Colorimetric ELISA measurement of specific mRNA on immobilized-oligonucleotide-coated microtiter plates by reverse transcription with biotinylated mononucleotides

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In this study, α-globin mRNA was captured on plastic plates to which α-globin-specific oligonucleotides had been covalently immobilized; the captured mRNA was immediately reverse-transcribed into cDNA in the presence of biotinylated dUTP. Addition of alkaline phosphatase-conjugated streptavidin to react with the biotin was followed by addition of substrate (p-nitrophenyl phosphate), and the resulting colorimetric development was measured at 405 nm. Because multiple biotin molecules are incorporated into cDNA during reverse transcription, and because synthesized cDNA is more stable than mRNA, we could successfully quantify by conventional colorimetry the amount of α-globin mRNA on the plate within a linear range of 100 pg to 10 ng. This is more sensitive than conventional Northern blotting. Therefore, the present method may be applicable to measurements of specific mRNAs in various test materials.

INDEXING TERMS: α-globin • biotin-streptavidin interaction • reverse transcriptase • cDNA

Quantification of various specific mRNA molecules in cells and tissues is an attractive field in diagnostic molecular pathology; the concentrations of each specific mRNA are different in normal and disease states and change rapidly in response to various clinical treatments. Among the technologies for assays of mRNA are Northern blotting [1], RNase protection assay [2], reverse transcription followed by polymerase chain reaction (RT-PCR) [3], in situ hybridization [4], and in situ PCR [5], and a variety of detection labels can be used, e.g., radioisotopes, fluorescence [6], and chemiluminescence [7]. Furthermore, once mRNA is reverse-transcribed into cDNA, various gene amplification techniques [8–10] may be applicable.

However, because each assay has its own problems, no assay has been accepted as routine for diagnostic purposes. For example, Northern blotting is labor intensive and is not suitable for quantification of mRNA because of uncertainty as to which fraction of applied mRNA is immobilized on the membranes, and most importantly, because some regions of mRNA may be used for immobilization rather than hybridization. RNase protection assay is more sensitive than Northern blotting but usually requires radioactive materials and labor-intensive steps, which may not be suitable for assaying large numbers of clinical specimens. PCR and other gene-amplification procedures may give problems in quantification and reproducibility, although such assays provide the best sensitivity. In situ hybridization and in situ PCR are the only available technologies for localization of gene expression; however, each specimen must be examined microscopically by expert pathologists with expensive imaging equipment for quantification. Fluorescence and chemiluminescence assays require additional expensive detection devices. And none of these assays allows researchers to use conventional colorimetric ELISA meters, which are widely available in any laboratory.

In previous studies [11], we introduced a unique research system of microtiter plates to which oligonucleotides containing oligo(dT) sequences were covalently immobilized at their 5' ends (GenePlate™). In crude cell lysates applied to the plate,

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4 Nonstandard abbreviations:RT-PCR, reverse transcription-polymerase chain reaction; FCS, fetal calf serum; PBS, phosphate-buffered saline; DTT, dithiothreitol; ds, double-stranded; MOPS, 3-(N-morpholino)propanesulfonic acid; and SSC, saline sodium citrate.
poly(A)$^+$ mRNA was captured on the plate by oligo(dT) sequences of the immobilized oligonucleotides, without previous preparation of mRNA; this was followed by reverse transcription and synthesis of double-stranded (ds) cDNA on the plate [11]. In the present study, $\alpha$-globin mRNA was captured on the plate by specific sequences of the immobilized oligonucleotides and was immediately reverse-transcribed into cDNA in the presence of biotinylated mononucleotides. Because multiple biotin molecules are incorporated into cDNA during reverse transcription, and because the synthesized cDNA is more stable than the mRNA, the amount of $\alpha$-globin mRNA in the test samples can be successfully quantified on the plate by conventional ELISA, at a sensitivity comparable with that of conventional Northern blotting.

**Materials and Methods**

**Materials**

We used the following reagents from the suppliers named: U937 and HL-60 cells (American Type Culture Collection, Rockville, MD); MagneSphere mRNA purification kit, Taq polymerase, and buffer (Promega, Madison, WI); cell-culture media and fetal calf serum (FCS), phosphate-buffered saline (PBS), diethiothreitol (DTT); rabbit globin mRNA, vanadyl–ribonucleoside complex, guanidine isothiocyanate, and MMLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD); tRNA and rRNA (Boehringer Mannheim, Indianapolis, IN); biotin–dUTP and calf thymus DNA (Clontech, Palo Alto, CA); T4 polynucleotide kinase (New England Biolabs, Beverly, MA); $[^{32}P]-\gamma$ATP (3000 kCi/mol; Dupont, Boston, MA); $p$-nitrophenyl phosphate tablets and 5 mol/L (5×) diethanolamine buffer (Kirkegaard & Perry Labs., Gaithersburg, MD); and oligonucleotides immobilized onto plates (Hitachi Chemical Research Center, Irvine, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

**Primer/probe sequence design and synthesis.** Primer and probe sequences were determined by using the computer program HYBrimulater (Advanced Gene Computing Technologies, Irvine, CA) [12, 13]. In brief, oligonucleotide sequences with a melting temperature ($T_m$) of 55 °C were extracted from every position of the target gene of interest; then, each oligonucleotide sequence was screened for possible cross-hybridizable genes and their binding strength against the gene sequence database in GenBank (National Center for Biotechnology Information, Bethesda, MD). Oligonucleotide sequences were selected mainly on the basis of maximum specificity (minimum chance of cross-hybridization with other gene sequences in GenBank) and their secondary structures [14, 15]. We used a DNA synthesizer (380 B; Applied Biosystems, San Jose, CA) to obtain the following oligonucleotide probes:

- **rabbit $\alpha$-globin:**
  5'-aagcggatctcgggctttgcttg-3', #1
  5'-ataacctggagtacgctggg-3', #2
  5'-cttggctcaggtggccctgac-3', #3
  5'-cttgctggagatctgctcagag-3', #4
  5'-agtctgctgcctgtc-3', #5
  5'-aagcggatctcgggctttgcttg-3' (1 mismatch, indicated by capital letter)
  5'-aagcggatctcgggctttgcttg-3' (2 mismatches)
  5'-aagcggatctcgggctttgcttg-3' (3 mismatches)
  5'-aagcggatctcgggctttgcttg-3' (5 mismatches)
  5'-aagcggatctcgggctttgcttg-3' (8 mismatches)

- **rabbit $\alpha$-globin-specific PCR primers:**
  5'-cgggaggtgtctcctg-3' (upper)
  5'-aagcggatctcgggctttgcttg-3' (lower).

After synthesis, the oligonucleotides were treated with ammonium hydroxide at 55 °C overnight, dried, resuspended in water at 0.1 g/L, and stored at -20 °C until use.

**Cell culture.** U937 and HL-60 cells were grown in RPMI 1640 containing 100 mL/L FCS and were subcultured 2–3 times a week. Viability was always >90%, as assessed by the exclusion of trypan blue. The number of cells was determined by hemocytometer.

**Purification of mRNA.** Total RNA was prepared by the acid guanidine–phenol–chloroform method [16]. In brief, animal tissues (100–150 mg) or $10^6$–$10^7$ cultured cells were suspended in 4 mL of guanidine isothiocyanate solution containing 20 mmol/L sodium acetate, 0.1 mol/L DTT, 5 g/L Sarkosyl (N-lauroylsarcosine), and 0.1 mol/L 2-mercaptoethanol, and then homogenized with a Polytron homogenizer (Brinkmann, Los Angeles, CA) for 1 min. Cell/tissue lysates were then mixed with 400 $\mu$L of 2 mol/L sodium acetate, 4 mL of water-saturated phenol, pH 5.0, and 2 mL of chloroform : isooamy alcohol (49:1 by vol), and incubated on ice for 15 min. Lysates were centrifuged at 9700g for 20 min at 4 °C. After centrifugation, supernatant solutions were transferred to fresh tubes, mixed with equal volumes of isopropanol, and incubated for an additional 1 h at -20 °C. RNA materials were pelleted by centrifugation at 9700g for 20 min at 4 °C, resuspended in 180 $\mu$L of guanidine isothiocyanate solution containing 4 mol/L LiCl, and incubated at 4 °C overnight. On day 2, the samples were centrifuged at 18 000g for 10 min at 4 °C, and the pellets were washed once with 70% ethanol and dried by centrifugation under reduced pressure.

**Purification of mRNA.** Total RNA (in the pellets) was suspended in hybridization buffer (10 mmol/L Tris, pH 7.6, containing 1 mmol/L EDTA and 0.5 mol/L NaCl), mixed with 100 pmol of biotin–oligo(dT), and incubated at room temperature for 1 h. After incubation, each RNA solution was mixed with 500 $\mu$L of streptavidin–MagneSphere paramagnetic particles and incubated at room temperature for 30 min. Magnetic particles were captured by magnet, and the supernatant solutions were decanted. The particles were washed with 1 mL of 0.5× saline sodium citrate (SSC; 0.075 mol/L NaCl and 0.0075 mol/L sodium citrate) twice by the magnetic capture method. After the final washing, particles were mixed with 30 $\mu$L of heated diethyl pyrocarbonate water to dissociate mRNA from oligo(dT), and the resulting supernatant solutions were stored at -80 °C until use.
Fig. 1. Principle of colorimetric detection of mRNA.

Specific mRNA is captured by the oligonucleotides immobilized on microtiter plates (step I: hybridization). After hybridization, unbound materials are removed by aspiration and cDNA is synthesized on the plate by using reverse transcriptase and biotin-labeled mononucleotides (step II: cDNA synthesis). Biotin is reacted with enzyme-conjugated streptavidin (step III: enzyme binding), which is followed by addition of substrate and measurement with a colorimeter (step IV: colorization).

**Colorimetric assay for mRNA** (Fig. 1). RNA solution was resuspended in hybridization buffer containing 0.3 mol/L NaCl and 10 mmol/L vanadyl–ribonucleoside complex, and 50 μL of RNA solution was applied to each well of the immobilized oligonucleotide-coated plates (Hitachi) for hybridization. After hybridization at room temperature for 1 h, each plate was washed twice with low-salt washing buffer (10 mmol/L Tris, pH 7.6, containing 1 mmol/L EDTA and 0.1 mol/L NaCl), and the first strand of cDNA was synthesized on the plate by replacing the buffer with 50 mmol/L Tris, pH 8.3, containing 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, and 10 mmol/L DTT; 10 mmol/L each of dATP, dGTP, dCTP, and dUTP; and 400 U of MMLV reverse transcriptase at 37 °C for 1 h. We then mixed 1–5 μL of the template first-strand cDNA (synthesized in solution), or second-strand cDNA (synthesized on the plate), with 0.2 μL each of 10 mmol/L dATP, dGTP, dCTP, and dUTP; 0.5 μL each of sense and antisense primers; 0.5 μL of 25 mmol/L MgCl<sub>2</sub>; 1 μL of PCR buffer; and 0.1 μL of Taq polymerase (Promega) in a final volume of 10 μL. PCR was carried out in a DNA thermal cycler (Model 480; Perkin-Elmer, Norwalk, CT) for 40 cycles, each consisting of annealing at 55 °C for 1.5 min and extension at 72 °C for 4 min, followed by 95 °C denaturation for 1.5 min. After PCR, amplified genes were analyzed by electrophoresis on 1.0% agarose gel, followed by staining with ethidium bromide.

**Northern blotting.** RNA samples were loaded onto 1% agarose gel containing 67 mL/L formaldehyde, 1 × 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (0.02 mol/L MOPS, 8 mmol/L sodium acetate, 1 mmol/L EDTA). After the gel was run in 1 × MOPS buffer at 100 V, mRNA was transferred to a nylon membrane with a positive pressure of 2 kPa for 90 min (Posiblot; Stratagene, La Jolla, CA) followed by ultraviolet radiation-induced cross-linkage (Stratagene). The membrane was prehybridized in hybridization buffer (0.15 mol/L NaCl, 15 mmol/L sodium citrate, pH 7.0, 20 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 70 mL/L sodium dodecyl sulfate, 10 × Denhardt solution, 100 g/L dextran sulfate, and 100 mg/L denatured herring sperm DNA) at 37 °C for 2 h, then hybridized with 32P-labeled oligonucleotides overnight at 37 °C, as previously described [17]. Labeled probes were prepared by using 20 U of T4 polynucleotide kinase with 50 pmol of oligonucleotides and 150 μCi of [γ-32P]ATP (3000 kCi/mol). After we washed the membranes with washing solution (1 × SSC, 10 mL/L sodium dodecyl sulfate, 0.3 mol/L NaCl), we exposed them to x-ray film at room temperature for 3 h.

**Results**

**Selection of oligonucleotides for immobilization**

We selected four candidate oligonucleotides from different regions of rabbit α-globin mRNA. All four oligonucleotides were 21–24mers with similar hybridization strength. Because the oligonucleotide sequences selected were specific to rabbit α-globin mRNA, but had some mismatches against human and rat α-globin mRNA, we successfully evaluated the specificity of each oligonucleotide by adding human or rat total RNA to the pure rabbit α-globin mRNA. Under low-stringency hybridization conditions (300 mmol/L NaCl at room temperature), the color intensity of rabbit α-globin mRNA in the wells of immobilized oligonucleotides (#1, #3) did not increase even

**Synthesis of ds cDNA on the plate.** After the first strand of cDNA was synthesized on the plate, we replaced the reaction buffer with 25 mmol/L Tris, pH 7.5, containing 100 mmol/L KCl, 5 mmol/L MgCl<sub>2</sub>, 10 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.15 mmol/L β-NAD<sup>+-</sup>; 250 μmol/L each of dATP, dGTP, dCTP, and dTTP; 1.2 mmol/L DTT; 65 000 U/L DNA ligase; 250 000 U/L DNA polymerase; and 13 000 U/L RNase H (Intermountain, Kaysville, UT); and incubated overnight at 16 °C to synthesize ds cDNA on the plate [11]. The synthesized second strand of the cDNA was removed for subsequent PCR analysis by adding 50 μL of boiling water for 10 min.

**RT-PCR.** Total RNA or mRNA was reverse-transcribed into cDNA in solution in 50 mmol/L Tris, pH 8.3, containing 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, and 10 mmol/L DTT; 10 mmol/L each of dATP, dGTP, dCTP, and dUTP; and 400 U of MMLV reverse transcriptase at 37 °C for 1 h. We then mixed 1–5 μL of the template first-strand cDNA (synthesized in solution), or second-strand cDNA (synthesized on the plate), with 0.2 μL each of 10 mmol/L dATP, dGTP, dCTP, and dUTP; 0.5 μL each of sense and antisense primers; 0.5 μL of 25 mmol/L MgCl<sub>2</sub>; 1 μL of PCR buffer; and 0.1 μL of Taq polymerase (Promega) in a final volume of 10 μL. PCR was carried out in a DNA thermal cycler (Model 480; Perkin-Elmer, Norwalk, CT) for 40 cycles, each consisting of annealing at 55 °C for 1.5 min and extension at 72 °C for 4 min, followed by 95 °C denaturation for 1.5 min. After PCR, amplified genes were analyzed by electrophoresis on 1.0% agarose gel, followed by staining with ethidium bromide.
after addition of human RNA, whereas the intensity did increase in the wells containing oligonucleotides #2 and #4 (Fig. 2).

Given that the four oligonucleotide sequences were selected from different regions of rabbit α-globin mRNA, the oligonucleotides near the 5' end (e.g., #4) have less space for extension during reverse transcription, whereas oligonucleotides located near the 3' end (e.g., #1) have a capacity for long cDNA synthesis (Fig. 2, inset). Because oligonucleotide #1 exhibited the highest value for mRNA without nonspecific increases on addition of human RNA, we used oligonucleotide #1 for all the remaining experiments.

**OPTIMIZED ASSAY CONDITIONS**

To determine the optimal hybridization stringency, we hybridized rabbit α-globin mRNA to plates where oligonucleotide #1 had been previously immobilized and conducted the hybridization with various NaCl concentrations, from 10 mmol/L to 0.5 mol/L. As shown in Fig. 3A, A405 increased with NaCl concentration, and was maximum at 300 mmol/L. Interestingly, even in low-stringency conditions of >300 mmol/L NaCl, A405 did not increase in the presence of nonspecific mRNA (Fig. 3A, ■). Furthermore, human RNA did not show any signal on the plate (Fig. 3A, △). Because A405 was maximum at 300 mmol/L without nonspecific increase by human RNA, we selected 0.3 mol/L NaCl for the assay concentration.

To test stringency and (or) reversibility of hybridization, we washed the hybridized rabbit α-globin mRNA on the plate with preheated hybridization buffer 5-20 times. As shown in Fig. 3B, the signals were stable even after 20 washes at room temperature but decreased substantially where wash buffer was preheated to 37-60 °C.

Study of the kinetics of the hybridization (Fig. 3C) indicated that the reaction reached equilibrium after ~1 h at room temperature.
COMPETITIVE ASSAY WITH FREE OLIGONUCLEOTIDES
To quantify the assay stringency or degree of cross-hybridization, we applied rabbit α-globin mRNA to the plate in the presence of free oligonucleotides, with or without mismatches, as competitors. In our previous study, using a new fluorimetric assay, we reported that ~1-2 pmol of oligonucleotides was immobilized on the plate \[18\]. In the present competitive assay, specific mRNA signals were decreased in the presence of 0.5–50 pmol of matched oligonucleotides, the 50% inhibition concentration (IC50) being ~3 pmol, a reasonable value considering the amount of immobilized oligonucleotides (Fig. 4). Interestingly, using the present assay conditions, we could not discriminate 1–2 mismatches, but oligonucleotides with >3 mismatches were much less inhibitive (Fig. 4).

ASSAY SENSITIVITY AND SPECIFICITY
The A405 values increased in proportion to the amount of applied rabbit α-globin mRNA, whereas tRNA or rRNA alone did not show any substantial increase in A405 even when present in 3–5 orders of magnitude excess (Fig. 5). Interestingly, if >1 μg of DNA was applied to the plate, the A405 increased notably, although we still do not know if the reverse transcriptase incorporates biotinylated dUTP into nonspecifically bound DNA on the plate. To avoid any misinterpretation of the assay results, we decided to purify RNA or mRNA from cells and tissues before application to the plate.

An increase in A405 was noticeable for 30 pg of rabbit α-globin mRNA; from the log-log plot in Fig. 5 (right inset), however, we conclude that the linear range of quantification of rabbit α-globin mRNA ranges from 100 pg to 10 ng.

In parallel experiments, we also conducted Northern blotting, using the same #1 oligonucleotide as a probe and the same stringency (300 mmol/L NaCl at room temperature). The lower detection limit under these conditions was 1 ng when radioactivity was exposed to x-ray film for 3 h (Fig. 5, left inset); it was 100 pg if membranes were further washed at high temperature and then exposed overnight (data not shown).

QUANTIFICATION OF RABBIT α-GLOBIN mRNA FROM TISSUES
Preliminary RT-PCR experiments confirmed that newborn rabbit liver contained α-globin mRNA, whereas adult rat liver and human U937 and HL-60 cells did not express detectable amounts of α-globin mRNA (data not shown). We also found α-globin mRNA in newborn heart, probably from contaminating erythroblasts (data not shown).

For hybridization, RNAs from these tissues were applied to the plates with immobilized oligonucleotides, followed by first-strand cDNA synthesis in the presence of biotinylated mononucleotides and ds cDNA synthesis in the absence of biotinylated mononucleotides on the plate (see Procedures). The resulting second strand of cDNA was removed from the plate by washing with 50 μL of boiling water for 10 min and was used for PCR. As shown in Fig. 6, the presence of α-globin mRNA was confirmed in newborn rabbit liver and heart; its absence in rat liver and human cells was also confirmed. This indicates that α-globin mRNA was successfully hybridized on the plate and that the corresponding cDNA was also synthesized on the plate.

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Fig. 4. Inhibition with mismatched oligonucleotides.
We mixed 10 ng of rabbit α-globin mRNA with various concentrations of mismatched oligonucleotides (no mismatch ○, 1 mismatch ■, 2 mismatches ▲, 3 mismatches △, 5 mismatches □, and 8 mismatches ▲) and applied the mixtures to each well of a plate to which oligonucleotide #1 had been immobilized. After hybridization at room temperature for 1 h, cDNA was synthesized on the plate in the presence of biotin-labeled dUTP, followed by reaction with alkaline phosphatase-conjugated streptavidin and colorimetric detection as described in the text. Each point represents the mean of triplicate determinations.

Fig. 5. Dose dependency of rabbit α-globin mRNA (●), calf DNA (+), tRNA (■), and rRNA (▲) applied to each well of a plate to which oligonucleotide #1 had been immobilized.

In parallel experiments, we also conducted Northern blotting, using the same #1 oligonucleotide as a probe and the same stringency (300 mmol/L NaCl at room temperature). The lower detection limit under these conditions was 1 ng when radioactivity was exposed to x-ray film for 3 h (Fig. 5, left inset); it was 100 pg if membranes were further washed at high temperature and then exposed overnight (data not shown).
Discussion

1.319
1755
280
0937
1
1.555
1.220

Fig. 6. RT-PCR determination of α-globin cDNA in solid-phase reverse
transcription reaction.

We applied 500 ng of mRNA purified from newborn rabbit liver and heart, adult
rat liver, and human U937 and HL-60 cells to plates coated with oligonucleotide
#1 for hybridization followed by reverse transcription in the presence of biotin
dUTP. After addition of RNaseH, DNA ligase, and polymerase, double-stranded
cDNA was synthesized on the plate at 16 °C overnight as described in the text.
Sense-stranded cDNA was removed from the plate by addition of boiling water
and was used for PCR amplification for α-globin gene. Left lane: 100-bp ladder;
N: negative PCR control (water); and P: positive PCR control of rabbit α-globin
cDNA.

even when biotinylated mononucleotides were included in the
reaction mixture.

Various concentrations of total RNA or mRNA purified from tissues and cultured cells were applied to the plate for
colorimetric determination of the amount of α-globin mRNA. Measurable signals were obtained from 50 ng of mRNA or 0.5–5
μg of total RNA from newborn rabbit liver (data not shown).

Therefore, by calibrating with known concentrations of purified rabbit α-globin mRNA, we determined the amount of α-globin
mRNA from each tissue. α-Globin mRNA constituted ~0.16% of total mRNA in rabbit liver and was <0.03% in newborn
rabbit heart (Fig. 7). Rat liver and two cultured human cell lines failed to show detectable amounts of α-globin mRNA (Fig. 7).

To confirm the results of colorimetric assay, we also con-
ducted Northern blotting with these RNA materials, using the
same oligonucleotide #1 as a radioactive probe under the same
stringency conditions as described in Procedures. As shown in
Fig. 7 (inset), three prominent bands were identified from
newborn rabbit liver and heart, suggesting mature α-globin
and two unprocessed α-globin mRNAs [19], whereas no band was
confirmed in rat liver and human cells. As a control, β-actin
mRNA was included and confirmed in all RNA samples on the
same Northern membranes (Fig. 7, inset). Furthermore, in spite
of the conditions of very low stringency hybridization and
minimum washing, the background noise of the membranes was
negligible, suggesting high specificity of the oligonucleotide
sequences.

PRECISION STUDIES

The amount of α-globin mRNA was repeatedly determined
from newborn rabbit liver in several independent experiments.
In each experiment, purified rabbit mRNA was used as the
calibrator. As shown in Table 1, the intra- and interassay results
were consistent and reproducible. Moreover, the amount of

mRNA determined by this method is linearly correlated with
the amount of applied total RNA; i.e., the amount of α-globin
mRNA measured from 280 μg of total RNA was ~3.7 ng,
2.8-fold higher than the amount measured from 100 μg of total
RNA (Table 1).

Discussion

The ELISA methodology [20] has been well established and
used widely in clinical diagnostic fields for many years and
provides qualitative and quantitative analysis of various peptides,
proteins, and small molecules in serum, exudates, and cellular
extracts. Although protein ELISA and mRNA analysis are
targeted to the same gene or gene products, these analyses
examine different aspects of disease mechanisms. For example, if
specific mRNA is not expressed in spite of an increase in the

Table 1. Intra- and Interassay quantification of α-globin
mRNA (ng) from newborn rabbit liver.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Experiments</th>
<th>100 μg</th>
<th>280 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1.555 ± 0.237 (10)*</td>
<td>3.721 ± 0.367 (10)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.319 ± 0.299 (8)</td>
<td>3.326 ± 0.319 (10)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.356 ± 0.260 (10)</td>
<td>3.725 ± 0.261 (9)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.220 ± 0.156 (10)</td>
<td>3.752 ± 0.619 (8)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.516 ± 0.403 (9)</td>
<td>3.766 ± 0.307 (10)</td>
</tr>
</tbody>
</table>

* Results are given as mean ± SE (and no. of wells).
corresponding protein, the protein may simply be being released from the cells unaccompanied by additional mRNA synthesis, or the assay antibody may be recognizing other gene products with a similar structure. In contrast, increases in specific mRNA with unchanged protein concentrations may indicate either enhanced protein catabolism or a very early event of cellular biological changes, because mRNA changes occur more rapidly than protein synthesis.

Furthermore, mRNA analysis has potential advantages over conventional ELISA. ELISA requires the lengthy process of production of specific antibodies and labor-intensive work for characterization of each antibody. In contrast, mRNA analysis can be established more quickly, through use of a computer program to design the specific oligonucleotide sequences once the nucleotide sequences have become public knowledge. More interestingly, unlike ELISA, specificity or cross-reactivity can be easily controlled in mRNA analysis by selecting gene-family-common probes or species-specific probes—again, by using computer programs. Moreover, because oligonucleotides can be synthesized by a synthesizer, transition from small pilot experiments to large-scale manufacturing is more easily established.

Although RT-PCR [3] and in situ hybridization [4] are becoming popular in molecular diagnostics, the major obstacles to using mRNA analysis are instability of mRNA, labor-intensive multiple steps, lack of sensitivity, and requirements for additional expensive devices and reagents. In the present study, we developed a colorimetric assay for measuring rabbit globin mRNA in a common microtiter plate format. The assay was quicker, more sensitive and quantitative, and less labor intensive than Northern blotting and provided reproducible results. Although the sensitivity of the present assay was less than that of RT-PCR, we think our method will be applicable to clinical diagnostics in cases where abundant mRNA species are the targets for analysis or when large quantities of materials are available (e.g., surgical specimens).

In our earlier experiments, we developed a sandwich assay of mRNA on microtiter plates with multiple oligonucleotides, one for immobilized capture probe, and others for fluorescence-labeled detection probes. Because of the use of multiple oligonucleotides, this sandwich assay provided good specificity and sensitivity. However, because unstable mRNA remained present during the entire assay, extra care was essential to keep mRNA hybridized with multiple oligonucleotides under reasonably stringent conditions and minimizing RNase contamination. Although the sandwich assay did not require a reverse transcription reaction, consistent and quantitative results were difficult to obtain. Furthermore, to detect fluorescence on microtiter plates, a relatively uncommon fluorescent plate reader was required. Therefore, in the present study, we wanted to obtain stable and reproducible quantification in the microtiter plate format with colorimetric detection.

One of the critical aspects of this new assay is the specificity of the immobilized oligonucleotide sequences. Unlike PCR, where the sequence specificity depends on the combination of two oligonucleotides, the sequence specificity in the present assay is totally dependent on a single oligonucleotide. Furthermore, unlike Northern blotting, where specific and nonspecific hybridization can be easily detected by the size of each band and background noise, we do not know whether the A_{405} values are derived from specific mRNA or nonspecific ones. Therefore, the specificity of immobilized oligonucleotides should be tested extensively by Northern blotting (see Fig. 7, inset) or by addition of nonspecific mRNA (Fig. 2). Moreover, an advanced computer program (e.g., HYBrimulator) may help researchers select candidate oligonucleotides with quantitative specificity from defined gene databases [12, 13]. Once the specificity of the immobilized oligonucleotides is confirmed, the present assay can be used to quantify the specific mRNA as an initial screening assay—alphanumeric to ELISA vs Western blotting [21]. For example, ELISA is more quantitative than Western blotting, but the cross-reactivity of the antibodies used can be assessed only by Western blotting.

This assay format was successfully adopted to other mRNA species, e.g., from β-actin and interleukins, and some mRNA could be quantified even from crude cell lysate with 10^7-10^5 tissue culture cells (manuscript in preparation). Therefore, mRNA ELISA in the present study may become an interesting tool for the analysis of mRNA in both basic research and clinical diagnostics.

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