Oligo(dT)-immobilized Pipette Tip: Efficient New Methodology for mRNA Preparation and Direct Gene Amplification, Keisuke Eguichi,† Yohei Hamaguchi,† Yoshi-nasa Aso,‡ Toshiki Shioiri,‡ Mieko Ogura,‡ and Masato Mitsuhashi‡ (Departments of 1 Pathology and 2 Psychiatry, University of California, Irvine, CA 92697 and 3 Hitachi Chemical Research Center, 1003 Health Sciences Road West, Irvine, CA 92612; * author for correspondence: fax 949-725-2727, e-mail mmitsuha@uci.edu)

One powerful gene amplification technology, reverse transcription-PCR (RT-PCR), allows researchers to derive functional genetic information from small amounts of clinical specimens; however, it is labor-intensive, and multiple steps are needed to prepare mRNA in good quality and in good yield. We have been developing rapid and efficient procedures for mRNA purification and detection in microplates (1–4). The oligo(dT)-immobilized polystyrene microplate (GenePlate) can capture mRNA from crude cell lysates (1), quantify it (2), synthesize sense and antisense strands of cDNA (1, 3), and preserve cDNA (cDNA bank) (4). The most recent oligo(dT)-immobilized polypropylene microplate (GenePlate-PP) has the additional advantage of allowing direct RT-PCR in the plate immediately after mRNA is captured (5). Here, we introduce an oligo(dT)-immobilized pipette tip for not only mRNA purification but also for direct RT-PCR in a single pipette tip.

Dispersible polypropylene pipette tips (tip volume, 1–200 μL; Fisher Scientific) immobilized with oligo(dT)20 via the 5’ end were kindly provided by Advanced Gene Computing Technologies (AGCT, Irvine, CA). Rabbit globin mRNA was purchased from Gibco-BRL, and total RNA was extracted from rat brain with guanidine isocyanate lysis and acid-phenol extraction (6). Rabbit globin mRNA (200 ng) or rat brain total RNA (5 μg) was suspended in hybridization buffer (10 mmol/L Tris, pH 7.6, 1 mmol/L EDTA, 500 mmol/L NaCl, 20 mmol/L vanadyl ribonucleoside complex) in a final volume of 70–100 μL in 10-mL tubes. The oligo(dT)-immobilized tips were attached to small disposable pipettes (length, 9.5 cm; weight, 6.1 g; Tricontinent), and were placed in 10-mL tubes to aspirate a fixed volume (50 μL) of mRNA solution. Each pipette was supported vertically within 10-mL tubes. Because 10-mL tubes contained a larger volume of mRNA solution than the tips can aspirate, the excess solution prevented evaporation from tips during incubation. To avoid evaporation of mRNA solution from the tips, each 10-mL tube contained ~20–50 μL of extra sample. After unhybridized materials were dispensed, these tips were washed three times by aspirating then dispensing wash buffer (10 mmol/L Tris, pH 7.6, 1 mmol/L EDTA, 500 mmol/L NaCl).

To visualize hybridized mRNA in the tips, Yoyo-1 (Molecular Probe) (7, 8) was diluted 1:1000 with wash buffer, and 50 μL of Yoyo-1 solution was aspirated into the tip. Yoyo-1 fluorescence was determined by a fluorescence image analyzer (FMBIO II, Hitachi Software). As shown in the upper inset of Fig. 1A, the Yoyo-1 signal in the mRNA hybridized tip was higher than that of the control tip. For quantification of mRNA hybridization, captured mRNA was dissociated from the tips by aspirating 50 μL of boiling diethylpyrocarbonate water into the tips, and then dispensing it into polypropylene sheets. The resulting mRNA was then stained with Yoyo-1 in diethylpyrocarbonate water in the final dilution of 1:1000, and the fluorescence was determined by FMBIO II. In parallel experiments, known concentrations of globin mRNA were used as calibrators. As shown in Fig. 1A, the Yoyo-1 fluorescence intensity was linearly correlated with the amount of mRNA in a range of 5–30 ng. When 250 ng of rabbit globin mRNA was applied to the tips, dissociated mRNA exhibited substantial Yoyo-1 fluorescence (Fig. 1A, lower inset), and the calculated amount of recovered mRNA was 91.1 ± 12.4 ng (mean ± standard error, n = 3). When 5 μg of rat brain total RNA was used, 99.7 ± 19.8 ng of mRNA was collected (Fig. 1A, lower inset).

To evaluate the quality of captured mRNA, 250 ng of globin mRNA was applied to the tips, and dissociated mRNA was then separated by 2% agarose gel electrophoresis. As a result, the appropriate mRNA band was detected at the same position as intact rabbit globin mRNA (Fig. 1B).

Captured mRNA was further processed by RT-PCR in the oligo(dT)-immobilized tips. In these experiments, we used crude cell lysates rather than purified RNA/mRNA. The K-562 chronic myelogenous leukemia cells (American Type Culture Collection) were grown in RPMI-1640 containing 100 000 units/L penicillin, 100 mg/L streptomycin, and 100 mL/L fetal calf serum (Gibco-BRL) at 37 °C in a 5% CO2 incubator. The viability of the cells was always >95% by the exclusion of trypan blue dye. Cells (1 × 10⁶) were suspended in 50 μL of lysis buffer (hybridization buffer containing 1 mL/L NP-40) and incubated on ice for 5 min to release cytosolic mRNA as described previously (2). The cell lysate was centrifuged at 15 000g at 4 °C for 5 min, and supernatants were aspirated into the oligo(dT)-immobilized pipette tips. After hybridization at room temperature for 30 min, unhybridized materials were dispersed, and the tips were washed with wash buffer three times. The cDNA was synthesized in the tips by aspirating 50 μL of reverse transcription buffer (1 mmol/L dithiothreitol, 125 μmol/L dNTP mix, and 400 U of MMLV reverse transcriptase, all supplied by Gibco-BRL) into the tips and incubating them at 37 °C for 1 h. The reverse transcription buffer was then replaced with 50 μL of PCR buffer (GeneAmp PCR buffer II, 1.5
mmol/L MgCl₂, 250 μmol/L dNTP mix, and 3.75 U of AmpliTaq DNA polymerase, all supplied by Perkin-Elmer) and primers for the human glyceraldehyde-3-phosphate dehydrogenase gene (Clontech); the bottom end of the tip was then sealed with a heated razor blade. Tips were detached from pipettes, and 1 drop of nuclease-free mineral oil was added from the top. PCR was started in a Robocycler (Stratagene), with 35 cycles of denaturation at 95 °C for 1 min, followed by annealing at 60 °C for 1 min and extension at 72 °C for 2 min. For negative PCR controls, the oligonucleotide in the tips was hybridized with mRNA but not reacted with reverse transcriptase. The resulting PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and analyzed by FMBIO II. As shown in Fig. 1C, the PCR products of the human glyceraldehyde-3-phosphate dehydrogenase gene were amplified in oligo(dT)-immobilized tips from the captured mRNA of K562 cell lysates, whereas appropriate PCR products were not amplified when the cDNA synthesis step was omitted.

In this study, we demonstrated the feasibility of our oligo(dT)-immobilized pipette tips for both mRNA preparation and RT-PCR. By using these tips, one can minimize labor-intensive steps for mRNA preparation, such as phenol–chloroform extraction, centrifugation, oligo(dT) cellulose columns, and/or magnet separation. Minimal use of consumables and reagents also represents lower costs. In this study, we used standard 200-μL tips; however, we can also use various tips, depending on the application. For example, long-neck tips used for sequencing gel electrophoresis may be better for large-scale mRNA preparation because of the wider surface area. Ten-microliter tips may be attractive for small-scale mRNA preparation or a miniaturized system. To conduct PCR, only Robocycler works for our tips at this moment, although some manufacturers may produce suitable thermal cyclers in the future. If these tips are attached to robotics for microplates, mRNA can be prepared from 96 different samples simultaneously with minimum handling. Furthermore, these tips may also contribute to a cleaner environment by reducing the amount of disposable plastic products.

Because of the variety of advantages, our oligo(dT)-immobilized pipette tips may be acceptable as a new methodology for various mRNA analyses in basic laboratory research and outdoor field research, as well as molecular pathological diagnostics.

**Fig. 1. Characteristics of the oligo(dT)-immobilized pipette tips.**

(A) Various concentrations (0–30 ng) of rabbit globin mRNA were stained with Yoyo-1 in a final dilution of 1:1000 in 20 μL of dimethylpyrocarbonate water and spotted on a polypropylene sheet. The Yoyo-1 fluorescence at 505 nm was measured by FMBIO II with excitation at 530 nm by a laser. (Insets) Two hundred nanograms of rabbit globin mRNA (top and bottom inset) or 5 μg of rat brain total RNA (bottom inset) was suspended in 50 μL of hybridization buffer and aspirated into the oligo(dT)-immobilized pipette tips (oligo(dT)+) or control regular tips (oligo(dT)−). After hybridization at room temperature for 30 min, the solution was dispensed, and the tips were washed three times by aspirating then dispensing wash buffer. Yoyo-1 solution (50 μL) in a final dilution of 1:1000 in wash buffer was aspirated into the tips, and the Yoyo-1 fluorescence was determined by FMBIO II (top inset). In parallel experiments, the hybridized mRNA was dissociated from the tips by aspirating 50 μL of boiling dimethylpyrocarbonate water and dispensing it into fresh tubes. Samples were diluted 1:4 with Yoyo-1 diluted 1:1000 in diethylpyrocarbonate water, and 20 μL of each sample was spotted on a polypropylene sheet to measure Yoyo-1 fluorescence (bottom inset). (B) Eight oligo(dT)-immobilized pipette tips were hybridized with 200 ng each of rabbit globin mRNA as described in the text. The captured mRNA was dissociated by aspirating boiling diethylpyrocarbonate water. All samples were mixed, lyophilized, and analyzed using 2% agarose gel electrophoresis. RNA bands were visualized by staining with ethidium bromide. S indicates mRNA recovered from the oligo(dT)-immobilized tips, and C indicates 1 μg of rabbit globin mRNA used as a control. (C) Lysate (50 μL) containing 1 × 10⁶ K-562 cells was aspirated into the oligo(dT)-immobilized pipette tips for hybridization as described in the text. The captured mRNA was further processed by cDNA synthesis followed by PCR to amplify the glyceraldehyde-3-phosphate dehydrogenase gene in the same tips as described in the text. PCR was conducted in the Robocycler (Stratagene) with 35 cycles of denaturation at 95 °C for 1 min, followed by annealing at 60 °C for 1 min and extension at 72 °C for 2 min. S indicates mRNA from K-562 cells, and PC represents control template supplied from Clontech. For negative controls (NC), we used the tips where mRNA hybridization, but not cDNA synthesis, occurred.

**References**

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Many enzymes involved in the biotransformation of xenobiotics have been shown to be genetically polymorphic, as reviewed recently (1). The rapidly growing list of polymorphic genes coding for xenobiotic-metabolizing enzymes includes various cytochrome P450s, flavin monooxygenases, epoxide hydrolase, NAD(P)H:quinone oxidoreductase, UDP-glucuronosyltransferases, N-acetyltransferases, glutathione-S-transferases, and paraoxonase. Among these are enzymes of great importance in the field of environmental and occupational toxicology; therefore, an effort should be made to develop valid genotyping techniques easily applicable on a large scale, e.g., in the framework of epidemiological studies in industrial settings.

Although in clinical practice genotyping is usually carried out on DNA extracted from white blood cells, the necessity of blood sampling probably contributes to limit the use of genotyping techniques in large cohort studies. The principal objective of this study is, therefore, to validate a reliable and robust protocol aimed to obtain, from urinary samples, a biological material on which molecular biology techniques such as restriction fragment length polymorphism (RFLP) analysis could be applied to determine the genotypic status of individuals. Indeed, in occupational and environmental medicine urine is a biological medium that is more easily sampled than blood and is very often obtained for biomonitoring purposes.

The protocols used for DNA isolation from whole blood and urine samples were as follows: Genomic DNA was isolated from whole blood with the QIAamp blood kit (QIAGEN, cat. no. 29104) according to the manufacturer’s instructions. To extract genomic DNA from urine, the procedure of the QIAamp Viral RNA kit (QIAGEN, cat. no. 29504) was followed: 4 mL of urine was centrifuged for 5 min at 20 000g. The supernatant was decanted, and the pellet was suspended in 140 μL of buffer AVL supplemented with carrier RNA (QIAamp Viral RNA kit). Nucleic acids were then extracted according to the manufacturer’s instructions and eluted in a final volume of 50 μL of water (urine extract).

A practical application of this urinary extraction protocol is illustrated by the determination of three RFLPs already described for an important drug-metabolizing enzyme in industrial toxicology, i.e., cytochrome P4502E1 (CYP2E1). RFLP analysis of the human CYP2E1 gene displays many polymorphisms (2), and we have selected three of them, detectable with the restriction endonucleases Rsal, Psil, DraI, and Taql for the validation process: polymorphism c1→c2 (3), polymorphism D→C (4), and polymorphism A1→A2 (5). Four Caucasian subjects were selected on the basis of their CYP2E1 genotype status, determined previously following classic blood extraction. Subject 1 is homozygous c1/c1 and D/D, subject 2 is heterozygous c1/c2 and D/C, subject 3 is homozygous A1/A1, and subject 4 is heterozygous A1/A2. Each subject was asked to provide a blood sample (blood a) and two different urinary samples. The first urine sample corresponded to the first morning void (urine b), which is usually more concentrated in exfoliated urinary tract cells; the second urine sample was an end-of-workday void or spot sample (urine c), such as the samples usually obtained in the framework of biomonitoring programs applied in occupational medicine. The extraction protocols described above were applied to the respective specimens. The protocol of this study was approved by the local ethics committee, and all subjects gave their informed consent.

Blood and urinary samples were amplified by PCR under the following conditions: PCR was carried out on a Perkin-Elmer 2400 thermocycler. The reaction mixture contained 1× PCR buffer (75 mmol/L Tris-HCl, pH 9, 20 mmol/L ammonium sulfate, 0.1 mL/L Tween 20), 2.5 mmol/L MgCl2, 200 μmol/L each dNTP, 0.5 μmol/L each primer, and 2.5 U of Goldstar Red DNA polymerase (Advanced Biotechnologies). The starting material was 10 μL of urine extract or 1 μL of blood extract, and the final reaction volume was 50 μL. The mixture was submitted to the following temperature profile: initial denaturation for 5 min at 95 °C, 40 cycles with denaturation for 30 s at 94 °C, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min 30 s; the final extension was at 72 °C for 5 min.

Restriction enzyme digestion. Twenty microliters of the PCR products was digested with 5 U of enzyme (New England Biolabs) in a final volume of 50 μL of 1× buffer, according to the manufacturer’s instructions. The reaction was at 37 °C for 1 h, except for Taql, which was at 65 °C for 1 h. The whole digestion mixture was electrophoresed in a 2% agarose gel for 1 h at 5V/cm in 1× Tris-borate-EDTA buffer.

Primers and restrictions for polymorphism c1→c2. DNA was amplified with primers c1pol1 (5’-CGTCAGTGTCATTTAGGATGTTCC) and c1pol2 (5’-GCCAGTGTGCTCTCATGTGTCAG). The product was 489 bp long and, depending on the allele considered, was cut into two fragments of 368 bp and 121 bp by the Psil enzyme (c2) or into two fragments of 354 bp and 135 bp by the Rsal enzyme (c1).

Primers and restrictions for polymorphism D→C. DNA was amplified with primers dra1pol1 (5’-TCTGTCAGTTCGCTGAAGCAG) and dra1pol2 (5’-AGTAGCTGTGACATTAGGGC). The product was 488 bp long and, depending on the allele considered, was either cut (D) or not cut.