MΩ-cm.
4. Oligonucleotide primers may be self-designed using primer design tools or may be purchased pre-designed. It has been suggested that RNA detected in human serum/plasma probably are present as short fragments (12). In this particular study, as example, we amplified products ranging from 127 to 383 bp in length, at various regions (5', 3', and – in some cases – amplifying most of the sequence) along the length of the mRNAs (full-length transcripts ranging from 0.294 to 3.111 kb in size). We were able to successfully amplify all regions along the length of cDNAs prepared using oligo(dT) primers targeting the poly(A) tail of mRNAs, suggesting that the CM mRNAs are not fragmented, but are full-length products.
5. In a study using saliva specimens, it was suggested (13) that microarray and qRT-PCR analysis might be detecting genomic DNA, rather than mRNA (14). Therefore, proper controls must be included to determine if the extracellular nucleic acids detected are either wholly or partly DNA in origin, and not from mRNA. As controls, RT-PCR analysis following DNase treatment; RNase treatment; and in the absence of RT enzyme should be performed. Amplified products detected following DNase treatment of samples, complete lack of product following RNase treatment, and in the absence of RT enzyme, supports that the nucleic acids detected are of RNA, not DNA, origin. The amplified products detected should be of the size expected for cDNA, and not of genomic DNA.
6. Glycogen acts as a carrier or co-precipitant in RNA purification. Glycogen is added and left overnight at –20°C, to ensure maximum RNA precipitation. However, for isolating RNA from cells only, a glycogen carrier is unnecessary due to the substantially greater amounts of RNA present. Chloroform helps in phase separation of the mixture into three phases: a red organic phase containing protein, an interphase containing DNA, and an upper colourless aqueous phase containing RNA.
7. A constant amount of total RNA (e.g. 1 µg, as determined by NanoDrop) is used for all cell analysis. In the case of CM, the levels of mRNA are lower and it is likely that all types of total RNA detected in the cell are not present in CM. The numbers

of cells seeded should be accurately counted, and the volumes of medium used kept constant for all replicates. Therefore, for analysis of mRNA in CM a constant volume (e.g. 4  $\mu$ L) of RNA suspension may be analysed.

8. 30 cycles of PCR is sufficient when amplifying gene transcripts from cell RNA. In some cases, very low intensity band or no bands are observed when CM RNA was amplified at 30 cycles of PCR. So it may be necessary to increase this to 45 cycles when using RNA extracted from CM.

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