

site towards establishing baseline information to serve as a repository for further monitoring and archaeological investigations.

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Isolation of RNA from cell scrapings and blood samples from cervical cancer patients

Cervical cancer is the third most prevalent form of cancer worldwide among women, with greater burden of the disease in the developing countries. Collection of biological samples for analysis has constraints and warrants generating maximum information. Cervical scrapings have been collected from patients where biopsy is difficult or not feasible, for example, in patients undergoing radiotherapy or follow-up patients. Isolation of RNA for gene expression studies is a cumbersome and time-consuming process. It is difficult to isolate from cervical cell scrapings due to three reasons: (i) The amount of cells obtained from the scraping is less; (ii) High levels of endogenous RNAase, and (iii) Contamination with DNA and proteins.

To overcome these problems, we developed a simple and efficient RNA isolation procedure by modifying the original method described by Chomczynski and Sacchi¹, which improves recovery of total RNA from small quantities of cells suitable for gene expression studies (PCR). Basically this was done by collecting the scraped cells in guanidine thiocyanate to inhibit RNAase activity and subsequent cooling to -20°C , and reducing the incubation time to 20 min in the reprecipitation step.

Scraped cervical cells, approximately 50 mg, were collected in 10 ml saline in a Falcon tube and stored on ice. The cells were centrifuged at 2000 g for 5 min. The

supernatant was discarded and cells were transferred into a 1.5 ml polypropylene tube. In case of cell scrapings with blood contamination, rewash with 5–10 ml saline was done. Buffy coat was separated from 5 ml of whole blood and used for RNA extraction. Next 500 μl of solution D (4 M guanidine thiocyanate; 25 mM sodium citrate, pH 7; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol) was added to the cells and vortexed until a viscous lysate was observed. To this, 100 μl of 2 M sodium acetate (pH 4) and 500 μl of phenol (water-saturated) were added with thorough mixing by inversion. Guanidinium thiocyanate and phenol facilitate immediate and effective inhibition of RNAase activity. Then 100 μl of chloroform–isoamyl alcohol mixture (49:1) was added to the homogenate and the final suspension was shaken vigorously for 20–30 s, cooled to -20°C for 5 min and centrifuged at 12,000 g for 15 min at 4°C . The homogenate separates into aqueous and organic phases by addition of chloroform–isoamyl alcohol mixture. On centrifugation RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. RNA was precipitated from the aqueous phase by the addition of 1 ml isopropyl alcohol and maintained at -20°C for at least 1 h for precipitation. RNA pellet obtained after centrifugation at 12,000 g for 10 min at 4°C was dissolved in 200 μl of solution D and re-

precipitated with 1 volume of isopropyl alcohol and incubated for 15 min in -20°C and centrifuged at 12,000 g for 15 min at 4°C . The pellet obtained was washed with 1 ml of chilled 75% ethanol, vortexed and centrifuged at 12,000 g for 10 min at 4°C . Ethanol was decanted and the pellet was dissolved in aliquot volume of sterile double distilled water for further analysis. For long-term storage we recommend storage in absolute ethanol at -70°C .

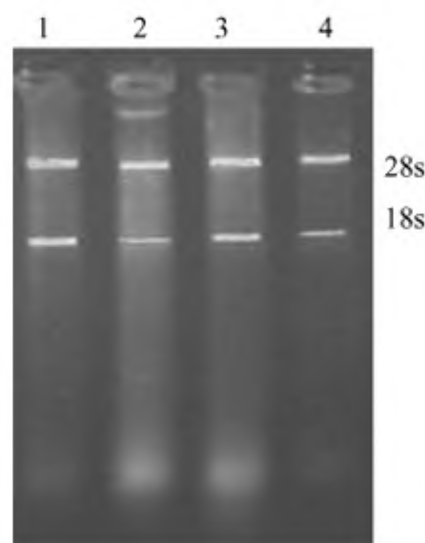


Figure 1. Agarose gel electrophoresis of RNA isolated from cervical cell scrapings (lanes 1 and 2) and blood samples (lanes 3 and 4).

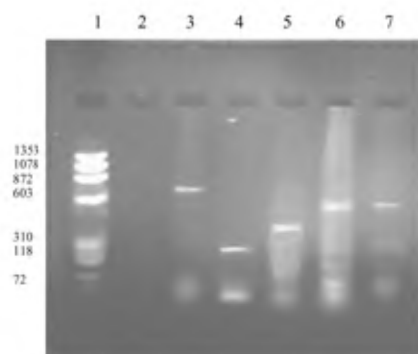


Figure 2. RT-PCR profile with cytokine primers in cell scrapings. Lane 1, Molecular marker; lane 2, Blank; lane 3, β -Actin; lane 4, CD3; lane 5, IL-6; lane 6, CD4, and lane 7, CD8.

Table 1. RNA purity and concentration from cervical scrapings and blood samples

Sample	260 nm	260/280 ratio	Yield (μ g)
Cervical scrapings	1.196	2.0	143
Cervical scrapings	0.607	2.0	121
Blood	0.591	2.0	118
Blood	0.479	1.9	96

The quality of the RNA isolated by the above procedure was analysed on 1.5% agarose gel using TBE (tris borate EDTA) buffer, pH 8.0. For this, 5 μ l of the RNA sample was mixed with 5 μ l of loading dye (0.25% bromophenol blue in 40% sucrose (w/v)) and electrophoresis was performed at 90v for 45 min. RNA samples gave sharp 28s and 18s bands on electrophoresis as shown in Figure 1.

The concentration and purity of RNA was determined by UV spectrophotometry.

Table 2. Primer sequence used for PCR amplification

Primer	Sequence
β -actin (fw)	5' TCG TCG ACA ACG GCT CCG GCA TGT GC 3'
β -actin (rv)	5' TTC TGC AGG GAG GAG CTG GAA GCA GC 3'
CD3 (fw)	5' TGT CTG AGA GCA GTG TTC CCA C 3'
CD3 (rv)	5' CCA GGC TGA TAG TTC GGT GAC C 3'
IL-6 (fw)	5' ACG AAT TCA CAA ACA AAT TCG GTA CA 3'
IL-6 (rv)	5' CAT CTA GAT TCT TTG CCT TTT TCT GC 3'
CD4 (fw)	5' GGA GTC CCT TTT AGG CAC TTG C 3'
CD4 (rv)	5' GAA CTC CAC CTG TTC CCC CTC 3'
CD8 (fw)	5' CTC CTC CTG GCC GCG CAG CTG 3'
CD8 (rv)	5' GCC GGG CTC TCC TCC GCC G 3'

Optical density readings were taken at three wavelengths, viz. 280, 260 and 230 nm. An aliquot of the final preparation of 10 μ l of the total extracted RNA was taken and diluted up to 2 ml with sterile water. A solution of 40 μ g/ml gives an OD of 1 at 260 nm and pure RNA exhibits A260/A280 ratio of 2.0. The results obtained are given in Table 1.

The RNA isolated was used for gene-expression studies with specific primers, viz. CD3 (220 bp), IL-6 (335 bp), CD4 (640 bp) and CD8 (587 bp) along with a housekeeping gene β -actin (688 bp; Table 2). The amplified products were analysed on a 2% agarose gel at 90v for 45 min. PCR products from cervical cell-scraping amplification gave sharp bands corresponding to their product size (Figure 2). Reference DNA molecular weight marker (72–1353 bp) was also used. The expression profile in case of blood was also the same.

This method inhibits RNAase activity during the process of extraction which resulted in optimal recovery of RNA

from small amount of cells; thus it also saves on time and reagents. Purity of RNA was reasonably good. This would help prognosticate and stratify response based on proinflammatory cytokines in cervical cancer patients and could be used on routine basis on follow-up cases to validate expression profile of cytokines of interest².

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