Quantitative Detection of Messenger RNA by Solution Hybridization and Enzyme Immunoassay*

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A novel nucleic acid detection technique is described for the quantitative measurement of eukaryotic mRNA in biological samples. The procedure involves two steps: a hybridization reaction in solution with a biotinylated cDNA probe, and a conventional enzyme immunoassay that uses a monoclonal antibody for DNA-RNA hybrids to detect the specific mRNA-cDNA complexes. The method has comparable sensitivity to 32P-based methods and yields results that are quantitative and highly reproducible. Furthermore, the test can be performed using unfractionated cytoplasm without the need for extraction with organic solvents. This technique provides a rapid and quantitative method for studying changes in cellular mRNA levels, and it is suitable for testing large numbers of samples.

Nucleic acid hybridization assays are powerful tools for analyzing the transcriptional activity of genes. Messenger RNA (mRNA) has most frequently been detected by the labor-intensive technique of mixed phase hybridization assay (1, 2). The utility of this method is limited by the fact that nucleic acids bound to nitrocellulose filters have unfavorable reassociation kinetics, and that nonspecific binding of probe can interfere with detection of small amounts of specific mRNA (3, 4). It has been difficult to standardize assays from experiment to experiment because the preparation of filters and their performance characteristics are highly variable. A further disadvantage is that standard assays rely on the use of radiolabeled probes that have a short functional half-life and must therefore be regenerated regularly.

We have devised a method for measuring individual species of cytoplasmic mRNA which overcomes many of these problems. With this method, mRNA is rapidly recovered from unfractionated cytoplasm by detergent treatment and proteolysis. Specific mRNA is hybridized to biotin-labeled cDNA probes in the liquid phase, and labeled DNA-RNA hybrids are detected by enzyme immunoassay. We describe herein the application of the method to the quantitative analysis of

interleukin-1 (IL-1β) and interleukin-2 (IL-2) mRNA in stimulated monocyte and T lymphocyte cell lines.

EXPERIMENTAL PROCEDURES

Probes

HUM IL-1, a 1.3-kilobase fragment of the human IL-1α gene cloned in pBR322, was obtained from Smith, Kline, and French (5). A 1.1-kilobase fragment of the murine IL-2 gene, inserted in pUC13, was provided by Dr. Gordon D. Mille, Toronto, Canada (6). pGEM-3 was purchased from Promega Biotec (Madison, WI). The fragments were cleaved from vector sequences with appropriate restriction enzymes, electrophoresed in an agarose gel, and recovered by electroelution with NA-45 DEAE-cellulose membranes (Schleicher & Schuell) according to standard procedures (7). The purified inserts were labeled with bio-11-dUTP or 100 μCi of [32P]dCTP (3000 Ci/mmol, Amer sham International) in a nick translation reaction (8, 9).

Cell Lines

The THP-1 cell (TIB 202, American Type Culture Collection, Rockville, MD) is a human monocyte leukemia cell line that secretes IL-1 when stimulated (10). The EL4-6.1 cell is a murine thymoma cell line (provided by H. Robson MacDonald, Epalinges, Switzerland) that secretes IL-2 after exposure to phorbol 12-myristate 13-acetate plus IL-1 (11). Cells were maintained in RPMI 1640 medium containing 7% bovine calf serum and 3% fetal bovine serum (THP-1 cells) or 10% fetal bovine serum (EL4 cells), glutamine, 5 x 10^-5 M β-mercaptoethanol, penicillin, and streptomycin in an atmosphere of 95% air, humidified 5% CO2. Harvested peripheral blood specimens were obtained from healthy donors, and mononuclear cells were isolated by centrifugation over Ficoll-Hypaque. Cells were allowed to adhere overnight at 37°C in a tissue culture dish. After two washes with RPMI, the monolayer of cells was scraped off, washed, and resuspended.

Cell Activation Conditions

THP-1 cells (2 x 10^6/ml) were stimulated with 10 μg/ml endotoxin (Escherichia coli, Sigma) for 2 h at 37°C. EL4 cells (2 x 10^6/ml) were cultured in the presence of phorbol 12-myristate 13-acetate (10 ng/ml) and human recombinant IL-1α (2 ng/ml; kindly supplied by Dr. Charles Dinarello, Boston, MA) for various time periods at 37°C. Peripheral blood adherent cells were activated with 10 μg/ml endotoxin for 3 h at 37°C. In each case, activated cells were subjected to centrifugation at 250 x g for 5 min and washed once with cold phosphate-buffered saline (pH 7.4) before processing for mRNA.

Preparation of RNA

Messenger RNA was extracted in three ways.

1. A Modified Guanidinium Thiocyanate/CsCl Centrifugation Method (12)—Total cellular RNA was obtained by solubilizing cell pellets in 4 x guanidinium isothiocyanate followed by centrifugation over a cushion of 5.7 M CsCl for 18 h. Pelleted RNA was redissolved

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Quantitative Detection of mRNA by Enzyme Immunoassay

in TE (10 mM Tris (pH 7.4), 1 mM EDTA) with 1% SDS, extracted with chloroform:1-butanol (1:1), precipitated in 70% ethanol, redissolved in DEPC-treated H2O, then quantitated spectrophotometrically.

2. The One-step Acid Guanidium Isothiocyanate/Phenol Chloroform Extraction Method of Chomczynski and Sacchi (Rapid Guanidinium Method (13))—Briefly, cells were pelleted and resuspended in 1 ml 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol (solution D). This mixture was mixed with 0.1 ml of 2 M sodium acetate (pH 4), 1.0 ml of water-saturated phenol (pH 5.0), and 0.2 ml of chloroform/isoamyl alcohol (24:1), vortexed for 10 s, and cooled on ice for 15 min. After centrifugation at 4 °C for 20 min at 10,000 × g, the aqueous phase was removed, mixed with an equal volume of chloroform/isoamyl alcohol, and incubated at −20 °C for 1 h. After another centrifugation at 10,000 × g, the RNA pellet was resuspended in 0.3 ml of solution D and precipitated with 2 volumes of 95% cold ethanol and sodium acetate at a final concentration of 0.3 M for 1 h at −70 °C. After a final centrifugation at 10,000 × g, the RNA pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated water with 0.5% sodium dodecyl sulfate (SDS) as a weak RNase inhibitor. Concentrations of total RNA were measured by UV spectrophotometry.

3. A Single-step Cytoplastim RNA Isolation Method That Was Modified from the Procedure of Gough (14) and That of White and Bancroft (15)—Cell pellets were resuspended in 50 μl of 10 mM Tris, (pH 7.4), 1 mM EDTA, and 0.5% Nonidet P-40, vortexed for 10 s, incubated for 10 min on ice to lyse the cell membrane without disrupting the nuclear membrane, and centrifuged at 15,000 × g for 4 min to pellet nuclei. The supernatant was mixed with 50 μl of 2 × proteinase K buffer containing 0.2 M Tris, (pH 7.5), 25 mM EDTA, 0.5 M NaCl, 2% SDS, and 400 μg/ml proteinase K. The sample was incubated for 30 min at 35 °C and tested immediately in the hybridization assay.

Solution Hybridization

RNA preparations, as described above, were mixed with 2.5–10 ng of a biotinylated DNA probe in a total of 200 μl of hybridization buffer containing 2 × SSC (1 × SSC = 150 mM NaCl plus 15 mM sodium citrate (pH 7.0)), 10 mM HEPEs buffer (pH 7.4), 1 mM EDTA, and 0.3% SDS. The mixture of nucleic acids was heated to 100 °C for 10 min, quick cooled on ice, and hybridized overnight in a water bath at 78 °C. After completion of the hybridization reaction, samples were cooled to room temperature, and 40 μl of 10% Triton X-100 was added to form micelles with SDS.

Enzyme Immunoassay for Detection of DNA-RNA Hybrids

Fifty-μl aliquots of the hybridization reaction mixture were tested in 96-well black microtiter plates (MicroFLUOR B 96 U-microplate 011-010-7201 from Dynatech Laboratories). Plates were prepared by incubation overnight at 4 °C with 50 μl/well streptavidin (or anti-biotin antibody) at a concentration of 1 μg/ml in 0.06 M carbonate buffer (pH 9.4). Before use, plates were washed six times with 10 mM sodium phosphate buffer (pH 7.2), 0.15 M NaCl, and 0.05% Tween 20 (PBST). Following addition of samples, plates were incubated at 37 °C for 2 h and then washed with PBST. Fifty μl of a solution containing Fab' fragments of a monoclonal antibody to DNA-RNA hybrids conjugated to β-galactosidase (gift of Robert Carrico, Miles Laboratories, Elkhart, IN) at a concentration of 0.025 μg/ml in PBST with 0.5% gelatin and 0.5% mouse serum was added to each well, and plates were incubated for 1 h at 37 °C. Plates were washed three times with PBST, and then 50 μl/well substrate solution containing 0.1 mM 4-methylumbelliferyl β-D-galactoside (Sigma) in PBS with 1 mM MgCl2 and 50 μg/ml luminov (a luminous albumin) was added. After a 2.5-h incubation at room temperature, the amount of fluorescent methylumbelliferone that was generated by the enzymatic degradation of substrate was measured in a Dynatech MicroFLUOR microtiter plate fluorometer (detection wavelength of 365 nm, emission wavelength of 450 nm).

Statistical Analysis

Semiquantitative results can be obtained by simple inspection of graphs of log fluorescence for various RNA dilutions. Quantitative comparisons of any two samples can be done by a parallel line assay method (16). This provides both a ratio of mRNA content and confidence limits for that ratio.

RESULTS AND DISCUSSION

Description of the Hybridization Method—The assay format is based on a hybridization reaction in solution between a biotinylated DNA probe and a complementary mRNA target sequence. Optimum hybridization conditions are determined by reactions of positive and negative control samples with various probe concentrations at different incubation temperatures. Previous studies with plasmid DNA and complementary RNA transcripts have shown that the temperature optimum for perfectly matched targets is 78 °C and that probe concentrations in the range of 20–1000 ng/ml give optimal results (17). Biotin-labeled DNA-RNA hybrids are captured on microtiter plates coated with streptavidin or anti-biotin antibody. (Triton X-100 is added to samples before this step to form micelles with the SDS and prevent desorption of protein from the solid phase.) Following removal of unbond nucleic acids by washing, the amount of biotin-labeled duplexes bound to the solid phase is detected by reaction with an enzyme-labeled monoclonal antibody directed at DNA-RNA hybrids. The preparation and binding properties of the monoclonal antibody have been described previously (18–20). Of note, the reactivity of the antibody exhibits little dependence on the base composition of the nucleic acid hybrid, and the antibody has a very low affinity for either single- or double-stranded DNA. Bound DNA-RNA hybrids are measured by the addition of a fluorogenic substrate. For concentrations of RNA between 10 and 3000 pg/ml, the amount of fluorescence generated by the enzymatic reaction is proportional to the amount of captured hybrid. Signals are dependent on probe length, but DNA-RNA hybrids as short as 25 base pairs can still be recognized by the monoclonal antibody.

Comparison of Solution Hybridization/Enzyme Immunoassay Method to Isotopic Slot Blot for Detection of mRNA—The performance of the method for detection of eukaryotic mRNA was first evaluated in an assay for IL-β mRNA in LPS-stimulated THP-1 cells. The solution phase nonisotopic method was compared with a standard slot blot method (21) using a 32P-labeled probe (specific activity, 2 × 106 cpm/mg). THP-1 cells were passed for 24 h to provide a stable baseline and then stimulated for 2 h with LPS. Total RNA was extracted by the rapid guanidinium method. One hundred μg of extracted RNA was treated with 23 units of RNase-free DNase I for 15 min at room temperature and then boiled for 3 min. A titration curve of the extracted RNA was prepared in DEPC-treated water and subjected to analysis by either solution phase hybridization followed by enzyme immunoassay (EIA) detection, or slot blot assay with autoradiogram (Fig. 1). The nonisotopic assay could detect an IL-1 message in as little as 50 ng of total RNA with a signal of 16 ± 6 fluorescence units (fu). The background reactivity generated by the probe was 3 ± 1 fu. The standard filter assay with an isotopic probe and overnight autoradiogram had a comparable detection limit.

Specificity of the Assay for DNA-RNA Hybrids—To prove that the EIA was detecting DNA-RNA hybrids, a separate experiment was performed with human peripheral blood adherent cells as the source of IL-β mRNA. Total cellular RNA, before and after treatment with 1′ RNase, was hybridized with the biotin-labeled IL-β probe, and the reaction products were tested by EIA (Table I). A total of 4 μg of RNA, extracted by the rapid guanidinium method, was incubated with 1000 units of T1 RNase for 45 min at 37 °C. The nucleic acids were boiled for 10 min and tested in the assay for IL-β mRNA. Stimulated cells gave a reactivity of 2180 ± 22 fu which, after digestion with T1 RNase, was reduced to 51 ± 6 fu. Controls to demonstrate the specificity of the assay in
Quantitative Detection of mRNA by Enzyme Immunoassay

**Table I**

Specificity of the enzyme immunoassay for detection of DNA-RNA hybrids

Human peripheral blood mononuclear adherent cells were cultured with or without LPS for 2 h. Total cellular RNA was extracted by the rapid guanidium method and then treated as indicated. Each sample (4.0 μg) was hybridized with biotin-labeled probes, as indicated, for 18 h at 78°C. Reaction products were tested by EIA.

<table>
<thead>
<tr>
<th>RNA source</th>
<th>Treatment</th>
<th>Probe</th>
<th>Fluorescent units (± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated cells</td>
<td>IL-1β</td>
<td>2,180 ± 22</td>
<td></td>
</tr>
<tr>
<td>Stimulated cells</td>
<td>T1-RNase</td>
<td>61 ± 6</td>
<td></td>
</tr>
<tr>
<td>Stimulated cells</td>
<td>pGEM</td>
<td>38 ± 6</td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>IL-1β</td>
<td>45 ± 1</td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>T1-RNase</td>
<td>32 ± 4</td>
<td></td>
</tr>
<tr>
<td>E. coli tRNA</td>
<td>IL-1β</td>
<td>27 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

RNA (μg) | Solution Phase | Slot blot |
<table>
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<th></th>
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<tbody>
<tr>
<td>12</td>
<td>950 ± 35</td>
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</tr>
<tr>
<td>6</td>
<td>605 ± 43</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>318 ± 4</td>
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</tr>
<tr>
<td>1.5</td>
<td>191 ± 4</td>
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</tr>
<tr>
<td>0.75</td>
<td>101 ± 5</td>
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<tr>
<td>0.38</td>
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<tr>
<td>0.19</td>
<td>40 ± 1</td>
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<td>0.10</td>
<td>21 ± 2</td>
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<td>0.05</td>
<td>16 ± 6</td>
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<td></td>
</tr>
<tr>
<td>0.005</td>
<td>4 ± 1</td>
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</table>

**Fig. 1.** Comparison of solution hybridization/enzyme immunoassay and 32P-based slot blot assay for detection of IL-1β mRNA. Total cellular RNA was extracted from LPS-stimulated THP-1 by the rapid guanidium method. One hundred μg of extracted RNA was treated with 23 units of RNase-free DNase I for 15 min at room temperature and then boiled for 3 min. Two-fold dilutions were prepared in DEPC-treated water with 0.5% SDS. The presence of IL-1β mRNA was detected either by solution hybridization and enzyme immunoassay or slot blot assay with 32P-labeled probe. Data for the solution hybridization method are expressed as the mean fluorescence ± S.D. for three replicates. (Background reactivity generated by probe in the absence of RNA samples was 3 ± 1 fluorescent units.)

**Fig. 2.** Comparison of RNA extraction methods for detection of IL-1β mRNA in THP-1 cells. THP-1 cells (2 x 10⁶) were stimulated with LPS (10 μg/ml) and then incubated at 37°C. At indicated times after stimulation, cells were harvested, and RNA was extracted by one of three methods: (a) purification by guanidinium isothiocyanate/cesium chloride centrifugation (Guan/CsCl); (b) extraction by the one-step acid guanidium isothiocyanate/phenol-chloroform method (Guan/Phe); or (c) recovery of cytoplasmic RNA by a single-step method (cytosol). In all cases, the RNA equivalent of 1.0 x 10⁶ cells was mixed with biotinylated IL-1β probe and hybridized in solution. DNA-RNA hybrids were detected by EIA. Data are expressed as fluorescent units (mean ± S.D.). They represent the values obtained in three different experiments, each performed in triplicate.

**Table II**

Comparison of RNA extraction methods

THP-1 cells (2 x 10⁶) were stimulated with LPS (10 μg/ml) and then incubated at 37°C. At indicated times after stimulation, cells were harvested, and RNA was extracted by one of three methods: (a) purification by guanidinium isothiocyanate/cesium chloride centrifugation (Guan/CsCl); (b) extraction by the one-step acid guanidium isothiocyanate/phenol-chloroform method (Guan/Phe); or (c) recovery of cytoplasmic RNA by a single-step method (cytosol). In all cases, the RNA equivalent of 1.0 x 10⁶ cells was mixed with biotinylated IL-1β probe and hybridized in solution. DNA-RNA hybrids were detected by EIA. Data are normalized to the baseline fluorescent signal at t = 0 min.

**Table III**

IL-1β mRNA Quantitation in RNA Extracted from Cells by Three Different Methods—THP-1 cells were incubated overnight in 2% fetal bovine serum. Equal numbers (2 x 10⁶) of cells were stimulated the next day with LPS (10 μg/ml) and harvested at various times after stimulation. RNA was extracted by one of three methods: (a) purification by guanidinium isothiocyanate/cesium chloride centrifugation; (b) extraction of RNA by the one-step acid guanidinium isothiocyanate/phenol-chloroform method; or (c) recovery of cytoplasmic RNA by a single-step method. In all cases, the RNA equivalent of 1.0 x 10⁶ cells was mixed with biotinylated IL-1β probe and hybridized in solution. DNA-RNA hybrids were detected by EIA. Data are normalized to the baseline fluorescent signal at t = 0 min.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Guan/CsCl</th>
<th>Guan/Phe</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>30</td>
<td>0.50 ± 0.10</td>
<td>0.30 ± 0.05</td>
<td>0.20 ± 0.00</td>
</tr>
<tr>
<td>60</td>
<td>1.00 ± 0.20</td>
<td>0.60 ± 0.10</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>120</td>
<td>1.50 ± 0.30</td>
<td>1.00 ± 0.20</td>
<td>0.75 ± 0.10</td>
</tr>
</tbody>
</table>

The elimination of reactivity by T1 RNase treatment as well as the low level of reactivity of the labeled probe in the absence of specific target sequences illustrate the specificity of the monoclonal antibody for probe-specific DNA-RNA hybrids.

**IL-1β mRNA Quantitation in RNA Extracted from Cells by Three Different Methods—**THP-1 cells were incubated overnight in 2% fetal bovine serum. Equal numbers (2 x 10⁶) of cells were stimulated the next day with LPS (10 μg/ml) and harvested at various times after stimulation. RNA was extracted by one of three methods: (a) purification by guanidinium isothiocyanate/cesium chloride centrifugation; (b) extraction of RNA by the one-step acid guanidinium isothiocyanate/phenol-chloroform method; or (c) recovery of cytoplasmic RNA by a single-step method. In all cases, the RNA equivalent of 1.0 x 10⁶ cells was mixed with biotinylated IL-1β probe and hybridized in solution. DNA-RNA hybrids were detected by EIA. Data are normalized to the baseline fluorescent signal at t = 0 min.
Quantitative Detection of mRNA by Enzyme Immunoassay

Fig. 3. Titration of extracted RNA from EL4 cells, assayed for IL-2 mRNA by the solution hybridization/enzyme immunoassay method. EL4 cells (2 x 10^6) were cultured for 4 h in the presence of phosphor 12-myristate 13-acetate and IL-1, and cytoplasmic mRNA was recovered by the single-step isolation method. The RNA extract (solution A, •—•), 2-fold (solution A/2, □—□), and 4-fold dilutions (solution A/4, △—△) were prepared. Each solution was then serially diluted and assayed for IL-2 mRNA. Results represent mean ± S.D. for three replicate samples.

Prepared in DEPC/water with SDS, and then each sample was hybridized with biotinylated IL-2 cDNA at a final concentration of 12.5 ng/ml. Labeled hybrids were detected by EIA (Fig. 3). The peak signal in stimulated cells (2 x 10^6) was 939 ± 87 fu, compared with 27 ± 1 fu from an equal number of unstimulated cells and 3 ± 1 fu from probe reacted with buffer alone. Standard deviations for mean fluorescent signals of three replicates were 5 ± 2.6% of the mean. The assay was linear over 2 log_10 dilutions. Semiquantitative results, obtained by direct inspection of the graph, demonstrate that each of the dilution curves is parallel to the others and shifted by a factor of approximately 2.

Quantitative results were obtained by analyzing the fluorescence data for undiluted mRNA, 1:2 mRNA, and 1:4 mRNA by the parallel line method (16). Six-point calculations were made, selecting fluorescence values between 100 and 500 for each dilution (Fig. 3). This range was selected because the lines appeared to be steepest there and because there was a suggestion of nonlinearity with fluorescence values over 500.

The comparisons made were undiluted to 1:2, undiluted to 1:4, and 1:2 to 1:4. The ratios for these comparisons should have been 2.0, 4.0, and 2.0, respectively. The calculated values were 2.26 with confidence limits of 2.12 and 2.41; 4.77 with suggestion of nonlinearity with fluorescence values over 500. These values are close to but not identical with the theoretical ones, which confirms the visual impression that concentrated samples give slightly lower fluorescence values than would be expected. The analyses of variance showed no serious deviations from parallelism and no evidence of line curvature. The g values were 0.004, which confirmed assay validity.

A novel nucleic acid detection technique is described for the measurement of eukaryotic messenger RNA in biological samples. The procedure involves two steps: a hybridization reaction in solution with a biotinylated DNA probe, and a conventional enzyme immunoassay to detect specific, labeled mRNA. The method is practical and yields results that are highly reproducible and objective. The nonisotopic detection method avoids the biohazards, inconvenience, and cost associated with use of radioactivity while achieving a sensitivity comparable to ^32P-based methods. It should be noted that although a fluorogenic substrate was used in these experiments, equal sensitivity can be achieved with an alkaline phosphatase-labeled antibody and a colorimetric substrate (22). A second major advantage of the assay over techniques that use autoradiographic detection is the ability to quantitate results easily and accurately. The assays for IL-1β and IL-2 mRNA were generally linear over two log_10 dilutions, and samples containing equivalent amounts of mRNA gave similar fluorescence values. Another advantage of this method is that samples can be tested without the need to extract RNA with organic solvents. However, when maximum sensitivity is required, extracted samples may be required to reduce background reactivity of negative controls.

The methods described in this report can in principle be applied to measure the expression of any gene whose sequence is known. The minimal requirement for the assay is a sequence of 25-50 bases although a probe of 250-350 bases is preferred for maximal sensitivity. When used in combination with a rapid method for recovery of cytoplasmic RNA, the solution hybridization/enzyme immunoassay technique offers a rapid and quantitative means of studying changes in cellular mRNA levels.

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REFERENCES