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Methods for RNA extraction, cDNA preparation and analysis of CFTR transcripts

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Abstract

The scope of this article is to outline some of the basic methods for good quality RNA preparation from mammalian tissues and cells (including epithelial cells). Additionally, we give an outline of common techniques of measuring CFTR gene expression such as quantitative and semi-quantitative reverse transcription (RT) PCR and ribonuclease protection assay (RPA). These methods are designed to detect low abundance transcripts, which apply to CFTR mRNA in most cell types and tissues.

Keywords: Cystic fibrosis; CFTR; Epithelial cells; mRNA; RT-PCR; Ribonuclease protection assay

1. Transcript analysis

The fundamental question in the analysis of gene expression at the RNA level is whether messenger RNA (mRNA), derived from a gene of interest, is present in cells or tissues.

Ordinarily, mRNA makes up less than 6% of the total RNA content of a cell or tissue. When RNA is isolated from these sources, all RNA species are present, that is, ribosomal, transfer small RNA molecules besides mRNA. Many methods and kits exist for RNA extraction from cells and tissues as well as for cDNA preparation and analysis of gene transcripts. All protocols included here are also described in detail elsewhere [1].

Abbreviations: ATC, aurintricarboxylic; BSA, bovine serum albumin; cpm, counts per minute; DEPC, diethyl pyrocarbonate; RPA, ribonuclease protection assay; RT, reverse transcriptase; TE, Tris–EDTA buffer.

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2. Methods for RNA extraction

2.1. Preservation of biological samples for RNA extraction

A ribonuclease (RNAse)-retarding solution (consisting of 25 mM sodium citrate; 10 mM EDTA; 10 M ammonium sulphate; pH 5.2 by addition of 1 M H_2SO_4), presents an alternative to the commercially available RNAlater™ (Qiagen, Hilden, Germany) preservation solution. It can be used for storage of cells from a variety of sources (nasal brushing cells or harvested cultured cell lines). It penetrates harvested cells and retards RNA degradation to a rate dependent on the storage temperature (1 day at 37 °C, 1 week at room temperature, and 1 month at 4 °C or indefinitely at −20 or −80 °C). Following storage, cells may be recovered in a pellet for RNA extraction by centrifugation at 3000 × *g* for 5 min at 4 °C.

2.2. Protocol for RNA extraction from nasal epithelial cells

This RNA extraction procedure is appropriate for the preparation of RNA to be used as a substrate in a variety of

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reactions, e.g. cDNA synthesis, RT-PCR and primer extension [2,3]. The protocol is adapted from the one provided with the RNeasy mini kit (Qiagen, cat no. 74104). Nasal epithelial cells are collected from the nasal epithelium by gently brushing the inferior turbinates of both nostrils [4] using interdental brushes (2 or 3 mm supplied by Paro-Isola, Thalwil, Switzerland). The brush is introduced into a microcentrifuge tube containing 350 μl extraction buffer RLT (Qiagen), supplemented with 1% (v/v) β-mercaptoethanol. Cell lysates can be stored in the buffer at −80 °C for several months. Frozen lysates are incubated at 37 °C for 10 min to dissolve all salt. The lysate is transferred to a QIAshredder column (Qiagen), ensuring that all liquid from the brush is removed by additionally centrifuging the microcentrifuge tube containing the brush. Homogenization is achieved by spinning for 2 min at 3000 × *g*. Alternatively cells are homogenized by passing the lysate 10 times through a 20-gauge (G) needle fitted to a syringe. Three hundred and fifty microliters of 70% (v/v) ethanol is added to the column eluate, mixed well by pipetting, and transferred to an RNeasy mini column (Qiagen) and spun for 15 s at 3000 × *g*. Seven hundred microliters of RW1 buffer (Qiagen) is pipetted to the RNeasy column and centrifuged as above. The column is transferred to a new tube and 500 μl of RPE buffer (Qiagen) is added onto the column and spun as above to wash the column. The procedure is repeated with buffer RPE, the column is transferred to a new tube and centrifuged for 2 min at 3000 × *g* (all ethanol should be washed out from the membrane). Finally, the column is transferred to a new 1.5-ml collection tube and 50 μl of RNAse-free water is pipetted to the middle of the membrane, incubated for 1 min at room temperature and centrifuged for 1 min at 3000 × *g*. The RNA sample is stored at −80 °C.

2.3. Extraction of total RNA from mammalian cells or tissue using cesium chloride

The procedure for total RNA extraction using guanidine thiocyanate and cesium chloride centrifugation was initially applied to pancreatic tissue, which is very rich in RNAses [5]. It yields RNA of superior quality that can be used for many applications including Northern blotting, RT-PCR, ribonuclease protection assay (RPA). Cell pellet (10^8 cells in 2–3 ml) or tissue (1 g/16 ml) is lysed in 4 M guanidine thiocyanate buffer (for cells: 4 M guanidine thiocyanate; 0.5% (w/v) sarkosyl; 7 mM sodium citrate; pH 7.0; or for tissue: 4 M guanidine thiocyanate; 0.1 M Tris–HCl pH 7.5). The solution is filtered and stored in 10-ml aliquots at room temperature. For tissue homogenizations, 0.5% (w/v) sodium *N*-laurosylsarcosinate and 1% (v/v) β-mercaptoethanol are added to the homogenate. The cell lysate or tissue homogenate is passed through sterile syringe needles of decreasing gauge size (18G > 23G>26G) to reduce the solution viscosity. The lysates can be stored at −80 °C for up to 2 months. Two to three milliliters of cell lysate are loaded onto 8 ml of cesium chloride gradient (0.1 M sodium acetate pH 5.0; 5 mM sodium EDTA; 4.7 M cesium chloride; pH not adjusted; filtered and autoclaved) in a sterile centrifuge tube (Beckman 331374 for SW40 rotor, L8-80M ultracentrifuge), and spun at 155,000 × *g* for 16 h at 20 °C. The supernatant is discarded by carefully pipetting from the top to about 1–2 cm from bottom of the tube and slowly inverting the tube onto a clean filter paper. The residual liquid from the tube walls is removed using sterile cotton swabs and the pellet dried for another 2–5 min (pellet may be invisible). It is then re-suspended in 150 μl elution buffer (10 mM Tris–HCl; 5 mM EDTA; 1% (w/v) SDS) and extracted with one volume chloroform/1-butanol (4:1). The aqueous phase is recovered and the organic phase re-extracted with 150 μl elution buffer. The two aqueous phases are pooled and 30 μl of sterile 3.3 M sodium acetate, pH 5.2 and 660 μl of 100% ethanol are added and precipitated for a minimum of 6 h at −20 °C. The solution is centrifuged at 10,000–12000 × *g* at 4 °C in a microfuge, the supernatant is discarded and the pellet washed in 70% (v/v) ethanol and dried under vacuum. The RNA pellet is finally re-suspended in 50–200 μl sterile diethyl pyrocarbonate (DEPC)[1]-treated water and the absorbance read at 260 nm and 280 nm. The ratio $A_{260}/A_{280} \geq 1.5$ is indicative for good quality RNA [5].

2.4. Protocol for extraction of total cytoplasmic RNA from cultured cells

This extraction protocol produces excellent quality RNA for Northern blot analysis[2] [6] and avoids contamination with both DNA and hnRNA, as nuclei remain intact. Cells are washed twice with PBS, and adherent cells are collected by using a "rubber policeman" and centrifuged in a microfuge for 1 min at 8000 × *g* at 4 °C. The supernatant is discarded and the cell pellet washed with 10 mM Tris–HCl, pH 7.5 and re-suspended in 500 μl buffer M (100 mM Tris–HCl; 1 mM EDTA; 100 mM β-mercaptoethanol; 50 μM aurintricarboxylic (ATC) acid (as an RNase inhibitor); pH 8.5). Cells are then lysed by addition of non-ionic detergent Nonidet P-40 (NP-40) to a final concentration of 1% (v/v), and centrifuged for 6 min at 14,000 × *g* at 4 °C. SDS at a final concentration of 0.2% (w/v) is added to the post-mitochondrial supernatant thus obtained. This is then extracted twice with phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v) and centrifuged for 5 min at 14,000 × *g* at 4 °C (the aqueous-upper phase is collected), then once with chloroform/isoamyl alcohol (24:1, v/v) and centrifuged as

[1] DEPC water: Add 0.2% diethyl pyrocarbonate (DEPC) to distilled water. Stir for 30 min. Heat to boiling while stirring until 1/4 volume has evaporated. Autoclave for 20 min. DEPC-treated water needs to be prepared in a chemical hood with proper exhaust ventilation.

[2] This procedure cannot be adopted when the RNA is tube used in any enzymatic reaction (e.g. cDNA synthesis, in vitro translation, etc.) as ATC inhibits these reactions.

above. Finally, 0.1 volume of 3 M sodium acetate, pH 4.6 and 2.5 volumes of ice-cold 100% ethanol are added to the aqueous phase and left overnight at −20 °C for RNA precipitation. RNA is collected by centrifugation at 10,000 × *g*, 4 °C for 30 min, the pellet is re-suspended in 20–30 μl of DEPC-treated water (see Footnote 1) containing 50 μM ATC acid and the absorbance is measured as above.

3. RNA analysis by RT-PCR preparation

3.1. Analysis of the CFTR gene transcript by RT-PCR

Good quality RNA can be prepared from any of the above methods, if the RNA is stored under ethanol at −20 °C, prior to using centrifuge (10,000 × *g*) at 4 °C for 15 min and after removing the supernatant, the pellet is allowed to air-dry on ice. RNA is then re-suspended in DEPC-treated TE (10 mM Tris; 0.1 mM EDTA) or water to a concentration of 0.2–1 μg/μl. One microgram RNA is added to 50 ng reverse PCR primer in DEPC-treated water or TE in a total volume of 9.5 μl, covered with mineral oil and incubated at 65 °C for 10 min. "No RNA" and "no reverse transcriptase (RT)" controls should always be included. The tubes are snap-cooled on ice and 10.5 μl of RT reaction mix is added to give final concentrations of 1 × RT buffer (5 × RT: 250 mM Tris–HCl; pH 8.5; 375 mM KCl; 15 mM $MgCl_2$), 1 U/μl RNAse inhibitor and 1.25 mM dNTPs. MMLV reverse transcriptase (200 U) or Superscript (20 U) is added to all tubes except the "no-RT" control tube. First-strand cDNA can also be synthesized using hexanucleotide primers and MuLV reverse transciptase (GeneAmp Kit, Roche, Basel, Switzerland) in a final reaction volume of 20 μl containing 5 mM $MgCl_2$; 1 × PCR buffer; 1 mM of each of the dNTPs; 1 U/μl MuLV reverse transcriptase; 2.5 μM random hexamers and 0.5–1 μg mRNA. The reactions are incubated at 42 °C for 1 h and then snap-chilled on ice. PCR pre-mix is then added to give final concentrations of 1 × PCR buffer (10 × PCR buffer: 0.67 M Tris–HCl (pH 8.8); 67 mM EDTA; 0.17 M $(NH_4)_2SO_4$); 100 ng/μl each of forward and reverse primers; 170 μg/ml bovine serum albumin (BSA); 10 mM β-mercaptoethanol; 3 U Taq polymerase, e.g. Amplitaq from ROCHE). The conditions for the PCR included initial denaturation for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 2–3 min (see Table 1) at 72 °C and 5 min at 72 °C for the final extension. The sequences of the CFTR cDNA primer pairs A–F [7] used for the PCR are included in Table 1 and elsewhere [1]. Alternatively, PCR is performed using HotStar Taq polymerase mastermix (Qiagen) in a final volume of 50 μl which includes 5–10 μl of first strand cDNA, 25 μl HotStar mastermix and 0.1 μM of each primer. The sequences of the CFTR cDNA primer pairs 1–5b (most of which are internal to primer sets A–F) are included in Table 1. The PCR thermal cycling conditions are as follows: initial denaturation at 95 °C for 15 min; 30–36 cycles of 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 2 min followed by a final extension at 72 °C for 8 min. The success of the PCR is tested by running 5 μl of the product on a 1.5% agarose gel and staining with ethidium bromide. For fluorescent detection applications, one of the primer pairs is labelled with fluorescent dyes, e.g. Fam, Texas Red, Cy3, Cy5 [3,8].

Table 1
RT-PCR primers and conditions used for PCR

Region	5′ and 3′ primer[a]	Primer sequences (5′ –3′ s)	Product size (bp)
A	A1R 124	CGAGAGACCATGCAGAGGTC	1108
	A1L 1232	GCTCCAAGAGAGTCATACCA	
B	B1R 998	GACAAACAGAACTGAAACTG	974
	B1L 1972	GACAAACAGAACTGAAACTG	
C	C1R 1777	GTGGAGGTCAACGAGCAAGA	849
	C1L 2626	ACTCCTTTAAGTCTTCTTCG	
D	D1R2490	GACAACAGCATCCACACGAA	793
	D1L 3283	AATTGGACTCCTGCCTTCAG	
E	E1R 3055	AGATTCTCCAAAGATATAGC	768
	E1L 3824	GAAATGTTGTCTAATATGGC	
F	F1R 3722	ACGTGAAGAAAGATGACATC	950
	F1L 4672	CGAGCTCCAATTCCATGAGC	
1	F5for −53	TCGGCTTTTAACCTGGGCAGTG	476
	B1rev 425	TGTACTGCTTTGGTGACTTCCCC	
2	F12for 399	TTTAGGGGAAGTCACCAAAGCAG	705
	B15rev1104	GGGAAGCACAGATAAAAACACCAC	
3a	F23for 1051	AGCTCAGCCTTCTTCTTCTCAGG	1082
	B21rev 2133	GTGTAAGGTCTCAGTTAGGATTG	
3b	F2for 1616	CTGGATTATGCCTGGCACCATTAA	517
	B21rev 2133	GTGTAAGGTCTCAGTTAGGATTG	
3c	F3for 1714	GAGGACATCTCCAAGTTTGCAGAG	800
	B10rev 2514	CACTTTTCGTGTGGTTGCTGTTG	
4a	F7for 2478	CATTCACCGAAAGACAACAGCATC	1043
	B19rev 3521	CCAACTCTTCCTTCTCCTTCTCCTG	
4b	F8for 2782	CTTGGAAACACTCCTCTTCAA	739
	B19rev 3521	CCAACTCTTCCTTCTCCTTCTCCTG	
5a	F40for 3591	GGATAGCTTGATGCGATCTGTGAG	1028
	B40rev 4619	CAATTCCATGAGCAAATGTCCC	
5b	F10for 4655	TCGTGGAACAGTTACCTCTGCC	738
	B11rev 5393	AGGTCTGTGAGCTTGTCACTTTCTG	

Annealing temperature for all primer sets is 60 °C; extension time 3 min for primer pairs A–F and 2 min for primer pairs 1–5b.

[a] Number represents the 5′-end base on cDNA sequence based on EMBL/GenBank accession nos. M55106–M55131 and Riordan et al. [9].

3.2. OneStep RT-PCR method to determine low levels of CFTR mRNA in real time: application to nasal epithelium cells

Cells from respiratory epithelium are obtained by nasal brushing of both nostrils [4]. Using the OneStep RT-PCR kit (Qiagen, cat. no. 210210) 2 μl of total RNA (20–200 ng) are reverse-transcribed and directly amplified in the same glass capillary of a LightCycler™ system (Roche). Online fluorescence monitoring is performed by adding SYBR Green I (Sigma, St. Louis, USA). The final reaction volume is 20 μl (1 × RT-PCR buffer: 12.5 mM $MgCl_2$, 400 μM of each dNTP, 0.6 μM of each primer, $1{:}2 \times 10^5$ dilution of freshly prepared SYBR Green I. 0.375 U/μl RNase inhib-

itor, 1 μl of the Qiagen OneStep RT-PCR enzyme mix). The LightCycler™ RT conditions are as follows: 30 min at 50 °C, 15 min at 95 °C followed by the quantitative real-time PCR for 45 cycles of 95 °C for 0 s, 58 °C for 30 s and 72 °C for 15 s. Fluorescence is measured at the end of the elongation phase with the final melting curve analysis done from 58 to 95 °C with a slope of 0.1 °C/s. Primers used for the detection of CFTR transcripts with exon 9 are RTCF9/10 and for the CFTRex9del transcripts (exon 9 omitted) RTCF 8/10 with RTCF10 reverse primer for both (RTCF9/10:5′ CACTGGAGCAGGCAAGACTTC3; TCF8/10:5′ AGCCTTCTGGGAGGAGACTTC3′; RTCF10: 5′ CATGCTTTGATGACGCTTCTG-3′).

4. Ribonuclease protection assay for CFTR RNA analysis

4.1. Introduction

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection, quantification and characterization of mRNA species. The assay exploits the DNA-dependent RNA polymerases from the bacteriophages SP6, T7 and T3. A cDNA fragment of interest (e.g. a CFTR sequence) [10] is first subcloned into a plasmid that contains bacteriophage promoters (e.g. TA vector (Invitrogen, Paisley, UK) or the Bluescript KS (Stratagene, California. USA), and the construct is then used as a template for the synthesis of radiolabelled antisense RNA probes. The cloned fragments should range in size from 100 to 300 nucleotides. The orientation of the insert must direct the synthesis of complementary (antisense) RNA. The construct should also be digested with a restriction enzyme producing 5′ overhanging ends, to create a linear template that will be transcribed into a 100- to 300-base run-off transcript. As internal control probes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin mRNA fragments can be used. The probe is hybridized in excess to target RNA, in solution after which free probe and other single-stranded RNA are digested with RNases. The remaining ''RNase-protected'' probes are purified, resolved on denaturing polyacrylamide gel, and quantified by autoradiography or phosphorimaging. The quantity of each mRNA species in the original RNA sample can then be determined based on the intensity of the appropriately sized, protected probe fragment. The assay is highly specific and quantitative due to the RNase sensitivity of mismatched base pairs (or non-paired bases) and to solution-phase hybridization driven towards completion by usage of excess probe.

4.2. Assays

First a single-stranded cRNA probe labelled with α-^{32}P-UTP (or α-^{32}P-GTP) is generated and purified from 10 μg of linearized plasmid and re-suspended in an equal volume of 2× proteinase K buffer (200 mM Tris–HCl; 25 mM EDTA; 300 mM NaCl; 2% (w/v) SDS; 200 mg/ml proteinase K; pH 7.5). The mixture is incubated for 30 min at 37 °C, extracted with phenol/chloroform followed by ethanol precipitation at −20 °C and re-suspended at 1 mg/ml in water. For in vitro transcription, the MAXIscript in vitro Transcription Kit (Ambion, Austin, TX, cat. no. 1308) is used according to the manufacturer's instructions. The probe is purified with the QIAquick nucleotide removal kit (Qiagen, cat. no. 28304) or alternatively with a protocol available online[11]. The probe is checked in a scintillation counter α-^{32}P channel (good yield for 1 μl in the range of 5×10^5 to 6×10^6 counts per minute (cpm); for low abundance CFTR mRNA expect 0.5×10^5 cpm of the labelled antisense probe to 20 μg of total RNA) and stored at −20 °C until needed. Alternatively, the probe can be purified by electrophoresis in a 5% (w/v)/8 M urea polyacrylamide gel [12]. The relevant band is excised and eluted overnight at room temperature in 350 μl elution buffer. The specific radioactivity is determined and the solution is stored at −20 °C without removal of the gel fragment. For the (CFTR) RNA preparation procedures that generate total RNA of high quality and purity (RNeasy™ Qiagen kit, or Trizol™ from Invitrogen followed by phenol/chloform extraction) can be used, always performing DNase I digestion. For the hybridization experiment, the RPAII™ or the RPAIII™ kit (Ambion, cat. no. 1410 or 1415, respectively) can be used, according to the manufacturer's instructions. Alternatively, the desired amount of target RNA (1–20 μg) is solubilized in 8 μl of hybridization buffer (40 mM PIPES; 0.4 M NaCl; 1 mM EDTA; pH 8.0; 80% (v/v) formamide; pH 6.4), in a microfuge tube, a negative control containing yeast tRNA is also included. The probe is diluted with hybridization buffer to the appropriate concentration (the probe must be in excess for accurate quantification) and 2 μl of the diluted probe is added to each RNA sample and mixed by pipetting. The hybridization mix is heated to 95 °C for 3 min and incubated at 44 °C/55 °C overnight. The tubes are placed in a heat block at 37 °C for 15 min prior to the RNase treatment. For the RNase assay, the samples are removed from the heat block and 200 μl of the RNase buffer (300 mM NaCl; 10 mM Tris; 5 mM EDTA; pH 8.0; 40 μg/ml RNaseA; 2 U/ml RNaseT1; pH 7.5) is added. The samples are spun in the microfuge for 10 s and incubated at 37 °C for 20 min, followed by the addition of 1.7 μl proteinase K (20 mg/ml) and 13.6 μl 10% (w/v) SDS, vortexed quickly, re-spun and incubated at 37 °C for a further 20 min. The solution is cleaned up by extracting with 500 μl phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), vortexed and spun for 10 min. The aqueous phase is transferred to a new tube and 4.5 μl (30 μg) of tRNA (or 20 μg of glycogen) and 500 μl of 100% ethanol is added and immediately spun for 15 min at room temperature. The ethanol is aspirated and the RNA pellet is allowed to air dry and re-suspended in 5 μl of loading buffer (80% (v/v)

formamide; 0.1% (w/v) bromophenol blue; 0.1% (w/v) xylene cyanol; 1 mM EDTA; pH 8.0). The samples are denatured at 100 °C for 5 min and chilled on ice prior to loading 5–10 μl on an 8 M urea denaturing 6% (w/v) polyacrylamide gel and electrophoresis proceeded at 20–25 V/cm. The gel is dried at 80 °C under vacuum and exposed to an X-ray film overnight. The results are visualized by autoradiography.

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