# Optimum Conditions for Preparation of Diagnostic Fish Rhabdovirus DNA Chip

# 어류 랩도바이러스의 진단용 DNA Chip 제작 최적 조건

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by

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# 김영주의 이학석사 학위논문을 인준함

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주 심 농학박사 박 수 일 

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# Optimum Conditions for Preparation of Diagnostic Fish Rhabdovirus DNA Chip

A Dissertation

by

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## ABSTRACT

For rapid and accurate detection of rhabdovirus from infected fish, DNA chip technology, which can search for various and specific gene, was applied.

Three strains involving infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicaemia virus (VHSV) and hirame rhabdovirus (HIRRV) were selected as objective viruses.

Sequences for probes were determined by sequence analysis of structural proteins of each virus which have been published on GenBank. Probes were prepared by Polymerase Chain Reaction

(PCR) with each of specific primer set. The specificity of probes were identified by blast alignment in NCBI, and the homologies are above 95%. Prepared probes were spotted on the amine and poly-L-lysine coated slide glass using self-developed microarrayer system.

Target DNA were obtained from total RNA of virus-infected cell lines by reverse transcription. During reverse transcription, target DNA (cDNA) were labelled with fluorescent dye (Cy5-dUTP). The hybridization conditions such as target DNA concentration, hybridization temperature and kind of slide glass were investigated.

Two kinds of slide glasses, which were coated with poly-L-lysine and aminosilane, were tested for probe spotting. Target DNA, which were prepared from IHNV, VHSV and HIRRV-infected cell lines, showed hybridization signal on the both of slide glasses. In this experiment used probe concentration 200 ng/$\mu\ell$. Humidity condition for spotting was investigated with poly-L-lysine slide glass, 65- 65% of humidity was considered to effective for probe spotting. The hybridization temperature of poly-L-lysine and aminosilane coated slide glass were respectively 65℃ and 62℃. Total RNA concentration for poly-L-lysine coated slide glass was adjusted 80 $\mu$g, 50 $\mu$g and 50 $\mu$g in accordance with IHNV, VHSV and HIRRV.

While, its concentration was 100 $\mu g$, 60 $\mu g$ and 50 $\mu g$ in the case of aminosilane coated slide glass.

It was tested the *in vivo* detection ability of cDNA chip using virus-infected fishes, olive flounder and rainbow trout. The results showed the successful detection of IHNV and VHSV gene in the virus-infected fishes.

# INTRODUCTION

Fish rhabdovirus has negative sense ssRNA genome and viral RNA is transcribed into positive sense mRNA. The genomic RNAs are composed of 11,000 - 13,000 nucleotides (Schutze *et al.*, 1995). There are five mRNAs encoded the viral structural proteins such as nucleotide capsid protein (N), two matrix proteins (M1 and M2), envelope glycoprotein, RNA polymerase (L) and non-viral protein (NV).

Fish rhabdoviruses such as viral haemorrhagic septicemia virus (VHSV), infectious hematopoetic necrosis virus (IHNV) and hirame rhabdovirus (HIRRV) are significant pathogens for salmonids (Oh, 1999). IHNV, VHSV and HIRRV often cause diseases with high mortality among salmonids and sea trout (Miller *et al.*, 1998). Especially rhabdoviruses caused many fish viral diseases in large scale nurseries on several ways. Diseased rhabdovirus with natural infection show gonad congestion, abdominal distention and ascites. Overall, rhabdovirus are prominent in fins, skeletal muscle and internal organs, as a consequences the gills are pale. The gall bladder is pale yellow, and histologic changes include pyknosis and necrosis of hematopoietic tissue (Wolf, 1988).

Several molecular biological techniques such as enzyme linked immunosorbent assay (ELISA), western blot, fluorescent antibody test have been used to detect virus at early stage of infection with low concentration of virus. Reverse transcriptase-polymerase chain reaction (RT-PCR) also has been employed in the detection of viral RNA. However, these methods are hard to apply at the early stage of infection in which the virus titer is low (Kim, 1999). Also RT-PCR requires highly purified RNA. It is important to rapidly identify and differentiate the causative virus in infected fish, because rapid determination of the origin of an outbreak may help to prevent the futher spread of the disease (Willams *et al.*, 1999).

A DNA chip or DNA microarray means a small surface which was spotted with a numerous of different genes (probe). Usually glass or silica, approximately 1 ㎠, has been used as matrix for spotting (Vernet, 2002). Each of probe consisted with different genes, which has specificity to test organism. These chips have been used in hybridization reactions to detect nucleic acids generated from a sample by an amplification technique (RT-PCR, PCR and TAM).

Especially, DNA chip technology is used for biomedical analysis such as gene expression analysis, polymorphism or mutation detection, DNA sequencing and gene discovery. Biological chips

offer a more rapid and accurate analysis than traditional research methods based on gels, filter and purification columns (Shena, 2003).

Following the completion of the Human Genome Project, the post-genomics era has been focused on the gene expression patterns of all kinds of organisms and gene functional research. The gene chip technique has become a powerful tool for completing this task, because the chip technology has been characterized as the platform of high output, sensitivity, speed and accuracy.

Microarray fabrication include spotting of DNA onto nylon membranes or glass slides by robots with pin or inkjet printers. The spotted DNA corresponds to fragments of genomic DNA, cDNA, PCR products or chemically synthesized oligonucleotides. Among of them, cDNA arrays are often used for RNA expression analysis, while oligonucleotide chips are used for sequence analysis (Zammatteo *et al.*, 2000).

It has been supposed that DNA chip technology is ideal for an extensive parallel identification of nucleic acids and analysis of gene expression. Simultaneous analysis for the presence of multiple markers makes it possible to determine a complete genetic profile of a single strain or distinguish one strain from a very large collection of possible alternatives in one experiment. Therefore, this approach is

potentially useful for the screening of multiple microbial isolates in a diagnostic assay (Bowtell and Sambrook, 2003).

At present, the best way of viral disease control is avoidance of virus introduction through rapid detection. In this study, we applied DNA chip technology to detection and identification of rhabdovirus in infected fishes. For this purpose, we investigated the optimization of conditions for preparation of rhabdovirus DNA chip.

# MATERIALS AND METHODS

## 1. MATERIALS

### 1) Viruses and cell line

IHNV, VHSV and HIRRV obtained from Department of Aquatic Life Medicine, Pukyong Natl. Univ. (Korea), were used as the objective viruses for development of DNA chip in this study. Chinook salmon embryo cells-214 (CHSE-214) was used for IHNV culture and epithelioma papulosum cyprini (EPC) were used for VHSV and HIRRV culture.

### 2) Oligonucleotide primers

The probes corresponding to structural proteins of objective viruses were amplified by PCR, and β-actin gene was used as positive control. Primers for reverse transcription-polymerase chain reaction (RT-PCR) were designed based on the gene sequences of each viruses and purchased from Bioneer, Inc., Korea. Table 1 represented the used primer sets.

Table 1. Specific primer sets used in amplification of probes

| Virus strain | Code protein | | Primer (5'→3') | Product size (bp) |
|---|---|---|---|---|
| VHSV | N | up | gcactgtccgtacttctct | 567 |
| | | down | aagagattccatgcacaga | |
| | G | up | ggtcgagaacatctcaaca | 585 |
| | | down | ttaagcgtttctgaggtag | |
| | M1 | up | cagatcagctctcttttcg | 582 |
| | | down | tgtccctactccaacttgt | |
| HIRRV | N | up | ccaacaaagcactaggaat | 575 |
| | | down | gatggacttcatgcagaga | |
| | G | up | atcccctgtttacataccc | 575 |
| | | down | ggaggtcttgtccacaaat | |
| | M1 | up | tgatattcccaagaatgct | 571 |
| | | down | gattgccatgttcttgact | |
| IHNV | N | up | aatgtccttgaggttgtga | 575 |
| | | down | ccatcgtcatacctatcgt | |
| | G | up | gatagagaaggcgcttgta | 594 |
| | | down | tcaagacattcctctctgc | |
| | M1 | up | agaaggcgaagacatactg | 598 |
| | | down | acttccctcgtattcatcc | |
| β-actin | | up | actacctcagaagatcctg | 564 |
| | | down | actacctcagaagatcctg | |

### 3) Slide glasses for spotting

There are numerous commercial vendors for coated slide glass, and three kinds of coated slide glass were used in this study. NC-AMCS coated with aminosilane (Nuricell, Korea) and poly-L-lysine coated slide glass (Sigma, USA) were used to fabricate cDNA chip using Genomics microarrayer. CMT-GAPS coated with aminosilane (Corning, USA) were used in the case of fabricating cDNA chip by MGⅡ TAS microarrayer (BioRobotics, UK).

### 4) Microarrayer

The microarrayer which can fabricate DNA chips was developed by General microbiology laboratory and Department of Mechatronics Engeering, Pukyong Natl. Univ. (Korea) (Fig. 1). It realized a typical, low-cost and efficient microarrayer for generating low density microarray. The microarrayer was developed by using a prependicular type robot with three axes. It is composed of a computer controlled three axes robot and tip assembly.

The microarrayer was designed to automatically collect probes from two 96 well plates with up to 32 pins at the same time. It

takes 4 hours to operate the microarrayer when single pin was used. However, operating time will be decrease depending on the pin number. It has sufficient performance for the production of low intergrated DNA chip consisted of 96 spots within 1 ㎠. And software was also developed for microarrayer on the slide glasses corresponding to the prescribed arrays, for turning on and off of various devices, for monitoring all the procedure of the operating processes. The self-developed microarrayer was named Genomics microarrayer.

At this time, MG Ⅱ TAS microarrayer (BioRobotics, UK) was used to confirm the efficiency of self-developed Genomics microarrayer.

### 5) Scanner

Hybridized slides were scanned with the ScanArray (Perkin Elmer, USA), which generates Tiff images of Cy5 channel.

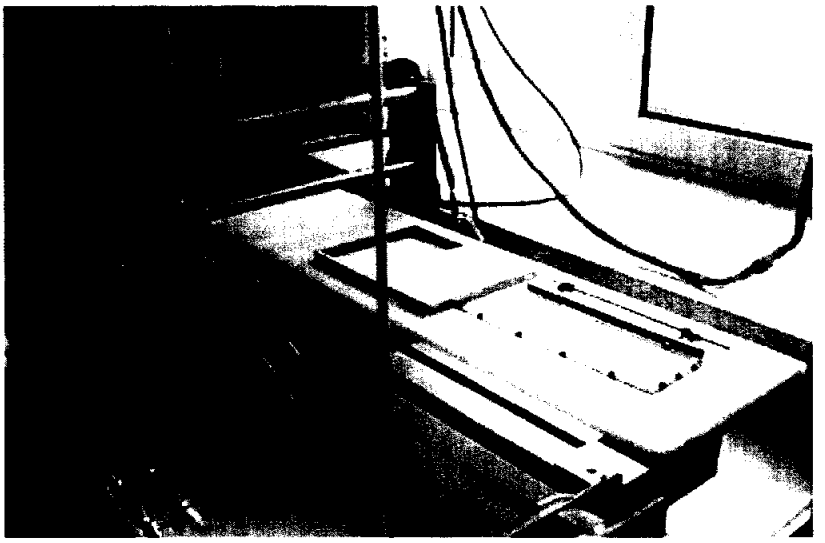

Fig. 1. The self-developed Genomics microarrayer.

## 2. METHODS

### 1) Culture of viruses

CHSE-214 and EPC were grown at 18℃ using Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% antibiotic (Gibco BRL) (Kim, 2003). Temperature for virus propagation was 15℃.

### 2) Cytopatic effects and virus-infected cell counts

To determinate whether viruses were grow, viral cytopatic effects (CPE) were measured by observation using phase contrast microscopes (Raouf and Seth, 2003).

The number of cells was determined using hemacytometer. At first virus-infected cell lines were suspended with 1 ㎖ phosphate buffered saline (PBS, pH 7.2), 20 ㎕ of diluted cells was allowed to flow under the cover glass of the hemacytometer. The number of cells were counted, seeing the ocular lense on phase contrast microscopes (Benson, 1994).

### 3) Design of probes for cDNA chip

Nucleocapsid protein (N-protein), glycoprotein (G-protein) and matrix protein 1 (M1-protein) were selected as probe sequences for cDNA chip through GenBank analysis (Schutze *et al.*, 1999).

Probes were amplified by PCR from a cDNA using Mygenie 32 thermal block (Bioneer, Korea). PCR was performed in 20 ㎕ of PCR mixture using premix containing 1 U Taq polymerase, dNTPs (0.25 mM each of ATP, TTP, GTP and CTP), 10 mM Tris·Cl, 40 mM KCL and 1.5 mM $MgCl_2$. 10 pmole of specific primers and cDNA template were added. A temperature profile was on prereaction at 94℃ for 5 min ; 30 cycle reactions on denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, extension at 72℃ for 30 sec; and finally for a 7 min post-extension at 72℃ (Kim, 2003). The amplified PCR products were analyzed on 1% agarose gel electrophoresis.

### 4) Fabrication of cDNA chip

cDNA chips were prepared by spotting 200 ng/㎕ of probe suspended in 50 % dimethylsulfoxide (DMSO) on the surface of

poly-L-lysine coated slide glass and aminosilane coated slide glass using Genomics microarrayer. N, M1 and G genes of IHNV, VHSV and HIRRV and ß-actin were arrayed from left to right, respectively. Conditions of humidity for spotting were tested in 60-65 % and 65-70 %.

On the other hand, the same cDNA chips were also fabricated in BMS Korea Institute to compare the efficiency of Genomics microarrayer. At this time, used matrix and spotter were aminosilane coated slide glass (Corning, USA) (Southern *et al.*, 1999) and MG II TAS microarrayer (BioRobotics, UK). cDNA chips were arrayed 10 genes with duplicate, the N, G and M1 gene of IHNV, VHSV and HIRRV were arrayed in the first, second and third line, respectively. Also, the final line are arrayed ß-actin with duplicate.

### 5) Preparation of target DNA

① Total RNA isolation

To isolate total RNA, virus-infected cell lines were homogenized in 1 ㎖ of TRIzol reagent (Invitrogen, USA) per $10^5$-$10^6$ cell/㎖. These mixture was standed at room temperature for 5 min. 200 ㎕

of chloroform was added, and the mixture was vigorously shaked for 15 sec. Then, the sample was standed at room temperature and centrifuged for 15 min at 12,000 xg. The aqueous phase was transferred to a new tube, 0.5 ㎖ of isopropanol was added and mixed by inverting. The samples were centrifuged for 15 min at 12,000 xg and then the supernatant was discarded. The RNA pellets were dried for 10 min under vacuum and dissolved in RNase free water (0.1% DEPC treated water). RNA ratio ($A_{260/280}$) was checked by spectrophotometer (Perkin Elmer, USA). Degradation of RNA was checked on 1% agarose gel electrophoresis. For electrophoresis, total RNA was denaturd at 65℃ for 15 min and then 3 ㎍ was loaded into the well (Bowtell and Sambrook, 2003).

② First strand cDNA labelling with reverse transcription

Approximately 50-100 ㎍ of total RNA was used to reverse transcription reactions with oligo$(dT)_{18}$. M-MLV transcriptase (Ambion, USA) was used, and total reaction mixture was 40 ㎕. The components of reverse transcription and condition were shown in Fig. 2 (Bowtell and Sambrook, 2003; Tran *et al.*, 2002). To remove extra RNA, samples were incubated with 2 ㎕ of 0.5 M EDTA at

98℃ for 3 min and treated with 5 ㎕ of 1 N NaOH at 37℃ for 10 min. This products were neutralized by addition of 10 ㎕ of 1 M Tris-Cl buffer (pH 8.0). Finally, Micro Bio Spin 30 columns (BioRad, USA) were used to remove the uncooperated dyes and free nucleotides (Call *et al.*, 2001).

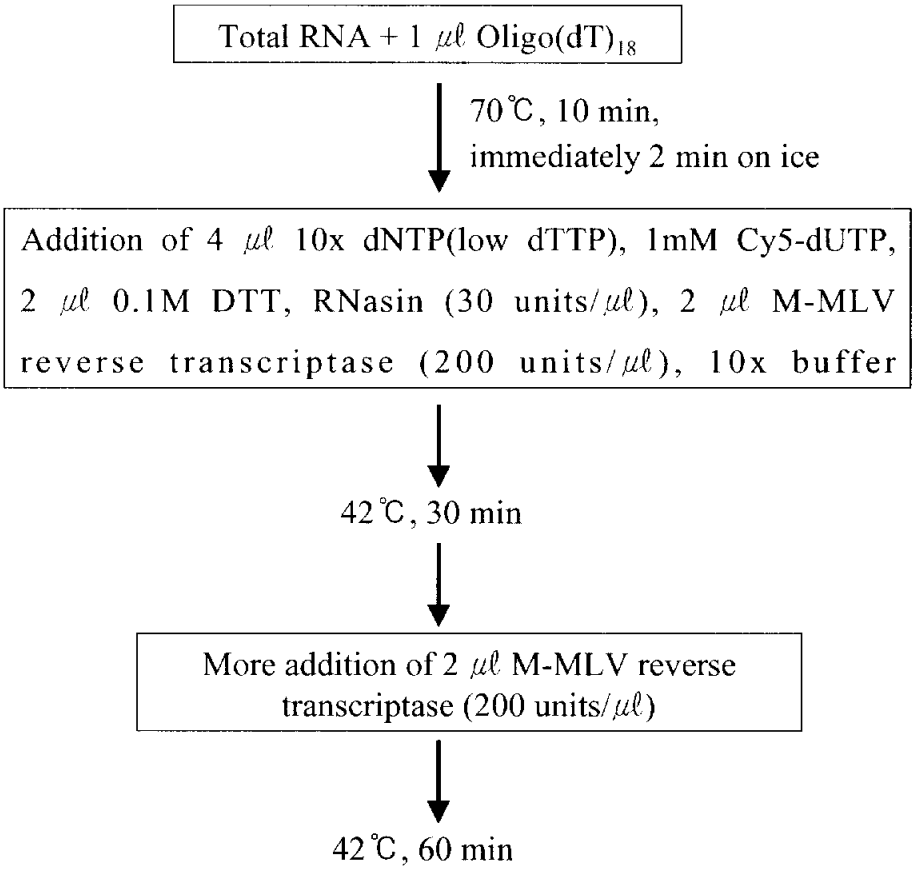


Fig. 2. The precedure of reverse transcription for cDNA synthesis.

### 6) Hybridization and scanning

The labeled cDNAs were denatured at 100℃ for 2 min after addition of hybridization solution (5X SSC/ 0.2% SDS) (Zhang *et al.*, 2002) and then applied to the microarray. Hybridization of target cDNA and probe was proceeded in hybridization cassette (Telechem International Inc., USA) to prevent drying of the spotted probes. After hybridization, the slides were washed two times with 2X SSC/ 0.2% SDS solution for 10 min, once with 0.2X SSC for 10 min, and finally once with 0.1% SSC for 5 min (Hegde *et al.*, 2000). And then immediately, the hybridized slides were dried by centrifuging at 1,000 xg for 1 min (Chizhikov *et al.*, 2001; Dihel *et al.*, 2001), scanned and analyzed.

### 7) *In vivo* test

① Isolation of total RNA from olive flounder and rainbow trout

To evaluate the efficiency of cDNA chip for virus detection *in vivo*, virus-infected flounder and rainbow trout samples were collected. The former was collected in Gangneung and the latter

collected in Sangju. Total RNA were isolated from virus-infected fish brain, liver and kidney tissues using TRIzol reagent (Invitrogen, USA). Tissues were homogenized with 3 ㎖ of TRIzol reagent and the RNA was extracted with method used in 4-1.

② Hybridization and scanning

60-150 ㎍ of total RNA was used in reverse transcription reactions with oligo(dT)$_{18}$. Cy5-dUTP (Amersham Biosciences, UK) was used as fluorescent for labelled cDNA (Yu *et al.*, 1994), and next procedures were performed with the same methods in prior experiments. Hybridization, washing and scanning were also processed according to the prior experiments.

# RESULTS AND DISCUSSION

## 1. Design of cDNA microarray

The specific primer sets for rhabdovirus DNA chip were arranged considering the GC contents, $T_m$ and length. Total RNA extracted from the viruses was subjected to reverse transcription and PCR amplification was carried out using viral structural gene specific oligonucleotide primers. The sequences of these primers were based on the structural gene of virus. Fig. 3 showed the results of RT-PCR. The RT-PCR products of approximately 600 bp were obtained by this reaction.

To verify the specificity of primers, cross PCR reactions of the synthesized cDNA were performed (primers were represented in Table 1). In IHNV PCR reaction, N, M1 and G protein of IHNV of about 600 bp were detected, however the PCR products were not detected with primers of VHSV and HIRRV (Fig. 4). Likewise, Fig. 5 showed the same case of amplified VHSV gene, when cDNAs of VHSV were obtained by PCR, the bands were not appeared in PCR using IHNV and HIRRV primers. Fig. 6 showed PCR products of cDNA of HIRRV .

The sequences of N, G and M1 proteins of IHNV, VHSV and HIRRV were presented in Fig. 7-9.

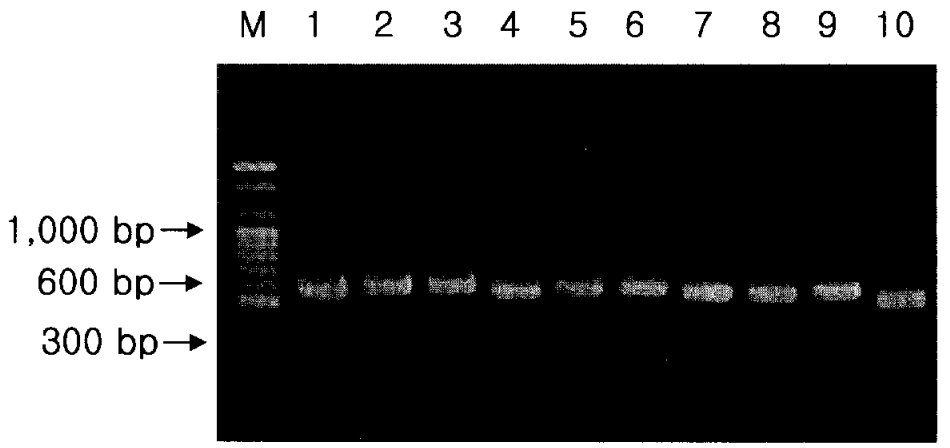


Fig. 3. cDNA probes prepared from IHNV, VHSV and HIRRV by RT-PCR using viral structural gene specific primers. Marke r(M), 100 bp DNA ladder (Bioneer, Korea). Lanes 1-3: IHNV. 1, N-gene; 2, M1-gene; 3, G-gene. Lanes 4-6: VHSV. 4, N-gene; 5, M1-gene; 6, G-gene. Lanes 7-9: HIRRV. 7, N-gene; 8, M1-gene; 9, G-gene. Lane 10, ß -actin.

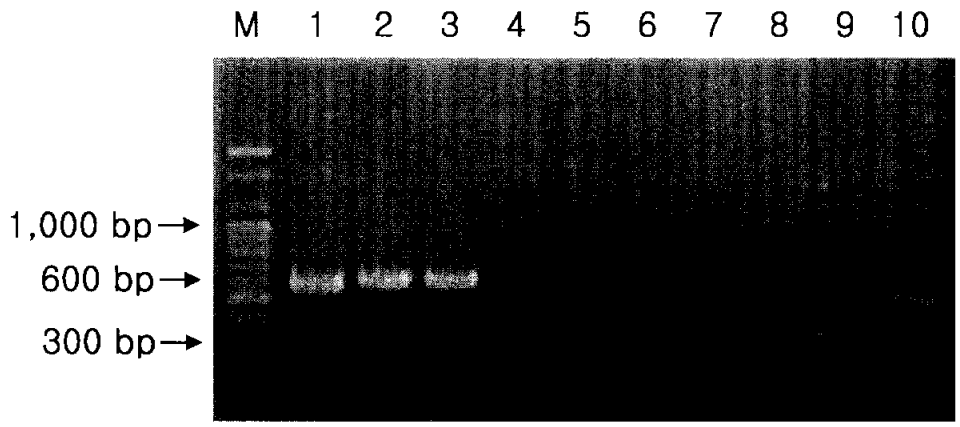


Fig. 4. Identification of specificity of IHNV cDNA probes by cross PCR reaction with specific primer sets. Marker (M), 100 bp DNA ladder. Lane 1, IHNV N primer; 2, IHNV M1 primer; 3, IHNV G primer; 4, VHSV N primer; 5, VHSV M1 primer; 6, VHSV G primer; 7, HIRRV N primer; 8, HIRRV M1 primer; 9, HIRRV G primer; 10, ß-actin primer.

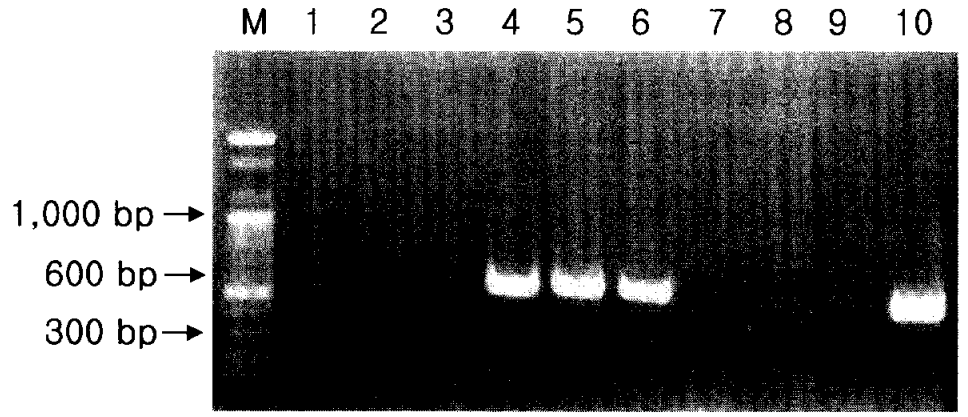


Fig. 5. Identification of specificity of VHSV cDNA probes by cross PCR reaction with specific primer sets. Marker(M), 100 bp DNA ladder. Lane 1, IHNV N primer; 2, IHNV M1 primer; 3, IHNV G primer; 4, VHSV N primer; 5: VHSV M1 primer; 6, VHSV G primer; 7, HIRRV N primer; 8, HIRRV M1 primer; 9, HIRRV G primer; 10, ß-actin primer.

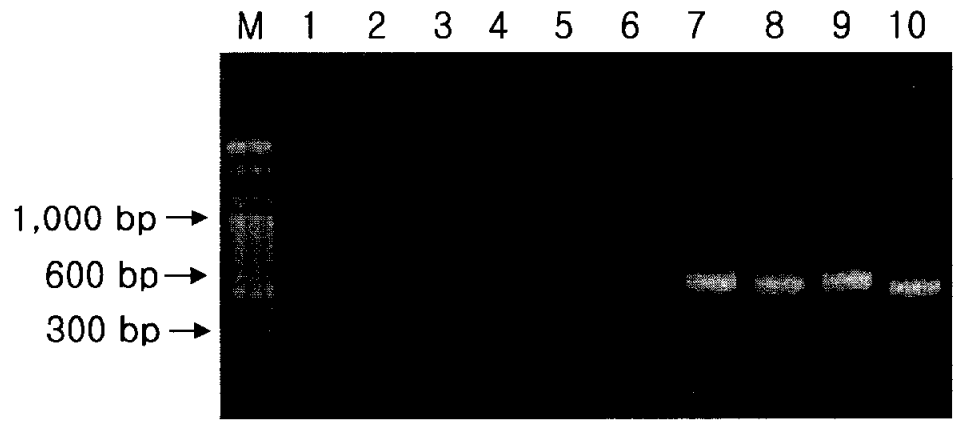


Fig. 6. Identification of specificity of HIRRV cDNA probes by cross reaction with specific primer sets. Marker(M), 100 bp DNA ladder. Lane 1, IHNV N primer; 2, IHNV M1 primer; 3, IHNV G primer; 4, VHSV N primer; 5, VHSV M1 primer; 6, VHSV G primer; 7, HIRRV N primer; 8, HIRRV M1 primer; 9, HIRRV G primer; 10, ß-actin primer.

5'-aatgtccttgaggttgtgaccggcctcctcttcacctgcgccctactgactaagta
tgatgtggacaagatggccacatactgccaaaacaagctcgagcgtcttgcaaccagcc
aagggattggcgagttggttaacttcaacgccaacaggggagtcctggccaagatcggg
gcggtgcttagacccggacagaagctcaccaaggctatctatgggatcattctcatcaa
cctgtccgacccagccatcgctgccagagccaaggcactgtgcgccatgagactgagcg
ggacaggaatgacaatggtggggctgttcaaccaagccgcaaagaacctgggcgccctt
ccagccgacctttagaggatctgtgcatgaagtcagtggtggagtccgccagacgcat
tgtcagactgatgaggatcgtagcagaggccccaggggtagcagcaaagtacggtgtca
tgatgagcaggatgctcggggaggggtacttcaaggcctacgggatcaacgagaacgcc
aggatcacctgcattctcatgaacatcaacgataggtatgacgatgg-3'

A. N protein.

5'-gaaggcgaagacatactgaggctagaatcccgtctgaaaaccccacgaaatgacggg
caaatcggcaagaaccccaggcgacggaaggaggaccaggcgcctcaagaggaaccaaag
aaaaccaccaggaggcccgacaagaacaagggtctatctcaactggagcaactcatccta
aagtacgttgaggaggagagctgtcaggatgccctgaaggacttcggaggtctaattgcc
aacatcagacaggcccaccaggccgaaatgacatctcacctagaaaaggttgctacggaa
caccgagccaatcttcaggctcttacaaagtcccagcaagagcacgagaaagtctcgaag
gagattttgtctgcggtaattgctattcggtccaacctcaacgagaaccacagtccccta
cccaagccactcgacccggatcaggtgaaggccgctcgtgcccttggattcggaattggg
tatcgaacggccctcaatgtcttcgaccgaatcaagggagtcaccccagacaacgcagga
tcccaagaggtgaagaacttggccattcgggcggcggaagaggatgaatacgagggaagt
-3'

B. M1 protein.

5'-gatagagaaggcgcttgtaaaaatgaaactctctacgaaagaagcaggggcgtatga
caccacaaccgcagccgctctgtacttcccagctccccgatgccaatggtacaccgacaa
cgtacaaaacgatctcatcttctactacacaacacaaaagagtgttcttagagatcccta
caccagagactttctggactcagattttattggaggaaaatgtaccaaatcaccctgcca
gactcattggtccaacgtagtttggatgggtgatgcagggataccagcctgtgattccag
ccaagagataaaagctcacctctttgttgataaaatctccaatcgagtcgtgaaggcaac
gagctacggacaccacccctggggactgcatcaggcctgtatgattgaattctgtgggca
acagtggatacggacagatctcggtgacctaatatctgtcgtatacaattctggatcaga
aatcctctcgttcccgaagtgtgaagacaagaccgtgggaatgaggggaaact
tggatgactttgcctatctagacgatctggtgaaggcctctgagagcagagaggaatgtc
ttga-3'

C. G protein.

Fig. 7. Nucleotide sequences of IHNV structural protein.

5'-gcactgtccgtacttctctcctatgtactccaaggaagtgcacaaggagatctggag
gcaaagtgcaagatcctcactgacatgggcttcaaggtgacacagtcaccccgggcaact
ggtatcgaggccggaatccttatgccgatgagggaactggcccagactgtcaacaacgac
aacctcatggacatcgtcaaggggccctgatgacgtgttcccttcttgacaagtactcg
gtggacaagatgatcaagtacatcaccaagaagcttggggagctgggcagcacacagggg
gttggagaactgcagcacttatccgccgacaaagcagccatcagaaagctcgcaggatgt
gtgcgtcctggacagaagatcaccaaggccctctacgccttcatcctgactgagatcgcg
gaccccaccacccagtcaagggtccagagcatgggggcgttgaggctcaatgggacagga
atgaccatgatcggactgttcacccaagccgccaacaacctgggcattccgccggcgaag
ctgctggaggatctgtgc atggaatctctt-3'

A. N protein.

5'-cagatcagctctcttttcgtcctccccagggtcctccaaacagaagccaagccccaa
gaagaaatccgctccgacaacgctcgaggagatcattggacacttcgtccccgaagatct
tcaattggatgcacgaaggccctcggacaactcctaagacgtatcaagctgtcccatcag
gaagaactcactcaacacctagagaaggtcaatggggagaatcgagccaagatgggggcc
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tccatgagaggagaaggagcggaaaatgcatccaagaagcccaaagttctagacggggac
caggtccggaatgagagagcacttggattcaaccgaggactaaccacggctgccattgcc
atgaagaagttcaagttggaggatccccttgtactctgcaagggctcagtcaagcgagca
gccctctccgccatggaaaaagaggaatacgacgggggggcgagagacttactccacggtc
gcaaaggcaaccaaggcggagttggacaagttggagtagggaca-3'

B. M1 protein.

5'-ggtcgagaacatctcaacataccatgtagactgggacactccactgtatactcaccc
ctccaactgcagaaaaaactcctttgttccgattcggccagatcaactcaggtgtcccca
tgagttcgaggacacaaacaagggcttggtctctgtcccagcccagatcatccatcttcc
gttatcagtcaccggcgtctcagcagtcgcaaatggccactacctacacagagtgaccta
ccgggtcacctgctcaaccaatttctttggaggacaaaccattgaaaaaaccatcctgga
ggcaaagctgtcccgtcaagaggccatcaatgaggctggcaaggatcacgagtacccttt
cttccccgaaccttcctgcatctggatgaaggacaatgtccacaaggacataa
cccactattacaagaccccaaagacggtgtccattgatctctacagtagaaagtttctaa
accctgacttcatagaggggtttgtacaacatcaccctgcccaacccactggcaaggag
tctactggatcggtgctacacctcaggcccattgccctacctcagaaacgcttaa-3'

C. G protein.

Fig. 8. Nucleotide sequences of VHSV structural protein.

5'-caaaccaacaaagcactaggaatcctttgtgcttttgtcacgtcagagaacaaccct
gacatgacagatgcagcagtcaagctcctggtggacatgaggttcaaggtggacgtggtg
cccgtggacgacaggctcggtgacaatctggatgaccccaactcaaagctggcggaggtc
ctgacggaagagaacatggtggacctggtgaaaggccttctgttcacttgtgccctgatg
gtcaagtacgatgtggacaagatggccacatactgccagcagaagctggagcgactggcc
aacagtcagggactcaacgagctgaccctgataagcacgagtagggcagttctggctcgg
attggggcagcggtaagaccggggcagaagttaaccaaggccatctacgggatcattctt
atcaacctgatggatcccgccactgctgccagggcaaaggccctttgtgccatgaggctg
agcggaaccggaatgaccatggtgggtctattcaatcaggcctctaagaaccttggagct
ccacctgcagatctgctggaagatctctgcatgaagtccatc-3'

A. N protein.

5'-acagttctttgatattcccaagaatgctctggacagagttgaggcacggacgatgtg
tcccagggaggatggaaaggttgtccggaaacaagcgcctctaaaagaggaaccaagact
ggaggcagagcagaagcggtctccaaagaagcaggagaaaccccggggaatgctccctct
ggaacaacttgttctgaagtatgtggttgtggtctgctcccttgatgcgctccgagagtt
cggaggactaattgcccagatcaggcagtctcatcaggccgatatgactcgtcatctgga
ggcagtggcaacagagcaccgggccaatctccaggcgctcaccaagtctcagcaggagca
cgagaaagtctccaaagagatcctctcggcagtcatctccatccggtccaacctcaacga
gaactccagtccccgacacaaacccctggacctggaccaggtcaatgcggagagagccct
cggatttggagtcgggtaccggaccgccttgaacgtctttggcaaactacggggaatcac
accagaagaggcaggctcgcaagaagtcaagaacatggcaatc-3'

B. M1 protein.

5'-atcccctgtttacataccctgtggactgtcctgcggctaagctatccaaggtgagtc
cctcacagttgaggtgcccacgcatatttgatgatgaaaaccgaggactcgttgcctacc
ctgctgtcatcagatccctatcggtagggaacaatcttggggacattcatactcaggggg
aatacgtccacaaggtcctttaccgaaccacatgttcaacagggttctttgggggccaaa
ccatagagaaggccctagtagagatgaaactggccccccagggaggtgggagtgtatgaca
ccacgacagcctcagcactgtacttcccagctccaaggtgccaatggtacacggacaatg
tacataacgacctgacattctactatacgaccgccaagagtgtccttagagatccttaca
ccctgggattccttgattctgattttattgaagggaagtgctccaagtcgccgtgtcaga
cccattggtcaaatgtggtgtggaagggagactcgggagttgccgcctgtgacacggggt
ctgagataaagggtcacatatttgtggacaagacctcc-3'

C. G protein.

Fig. 9. Nucleotide sequences of HIRRV structural protein.

## 2. Determination of humidity for spotting

The effect of humidity for probe spotting on slide glass was investigated HIRRV. The chips were prepared on poly-L-lysine coated slide glass (Sigma, USA) using Genomics microarrayer and the concentration of total RNA was 50 $\mu$g. As shown in Fig. 10, the humidity condition of 60-65% was better than 65-70% of condition for spotting on the poly-L-lysine. The spot signals did not appear in 65-70%, however, were clear and accurate in 60-65% humidity. Humidity is important condition for spotting because constant humidity is likely to emerge from the pin.

The scanned images were speckled using a graphics program and then analyzed using a custom image gridding program. This program created a spread sheet of average red and green hybridization intensities which corrected for optical cross talk between the fluorescein and lissamine channels, using experimentally determined coefficients. Nowadays the rainbow palette is often used as graphics program for analysis. Microarray data which obtained from a fluorescent scan are represented in a rainbow palette coded to signal intensities from 1 to 65,536 counts as shown in the color bar. So, it means that the relative signal intensity corresponds spot to spot.

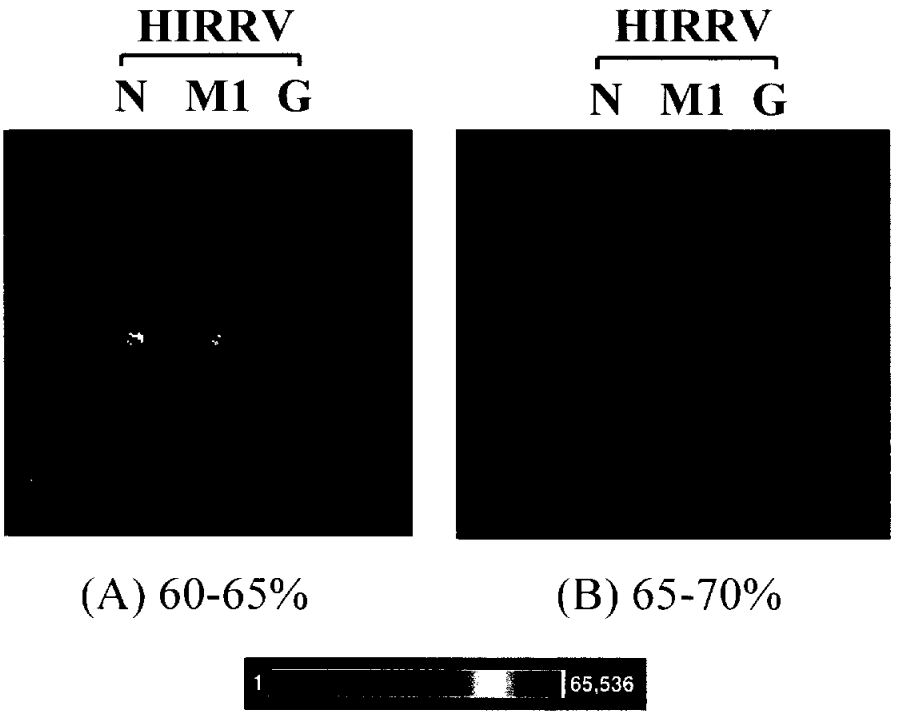


Fig. 10. Effect of humidity for probe spotting on poly-L-lysine coated slide (Sigma, USA). The concentration of total RNA of HIRRV was 50 ㎍ and chips were performed using Genomics Microarrayer. The laser strength is 75, PMT (Photomultiplier tubes) is 65. The color bar represent the intensity of signal.

### 3. Effect of the coated materials on slide glass

It has been reported poly-L-lysine is effective coating agents for slide glass used in microarray analysis, as coating method is easy and low-priced. However, poly-L-lysine coated slide glass often binds dust and other materials in room circumstances. In addition, it has disadvantage that DNA on slide glass sometimes detached during hybridization (Bowtell, 2003 ; Zammatteo *et al.*, 2000).

Fig. 11 showed the actual signal on cDNA chip using poly-L-lysine. IHNV showed the lower signal intensity than those of other viruses, it was considered that IHNV expression rate is relatively lower. Also, β-actin was difficult to get the signal in experiment because β-actin has the low expression rate compared with other virus genes.

To increase interaction between slide glass and probes, aminosilane coated slide glass (Nuricell, Korea) was substituted by poly-L-lysine (Fig. 12). At present, aminosilane slide glass was considered as proper matrix for cDNA chip than poly-L-lysine slide glass. Probes are bound to the substrate by an electrostatic interaction between the amine groups of silane, which are positively charged, and the negatively charged phosphodiester backbone of the DNA. Fig. 12

showed the correct signal and low background in cDNA chip with aminosilane slide glass. All genes of viruses and ß-action were detected by hybridization. So, aminosilane coated slide glass were represented more effective as substrate for probe attachment.


Fig. 11. Results of hybridization of IHNV (A), VHSV (B) and HIRRV (C) in cDNA chip using poly-L-lysine coated slide glass (Sigma, USA). Hybridization temperature was 65℃ and the spotting was performed using Genomics microarrayer. The color bar represent the intensity of signal.

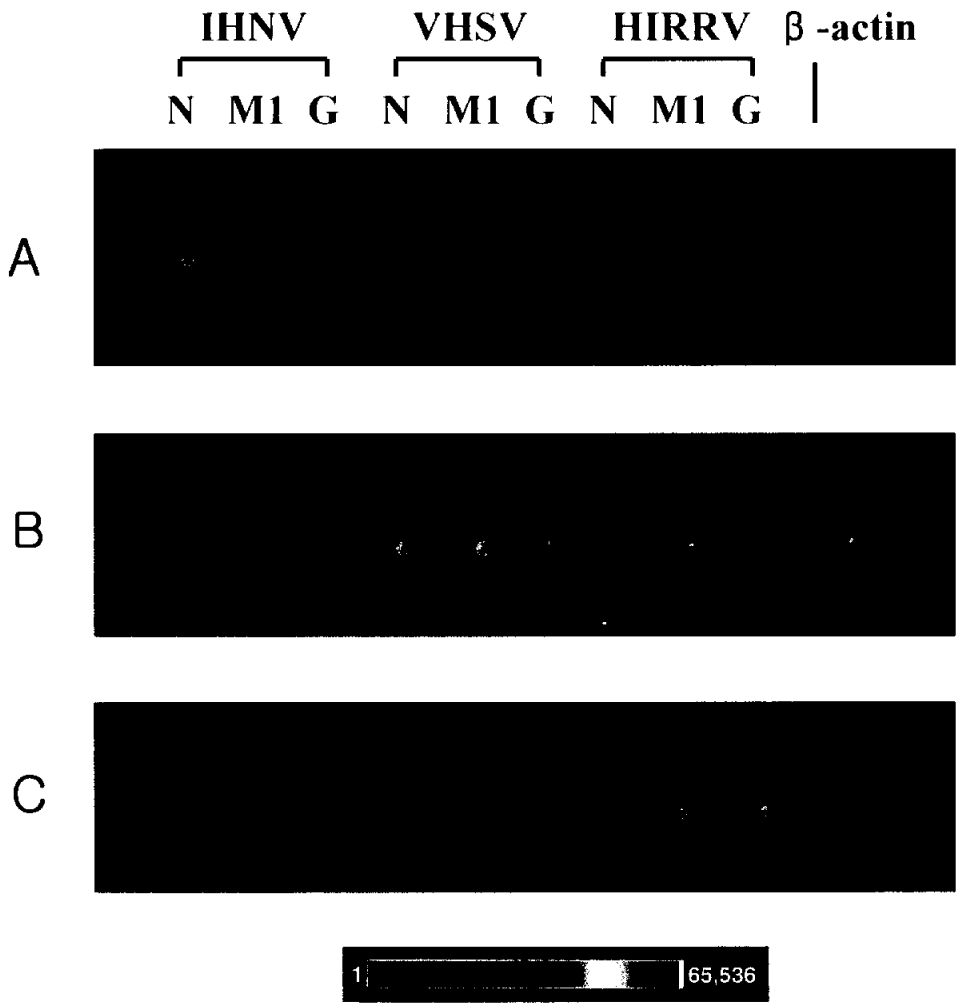


Fig. 12. Results of hybridization of IHNV (A), VHSV (B) and HIRRV (C) in cDNA chip using aminosilane coated slide glass (Nuricell, Korea). Hybridization temperature was 62℃ and the spotting was performed using Genomics microarrayer. The color bar represent the intensity of signal.

## 4. Determination of hybridization temperature

Fig. 13 and 14 showed cDNA chip of IHNV, VHSV and HIRRV which were hybridized at 62℃ and 65℃ using aminosilane coated slide glass (Nuricell, Korea).

Hybridization were carried out at 62℃ and 65℃ overnight in all viruses. In the case of 62℃ (Fig. 13) N, M1, G and ß-actin were accurately and strongly detected. However, in 65℃ only M1 was detected in VHSV (Fig. 14 (B)), and no spot signal was observed in HIRRV (Fig. 14 (C)). Therefore, the temperature for hybridization was more effective at 62℃ than 65℃.

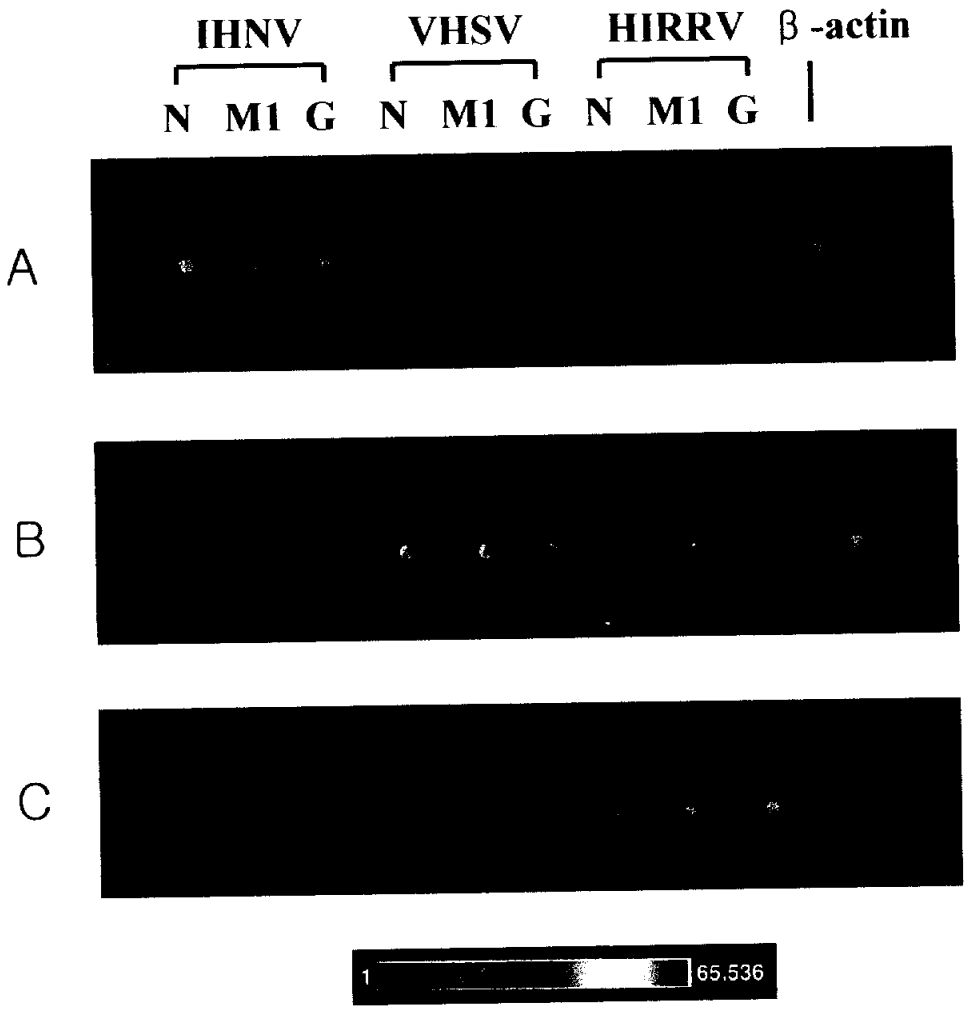


Fig. 13. Effect of hybridization temperature on probe spotting Hybridization temperature was 62℃ and chips were prepared on the aminosilane coated slide glasses. Spotting was performed by Genomics microarrayer. The color bar represent the intensity of signal.

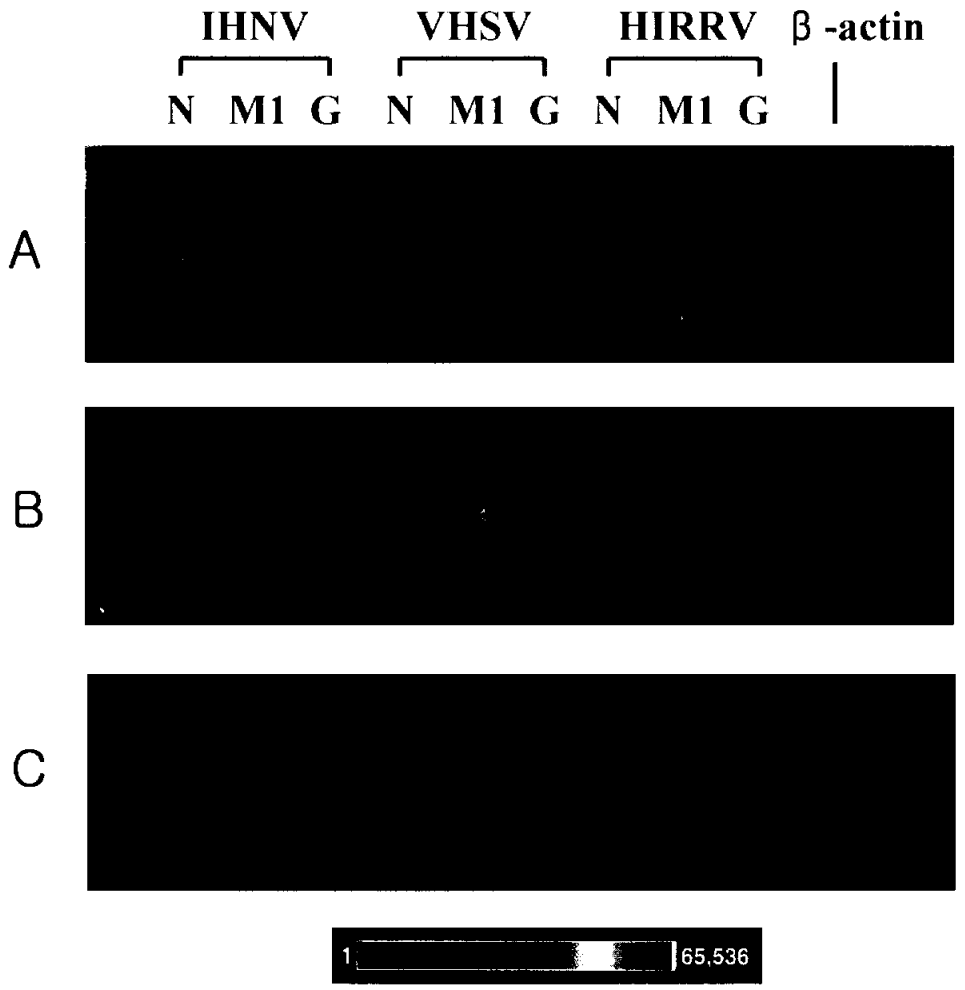


Fig. 14. Effect of hybridization temperature on probe spotting Hybridization temperature was 65℃ and chips were prepared on the aminosilane coated slide glasses. Spotting was performed by Genomics microarrayer. The color bar represent the intensity of signal.

## 5. Efficiency confirmation of Genomics microarrayer

To evaluate the efficiency of self-developed Genomics microarrayer, cDNA chips were prepared by Genomics microarrayer and MG Ⅱ TAS microarrayer (BioRobotics, UK), respectively. The chips were fabricated on the aminosilane coated slide glass (Corning, USA). A rainbow type showed the strength of relative strength spot to spot. Used total RNA concentration were respectively 100 ㎍, 60 ㎍ and 50 ㎍ for IHNV, VHSV and HIRRV. As shown in Fig. 15, the relative signal intensity of G gene and ß-actin were faint in all viruses. In case of IHNV total RNA is always need to use more than of other viruses according to the results of other experiments. Reference results of Genomics microarrayer was shown in Fig. 12.

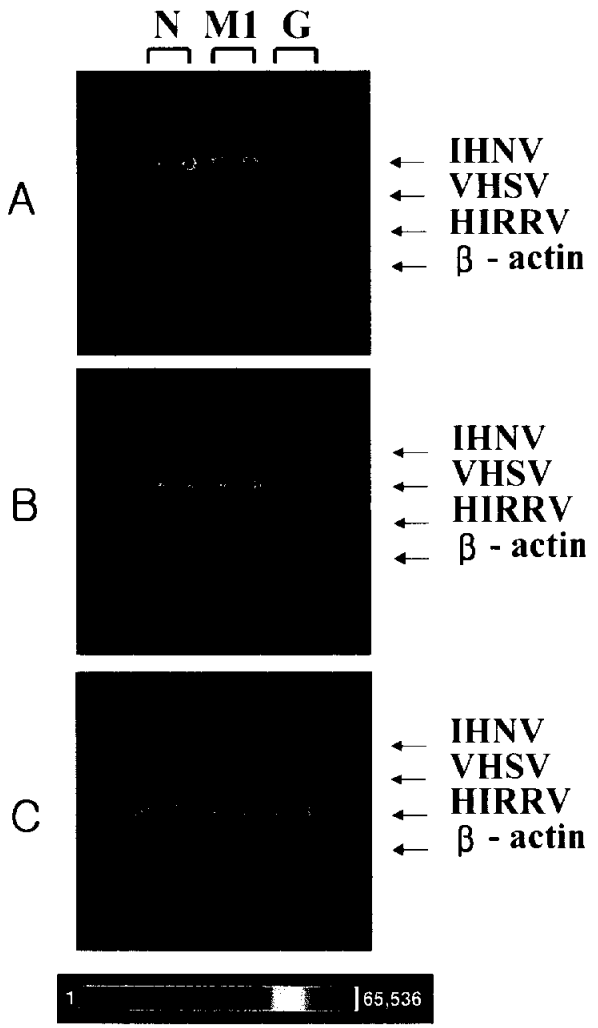


Fig. 15. cDNA chip prepared by MG II TAS microarrayer. Aminosilane coated slide glasses (Corning, USA) were used as matrix. A, cDNA chip for IHNV; B, cDNA chip for VHSV; C, cDNA chip for HIRRV. The color bar represent the intensity of signal.

## 6. Effect of total RNA concentration

Effect of total RNA concentration required for cDNA chip was investigated in the range of 40~100 ㎍. The results were shown in Fig. 16-18. When 40 ㎍ of total RNA was used for IHNV cDNA chip, N and M1 genes were detected, however G and ß-actin did not appear (Fig. 16). Signal of G and ß-actin appeared when total RNA concentration increased to 100 ㎍, this means that much of total RNA was required for cDNA chip of IHNV virus.

In the case of VHSV cDNA chip, when 40 ㎍ of total RNA was used, G protein and ß-actin were not detected (Fig. 17). N gene also represented hybridization signal regardless of total RNA concentration. The strength of M1 gene was strong in all tested concentration.

The result of HIRRV was shown in Fig. 18, with the concentration of 50 ㎍, all genes of HIRRV were hybridized on cDNA chip.

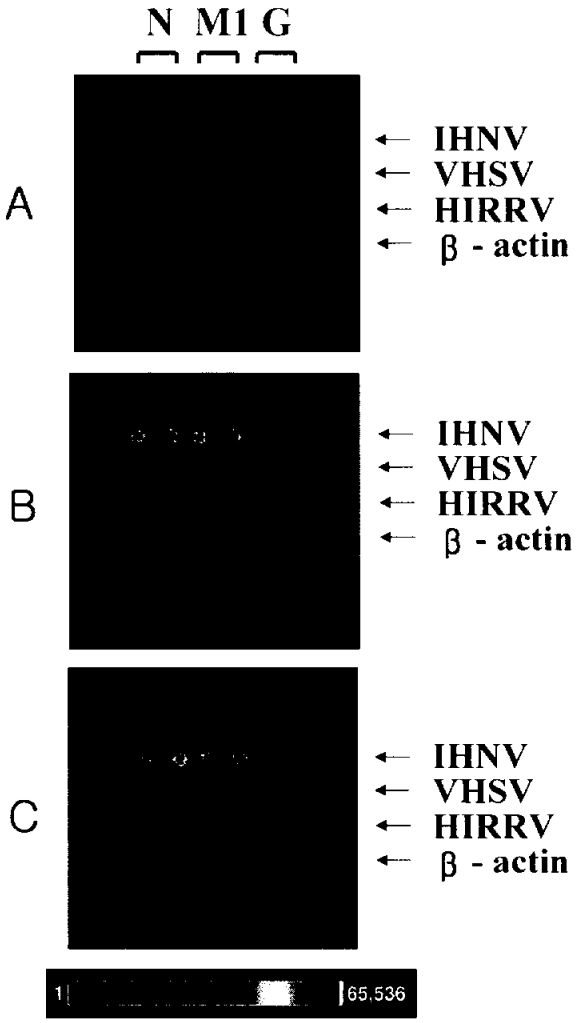


Fig. 16. Effect of total RNA concentration of hybridization of IHNV cDNA chip. Aminosilane coated slide (Corning, USA), and MG II TAS microarrayerwere used for preparation of DNA chip. A, 40 ㎍; B, 80 ㎍; C, 100 ㎍. The color bar represent the intensity of signal.

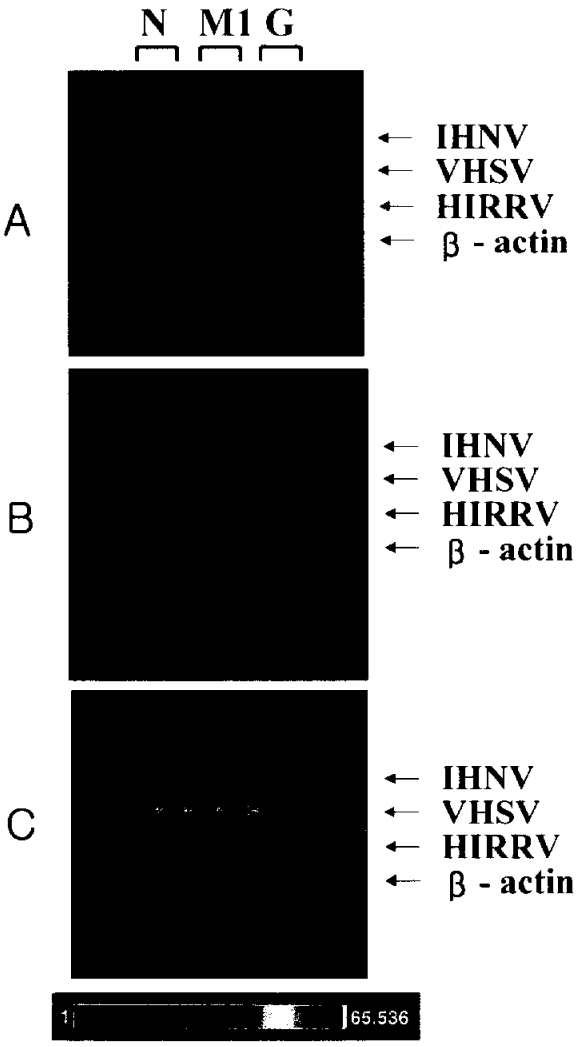


Fig. 17. Effect of total RNA concentration of hybridization of VHSV cDNA chip. Aminosilane coated slide (Corning, USA), and MG II TAS microarrayerwere used for preparation of DNA chip. A, 40 ㎍; B, 50 ㎍; C, 60 ㎍. The color bar represent the intensity of signal.

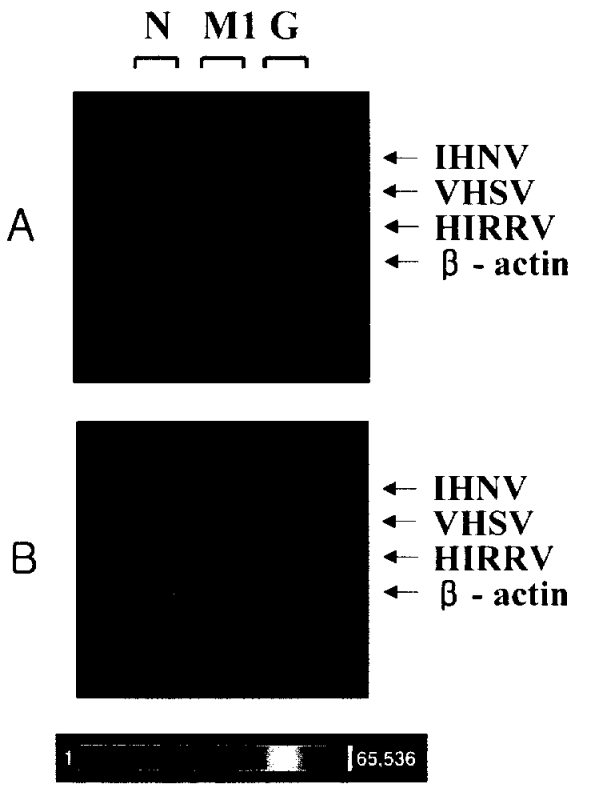


Fig. 18. Effect of total RNA concentration of hybridization of HIRRV cDNA chip. Aminosilane coated slide (Corning, USA), and MG Ⅱ TAS microarrayerwere used for preparation of DNA chip. A, 40 ㎍; B, 50 ㎍. The color bar represent the intensity of signal.

## 7. *In vivo* efficiency of cDNA chip for detection of fish pathogenic virus

To evaluate the efficiency of cDNA chip for virus detection *in vivo*, two virus-infected flounder and rainbow trout samples were used. Total RNA was extracted from samples and cDNA were synthesized and hybridized with prepared cDNA chip. Fig. 19 showed the virus detection results in olive flounder. Total RNA concentration of 60 $\mu$g, 100 $\mu$g and 150 $\mu$g. The signal spots only appeared in VHSV, but not in IHNV and HIRRV, although the intensity was different according to the RNA concentration. This result lead to the conclusion that this fish sample was infected only by VHSV.

Fig. 20 represented the virus detection result of the rainbow trout. Total RNA concentration was adjusted to 70 $\mu$g. The signal spots only appeared in IHNV, although the intensity was stronger in N and M1 gene, but lower is G gene. No signal spot appeared in VHSV and HIRRV. Consequently, this sample was infected by IHNV.

Positive control have to present for correct experiment for DNA chip. Under conditions of probe excess, there is a selective saturation

of targets corresponding to abundant species, so that successive more increases in signal intensity at only some spot location, especially in N and M1 gene. Positive control genes are a set of predefined genes required for fundamental cellular processes in a wide range of cell types. Tissues and whose expression is not general dependent on the developmental stage or physiological or pathological state of the tissue. Designated positive control genes tend to be highly expressed and may not be representative of genes of interest that are expressed at lower levels and may be subject to and intensity dependent bias. ß-actin was enable to represent as positive control.

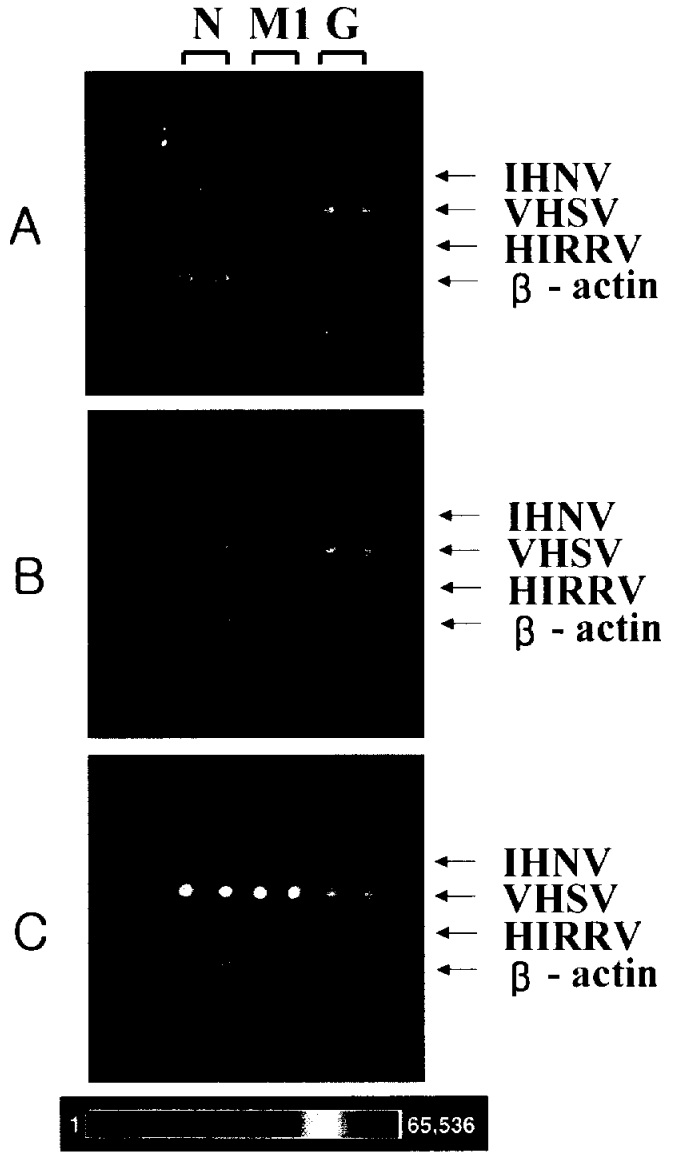


Fig. 19. Detection of VHSV in infected olive flounder using cDNA chip. A: 60 ㎍ of total RNA, B, 100 ㎍ of total RNA; C, 150 ㎍ of total RNA. The color bar represent the intensity of signal.

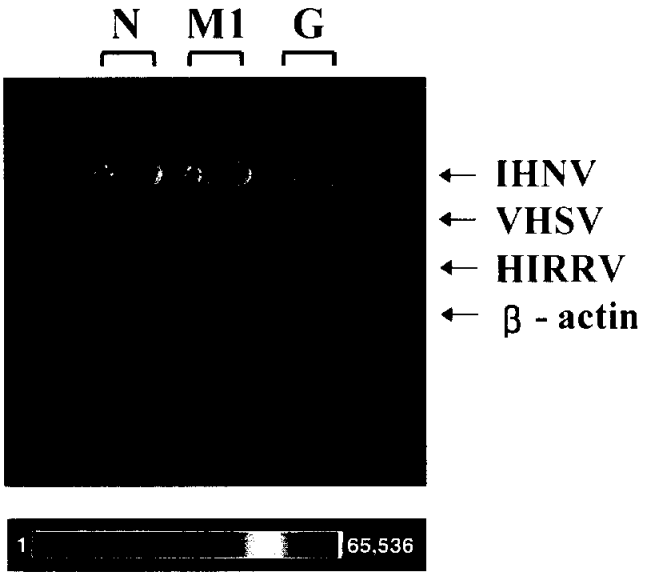


Fig. 20. Detection of IHNV in infected rainbow trout using cDNA chip. Used total RNA concentration was 70 ㎍. The color bar represent the intensity of signal.

# 국 문 초 록

랩도바이러스에 감염된 어류로부터 바이러스를 신속하고 정확하게 검출할 수 있는 진단용 DNA chip의 제작을 위한 최적 조건을 조사하였다.

이를 위해 IHNV, VHSV 그리고 HIRRV를 대상바이러스로 채택하였고, 각 바이러스의 구조 단백질의 염기서열을 분석하여 probe를 선정한 다음, 이를 GenBank를 이용하여 분석하였다. 각 probe는 특이 primer로 PCR을 실시하였고, NCBI의 blast alignment를 통해 특이성을 확인하였다.

또한 target DNA는 바이러스가 감염된 cell line으로부터 total RNA를 분리하여 reverse transcription에 의해 Cy5가 결합된 cDNA를 제작함으로써 준비되었다. 선정된 probe를 자체 개발된 Genomics microarrayer를 이용하여 slide glass에 spotting 한 다음, probe와 target DNA를 hybridization 시킨 후 스캐닝을 실시하였다. 이 때, spotting에 필요한 microarrayer chamber 내의 습도, 사용된 슬라이드 글라스의 종류, total RNA 농도 그리고 hybridization 온도 등의 최적 조건을 검토하였다.

그 결과 poly-L-lysine과 aminosilane이 코팅된 슬라이드 글라스에서 효과적인 결과를 얻을 수 있었으며, spotting에 필요한 Genomics microarrayer chamber 내의 습도는 두 슬라이드 글라스

모두 60-65%로 선정되었다. 또한 hybridization 온도는 poly-L-lysine의 경우 65℃, aminosilane 슬라이드 글라스의 경우 62℃에서 정확한 결과를 얻을 수 있었다. Target DNA의 제작에 필요한 total RNA의 농도는 aminosilane이 코팅된 슬라이드 글라스의 경우 IHNV가 100 ㎍, VHSV는 60 ㎍ 그리고 HIRRV는 50 ㎍이 최적인 것으로 나타났으며, poly-L-lysine이 코팅된 슬라이드 글라스의 경우는 IHNV와 VHSV, HIRRV의 순서대로 각각 80 ㎍, 50 ㎍, 50 ㎍에서 결과를 얻을 수 있었다.

cDNA chip의 효과를 검증하기 위하여, 실제 바이러스에 감염된 어체로부터 분리한 total RNA를 이용하여 제작된 target DNA로 바이러스 검출을 실험하였을 때, VHSV와 IHNV의 구조단백질에 관련된 gene이 검출되는 것을 확인할 수 있었다.

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먼저 학문에 있어서는 물론 생활면에서도 많은 가르침을 주신 저의 지도교수님 이명숙 교수님께 감사드리며, 앞으로도 저의 생활에 소중히 기억될 것입니다. 부족한 논문을 검토하여 지도해 주신 박수일 교수님과 최태진 교수님께 감사드립니다. 항상 지켜봐 주시고 가르침을 주신 이원재 교수님, 김진상 교수님, 이홍구 교수님, 송영환 교수님, 김영태 교수님 감사드립니다.

CIMEC Lab.의 김상봉 교수님께도 감사의 말을 전해야 할 것 같습니다. 그리고, 어병학과 수미언니 많이 고마워요. 은전이, 석열오빠, 재성선배, 영규선배 감사드리고, DNA chip에 좋은 결과 있으리라 믿습니다.

학부 때부터 함께하며 학문이나 실험실 생활에 있어서 항상 이끌어준 지희언니 고맙습니다. 멀리서도 잘 챙겨봐 주시고 격려해주신 용운선배도 물론입니다. 힘든 의사소통에도 불구하고 옆에서 많이 도와준 Duong 언니, 일반미생물실험실이라는 곳에서 함께 할 수 있어 좋았던 은미, 경택, 아영, 정미에게도 고맙다는 말과 함께 원하는 일이 다 잘되기를 바라겠습니다. 그리고 우리 실험실 3학년들 소언, 해숙 그리고 수연이 실험실에서 많이 배워나가길 바랍니다.

대학에 와서 큰 소득이라면 소중한 친구들을 여럿 만났다는 것이라고 자신있게 말할 수 있는 나의 친구들 - 수미, 소현, 지선, 미주, 선영, 윤정 그리고 명희에게도 고맙다는 말을 전합니다. 지금 모두 인사드리지는 못하지만 항상 자기 일처럼 걱정해주고 도와주시는 7호관의 미생물학과 선배님들, 후배들 그리고 동기 여러분들께도 깊은 감사드립니다.

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