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DARFA: A novel technique for studying differential gene expression and bacterial comparative genomics

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Abstract

We have developed a powerful method, named differential analysis of restriction fragments amplification (DARFA), which enables researchers to perform comprehensive transcriptome analysis as well as bacterial DNA fingerprinting. The key feature of this novel technique lies within the usage of a type IIS enzyme, Hpy188III, which cleaves cDNA or genomic DNA at a TC^NNGA recognition sequence. Cleavage at this particular site results in the production of a pool of restriction fragments which can be divided into 120 subsets based on the 2-nt 5′-overhang sequence. Each subset of restriction fragments is then selectively amplified by PCR after ligation with a pair of hairpin adaptors containing 2-nt overhangs which are complementary to those in the subset of fragments that are to be analyzed. The results obtained from the analysis of strain-specific and tissue-specific differences using DARFA and further confirmation by DNA sequencing and Northern analysis have demonstrated that the DARFA technique provides a novel tool for expression profiling, as well as bacterial DNA fingerprinting.

Keywords: DARFA; Expression profiling; DNA fingerprinting

After completion of the Human Genome Project, one of the most important tasks at hand in the post-genomics era is to analyze the tissue-specific and disease-specific expression patterns of 30,000–40,000 genes predicted from sequencing projects. As of today, various methods have been developed to investigate gene expression such as: serial analysis of gene expression (SAGE) [1–3], differential display (DD) analysis [4,5], restriction endonucleolytic analysis of differentially expressed sequences (READS) [6,7], total gene expression analysis (TOGA) [8], amplified restriction fragment-length polymorphism (AFLP) [9], suppression subtractive hybridization (SSH) [10,11], and micro-array analysis [12,13].

Among the aforementioned techniques, DD analysis, SAGE, and micro-array analysis have been most widely used for studies on a genomic level. Although DD analysis can be performed with a small amount of RNA and it is relatively inexpensive, it has disadvantages in that significant false positive bands may result from nonspecific amplification using arbitrary primers. Furthermore, it requires 300 reactions in order to detect 90% of all mRNAs [14]. SAGE is a high-throughput expression profiling technique that is used to determine the existence of transcripts and their relative frequencies by cloning short (9–10 bp) nucleotide sequences (tags) and then sequencing them. Although methods such as microSAGE [15] and SAGE-lite [16] have been invented to perform SAGE using a relatively small amount of RNA, it is generally recognized that SAGE is a method which has several drawbacks. Specifically, for SAGE analysis, it is necessary to use a relatively significant amount of RNA, it may be seriously affected by sequencing errors and it is expensive to perform

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numerous sequencing reactions. The micro-array method is a closed system which essentially needs cDNA or oligonucleotide probes to execute the necessary hybridization reactions. Considering the great importance of expression profiling techniques in identifying biomarkers and disease targets for medical treatment through studies of tissue-specific as well as disease-specific gene expression, it is necessary to develop a more effective technique which compensates the aforementioned disadvantages for all previously established techniques.

In the current study, we present a novel expression profiling technique, named DARFA. This technique is an open system, able to perform without prior sequence information, and uses stringent PCR conditions which may lead to less probability to produce false positives. Furthermore, it has the advantage of ideally displaying entire transcriptome and may be accordingly used for analyzing expression profiles in various organisms.

Materials and methods

Bacterial genomic DNA preparation. Genomic DNA was extracted from *Staphylococcus aureus* strains using the DNeasy tissue kit (Qiagen, Hilden, Germany). Cells were treated with lysozyme (5 mg/ml culture) at 37 °C for 1 h prior to the addition of lysis buffer. Subsequently, genomic DNA was purified following manufacturer's instructions.

cDNA preparation. Human spleen and small intestine total RNAs were obtained from Clontech (Palo Alto, CA). mRNA was purified from total RNA using the Oligotex mRNA mini kit (Qiagen, Hilden, Germany) and double-stranded cDNA was synthesized from mRNA using AccuRapid PCR cDNA library kit (Bioneer, Daejeon, Republic of Korea). Briefly, first strand cDNA was synthesized from 300 ng mRNA using 50 U MMLV reverse transcriptase (Roche, Mannheim, Germany) in a buffer containing 10 pmol of oligo(dT) primer with 24 nt 'heel' sequence toward the 5′ end (5′-CTG ATC TAG ACC TGC AGG CTC GAG TTT TTT TTT TTT TTT TTT TT-3′). The reaction was performed for 60 min at 42 °C in a 20 μl reaction volume. Subsequently, *Escherichia coli* DNA polymerase I (20 U; Takara bio, Otsu, Japan) and RNase H (5 U; Takara bio, Otsu, Japan) were added to the reaction mixture and the reaction was carried out for 120 min at 12 °C in a volume of 100 μl. The reaction was terminated by incubation at 70 °C for 10 min and *E. coli* ligase (120 U; Takara bio, Otsu, Japan) was added to the reaction and the mixture was held at 20 °C for 15 min. To make blunt-ended cDNA, T4 DNA polymerase (8 U; Takara bio, Otsu, Japan) was added and the reaction mixture was incubated for 15 min at 20 °C. After phenol/chloroform extraction and isopropanol precipitation, double-stranded cDNA was resuspended in 6 μl H_2O and ligated with 10 pmol of cassette adaptor for 2 h at 20 °C in a 20 μl reaction volume. The sequence of the long strand of the cassette adaptor was 5′-AGC GCG TGG TAC CAT GGT CTA GAG TCG ACT AAG TAG GT-3′ and the sequence of the short strand was 5′-ACC TAC TT-3′. After ligation with the cassette adaptor, the resulting double-stranded cDNA was purified by isopropanol precipitation and subsequently amplified twice by PCR using CP and RP primers that were provided in the kit. The sequences of the CP and RP primers were 5′-CGT GGT ACC ATG GTC TAG AGT-3′ and 5′-CTG ATC TAG ACC TGC AGG CTC-3′, respectively. Cycling conditions for both PCRs consisted of an initial denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min. The total reaction volumes for the first and second PCRs were 50 and 800 μl, respectively. The final PCR product was purified by phenol/chloroform extraction and ethanol precipitation, and was subjected to DARFA reaction.

DARFA reaction. The DARFA reaction was performed using a DARFA kit (Bioneer, Daejeon, Republic of Korea) following manufacturer's recommendation. Briefly, 30 μg cDNA or bacterial genomic DNA was digested with Hpy188III (2 U/μg DNA; New England biolabs, Beverly, MA) at 37 °C overnight in a reaction volume of 400 μl. After purification by phenol/chloroform extraction and ethanol precipitation, 200 ng of Hpy188III-digested DNA was ligated to each pair of total 120 hairpin adaptor sets for 2 h at 25 °C in a ligation volume of 10 μl containing 10 pmol of each hairpin adaptor. In order to remove unligated hairpin adaptors and restriction fragments, exonuclease III (20 U; New England biolabs, Beverly, MA) was added to the ligation mixture and incubated at 37 °C for 1 h. Finally, Hpy188III fragments that were ligated with hairpin adaptors at both ends were amplified by PCR in a reaction volume of 50 μl using an amplifying primer which is complementary to the 18-nt sequence in hairpin adaptors (Reamp; 5′-GTG TCG ACC TTA ATT CTA-3′) (Fig. 2). The PCR cycling conditions included pre-denaturation at 94 °C for 5 min followed by 10 cycles of the amplification at 94 °C for 50 s, 53 °C for 50 s, and 72 °C for 50 s. The PCR was continued with 30 cycles at 94 °C for 50 s, 43 °C for 50 s, and 72 °C for 50 s. The final extension was carried out at 72 °C for 5 min. To visualize amplified Hpy188III fragments, 2 μl PCR products was fractionated on a 5% denaturing acrylamide gel and was subsequently stained by using a Silverstar staining kit according to supplier's instructions (Bioneer, Deajeon, Republic of Korea). The band of interest was eluted from the gel, reamplified by PCR using an amplifying primer, Reamp, and sequenced after cloning into a pGEM-T Easy vector (Promega, Madison, WI).

Hairpin adaptor preparation. Hairpin adaptor oligonucleotides (58-nt) were synthesized and PAGE purified (Bioneer, Deajeon, Republic of Korea). Each adaptor contains a 25 bp stem, a 6-nt loop, and a 2-nt 5′ overhang (Fig. 2). The stem and loop sequences are common for all hairpin adaptors. In order to make the hairpin adaptor, oligonucleotides were denatured at 99 °C for 10 min and were subsequently allowed to cool slowly to room temperature as a means to form the hairpin structure.

Northern blot analysis. Poly(A)$^+$ RNA from human spleen and small intestine was fractionated on a 1% agarose/formaldehyde gel, transferred to a Hybond-N+ membrane (Amersham, Piscataway, NJ), and hybridized with a ^{32}P-labeled probe at 65 °C in a buffer (1% bovine serum albumin, 2 mM EDTA, 0.5 M sodium phosphate, pH 7.2, and 7% $NaDodSO_4$) for 18 h [17]. The blot was washed twice at room temperature for 10 min in 1× SSC (standard saline citrate), 0.1% $NaDodSO_4$ and at 65 °C for 1 h in 0.5× SSC, 0.1% $NaDodSO_4$. Probes were prepared by PCR amplification of DARFA DNA fragments from a recombinant pGEM-T Easy vector containing DARFA fragment as an insert using an amplifying primer (Reamp). All probes were labeled by a random-primer extension as previously described [18].

Results and discussion

DARFA technology

The characteristic of DARFA lies in its ability to analyze DNA by dividing the total pool of Hpy188III fragments into 120 subpopulations based upon 2-nt 5′-overhang sequences. As illustrated in Fig. 1, 30 μg cDNA amplified by PCR or bacterial genomic DNA is digested with Hpy188 III which recognizes "TC^NNGA" (step 1). The resulting fragments contain a NN sequence at their 5′ protruding ends. With this NN, 120 types of

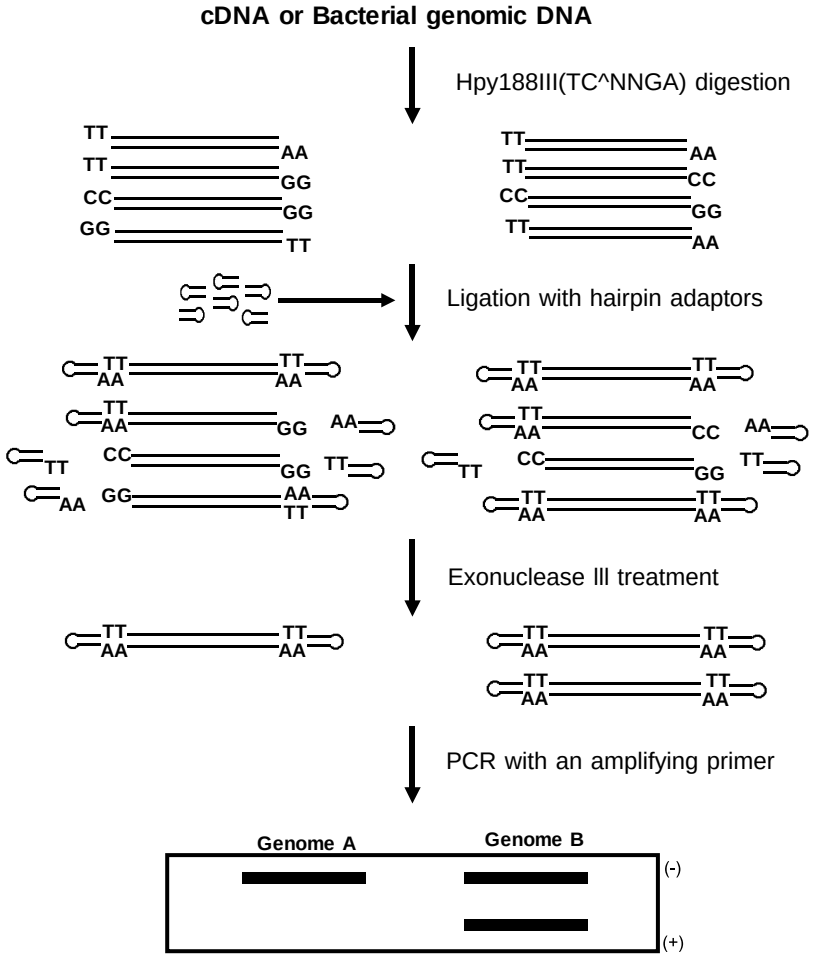

Fig. 1. Schematic representation of DARFA technique. cDNA or bacterial genomic DNA is digested with Hpy188III restriction enzyme (step 1). Hairpin adaptors containing AA or TT overhang sequences are ligated with Hpy188III fragments (step 2). Unligated hairpin adaptors and Hpy188III fragments are then removed with exonuclease III (step 3). The remaining Hpy188III fragments containing hairpin adaptors at both ends are amplified by PCR using a Reamp primer which is complementary to 18 nt sequence in hairpin adaptors (Fig. 2), separated on 5% denaturing sequencing gel, and visualized by silver staining (step 4).

fragments that are different at their ends are produced ($[4^2 \times 4^2]/2 - 8 = 120$). The Hpy188III fragments are then ligated with each pair of a total of 120 combinations of hairpin adaptor sets within separate tubes (step 2). The ligation reaction takes place only in the fragments containing NN sequences complementary to corresponding NN sequences of hairpin adaptors at 5′ protruding ends. In doing so, total fragments are classified according to NN base sequences at 5′ protruding ends resultant from Hpy188III digestion. Unligated fragments and remaining non-reacted hairpin adaptors are removed with exonuclease III, which leaves only the ligation products containing hairpin adaptors at both ends intact (step 3). The ligation products are then amplified using a Reamp primer that is complementary to the 18-nt sequence located in hairpin adaptors (Fig. 2), separated on 5% denaturing acrylamide gel, and visualized by silver staining (step 4). Finally, DNA is eluted from desired bands, amplified using PCR, cloned into pGEM-T Easy vector, and identified by sequencing.

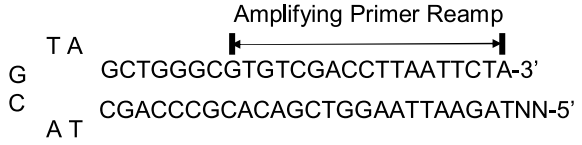

Fig. 2. The structure of a hairpin adaptor. The hairpin adaptor (58 nt) consists of a 25-bp stem, a 6-nt loop, and a 2-nt 5′ overhang. The sequence of a Reamp primer, which is used to amplify Hpy188III fragments, is indicated.

Bacterial DNA fingerprinting

In order to prove that DARFA is useful for bacterial DNA fingerprinting, genomes of methicillin-resistant (methicillin is one of the penicillin antibiotics) *S. aureus* (MRSA), vancomycin intermediate resistant *S. aureus* (VISA), and heterogeneously vancomycin-resistant *S. aureus* (hetero-VRSA) such as Mu3 strain are compared and analyzed using DARFA [19–21]. Thirty micrograms of genomic DNA isolated from each strain

was digested with Hpy188III and then ligated with a total of 120 hairpin adaptor sets. After digestion with exonucleaseIII, the remaining ligation products are amplified using PCR, separated on 5% denaturing acrylamide gel, and visualized following silver staining of the displayed bands on the gel.

Fig. 3 shows the results of analysis with 9 hairpin adaptor sets. Some are enlarged where the bands showing differences between strains are pointed out with arrows. For *Staphylococcus* of 2.8 Mbp genome size, approximately 91 bands per lane are produced following digestion with Hpy188III and these bands were readily isolated with high resolution as shown in Fig. 3 (2.8 Mbp/256 bp/120 = 91 fragments/hairpin adaptor set). Analysis of differences in DARFA patterns between strains can help investigate genes relating to antibiotic resistance and further be used in tracing variation between strains. It is also envisaged that DARFA can be employed as a useful tool for monitoring the quality control of bacterial strains.

Expression profiling

To demonstrate that DARFA technology can be used to analyze differential gene expression of eukaryotic mRNAs, cDNA was prepared from mRNA (300 ng) of human spleen and small intestine using the AccuRapid PCR cDNA library kit (Bioneer), and the resulting cDNA was analyzed by the DARFA technique. If we consider that there are approximately 35,000 genes in human beings, about 40% of the total genes are expressed in a given tissue at a given time, and that the average size of each mRNA is 1.2 kb, about 314 Hpy188III fragments are produced per each hairpin adaptor set (1200/256 bp = 4.69 Hpy188III fragments/mRNA, if fragments of both ends are excluded, 2.69 fragments/mRNA, $2.69 \times 35{,}000 \times 0.4/120 = 314$ fragments/adaptor set) [14,22].

As shown in Fig. 4, DARFA patterns from spleen and small intestine RNAs analyzed using 8 hairpin adaptor sets revealed numerous differentially expressed genes reflecting a high level of tissue-specific regulation. To determine the identity of DARFA bands, DNA was eluted from 7 bands, amplified by PCR using Reamp primer, cloned into the pGEM-T easy vector, and 5–10 clones per each DARFA band were analyzed by sequencing. As shown in Table 1, a search of the GenBank database with the sequence of insert fragments revealed the identity of DARFA fragments. The size of fragments correlated well with the size estimated from the display gel. With the exception of fragment No. 4, in one end of which the Hpy188III enzyme site did not

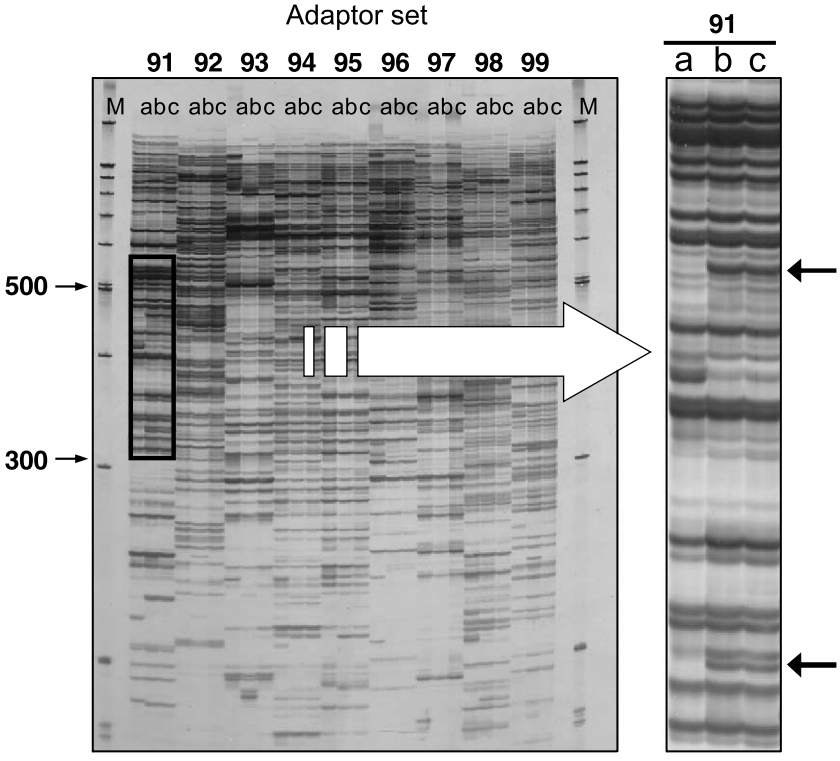

Fig. 3. Bacterial DNA fingerprinting using DARFA. Genomic DNA from three *S. aureus* strains was subjected to DARFA analysis. Lane a, methicillin-resistant *S. aureus* (MRSA); lane b, heterogeneously vancomycin-resistant *S. aureus* (Mu3); lane c, vancomycin intermediate resistant *S. aureus* (VISA). Hairpin adaptor sets used are 91 (CT + TG), 92 (CT + TT), 93 (GA + GC), 94 (GA + GG), 95 (GA + GT), 96 (GA + TA), 97 (GA + TC), 98 (GA + TG), and 99 (GA + TT). Arrows in the blowup on the right indicate differences between strains. Molecular weight markers are indicated on the left in base pair.

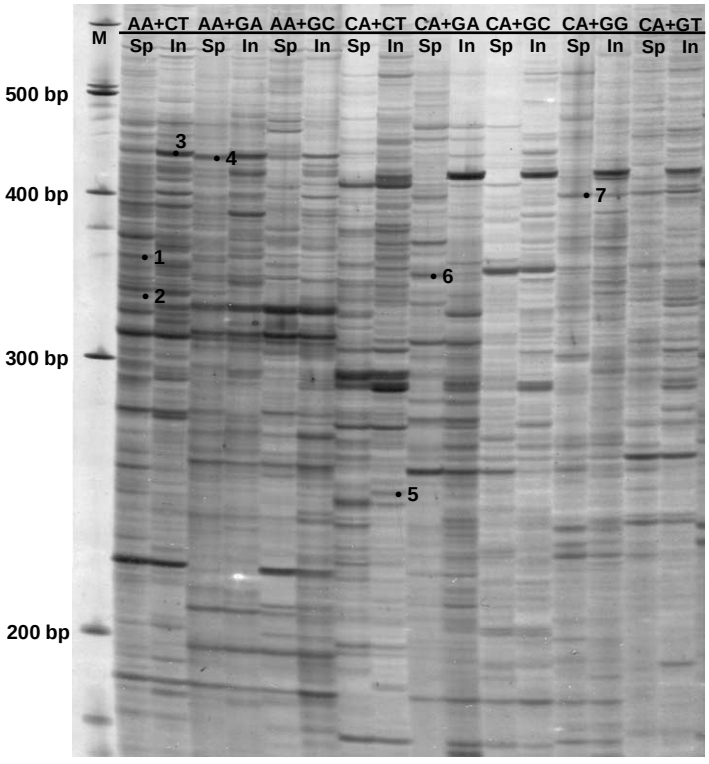

Fig. 4. Analysis of the differential gene expression using DARFA. cDNA from human spleen (lane Sp) or small intestine (lane In) was analyzed by DARFA. Hairpin adaptor sets used are AA + CT, AA + GA, AA + GC, CA + CT, CA + GA, CA + GC, CA + GG, and CA + GT. DNA was eluted from 7 bands which are indicated by dots, amplified by PCR, cloned into pGEM-T easy vector (Promega), and analyzed by sequencing. Molecular weight markers are indicated on the left.

Table 1
Identification of DARFA bands by sequencing

Fragment No.	NCBI Accession No.	Fragment length (bp)	Adaptor set	Frequency
1	BC048264 (*Homo sapiens* hypothetical protein LOC283666)	360	AA + CT	10/10
2	NM_005926 (*Homo sapiens* microfibrillar-associated protein 1)	337	AA + CT	5/7
3	NM_133503 (*Homo sapiens* decorin (DCN) transcript variant A2)	441	AA + CT	6/7
4	NM_030881 (*Homo sapiens* DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, transcript variant 2)	439	AA + GA	10/10
5	U75688 (*Homo sapiens* WF-1)	252	CA + CT	4/5
6	BC027885 (*Homo sapiens* cDNA clone IMAGE:5221584)	353	CA + GA	8/10
7	NM_005105 (*Homo sapiens* RNA binding motif protein 8A)	406	CA + GG	4/5

exist, the size of fragments was shown to be identical with the size that was expected from the database. The 5′ overhang sequence at both ends of fragment Nos. 1, 2, 3, and 7 was identical with the sequences expected from the database. However, in the case of fragment Nos. 5 and 6, only one of 2 nt 5′ overhang sequence on one end was identical with the sequence expected from the database.

As a next step, Northern blot analysis was conducted using PCR-amplified DARFA fragments for quantitative validation of DARFA fragments. As shown in Fig. 5, the relative expression level in spleen and small intestine poly(A)$^+$ RNA samples was highly correlated with the intensity of each band in the DARFA gel. Thus, bands in blots 1 and 3 were more intense in the spleen sample when the membranes were probed with fragment Nos. 1 and 4 which were isolated from the spleen sample in the DARFA gel. Similarly, bands were more intense in the small intestine samples in blot Nos. 2 and 4 which was probed with fragment Nos. 3 and 5 isolated from the small intestine sample in the DARFA gel.

Taken from these results, it is necessary to consider two potential complications associated with DARFA. One of them is the specificity of the ligation reaction between the hairpin adaptor and the Hpy188III fragments. When the specificity of the ligation reaction was tested by using the hairpin adaptor and an oligonucleotide containing 2-nt overhang sequence in both ends, it was

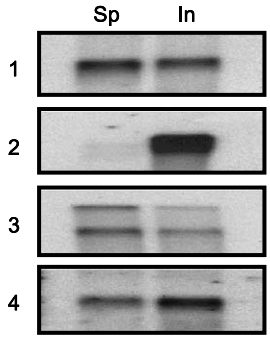

Fig. 5. Northern blot analysis of DARFA fragments. One microgram of poly(A)$^+$ RNA from spleen (lane Sp) and small intestine (lane In) was loaded in each lane. Blots were analyzed with PCR-amplified DARFA fragments (Table 1) as a probe. Blot 1, probed with DARFA fragment 1; blot 2, probed with DARFA fragment 3; blot 3, probed with DARFA fragment 4, and blot 4; probed with DARFA fragment 5.

found that even if only one of 2-nt overhang sequence was complementary between them, ligation still took place despite its poor efficiency. This non-specific ligation reaction was noticeably reduced when the ligation temperature was increased to 50 °C. As a result of actually conducting an experiment with *E. coli* genomic DNA, the ligation specificity was increased to 90% at 50 °C. Therefore, it is possible to at least partially solve the non-specific ligation complication, if the ligation reaction temperature is increased to 50 °C in the DARFA reaction. An additional potential problem is the fact that more than one fragment may exist per each DARFA band. When the number of Hpy188III fragments was analyzed among genes in the Refseq database, the number of fragments existing between 200 and 500 bp was 89,346 in total. If approximately 40% of them are expressed in a single type of cell, nearly 300 fragments per hairpin adaptor exist on the average. Actually, the number of fragments per hairpin adaptor exhibited considerable variation ranging from 17 to 3620. Therefore, when the number of fragments is great, it is expected that more than several fragments will exist per each DARFA band. In order to solve this problem, a method that is designed to specifically amplify only the 3′ end fragment is currently under development. Through such improvements, a more comprehensive and predictable DARFA method, which is applicable to various biological systems, will be made available to the scientific community.

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