Fast Isolation of RNA to Detect Expression of Tumor Markers

Jörg A. Schenk,^{1*} Timo Hillebrand,² Lieselotte Lübke,³ Stephan Heymann,¹
Michael Böttger,¹ Burkhard Micheel,^{1,4} and Peter Bendzko²

¹Max Delbrück Center for Molecular Medicine (MDC) Berlin-Buch, Berlin, Germany

²InViTek GmbH, Berlin-Buch, Germany

³Carl-Thiem Klinikum, Cottbus, Germany

⁴Institute of Biochemistry and Molecular Physiology, Potsdam University, Potsdam, Germany

The expression status of several tumor-related proteins is of great interest in clinical examination and research. As a completion to conventional antibody staining, RT-PCR is often used today. Reliable isolation of RNA from a low number of cells is very often a critical stage of such an examination. We

demonstrate here a simple and fast method to isolate RNA from only 10,000 cells and applied it to the detection of CEA, c-ERB-B2, and *mdr-1* as often studied models for tumor markers. *J. Clin. Lab. Anal.* 11:340–342, 1997. © 1997 Wiley-Liss, Inc.

Key words: carcinoembryonic antigen; *mdr1*; c-erb-b2; PCR; carrier method; MaTu; breast cancer

INTRODUCTION: THE STUDY OF TUMOR MARKERS

Detection of tumor-associated antigens requires a great deal of effort in cancer research and treatment. The conventional method is to stain cell slices with monoclonal antibodies. As an alternative approach, RT-PCR is often used to measure the expression status of these proteins. Both methods together give a rather sure diagnosis, depending on the antigens.

First, RNA must be isolated from the starting material, cultured tumor cells, or biopsy material. Unfortunately, most isolation protocols described so far are not applicable for a very small number of cells, which is a major weakness of RT-PCR compared to antibody staining. There are two general methods of isolating total RNA: the acid guanidinium thiocyanate-phenol procedure (AGCP) (1) and the carrier method, first described by Vogelstein and Gillespie (2). Here, we modified this carrier system for detecting the expression status of tumor markers and avoiding contamination with genomic DNA. RT-PCR was carried out for three well-known, tumor-related antigens. (1) Human carcinoembryonic antigen (CEA), a cell surface glycoprotein, was first discovered in 1965 as a tumor antigen. Its serum levels were positively correlated with colorectal cancer (3). Up to now, it is one of the most widely studied markers for many tumors. In breast cancer, CEA is found to be elevated in 55–90% of cases in the diagnosis of metastasis (4). (2) c-erb-B2/HER2 is the human homolog of the rat proto-oncogene *Neu* and encodes a glycoprotein with high homology to the human epidermal growth factor (EGF)-receptor. Amplification of the c-erb-B2 gene has been found

in ~ 30% of primary breast carcinomas (5). Overexpression of this gene may directly contribute to malignant tumor development. (3) A major problem in treating tumors with anti-cancer drugs is the overexpression of the *mdr-1* gene. The P-glycoprotein, a 170-kDa cell membrane glycoprotein that is encoded by the *mdr-1* gene, works as an ATP-dependent efflux pump that pumps lipophilic drugs out of the cell and makes the cell resistant. It has also been shown that the promoter of human *mdr-1* gene may be a target for the c-Ha-ras-1 oncogene, suggesting that *mdr-1* might be associated with tumor progression (6).

MATERIALS AND METHODS

RNA Isolation

The protocol described here was applied to clones of tumor cell line MaTu (7) grown in 96-well microtitration plates for 48 hr prior to extraction of RNA. Total RNA was isolated either from ~ 1×10^4 cells or from 2×10^6 cells.

The cell suspension was centrifuged at 3,000 rpm for 10 min, and after removal of the supernatant the pellet was incubated with a lysis buffer (InViTek GmbH, Berlin) for 5 min at room temperature. The lysed cells were then transferred into a 1.5 ml centrifuge tube. Fifteen μ l of a carrier suspen-

*Correspondence to: J.A. Schenk, MDC, Robert-Rössle-Straße 10, D-13125 Berlin-Buch, Germany.

Received 18 November 1996; accepted 28 June 1997.

sion (InViTek GmbH) were added, and the mixture was incubated on ice for 5 min. The lysis buffer (10 M LiCl, 2.25% Triton X-100) is responsible for cell lysis and inactivation of endogenous RNases due to its cell and protein destructive properties and also enables the binding of total RNA to the carrier material. In contrast to other mineralic materials, the used carrier suspension contains nonporously and highly dispersed nanoparticles of pure silica oxide. This composition in combination with the lysis buffer allows very efficient binding of RNA molecules, even if only very small amounts of starting material are available.

After centrifugation, the carrier with bound RNA was washed three times with wash-buffer containing Tris/HCl, NaCl, and 70% ethanol and dried. Bound RNA may be eluted from the carrier in 20 µl diethylpyrocarbonate (DEPC)-treated water at 52°C and used for further experiments. To protect RNA from RNases 1 µl RNasin (USB, Cleveland, OH) was added to the samples. Total isolation time was ~ 20 min. Before elution, RNA bound to the carrier can be stored at -20°C for several weeks, which allows an interruption of isolation directly after lysing the cells and enables a later cDNA synthesis.

Reverse Transcription

For reverse transcription, 10 µl of isolated RNA (total RNA from ~ 5×10³ cells) was added to a reaction mixture containing 50 mM Tris/HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01% Tween20, 1 mM MnCl₂, 0.2 mM of each dNTP (Boehringer Mannheim, Mannheim, Germany), 1 µl RNasin (USB), 1 nmol of hexa-random primer, and 5 units of Tth-Polymerase (Perkin Elmer, Oak Brook, IL) in a total volume of 20 µl. Reaction was carried out at 70°C for 20 min. To stop the reaction, 0.4 µl EGTA (100 mM) were added after this incubation.

PCR

For amplification, 1 µl of synthesized cDNA was added to a reaction mixture containing the same buffer components as reverse transcription with the exception that MnCl₂ was replaced by 2 mM MgCl₂ and 0.2 mM of each dNTP, 25 pmol of primers HB28 and HB29 (for sequences, see Table 1) and 2 units of CombiPol-polymerase (InViTek GmbH) were used in a final volume of 70 µl, covered with mineral oil. Primer sequences correspond to exon 2 of the CEA gene, positions 93-115 (HB28) and 323-341 (HB29) of EMBL entry HSCEA (8). These primers are specific for CEA and do not recognize

related antigens such as NCA (nonspecific cross-reacting antigen). Primers for c-ERB-B2 and mdr-1 were derived from Lönn et al. (9) and Futscher et al. (10), respectively. Primers for mdr-1 correspond to different exons (4 and 7) in the gene. Each cycle of PCR included 30 s denaturation at 94°C, 30 s primer annealing at 55°C (for CEA), 59°C for c-ERB-B2, 60°C for mdr-1, and 20 s elongation at 72°C with a delay of 1 s for each cycle. The PCR was carried out for 30 cycles in a DNA thermal cycler (Eppendorf, Hamburg, Germany). The products were analysed on a 1.5% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

As shown in Figure 1, our isolation and amplification leads to unequivocal results. All three antigens could be detected. Tumor cell-line MaTu-p19 and MaTu19c2 express CEA in the same manner (Fig. 1A). For exact data, semiquantitative PCR could be carried out. In these cell lines, CEA expression seems not to correlate with the behaviour of the clones found in nude mice assay (7). Here, MaTu-p19 was much more tumorigenic than derived MaTu19c2. But, nevertheless, in many cases CEA expression might be a marker for tumorigenicity (11) and metastatic potential, too.

Expression of c-ERB-B2 also showed no differences in both probes (Fig. 1B). In the same way, both cell types have the same expression status for mdr-1/P-Glycoprotein (Fig. 1C). Because the primers are derived from different exons, disturbing contamination with genomic DNA should lead to larger PCR products. This also may be due to the fact that the copy number of RNA is much larger than those of DNA per cell.

The yield of RNA was sufficient to get amplification from low amounts of starting material. According to earlier measurements, we get ~ 80% of total cellular RNA. In serial dilutions of transcribed cDNA, we found amplification of CEA even at a 10⁻⁵ dilution (see Fig. 2).

Storage of RNA bound to the nanoparticle matrix is pos-

Fig. 1. Ethidium bromide-stained 1.5% agarose gel showing PCR products of different tumor associated antigens. PCR products are 249 bp for CEA (A), 217 bp for c-erb-B2 (B), and 402 bp for mdr-1 (C). Lane 2 is from MaTu-p19, lane 3 from MaTu19c2, and lane 1 shows the 100bp-DNA-Ladder (Gibco BRL, Gaithersburg, MD), respectively. RNA was isolated from ~ 10⁴ cells.

TABLE 1. Primer Sequences

CEA	HB28: 5' -AGC TCA CTA TTG AAT CCA CGC CG-3'
	HB29: 5' -CTG TGT CAT TCT GGA TGA T-3'
c-ERB-B2	ERB1: 5' -GGA TAT CCA GGA GGT GCA GGG CTA C-3'
	ERB2: 5' -CAC CTG TGA GGC TTC GAA GCT GCA G-3'
mdr-1	M1: 5' -CTG GTG TTT GGA GAA ATG ACA G-3'
	M2: 5' -CCC AGT GAA AAA TGT TGC CAT TGA C-3'

Fig. 2. Ethidium bromide-stained 1.5% agarose gel showing PCR amplification of CEA from a serial dilution of cDNA synthesized from $\sim 2 \times 10^6$ cells. The dilutions are 10-fold. Lane 2 corresponds to 1/20 of total cDNA, lane 3 to 1/200, etc., until lane 7 with $1/2 \times 10^6$. Lanes 1–8 show the 100bp-DNA-Ladder (Gibco BRL).

sible for several months. In another application we were able to amplify immunoglobulin light and heavy chain fragments from RNA stored for even 1 yr at -20°C (data not shown).

Our results show that this assay is useful for analysis of the expression status of tumor associated antigens, such as CEA,

c-ERB-B2, and *mdr-1*. It is a fast test, particularly if very small numbers of cells ($<10^3$) are available. The whole procedure takes ~ 4 hr, comparable to immunohistochemical methods. The isolation of RNA is much faster (20 min) and easier to handle compared to recently known methods, especially in the case of large numbers of samples. This indicates that this system is really useful for the isolation of total RNA from a large number of sample per day even if only a very small amount of starting material is available.

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