FUJIFILM

Application Note

SCIENCE IMAGING SYSTEMS

Application Note No.14

Fluorescent Differential Display Method (FDD)

FLA-2000 / FLA-3000

FLA

Foreword

The talk titled "Gene Expression Analysis Using The Fluorescent Differential Display Method" was presented by Mr. Tsuyoshi Nomura, Dr. Kaoru Azumi and Dr. Hideyoshi Yokozawa (The Graduate School of Pharmaceutical Sciences, Hokkaido University), at the Biotechnology Seminar held in 1998 as part of the 21st Meeting of the Japan Society of Molecular Biology.

In Application Note No. 14, Dr. Azumi explains the protocol for the Fluorescent Differential Display (FDD) method using a fluorescent image analysis (FLA) system.

Gene expression analysis has attracted widespread attention and this has led to increasing interest among researchers in finding an easy and fast way to apply the Differential Display (DD) method. The FLA Multi-Stage available for use with the FLA system permits fluorescent detection using a 20cm x 40cm glass plate. This means that detection can be conducted without removing the glass plate used for electrophoresis. This, plus the fact that sample reading takes only a few minutes, makes the FDD method easy to implement.

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2. Principle of the FDD method
3. Materials, methods and test results of the FDD method
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Summary

- This method can be used in any laboratory since RI is not used.
- Fast fluorescent readout and cutting of sample can be done after electrophoresis.
- The band pattern is reproducible and stable.
- False positive clones can be eliminated by using a retarder of DNA fragments in electrophoresis.

1 Introduction

Low-malignancy cancer cells are known to acquire a malignant character when subjected to various stimuli. This prompted us to investigate the differences between a low-malignancy cancer cell and descendants of the cell transformed into more malignant cells by stimulation in vitro or in vivo. We used two-dimensional electrophoresis to analyze the intracellular proteins of four types of cells: a parent cell with low metastasis and three types of cancer cells derived from the parent cell which differs in malignancy levels. After staining the proteins with silver, we looked for spots that differed among the four cell types but found no substantial differences.

We then decided to try the Differential Display (DD) method[1], which we knew had already proven to be capable of high-sensitivity detection of differences in expression mode among the mRNAs of different cells. At the time, the laboratories in our research division shared a fluorescent image analyzer (FLA-2000, product of Fuji Photo Film Co., Ltd.) but none used the DD method - a method that detects the PCR (polymerase chain reaction) product by fluorescent labeling rather than RI (radioisotope) labeling. Partly because we were inexperienced in molecular biology techniques, we looked into a number of DD method protocols starting with a study of methods that could be implemented with a simple electrophoresis unit without the use of RIs. It was then we learned that the FDD method protocol[2)-4] developed by Dr. Takashi Ito of the Human Genome Center at The Institute of Medical Science, University of Tokyo, would work with our equipment. Following this stroke of good luck, we set about trying to follow Dr. Ito's protocol so as to implement FDD method analysis using the FLA-2000. In the end, despite using only 78 primer combinations, we discovered 11 gene types whose expression was induced in the cancer cells with the highest degree of malignancy. These included seven known types and four unknown types.

This success demonstrates that the FDD method can be utilized with relative ease in any laboratory equipped with an image analyzer that, like the FLA-2000, permits mounting of a gel plate. It also shows that experience in molecular biology is not particularly a factor. Mr. Nomura, the member of our group primarily responsible for the development of the FDD method and the discovery of the new genes, was still a fourth-year undergraduate student at the time. We later applied this method in another project (research into the genealogical evolution of immunity) and, from invertebrate blood cells (body cavity cells), were able to detect a large number of genes whose expression amount varied under various types of stimuli. This leads us to believe that the method can be applied to basic life science research irrespective of the type of organism.

We hope the following explanation of our protocol and comments based on our limited experience will be of some help to those wishing to try the DD method.

In vivo and in vitro

The reaction systems inside an organism (in vivo) are complexly interrelated. A specific reaction system is therefore generally easier to analyze when experiments are conducted under artificial conditions using substances and cells extracted from the organism and placed in a test tube or petri dish (in vitro).

2 Principle of the FDD method

(1) First, the mRNA in the prepared total RNA is selectively reverse transcribed using anchor primers. The anchor primers are synthesized by bonding the fluorescent probe FITC to the 5' terminal and oligo dT or one of C, G or A to the 3' terminal side. When reverse transcription is effected using an anchor primer with C added to the 3' terminal, only mRNA whose sequence has G next to the 5' side of poly A is selectively reverse transcribed.

(2) Next, using cDNA as a template, PCR is selectively conducted using the same anchor primer as used for reverse transcription and a random primer of a random 10-mer sequence is used to selectively amplify only fragments with a sequence similar to the random primer. (Although we used 26 random primers obtained from Operon, many others are available. Nippon Gene, for example, offers 12-mers with more than 400 different sequences.) We used a low PCR annealing temperature of 40°C so that the random primer would tolerate a certain amount of mismatching during the annealing. By this, a large number of cDNA fragments could be amplified in a single reaction.

(3) The PCR product is separated by electrophoresis using denatured polyacrylamide gel and fluorescence of the FITC in the PCR product is analyzed with a fluorescent image analyzer (e.g., FLA-2000). A fingerprint composed of many bands is obtained. At this point, we compared the sample types in sets of two or more to find those exhibiting a change in the expression pattern of the PCR product bands. Those differing in band presence/absence or density were recovered from the gels, reamplified by PCR and cloned.

Fingerprint
When the amino acids and/or nucleic acids of a sample are separated on a thin layer of acrylamide, silica or other gel, they form a unique pattern that can be used to identify the sample. This concept is the same as identifying a person using one's fingerprint.

Experimental Tip

In the DD method using an isotope, the unlabeled substrate concentration has to be low in order to increase the incorporation efficiency of isotope-labeled substrate during RT-PCR. The dNTP concentration used in this case is lower than the optimum concentration in ordinary RT-PCR. This is thought to be the reason why the size of the amplified cDNA tends to be smaller and good reproducibility may not be obtained. In contrast, the FDD method, which uses direct anchor primer labeling, allows RT-PCR to be conducted under optimum conditions that ensure excellent reproducibility.

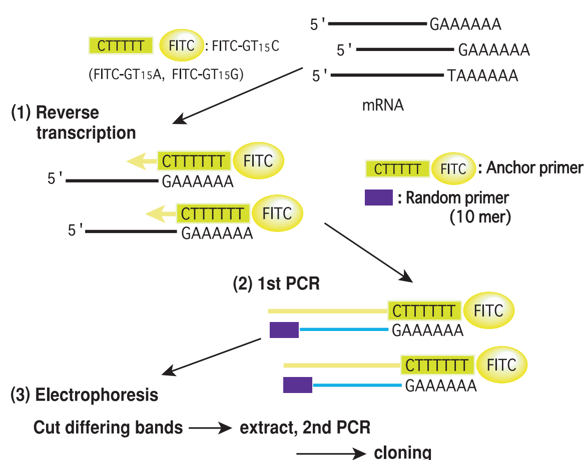

Fig. 1

Fig.1 Principle of the FDD method

3 Materials, methods and test results of the FDD method

What to prepare

<Equipment>

- Thermal cycler
- Set of electrophoresis equipment
  Inexpensive type is adequate
  Gel plates - e.g., 20 x 30cm nonfluorescent glass plates available from BIOCRAFT Co.,Ltd.
  Approx. 20 x 20cm heat radiator cut from aluminum plate
  Power supply with 1,500V output (SDS-PAGE power supply is insufficient)
- Fluorescent image analyzer (Fujifilm FLA-2000)

<Reagents>

- Anchor primer with FITC labeled 5' terminal (order to vendor); FITC-$GT_{15}X$ (X=A, C or G) prepare to 50µM with DEPC treated solution. Original solution usable for about 1 year if stored in dark at -20°C.
- Reverse transcriptase (preferably SUPER SCRIPT™II, product of GIBCO BRL)
- TE buffer :10mM Tris-HCl (pH8.0)/1mM EDTA
- Random primers (Operon 10mer kit (26 types); prepare to 10µM)
- Taq DNA polymerase (e.g., Gene Taq from NIPPON GENE Co.,Ltd., Ampli Taq from PE Biosystems)
- Denatured polyacrylamide gel solution; 6% acrylamide/8M urea/1x TBE
- 10x TBE buffer
- Denatured loading dye solution :95% formamide/10mM EDTA/0.1% BPB/0.1% XC
- H.A.-Yellow (Retarder of DNA fragments in electrophoresis according to contents in (A+T). Hanse Analitik)

Preparation of total RNA

We used TRIzol, a phenol-system preparation reagent (GIBCO BRL), to prepare total RNA from four types of cancer cells differing in malignancy. The AGPC method, CsTFA method or any of the other standard total RNA preparation methods described in laboratory handbooks should be usable. Phenol-system RNA preparation reagents like TRIzol are convenient for beginners because they permit preparation of high-purity total RNA from a small amount of cells in a short time simply by following the manufacturer's protocol. Very rarely, a tissue or cell may be encountered whose RNA cannot be extracted with phenol (extremely bad yield, coloration and failure to dissolve in water). We advise use of the CsTFA method in these cases, provided that the amount of sample is not too small. The total RNA was dissolved in DEPC treated solution to prepare a total RNA solution, which was stored at -80˚C after concentration (A260) measurement.

Treatment of extracted RNA with DNase

If any genome DNA is contained in the total RNA, it will act as a template and be simultaneously amplified in the ensuing PCR. We therefore carried out the following DNase treatment. Keep the sample on ice until incubation or otherwise stated.

Reaction solution (per tube)	
10x PCR buffer	2 µl
25mM $MgCl_2$	1.2 µl
RNase inhibitor (Toyobo)	0.5 µl
DNase I (Takara Shuzo)	0.5 µl
total RNA solution	15.8 µl (5µg)
Total	20 µl

Reaction was continued for 30min at 37˚C (DNA decomposition), followed by heat treatment for 5min at 95˚C (DNase deactivation).

AGPC (Acid Guanidinium-Phenol Chloroform) method

When phenol treatment is carried out under acid conditions, DNA enters the phenol layer because the acid conditions lower its hydrophilicity, but RNA, bearing the hydroxyl group of ribose, enters the water layer. The AGPC method uses this different behavior of DNA and RNA to separate the two.

CsTFA (Cesium Trifluoroacetate) method

This method uses the fact that the specific gravity of RNA is greater than those of biopolymers (DNA, protein etc.) to recover RNA by centrifugation. CsTFA is used as a substance to impart density gradient. The RNase inhibition activity of CsTFA makes it suitable for separating RNA. Employing an ultracentrifuge, the method is used mainly for large quantity RNA preparation.

Reverse transcription reaction using fluorescent anchor primer

(1) Preheat the thermal cycler to about 70˚C.
(2) Mix the following solution in PCR tubes and keep at 70˚C for 10min (to destroy the aggregation of RNA). Keep the sample on ice until incubation or otherwise stated.

Reaction solution (per tube)		
DNase treated RNA	10	µl (2.5 g)
Fluorescent anchor primer (50µM)	1	µl
Total	11	µl

(3) Prepare the following reaction solution while heating the solution of (2) is in progress. Successively add the listed solutions into a single tube in amounts equal to (amount per tube) x (required number of tubes plus one). (The extra solution is to cover for loss during pipetting.)

RT reaction solution (per tube)		
DEPC treatment solution	1.6	µl
10x PCR buffer	1	µl
25mM $MgCl_2$	1.4	µl
10mM dNTP	1	µl
0.1M DTT	2	µl
RNase inhibitor	1	µl
SUPER SCRIPT™II (200U/µl)	1	µl
Total	9	µl

Experimental Tip

An autopipette does not always dispense precisely the set amount. When the reaction solution is prepared in exactly the right amount for the required number of tubes, the amount left for the last tube may be insufficient. Preparing additional reaction solution to make up for the difference is time consuming and is apt to result in a slightly different composition. It is better to prepare a little more than needed from the start.

(4) Rapidly cool the tubes from (2) in ice water (1min). Centrifuge briefly to return the drops on the tube walls to the solution. Set the thermal cycler to 25˚C.
(5) Add the 9µl of RT reaction solution from (3) to each tube from (2) (bringing the total to 20µl / tube) and mix thoroughly by gentle pipetting.
(6) Place the tubes in the thermal cycler set at 25˚C. Incubate at 25˚C for 10min, 42˚C for 50min and 70˚C for 15min.
(7) Briefly centrifuge the tubes, add 80µl of TE buffer and mix thoroughly (cDNA solution). Store at -20˚C if not used immediately.

PCR using random primer

(1) Add 1µl of random primer (10µM) to each tube. Keep the sample on ice until incubation or otherwise stated.
(2) Add 2µl of cDNA solution to each tube from (1).
(3) Prepare following PCR reaction solution in an amount equal to (amount per-tube) x (required number of tubes plus one). (The extra solution is to cover for loss during pipetting.)

PCR reaction solution (per tube)	
Sterilized water	13.0 µl
10x Gene Taq buffer	2.0 µl
2.5mM dNTP	1.6 µl
Anchor primer (50µM)	0.2 µl
Gene Taq (5U/µl)	0.1 µl
Ampli Taq (5U/µl)	0.1 µl
Total	17.0 µl

Experimental Tip

In Dr. Ito's original protocol[3], Gene Taq, i.e., Taq polymerase lacking the N-terminal portion, appears to be more efficient for amplifying short fragments under 500bp and ordinary (full-length) Taq polymerase appears to be more efficient for amplifying long fragments. The original method therefore recommends mixing the two in 50:50 aliquots. We followed this advice. Also, the number of bands and the band pattern seems to differ with the type of Taq polymerase. If you feel too few bands appear, try a number of other enzymes for comparison.

(4) Add 17.0µl of the PCR reaction solution from (3) to each tube from (2) (bringing the total to 20µl/tube).
(5) Set the tubes in the thermal cycler and carry out reaction according to the following program.

(94˚C x 3min + 40˚C x 5min + 72˚C x 5min) x 1 cycle
(95˚C x 15sec + 40˚C x 2min + 72˚C x 1min) x 20-25 cycles
72˚C x 5min

Experimental Tip

Although this is the number of cycles specified by the original protocol, we currently use 30-35 cycles because we found that with certain samples no bands appeared at all. We advise trying different numbers of PCR cycles to find the best number for your sample.

Electrophoresis of denatured polyacrylamide gel

(1) Prepare 6% denatured polyacrylamide gel solution and filter to remove insolubles. Degas for 15-30min, add 19.5µl of TEMED and 225µl of 10% APS, mix thoroughly, pour between assembled gel plates (use nonfluorescent glass plates and nonfluorescent detergent (BIONOX)), and insert the shark comb. After the gel sets (about 2 hour), pre-electrophorese at 1,000V for 30min in 1x TBE buffer.

6% denatured polyacrylamide gel solution (per plate)

40% acrylamide gel	4.5 ml
Urea (8M)	14.4 g
5x TBE	6 ml
Sterilized water	As suitable
Total	30 ml

Fig.2

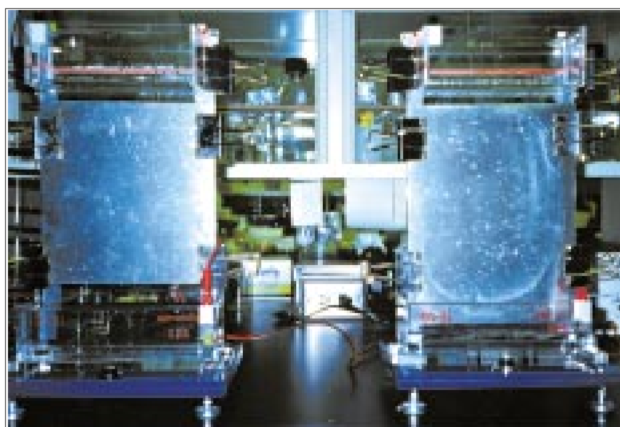

Fig.2 Electrophoresis unit

(2) Add 2.5µl denatured loading dye solution to 5µl of PCR product, mix, hold at 90˚C for 2min, and cool rapidly in ice water. Apply 5µl per lane. (Replace buffer on comb with fresh buffer before application. Otherwise the dissolved urea will prevent the sample sinking) (Fig.3)

Fig.3

Fig. 3 Application of sample
The sample etc. are easy to handle because no RI is used.

(3) After electrophoresing at 1,500V for 2.5-3 hour, the XC (xylene cyanol) will have reached the lower edge of the gel. Stop the electrophoresis, wash the gel with distilled water, remove a glass plate from one side of the gel plate. Neat removal may be difficult unless the inside of the glass plate to be removed has been pretreated beforehand with a coating agent such as Gel Slick (FMC Bioproducts). Carry out scanning with the fluorescent image analyzer (FLA-2000).

Experimental Tip

The original protocol also carries out first-stage scanning with both glass plates attached after about one hour of electrophoresis, i.e., when the BPB has reached the lower edge of the gel. After that, carrying out second-stage electrophoresis and second-stage scanning successively. Two-step scanning method enables detection of a larger range of PCR product sizes.
Fluorescent detection with the FLA-2000 can usually be completed in several minutes, even with a 20 x 36cm gel plate (close to the largest the scanner can handle) and at maximum resolution.

Fig.4

Fig.4 Set the gel plate in the FLA-2000

Fig.5

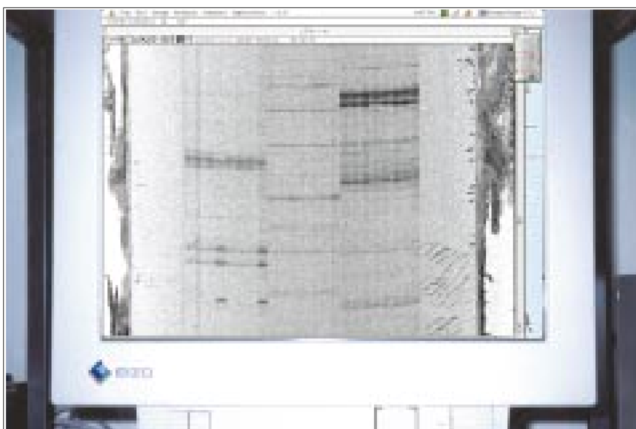

Fig.5 View results of scanning on display

Cutting bands of interest

(1) Print out a 1 x image of the gel (Fig.6) and lay the gel over the printout. Cut out nonidentical band pairs (including blank bands) with a spatula. Notching the gel edges at several points before imaging makes precise alignment easier. One advantage of this protocol is that fluorescent detection and band cutting are possible immediately after electrophoresing the sample.

Fig.6

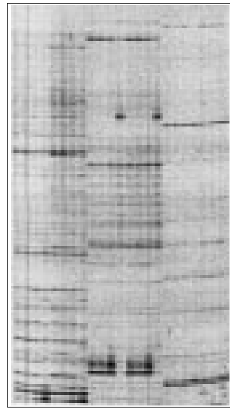

Fig.6 Fingerprint example
Around 20-30 bands per lane can be detected in one electrophoresis cycle.

Fig.7

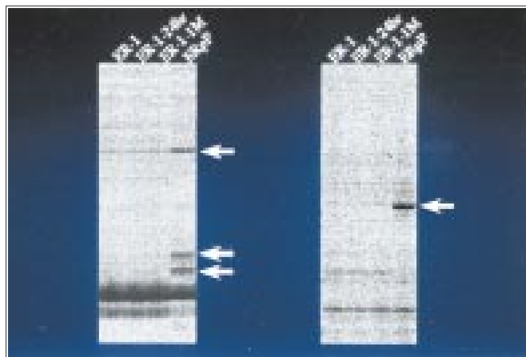

Fig.7 Enlarged view of portion of fingerprint where difference actually observed
The arrows indicate strongly expressed bands of the cell with the highest malignancy.

(2) Scan the gel again to make sure the bands of interest were properly cut.

Experimental Tip

After finding bands with different expression patterns, one can hardly resist moving directly into sequence analysis as soon as the bands are cut. Don't give into the temptation. First confirm that the band patterns are reproducible. If possible, start over from the beginning with independent RNA. If not possible, at least start over from the reverse transcription step. It is not at all uncommon that, after cloning and determining the base sequence, the expected differences completely fail to appear when RT-PCR or Northern blotting is tried. In the end it saves time by reconfirming the reproducibility of the differential expression on the acrylamide gel, and the reproducibility of nucleic acid separation by H.A.-Yellow, described on next page.

Band reamplification and nucleic acid separation by H.A.-Yellow

(1) Transfer each cut gel piece to a tube for PCR, add 78µl of sterilized water, hold at 94˚C for 10min (DNA extraction) and add the following reaction solution to conduct reamplification by 2nd PCR (the gel pieces can be left in). Keep the sample on ice until incubation or otherwise stated.

2nd PCR reaction solution (per tube)	
10x Gene Taq buffer	10 µl
2.5mM dNTP	8 µl
Anchor primer (50 µM)	0.5 µl
Random primer (10 µM)	2.5 µl
Gene Taq (5U/µl)	0.5 µl
Ampli Taq (5U/µl)	0.5 µl
Total	22 µl

Use the following program:

(94˚C x 3min + 40˚C x 5min + 72˚C x 5min) x 1 cycle
(95˚C x 15sec + 40˚C x 2min + 72˚C x 1min) x 10-13 cycles
72˚C x 5min

(2) Electrophorese 50µl each of the PCR product of the band of interest and the PCR product of the control band in 2.5% agarose gel containing 0.1% of H.A.-Yellow (Fig.8).

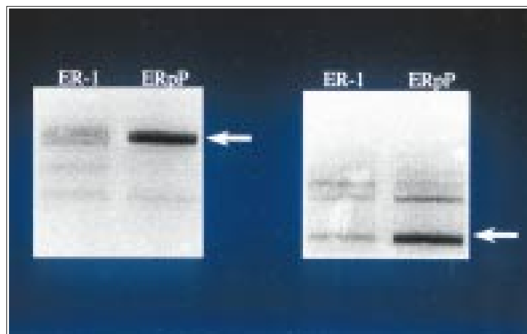

Fig.8

Fig.8 Nucleic acid separation with H.A.-Yellow

Experimental Tip

A single band detected on the polyacrylamide gel is usually composed of multiple DNA fragments of identical molecular weight but different base sequences. Being a polymer that binds specifically with DNA having a large AT content, H.A.-Yellow can help to separate such multiple DNA fragments in a band.

(3) Scan the agarose from (2) with the fluorescent image analyzer (FLA-2000) and cut out the bands with clearly differing expression amounts.

Experimental Tip

Some protocols call for these bands to be reamplified and separated with agarose containing H.A.-Red (Hanse Analitik), a polymer that binds specifically with DNA having a large GC content. Even a single electrophoresis cycle with H.A.-Yellow or H.A.-Red can narrow several types of mixed-in cDNA down to one or two types.

Cloning and checking expression pattern of cloned PCR product

(1) Recover DNA from agarose gel using QIAEX II Gel Extraction Kit (QIAGEN) and conduct TA cloning in the usual way.

(2) Select 4-6 clones and analyze inserted base sequences after PCR.

Experimental Tip

We generally obtained identical clones with identical base sequences but on rare occasions found two clone types. In such cases, the clone present in larger numbers is more likely to be the molecule of interest. Still, it is advisable to confirm by RT-PCR or Northern blotting.

(3) Finally, confirm whether the cloning resulted in the desired transcription product. The quickest way is to conduct quantitative RT-PCR using primer design based on the base sequence of the insert (Fig.9). The expression pattern should be confirmed by rigorous Northern blotting. In this case, use of a freshly pre pared RNA sample makes it possible to simultaneously check the reproducibility.

RT(reverse transcription)-PCR method

This method detects minute amounts of mRNA by first using reverse transcriptase to synthesize DNA with RNA as the template (reverse-transcription reaction) and then using the DNA as a template for PCR.

Fig.9

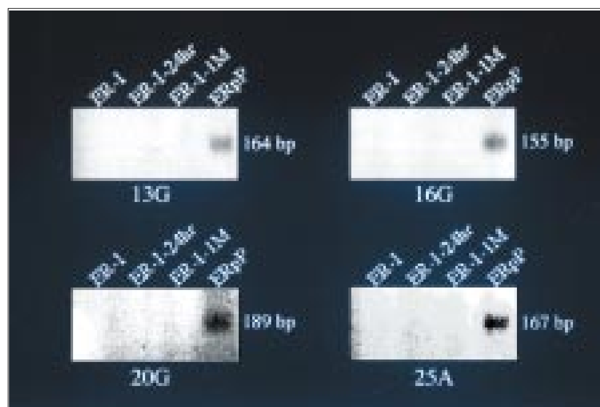

Fig.9 Example of RT-PCR

4 Conclusion

Using the method described here, we discovered 11 genes whose expression was induced in the cancer cells with the highest degree of malignancy. These included seven known types and four unknown types. We checked the reliability of the known genes by the quantitative RT-PCR method and of the new genes by both the quantitative RT-PCR method and Northern blotting. In all cases, the expression matched that of the PCR product detected on the acrylamide gel, verifying the high reproducibility of the protocol.

Several points we noted in using the protocol follows:

(1) The number of bands per lane is lower by the FDD method than by the DD method using RI-labeled primer but the band pattern that appears is stable and has high reproducibility.

(2) The random primers used in PCR have random base sequences, so many types of mRNA can be detected by increasing the number of random primers. It has been estimated that 450 or more primer combinations are necessary to cover all transcription products of higher organism cells.

(3) In cases such as when it is desired to isolate a gene having high homology with a known base sequence, PCR using degenerate primers (see PCR laboratory handbook) is more reliable than the DD method.

We wish to emphasize that the final objective of adopting the protocol introduced here is not to “discover” genes with different expression patterns but to ascertain the function of these genes at the protein level and the cellular level and through this to clarify the “true significance of differences in expression pattern.” Like many of you, we too will continue to focus on the challenging subjects of new gene functional analysis.

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July 2000

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