Fig. 2. Positions of the Capturing Oligos, PCR Primers, and Probe in *Le1*.
DNA sequences of the capturing oligos for *Le1* are indicated by open boxes. We used the DNA sequences of Le1n02-5' and Le1n02-3' as primers and Le1-Taq as the TaqMan probe for real-time PCR for *Le1*. The amplified region is shown with a bold line.

Table 1. List of Capturing Oligos

Capturing oligo	Orientation	Sequence
Upstream-s[a]	sense	5'-NH_2-GACGCTATTGTGACCTCCTC-3'
Upstream-a[a]	antisense	5'-NH_2-GAGGAGGTCACAATAGCGTC-3'
Le1n02-5'-s[a]	sense	5'-NH_2-GCCCTCTACTCCACCCCCA-3'
Le1n02-5'-a[a]	antisense	5'-NH_2-TGGGGGTGGAGTAGAGGGC-3'
Le1-Taq-s[a]	sense	5'-NH_2-AGCTTCGCCGCTTCCTTCAACTTCAC-3'
Le1-Taq-a[a]	antisense	5'-NH_2-GTGAAGTTGAAGGAAGCGGCGAAGCT-3'
Le1n02-3'-s[a]	sense	5'-NH_2-AAAAGGCTTGCAGATGGGC-3'
Le1n02-3'-a[a,b]	antisense	5'-NH_2-GCCCATCTGCAAGCCTTTT-3'
Downstream-s[a]	sense	5'-NH_2-GTCGTCGCTGTTGAGTTTGA-3'
Downstream-a[a]	antisense	5'-NH_2-TCAAACTCAACAGCGACGAC-3'
Le1n02-3'-a(−8 mer)[b]	antisense	5'-NH_2-GCAAGCCTTTT-3'
Le1n02-3'-a(+8 mer)[b]	antisense	5'-NH_2-GAAGGCAAGCCCATCTGCAAGCCTTTT-3'
SSIIb3-3'[c]	antisense	5'-NH_2-GATCAGCTTTGGGTCCGGA-3'
RRS-5'short-s[d]	sense	5'-NH_2-CCTTTAGGATTTCAGCATCA-3'

[a]Capturing oligos for *Le1* used to investigate the optimal location to bind target DNA.
[b]Capturing oligos for *Le1* used to investigate the optimal length of the capturing oligo.
[c]Capturing oligo for the starch synthase IIb gene in maize (negative control oligo).
[d]Capturing oligo for *RRS*.

oligo sequence for *RRS* was selected from a PCR primer. The PCR primer sequence for the starch synthase IIb gene (SSIIb) described in the previous report was used as the negative control oligo.[6]

Preparation of capturing oligo-immobilized PCR tubes. PCR tubes were treated with S-Bio® PrimeSurface® (Sumitomo Bakelite, Hyogo, Japan), providing a unique biocompatible phospholipid polymer and a highly active functional ester moiety to covalently bind the attachment site for amino-modified oligonucleotides under alkaline conditions.[5] The 5'-amino-modified oligo solution (Nippon Gene) was prepared at 10 μM with an alkaline solution, and was added to the PCR tube. After incubating for 90 min, the oligo-immobilized PCR tube was treated with a 0.1 M sodium hydroxide solution to block the remaining functional ester moieties, and then washed with water. To investigate the effects of different amounts of the capturing oligo, 0.1 and 1 μM oligo solutions were also used.

Single-tube real-time PCR. Each sample (25 μl) was added to the oligo-immobilized PCR tube. After preheating at 95 °C for 5 min, the tube was incubated at 50 °C for 30 min. To investigate the effects of incubation temperature and time, incubation was also carried out at 25, 40, and 60 °C for 15, 60, and 120 min. The tube was washed three times with 200 μl of a washing buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA, and 0.2 M NaCl). A 25 μl volume of the reaction solution containing 11 μl of nuclease-free water, 12.5 μl of the FastStart Universal Probe Master (Roche, IN, USA), 0.5 μl of a 10 μM probe solution, and 0.5 μl each of a 25 μM primer solution was added to the washed tube. The PCR system for *Le1* detection is shown in Fig. 2. The probe and primer sets had previously been used for the quantification of GM soybean based on real-time PCR.[7] The probe and primer set for *RRS* were purchased from Fasmac (Kanagawa, Japan). The real-time PCR assay was performed by using a 7300 Real-time PCR system (Applied Biosystems, CA, USA). The

thermal cycle program for all primers was as follows: 2 min at 50 °C, 10 min at 95 °C, and subsequent amplification of the DNA for 40 cycles of 30 s at 95 °C and 60 s at 59 °C. A standard curve was prepared from the GM Soybean (RRS) Detection Plasmid Set (Nippon Gene), and was run on each plate to validate the method and to determine the efficiency of the reaction which was taken into account in the final calculations. The standard curve for each gene was linear ($r > 0.99$).

Statistical analysis. Each data value is presented as the mean with standard deviation. A linear regression analysis of data for the correlation between Ct values and log of DNA or soybean lysate (%) was performed by the least-squares method. The real-time PCR efficiency was calculated by using the slope of the linear regression plot according to the equation $E = 10^{(-1/\text{slope})}$.[8] A PCR efficiency (E) of 2.00 corresponds to a doubling of the copy number per PCR cycle. Analyses were performed by using Sequence Detection Software version 1.4 (Applied Biosystems) and Excel 2000 (Microsoft, WA, USA).

Results and Discussion

DNA is generally isolated by using organic extraction and silica-membrane column extraction steps. However, these extraction steps are time-consuming and are difficult to automate and downscale to a small sample volume. The hybridization-bead method can resolve these problems. However, the hybridization-bead method is not directly used in real-time PCR, and the number of tube-to-tube transfers still needs to be minimized. We developed a novel real-time PCR method using hybridization as shown in Fig. 1. In this study, we first analyzed the reaction conditions, such as the capturing oligo design and hybridization conditions, by using soybean genomic DNA and lysate. We then used the method to detect GM soybean.

Capturing oligo

The design of capturing oligos is an essential factor in the specific recovery of target DNA in a hybridization system. We investigated the optimal location for target DNA, and the effects of its orientation, length and concentration when immobilized on PCR tubes. We chose capturing oligo sequences from the region between approximately 100-bp upstream and downstream of the PCR amplification region (Fig. 2). The capturing oligos were designed by using sequences from several regions and from both orientations (Table 1). Among all the capturing oligos, the highest copy number of *Le1*, 110 ± 16 copies, was detected by using Le1n02-3′-a (Table 2). Therefore, Le1n02-3′-a was used for all subsequent soybean DNA detection in this study. For comparison, DNA was extracted from soybean powder by using a silica membrane-type kit, and was directly assayed by a general real-time PCR method which was carried out with the PCR reagents and thermal cycle program described in the Materials and Methods section. The copy number of *Le1* in 100 ng of soybean DNA was 9068 ± 497 copies ($n = 4$). Accordingly, the recovery of soybean DNA by using an Le1n02-3′-a-immobilized tube was estimated to be 1.2%. *Le1* could not be detected by using an SSIIb3-3′-immobilized tube which was designed to capture maize DNA. These results suggest that the oligo orientation and secondary structure of target DNA are important factors for the selection of capturing oligos. Furthermore, this method could isolate target DNA by specific hybridization to the capturing oligo.

Table 2. Ct Values and Copy Numbers Measured for *Le1* from 100 ng of Soybean DNA Using Different Capturing Oligos

Capturing oligo	Ct value	Copy number
Upstream-s	34.2 ± 0.5	71 ± 23
Upstream-a	36.4 ± 0.5	15 ± 4
Le1n02-5′-s	33.9 ± 0.3	83 ± 14
Le1n02-5′-a	39.0 ± 0.4	3 ± 1
Le1-Taq-s	34.2 ± 0.2	69 ± 11
Le1-Taq-a	36.7 ± 1.0	13 ± 6
Le1n02-3′-s	36.8 ± 0.4	12 ± 3
Le1n02-3′-a	33.5 ± 0.2	110 ± 16
Downstream-s	36.6 ± 0.4	13 ± 4
Downstream-a	34.0 ± 0.2	76 ± 10
SSIIb3-3′	ND	ND
None	ND	ND

None, no oligo was immobilized on the tube.
Data are mean values ± SD ($n = 4$).
ND, not detected.

For a DNA microarray probe, the optimal oligonucleotide probe length in a hybridization system is typically 35- to 70-mer.[9] We examined the effect of oligonucleotide length for our method. Among the examined oligonucleotides, the 19-mer oligonucleotide gave the highest copy number of *Le1* (Le1n02-3′-a (−8 mer) [11 mer in length], 14 ± 4 copies; Le1n02-3′-a [19 mer in length], 157 ± 12 copies; Le1n02-3′-a (+8 mer) [27 mer in length], 32 ± 10 copies; $n = 4$). Consequently, this length was considered to be the most suitable. We also investigated the effect of different concentrations of the capturing oligo immobilized on the PCR tube. The copy number of *Le1* was correlated with Le1n02-3′-a concentrations from 0.1 to 10 μM (0.1 μM, 2 ± 1 copies; 1 μM, 12 ± 4 copies; 10 μM, 101 ± 28 copies; $n = 4$). Thus, 10 μM was considered to be the optimal oligo concentration for immobilizing on the PCR tube.

Incubation temperature and time

Temperature is an important factor for hybridization, and various temperatures from 42 to 65 °C are used with the hybridization bead methods.[1,10–12] We tested an incubation temperature from 25 to 60 °C. The copy number of *Le1* in an Le1n02-3′-a-immobilized tube was successfully detected at 40 to 65 °C, especially at 50 °C, but detection at 25 °C was poor (25 °C, 40 ± 5 copies;

40 °C, 115 ± 35 copies; 50 °C, 169 ± 43 copies; 60 °C, 104 ± 22 copies; $n = 4$). This result suggested 50 °C to be the optimum incubation temperature with this method.

We also investigated the effect of incubation time on the detection of *Le1*. The copy number of *Le1* in an Le1n02-3'-a-immobilized tube appeared to be correlated with the incubation time; however, there was little difference in the detected copy number among incubations ranging from 15 to 120 min (15 min, 111 ± 35 copies; 30 min, 119 ± 16 copies; 60 min, 151 ± 15 copies; 120 min, 185 ± 33 copies; $n = 4$). In previous reports, the hybridization times were 2 h for a one-step system and 4 h for a two-step system.[1,10] Our data show that this method can be carried out with a relatively short hybridization time. We subsequently used 30-min hybridization in this study.

Effect of food matrix on the determination of Le1

To investigate the food matrix effects, we detected *Le1* from 100 ng of soybean DNA in 50 mg/ml of wheat and corn lysates. The copy number of *Le1* in an Le1n02-3'-a-immobilized tube was not significantly affected by the presence of wheat and corn lysates (lysis buffer only, 74 ± 18 copies; corn lysate, 85 ± 35 copies; wheat lysate, 106 ± 25 copies; $n = 4$). This result indicates that the method can detect soybean DNA from a food lysate containing other food product; in this case, a wheat or corn extract.

Concentration-dependent detection

We investigated whether the copy number of *Le1* was detected from soybean DNA and lysate in a concentration-dependent manner. The copy number of *Le1* in the Le1n02-3'-a-immobilized tube was linearly correlated with the soybean DNA and lysate amounts (Table 3). A strong correlation was appeared between the log values for the soybean DNA and lysate amounts and the Ct values for *Le1* [DNA, $Ct = -3.41 \times \log(\text{soybean DNA ng}) + 39.19$, $r = 0.99$; lysate, $Ct = -3.14 \times \log(\text{soybean lysate (\%)}) + 35.19$, $r = 0.99$]. The detection limits were 10 ng of soybean DNA and 1% soybean lysate (data not shown). The PCR efficiency (E), where 2.00 corresponds to a doubling of the copy number per PCR cycle, was 1.96 in soybean DNA and 2.08 in the lysate sample. The PCR efficiency (E) in soybean DNA samples has reportedly ranged from 1.80 to 2.10 for various DNA extraction methods.[13] These results indicate that the proposed method allows concentration-dependent detection of soybean genes in both DNA and crude samples without inhibiting the PCR efficiency.

Effect of heat treating a sample on the determination of Le1

The DNA extracted from heat-treated soybean by using the silica membrane kit was degraded into small fragments, and a lower copy number of *Le1* was detected in the degraded DNA than that from the intact DNA.[6] To test the effect of heat treating a sample on the determination of *Le1*, we heated the soybean sample for various times. The copy number of *Le1* in the Le1n02-3'-a-immobilized tube was detected in all samples (0 min, 3578 ± 527 copies; 30 min, 15967 ± 800 copies; 60 min, 8011 ± 783 copies; $n = 4$). DNA microarray analyses are affected by the secondary structures and long nucleic acid molecules which reduce the hybridization efficiency.[14] The difference in copy number of *Le1* in the Le1n02-3'-a-immobilized tubes between the heat-treated samples is likely to have been due to differences in the secondary structures and lengths of degraded DNA. Our data suggest that this method can easily detect the copy number of *Le1* in a heat-treated soybean sample.

Table 3. Soybean DNA and Lysate Concentration Effects on Ct Values and Copy Numbers Measured for *Le1* by Using Le1n02-3'-a-Immobilized Tubes

Sample amount	Ct value	Copy number
Soybean DNA		
10 ng	35.8 ± 0.4	17 ± 5
100 ng	32.3 ± 0.2	174 ± 28
1000 ng	29.0 ± 0.4	1677 ± 493
Soybean lysate		
1%	35.7 ± 0.2	22 ± 3
10%	32.1 ± 0.2	285 ± 51
100%	28.7 ± 0.1	3089 ± 206

Soybean lysate (50 mg/ml) is defined as 100%.
Data are mean values ± SD ($n = 4$).

Table 4. GM Soybean Lysate Concentration Effects on Ct Values and Copy Numbers Measured for *RRS* by Using RRS-5'Short-s Immobilized Tubes

Sample amount	Ct value	Copy number
1%	36.5 ± 0.6	17 ± 7
10%	33.4 ± 0.3	143 ± 33
100%	29.5 ± 0.2	2111 ± 229

GM soybean lysate (50 mg/ml) is defined as 100%.
Data are mean values ± SD ($n = 4$).

GM soybean detection

To apply this method to detect GM soybean, an RRS-5'short-s oligo based on the *RRS* PCR primer was immobilized on the PCR tube. The copy number of *RRS* in the RRS-5'short-s immobilized tube was correlated with the GM soybean lysate amount in a concentration-dependent manner (Table 4). The log value for the GM soybean lysate amount was correlated with the Ct value for *RRS* [$Ct = -3.54 \times \log(\%\text{ GM soybean lysate}) + 36.66$, $r = 0.99$]. The PCR efficiency (E) was 1.92. Certified reference materials are frequently used as calibrators for GMO quantification by real-time PCR. The copy number of *RRS* in the RRS-5'short-s immobilized tube was also detected in the 50 mg/ml lysates of certified reference materials containing 1%, 2% and 5%

Roundup Ready soybean (1%, 17 ± 6 copies; 2%, 30 ± 9 copies; 5%, 63 ± 16 copies; $n = 4$). These results indicate that the method can detect specific genes in Roundup Ready soybean by using a corresponding capture oligo.

Conclusion

The real-time PCR method using capturing oligo-immobilized PCR tubes described in this study could detect soybean and GM soybean genomic DNA in samples comprising 1–100% soybean and GM soybean. In addition, this method could also detect GM soybean genomic DNA in certified reference materials containing 1, 2 and 5% Roundup Ready soybean. Many countries and areas have specified GMO labeling regulations to protect consumers' rights; labeling thresholds for GMOs are 3% in Korea and 5% in Japan.[15,16] The method described in this study can detect these threshold levels of GM soybean.

We have described in this study the development and validation of a novel real-time PCR method using capturing oligo-immobilized PCR tubes. This method is simple and specific, and can detect the copy number of target DNA from crude food matrices in a concentration-dependent manner. This method would be useful in many types of food analysis.

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All-in-One Tube Method for Quantitative Gene Expression Analysis in Oligo-dT$_{30}$ Immobilized PCR Tube Coated with MPC Polymer

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In this report, we have developed a novel quantitative RT-PCR protocol in which the procedure including mRNA purification can be performed in an all-in-one tube. To simplify gene expression analysis, oligo-dT$_{30}$ immobilized PCR tubes were used serially to capture mRNA, synthesize solid-phase cDNA, and amplify specific genes. The immobilized oligo-dT$_{30}$ can efficiently capture mRNA directly from crude human cell lysates. The captured mRNA is then amplified by one-step reverse transcription PCR (RT-PCR) with initial cDNA synthesis followed by PCR. In RT-PCR, this new reusable PCR tube device can be employed for multiple PCR amplifications with different primer sets from a solid-phase oligo-dT$_{30}$ primed cDNA library. This paper introduces a novel and highly reliable all-in-one tube method for rapid cell lysis, followed by quantitative preparation and expression analysis of target mRNA molecules with small amounts of sample. This procedure allows all steps to be carried out by sequential dilution in a single tube, without chemical extraction. We demonstrate the utility of this novel method by quantification of two housekeeping genes, β-actin and GAPDH, in HeLa cells. We believe this new PCR device can be useful as a platform for various mRNA expression analyses, including basic research, drug screening, and molecular toxicology, as well as for molecular pathological diagnostics.

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Introduction

Reverse transcription (RT) and polymerase chain reaction (PCR) have been widely used to assess the expression of specific genes in cells and tissues. RT-PCR involves several steps including mRNA preparation, complementary DNA (cDNA) synthesis by reverse transcription, and finally cDNA amplification by PCR. Quantitative real-time polymerase chain reaction (Q-PCR) in combination with reverse transcription (RT-Q-PCR), one of the most useful technologies for detecting transcriptional mRNA from cells, tissues and whole blood, is widely used by many basic research and medical institutions. However, in many cases, total RNA or mRNA must first be purified from a sample suspended in lysis buffer. The reliability of these measurements depends on the accuracy of each step, including preparation and recovery of total RNA, reverse transcription of mRNA into cDNA, and quantitative specific amplification of all target genes. As the optimizing of each of these steps is important, the number of tube-to-tube transfers should be minimized in order to avoid loss of templates and decrease the risk of contamination.[1] Contamination can occur due to environmental RNases or material carried over from sample to sample, as well as previously generated amplicons present on laboratory equipment. What is desirable is the sequential performance of several steps in an all-in-one tube, especially when starting with a small number of target mRNA molecules, such as a few virus particles.[2,3] An important limiting factor for the routine application of expression analyses is related to the method for preparing total RNA or mRNA from large numbers of samples. Several methods are in general use for mRNA purification, such as organic extraction or trapping on magnetic beads immobilized with oligo-dT.[4-10] The quantity of extracted total RNA is measured by the absorbance at 260 nm and the quality is assessed by gel electrophoresis to confirm the presence of 18S and 28S rRNA bands; however, whether the purified RNA represents all species of mRNA in the same proportions present in the original material remains unknown. The reporting of mRNA quantification results as the quantity of the target mRNA per microgram of total RNA may be inaccurate because mRNA represents only 1 - 5% of total RNA. Furthermore, mRNA concentrations can vary even when the total RNA concentration is constant. Yields of total RNA or mRNA also vary widely depending on the isolation method used. Commercial kits for RNA purification therefore commonly employ either chaotropic agents or lysis buffers containing strong detergents, or a combination of the two, in order to achieve rapid denaturation of proteins. Nucleic acids are then extracted to remove these chemicals, because their presence would interfere with subsequent enzymatic reactions. The chaotropic agent guanidine isothiocyanate (GuSCN) has long been the chemical of choice for nucleic acid preparation. It is particularly useful for mRNA studies,[11,12] because it rapidly denatures all cellular proteins, as well as serum proteins, including RNases, when added to culture media. GuSCN has also proven superior to all

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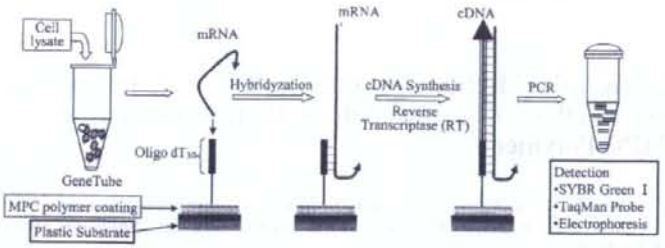

Fig. 1 The concept for the all-in-one tube method, S-BIO® GeneTube.

other tested methods for the recovery of RNA extracted from tissue.[13] Due to its strong chemical action, GuSCN at high concentrations offers the further advantage of allowing safe storage of the samples until they are processed for quantification. However, all traditional protocols require removal of GuSCN prior to RT and PCR to avoid inactivation of the enzymes.[14] Typically, this is done by extraction with phenol-chloroform and purification of the nucleic acids through alcohol precipitation cycles, or by absorption of the freed RNA to a matrix such as glass fiber filters, silica gel or aluminum oxide membranes, magnetic beads or proprietary compositions, usually followed by elution using a relatively large volume of wash medium. Both these approaches are time-consuming and involve a number of steps that can lead to incomplete RNA recovery.

In view of these limitations we devised an alternative strategy in which the sample can be collected and denatured in a minimal volume of a GuSCN solution. Rather than being washed out, this solution is then diluted and used for direct incubation in an oligo-dT_{30} immobilized PCR tube (S-BIO® GeneTube). After washing, the GeneTube is analyzed by performing quantitative RT-PCR (RT-Q-PCR). This new procedure is hereafter referred to as the all-in-one tube method in a GeneTube coated with biocompatible polymer.[15,16] It is being described here in full for the first time. At present, it is the only method that allows a whole sample to be processed from lysis to RT-PCR in the same tube under conditions that permit precise quantification of RNA copy numbers. This fully optimized method is sensitive enough to detect specific sequences. As shown in Fig. 1, the GeneTube is ideally suitable for fast and highly sensitive gene expression analyses of multiple samples including small whole specimens.

Experimental

Materials

Reagents for RT-PCR [Transcriptase and *Taq* polymerase (ExScript® RT reagent Kit and EX *Taq*™ Hot Start; TaKaRa Biosciences Co., Ltd., Ohtsu, Japan)], the HeLa cell line (American Type Culture Collection), One STEP Ladder50 (0.05 - 2 kbp, Nippon Gene Co., Ltd., Tokyo, Japan), Sepasol®-RNA I Super (Nacalai Tesque, Inc., Kyoto, Japan), and MagNA Pure LC RNA Isolation Tissue Lysis Buffer (Roche Applied Science) were purchased from the designated suppliers. All synthetic DNA oligonucleotides were ordered from Nippon Gene Co., Ltd. (Toyama, Japan) or Sigma-Genosys (Hokkaido, Japan). All chemicals and solvents were purchased from Wako (Tokyo, Japan) or Sigma/Aldrich (Tokyo, Japan), unless stated otherwise, and used without additional purification.

Support media and immobilization of mRNA capture probes

The new PCR device S-Bio® GeneTube (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) consists of a commercially available 200 µl PCR tube (conventional PCR tubes; PE Applied Biosystems), which has been grafted with an original biocompatible phospholipid polymer, poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl polyehyleneglycol methacrylate (MEONP)] (PMBN) hydrophilic polymer.[13,14] The biomembrane has a highly active functional ester moiety to covalently bind the attachment site for C_6-amino-oligonucleotides. The PMBN was synthesized from the mole functions of each monomer unit (MPC, BMA and MEONP), at about MPC/BMA/MEONP = 0.2/0.7/0.1. This activated tube is stable at room temperature and around 60% humidity for a few months (data not shown).

The 5′-amine-terminated oligo-dT_{30} is dissolved in immobilization buffer (Sumitomo Bakelite Co. Ltd.) to a final concentration of 100 nM as coating solution. Next, 100 µL of this oligo-dT_{30} coating solution is added to each PCR tube, and the tubes are incubated for 1 h at room temperature. The excess amine-reactive group (MEONP) is inactivated for 5 min at room temperature in the blocking buffer solution containing 0.1 M NaOH. Finally, the PCR tubes are washed three times with 300 µl room-temperature water and then dried by centrifugation for 2 min at 200*g*. The oligo-dT_{30} immobilized PCR tubes (S-Bio® GeneTube) are stored under desiccation at 4°C until use. These supports have been stored for 6 months without any reduction in binding capacity.

Preparation of cell lysate and mRNA capture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum and L-glutamine, and the number of cells was determined by hemocytometer. Before RNA preparation, the cells were washed in phosphate-buffered saline and suspended at a final concentration of approximately 10^4 to 10^5 cells/ml in 100 µl of lysis buffer (MagNA Pure LC RNA Isolation Tissue Lysis Buffer) at room temperature, mixed by pipetting up and down 10 times with the pipette tip at the bottom of the tube and then centrifuged at 13000*g* at 4°C for 5 min. Next, 1 µl of each supernatant solution was applied to the S-Bio® GeneTube and diluted in dilution buffer [100 mM Tris-HCl (pH 7.6), 0.4 M LiCl, 20 mM EDTA] to a final volume of 20 µl. After 15 min incubation at room temperature, unbound materials were removed by aspiration, and the PCR tubes were washed three times with 100 µl of wash buffer (10 mM Tris-HCl pH 7.6, 0.2 M LiCl, 1 mM EDTA) by mixing and aspirating as above. The tubes were immediately used for reverse transcription for the cDNA library.

Table 1 Primers used in RT-PCR analysis of characterization for the full length cDNA related to human GAPDH and β-actin genes

Human GAPDH	Sequence	Size
GAPDH(36-55)Forward	5'-TCCTCCTGTTCGACAGTCAG-3'	1252 bp
GAPDH(404-423)Forward	5'-TCTTCACCACCATGGAGAAG-3'	864 bp
GAPDH(876-895)Forward	5'-CAAGAAGGTGGTGAAGCAGG-3'	412 bp
GAPDH(1268-1287)Reverse	3'-GATGGTACATGACAAGGTGC-3'	
Human β-actin	Sequence	Size
β-actin(72-91)Forward	5'-CCATGGATGATGATATCGCC-3'	1668 bp
β-actin(426-439)Forward	5'-GCGAGAAGATGACCCAGATC-3'	1340 bp
β-actin(865-884)Forward	5'-TTCCTTCCTGGGCATGGAGT-3'	893 bp
β-actin(1347-1366)Forward	5'-ACTGGAACGGTGAAGGTGAC-3'	413 bp
β-actin(1740-1759)Reverse	5'-CAGTGTACAGGTAAGCCCTG-3'	

Multi-step total RNA extraction from HeLa cells

The total RNA of cultured HeLa cells as a reference was isolated with 1 ml of Sepasol-RNA I Super (Nacalai Tesque) according to the manufacturer's protocol. Total RNA was measured by a conventional UV (A_{260}) absorption method, and the quality was estimated with A_{260}/A_{280} nm.

General reverse transcription (RT) method using GeneTube

Total HeLa RNA was reverse transcribed to cDNA then subjected to PCR using either TaKaRa (ExScript® RT reagent Kit and EX *Taq*™ Hot Start Version). The incubation was performed in a Peltier Thermal Cycler PTC-200 (MJ Research). The GeneTube was transferred to total RNA solution (19 µl of dilution buffer and 1 µl of total RNA (10 ng/µl)), incubated for hybridization at room temperature for 15 min and then the PCR tubes were immediately washed three times with 100 µl of wash buffer (10 mM Tris-HCl pH 7.6, 0.2 M LiCl, 1 mM EDTA) by mixing, followed by its removal by aspiration. For the solid-phase cDNA synthesis, additional reagents (TaKaRa: ExScript® RT reagent Kit, RR035A) containing 1X ExScript® Buffer, 50 U ExScript® RTase, 0.5 mM dNTP, 10 U RNase Inhibiter were gently added to the GeneTube. The reverse transcription reaction was performed at 42°C for 15 min in 20 µl, and the reagents were removed by aspiration. Consequently, single strand cDNA was synthesized using the mRNA captured on the GeneTube as solid-phase oligo-dT_{30} primer. On the other hand, cDNA synthesis in conventional PCR tubes was performed in the solution phase using the above RT mixture plus 2.5 µM oligo-dT primer as a reference, and 2 µl of the cDNA products was transferred to a new tube for conventional PCR analysis.

General PCR analysis

The 25 µl amplification reactions contained 1X EX *Taq*™ buffer, 0.2 mM dNTP, 0.2 µM of forward and reverse primers and 0.625 U of DNA polymerase (Ex *Taq*™ Hot Start Version). The DNA polymerase was activated by a pre-incubation at 95°C for 10 s, followed by 95°C for 5 s, 62°C for 32 s for 35 cycles, performed in a Peltier Thermal Cycler PTC-200 (MJ Research). After the PCR was completed, the PCR products were analyzed by 2% agarose gel electrophoresis with 0.5 mg/l ethidium bromide in an electrophoresis chamber. Photographic images were recorded using FAS-III (Toyobo, Tokyo, Japan).

Primers for reverse transcription PCR (RT-PCR) studies using GeneTube

These forward and reverse primers were used to characterize the full length cDNA synthesized on the GeneTube by reverse transcriptase. Oligonucleotide primer pairs for human GAPDH (*ca.* 1.3 kb) and β-actin (*ca.* 1.9 kb) were listed in Table 1. Primers for GAPDH and β-actin were purchased from Nippon Gene (Toyama, Japan). Following PCR, primer pairs of human GAPDH and β-actin in Table 2 for repeatability of RT-PCR experiments were designed by ProbeFinder Version 2.20 (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) and purchased from Sigma-Genosys (Tokyo, Japan).

Table 2 Primers used in RT-PCR repeatable analysis of cDNA libraries

Primers set	Primers	Sequence	Size
β-ACTB-A	β-actin (414-433) Forward	5'-CCAACCGCGAGAAGATGA-3'	97 bp
	β-actin (491-510)Reverse	5'-CCAGAGGCGTACAGGGATAG-3'	
β-actin -B	β-actin (1687-1676)Forward	5'-CCCAGCACAATGAAGATCAA-3'	61 bp
	β-actin (1976-1997)Reverse	5'-CGATCCACACGGAGTACTTG-3'	
GAPDH-A	GAPDH(83-101)Forward	5'-AGCCACATCGCTCAGACAC-3'	99 bp
	GAPDH(159-140)Reverse	5'-GCCCAATACGACCAAATCC-3'	
GAPDH-B	GAPDH(955-972)Forward	5'-CTGACTTCAACAGCGACACC-3'	112 bp
	GAPDH(1043-1064)Reverse	5'-CTGTAGCCAAATTCGTTGTCAT-3'	

Reverse transcription quantitative PCR (RT-Q-PCR) protocol

Two-step protocol. After reverse transcriptional reaction, the reaction mixture was immediately removed by aspiration. The PCR thermal cycling protocol consisted of 95°C for 10 s, a DNA polymerase activation step followed by 35 cycles of 95°C for 5 s, 62°C for 32 s. The dissociation curve analysis consisted of 1 cycle of 60°C for 20 s and 1 cycle of 95°C for 15 s. Quantitative Real-time PCR (Q-PCR) was performed in a premade reaction mixture (TaKaRa SYBR® Premix EX *Taq*™, RR041A) in the presence of 200 nM of β-actin primers (TaKaRa ACTB HA031582-F:5'-ATTGCCGACAGGATGCAGA-3', HA031582-R:5'-GAGTACTTGCGCTCAGGAGGA-3': 89 bp), using SYBR® green chemistry and an ABI Prism 7000 (PE Applied Biosystems). The cycle threshold (Ct), the cycle at which certain amounts of PCR products are based on fluorescence, was generated and determined using analytical software (PE Applied Biosystems).

One-step protocol. One-Step RT-Q-PCR was performed using the One-Step SYBR® PrimeScript™ RT-PCR kit (TaKaRa: RR066A and an ABI Prism 7000). The RT-PCR reaction mixture contained 12.5 µl of 2 × One-Step SYBR® RT-PCR buffer III, 2.5 U of TaKaRa Ex *Taq*™ Hot Start (5 U/µl), 0.5 µl of PrimeScript™ RT enzyme Mix II, 200 nmol of PCR primers and distilled water to a final volume of 25 µl in the mRNA captured GeneTube. The following RT-PCR conditions were used: 1 cycle of 42°C for 10 min, followed by 1 cycle of 95°C for 10 s, 35 cycles of 95°C for 5 s, 62°C for 32 s, and for the dissociation curve analysis, 1 cycle of 60°C for 20 s, and 1 cycle of 95°C for 15 s.

Results

The oligo-dT_{30} immobilized PCR tube, S-BIO® GeneTube, has a wide range of useful applications, such as for capture of mRNA, solid phase cDNA synthesis, quantification of specific mRNA, quantitative PCR and RT-PCR, cDNA clone library construction. The use of conventional 200 µl PCR tubes offers major advantages over the recently available oligo-dT immobilized conventional cellulose and magnetic beads because of their rapid and simple washing procedures, requirement of less storage space, and applicability for high throughput liquid handling robotics. The GeneTube is capable of capturing mRNA in a cell lysate of a large amount of 10 cells. This quantity is sufficient for most gene expression experiments because of the capabilities of RT-Q-PCR.

Full-length cDNA synthesis

The cDNA library in HeLa cells was synthesized using the oligo-dT_{30} immobilized on the GeneTube. PCR analysis was

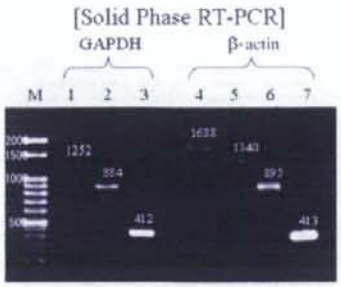

Fig. 2 Characterization of solid phase cDNA synthesized using GeneTube. Following mRNA capture in the GeneTube, a reverse transcription reaction and PCR were performed with oligo-dT_{30} immobilized in the same tube. The PCR products were separated by 2.0% agarose gel electrophoresis, followed by staining with ethidium bromide.

performed using each primer set that yielded amplicons of different sizes, such as 1252, 884 and 412 bp for GAPDH and 1688, 1340, 895 and 413 bp for β-actin. As shown in Fig. 2, all of the PCR products appeared very clearly on electrophoresis. The GeneTube offers a simple all-in-one tube method to capture mRNA and synthesize high-quality cDNA. cDNA synthesis up to 1.3 kb for GAPDH and 1.9 kb for β-actin could be performed on the surface. The PCR products corresponding to 1252, 884, and 412 bp for GAPDH and 1688, 1340, 895 and 413 bp for β-actin could be visualized in lanes 1 to 7. These findings indicate that nearly full-length cDNAs for the GAPDH and β-actin mRNAs were synthesized under these RT-PCR conditions.

Repeatability of sequential PCR analysis on GeneTube

Total RNA of HeLa cells, 10 ng, was suspended in RT buffer and transferred to the GeneTube for hybridization. The captured mRNA was converted to cDNA with the reverse transcriptase. GAPDH and β-actin specific polymerase chain reactions with two different primer sets were performed in sequence using the cDNA synthesized on the GeneTube. After each PCR, the tubes were washed 3 times with 100 µl of wash buffer (10 mM Tris-HCl pH 7.6, 0.2 M LiCl, 1 mM EDTA). The 25 µl amplification reactions contained 1X EX *Taq*™ buffer, 0.2 mM dNTP, 0.2 µM of β-actin-A, -B and GAPDH-A, -B specific primers and 0.625 U of DNA polymerase (EX *Taq*™ Hot Start Version). The DNA polymerase was activated by pre-incubation at 95°C for 10 s, followed by 95°C for 5 s, 62°C for 32 s for 35 cycles, performed in a Peltier Thermal Cycler PTC-200 (MJ Research). The PCR products were then analyzed by 2% agarose gel electrophoresis and the gel was stained with ethidium bromide (Fig. 3). PCR products appeared as clear and strong bands on the stained gel. The GeneTube could be reused for multiple PCRs with different primer sets because the synthesized cDNA was covalently attached to the inside surface of tubes at its 5′ end. This is very convenient as a molecular diagnostic tool because PCR reactions can be repeated in the same tubes if the results are uncertain and/or when different genes expression levels are to be estimated.

Sensitivity of two-step reverse transcription quantitative PCR (RT-Q-PCR)

HeLa cells (approximately 10^3 to 10^6 cells equivalents) were suspended in lysis buffer, and these extracts were applied to the GeneTube for hybridization as described in Experimental. Captured mRNA attached to the oligo-dT_{30} immobilized PCR tubes, GeneTube, was reverse transcribed to cDNA and then subjected to quantitative PCR (Q-PCR) using a pre-made reaction mixture (TaKaRa SYBR® Premix EX *Taq*™, RR041A) and an ABI Prism 7000, which was a two-step RT-Q-PCR reagent kit, used according to the manufacturer's instructions. After the PCR was completed, the bands visible after electrophoresis in 2% agarose gel and ethidium bromide staining correlated well with the obtained quantitative PCR results. The photographic image was recorded using ChemiDoc XRS (Bio-Rad, Tokyo, Japan). As shown in Fig. 4A, the β-actin gene was amplified in the captured mRNA GeneTube from 10 to 10^6 cells and real-time PCR product bands were detected by 35 thermal cycles. All this variability in the quantitative assay was determined from duplicate samples from HeLa cells. Interestingly, the β-actin transcript of HeLa cell could be clearly detected in a cell lysate containing only 10 cells.

The sensitivity of one-step RT-Q-PCR from synthesized cDNA on the GeneTube enabled detection with only 10 cells and was better than that of two-step RT-Q-PCR using the all-in-one tube method (data not shown). This was surprising because one-step RT-Q-PCR was conducted in an efficient solid phase RT reaction and liquid phase PCR by first dissociating mRNA from the GeneTube.

To test the sensitivity of the all-in-one tube method based on Ct values, as shown in Figs. 4B and 4C, calibration curves were prepared for β-actin cDNA from known quantities of HeLa cell (each diluted 10-fold from 10 to 10^4 cells). All calibration curves showed correlation coefficients >0.99, indicating a precise log-linear relationship. The detection limit was less than 10 cells for β-actin gene with the commercially supplied reagents. As shown Fig. 4D, melting behavior, expressed as a plot of the first negative derivative ($-dF/dT$) of the fluorescence *vs.* temperature, revealed high levels of purity and homogeneity of the PCR products. Single and sharply defined melting curves with narrow peaks were obtained for PCR products of the analyzed genes.

All-in-one tube one-step RT-Q-PCR in GeneTube

For comparison of quantification between conventional aqueous phase RT-PCR and the all-in-one RT-PCR method in GeneTube, we performed quantitative RT-PCR (RT-Q-PCR). HeLa cells (10^6 cells and 10-fold diluted 10^5 cells) were suspended in 1 ml of lysis buffer, and these extracts were applied to the GeneTube for hybridization as described in Experimental. Captured mRNA was then subjected to RT-Q-PCR using the One-Step SYBER Green RT-PCR Master Mix Reagents Kit and an ABI Prism 7000. In parallel experiments, known concentrations of total RNA, which were isolated by multi-step total RNA extraction from 10^6 cells or 10^5 cells of HeLa as described in Experimental, were also used as an aqueous phase in the RT-Q-PCR standard method. Both RT-Q-PCR methods were adjusted with the same number of HeLa cells as the starting specimen. As shown in Table 3, there were no differences on Ct values between the solid phase all-in-one tube method in the GeneTube and the conventional aqueous phase RT-Q-PCR.

Discussion

It has become increasingly clear that individual cells in a population do not exhibit identical patterns of gene expression and, hence, expression profiling is more informative if it is quantitative and carried out at the single cell level.[17-22] To

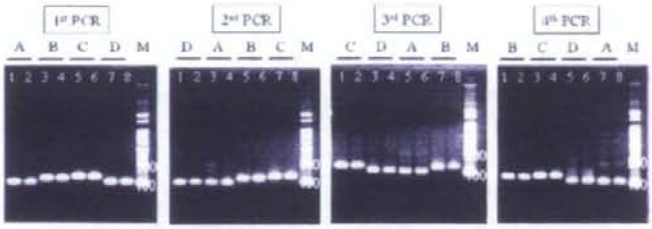

Fig. 3 Repeatability of PCR amplification. Sequential PCR was performed in various orders as shown in Table 3. Lane: A, β-actin-A; B, β-actin-B; C, GAPDH-A; D, GAPDH-B; M, a ladder marker. Wells 1 - 2, 3 - 4, 5 - 6 and 7 - 8 are duplicate samples. The PCR products were separated by 2.0% agarose gel electrophoresis, followed by staining with ethidium bromide.

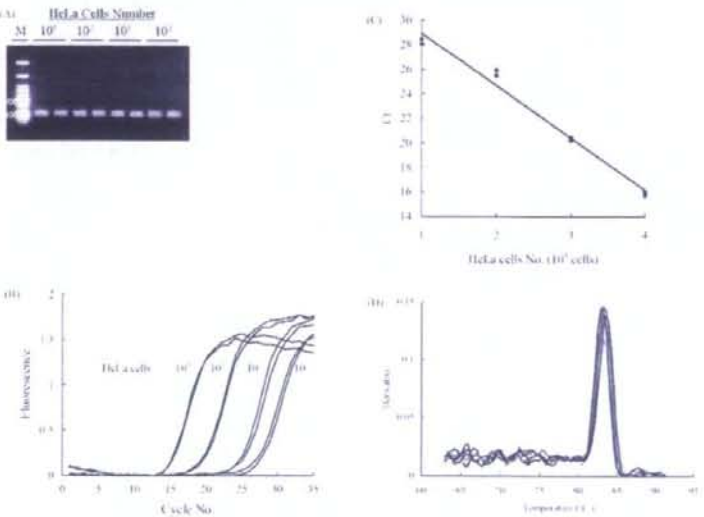

Fig. 4 RT-PCR analysis of gene expression with various quantities of HeLa cells. The mRNA was captured from a lysate of approximately 10 to 10^4 HeLa cells in the GeneTube and cell lines was used as template for RT-PCR assays for human β-actin. The amplicon size was 89 bp for the β-actin. (A) The RT-PCR products were electrophoresed in a 2% agarose gel, followed by staining with ethidium bromide. (B) Fluorescence data for β-actin calibrators together with the resulting calibration curve generated by the ABI Prism 7000 software. (C) The standard curve was showed a correlation between the Ct value and β-actin cDNA from known quantities of HeLa 10 to 10^4 cells. (D) Melting curves for all PCR products after β-actin amplification. Only one sharp peak for each sample was observed. This indicates that only one product was amplified.

increase the reliability of these measurements, we developed the S-BIO® GeneTube method. This completely all-in-one tube method is easy to use, eliminates loss of material, and improves the quantitative accuracy of gene expression studies.

In this method, cell lysis and protein denaturation occur very rapidly due to the high concentration of GuSCN, thus ensuring both protein removal from DNA and RNA and inactivation of cellular nuclease that would otherwise quickly degrade RNA.[14] The cell lysis allows dilution of the chaotropic agent before hybridization for the mRNA directly captured in the oligo-dT_{30} immobilized S-BIO® GeneTube after washing. This allows the reverse transcription to be performed on the whole sample and in the same tube in which it was collected without inhibition of the enzymatic activity. Finally, RT and PCR can be carried out immediately after cell lysis, rather than after cumbersome and lengthy nucleic acid preparation procedures, thereby further

Table 3 All-in-one tube RT-Q-PCR comparing with conventional RT-Q-PCR using the One-Step SYBER Green RT-PCR Master Mix Reagents Kit

Cells No.	Method	Ct	SD (n = 4)
10^6 cells/ml	GeneTube method	15.34	0.16
	Conventional method	15.14	0.16
10^5 cells/ml	GeneTube method	17.80	0.22
	Conventional method	17.71	0.11

reducing the time required to process many samples, as well as the risk of contamination.

Our quantitative measurements of β-actin or GAPDH mRNA levels in HeLa cells highlight one of several merits of the GeneTube method over the traditional, multi-step approach to nucleic acids purification.[8,11,23] This study has clearly shown that the very high initial concentration of GuSCN thoroughly breaks up protein-RNA interactions, but does not inhibit subsequent reverse transcriptase because the mRNA is captured in the oligo-dT_{30} immobilized GeneTube and can be completely recovered by washing. The phase separation based nucleic acid extraction method, in fact, presents several steps that require extreme care to avoid loss of material during liquid phase extraction and precipitation of total RNA, and repeated re-suspension and washing of barely visible pellets. All these manipulations render the results obtained with this technique operator-dependent, while the all-in-one tube method is much less operator-dependent because it simply requires sequential addition of reagents into the same tubes.

To validate the quantitative accuracy and reliability of the GeneTube method, we used it to measure β-actin and GAPDH mRNA levels in HeLa cells. The transcript levels in the individual cells were comparable to average RNA levels per cell calculated from whole cells. Based on these results, we anticipate that GeneTube will prove useful for quantification of RNA levels in small samples of tissue from many sources, as well as single cell and even fractions of cells such as neuronal dendrites and axons.[24] The small volume in which denaturation is carried out is also amenable to analysis of biological material isolated by laser capture microdissection or laser pressure catapulting.[25] These advantages of the all-in-one GeneTube method described here make it a very attractive choice, especially when working with very small amounts of material.

We have developed a novel all-in-one tube RT-PCR protocol in which the RT-PCR procedure (including cell lysis, cDNA synthesis by reverse transcription, and cDNA amplification and detection by Q-PCR) can be performed in an all-in-one tube. Using our system, once cell lysates are applied to the GeneTube for hybridization, the work can proceed to the direct RT-Q-PCR described in this study. It is also much less labor intensive than the previous multi-tube methods. A diagnostic assay is now being tested to consistently amplify the transcript from a single cell from whole blood.[26,27] Because of its simplicity and the fluorescence characteristics it gives to the products, our GeneTube should be applicable as a platform for various mRNA expression analyses in basic research, drug screening, and molecular toxicology, as well as molecular pathological diagnostics, with the potential for future automation. We believe that this protocol should prove useful for many single cell RT-PCR amplifications, particularly for mRNAs expressed at low levels.

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