CHAPTER III

RESULTS

pXGH 5 plasmids extraction and analysis

For comparing restriction sites with the map, the plasmid pXGH 5 was extracted and digested with restriction enzymes Hind III, Kpn I, Bgl II, EcoR I and BamH I. The extracted and digested plasmid was analysed by 0.7 % agarose gel electrophoresis, and the result was shown in Figure 14. The remaining incomplete digesting of fragment size as 6.7 kb was found in lane 3, 4 and 6. The result showed that the digested fragments of extracted plasmid were related to the expected size from the map (Table 4).

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Total plasmid pXGH 5 was prepared by large scale plasmid extraction and purified by CsCl ultracentrifugation. The extracted plasmid from large scale plasmid preparation was contaminated with bacterial chromosome and other proteins. After purification, many forms of plasmid was obtained. In order to obtain linearized plasmid DNA and did not disrupt the structure of growth hormone gene function, the pXGH 5 was digested with BamH I. The result was shown in Figure 15, the plasmid pXGH 5 was not completely linearized, a little circularized plasmid was still remained.

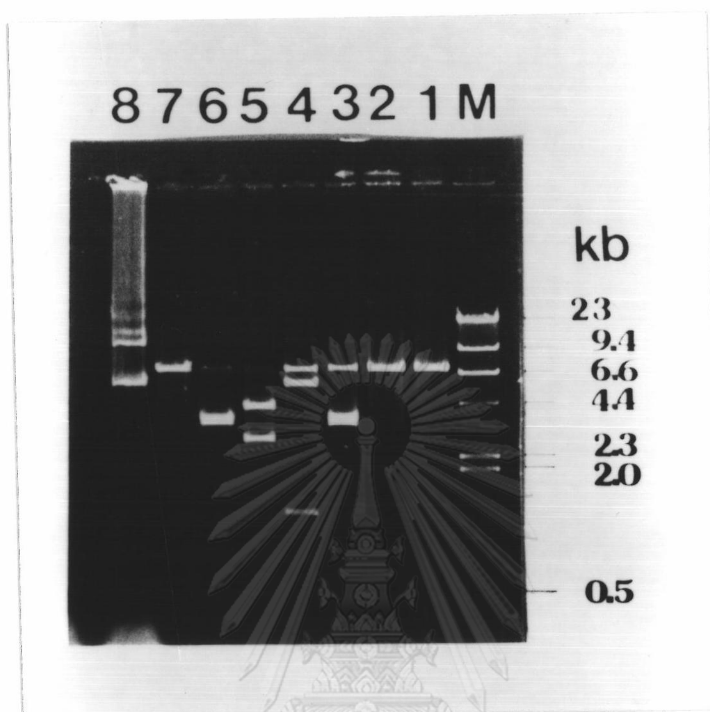

Figure 14 Gel analysis of plasmid pXGH 5 digested with restriction enzymes;

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Lane M = λ Hind III DNA size marker

Lane 1 = pXGH 5 digested with Hind III

Lane 2 = pXGH 5 digested with Kpn I

Lane 3 = pXGH 5 digested with Hind III and Bgl II

Lane 4 = pXGH 5 digested with Hind III and Kpn I

Lane 5 = pXGH 5 digested with EcoR I

Lane 6 = pXGH 5 digested with BamH I and Bgl II

Lane 7 = pXGH 5 digested with Bgl II

Lane 8 = Small scale extracted plasmid pXGH 5

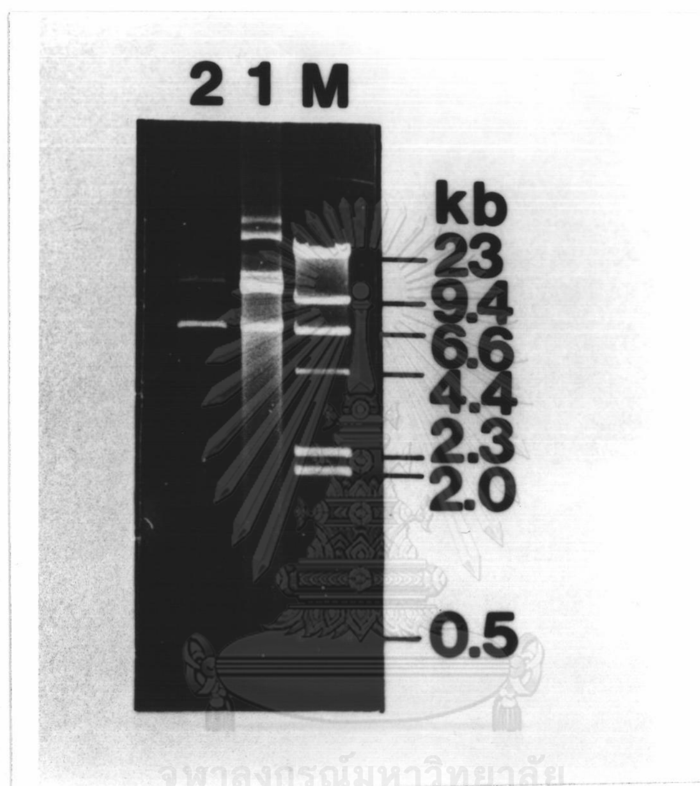

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Figure 15 Gel analysis of linearized plasmid pXGH 5

Lane M = λ Hind III DNA size marker

Lane 1 = Large scale extracted plasmid pXGH 5

Lane 2 = Linearized plasmid pXGH 5 for microinjection

Determination of microinjection flow rate

To determine the flow rate, volumes as 0.5, 1, 1.5 and 2 μl of the TE buffer was driven out of the microneedles by the pressure constant gas N_2 at 2,000 hpa for measuring time taken. Duration taken during each TE volume completely pass to the microneedle was determined and the flow rate of the injector was calculated. As showing in Table 5, the average flow rate of the microinjector was 240.41 pl/sec.

Preparation of plasmid pXGH 5 solution for microinjection

In order to inject 10^6 copies of plasmid pXGH 5 into a fish egg with a injected duration of 1 second, the calculation of this amount of plasmid was done as described in Appendix 2, and equal to 7.34 pg. 7.34 pg of plasmid pXGH 5 was resuspended in 240 pl of TE buffer pH 7.4, concentration of plasmid solution was consequently prepared as 30 ng/μl.

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Microinjection of plasmid solution into fish eggs

Microinjection was done through the germinal disc at 25 ^{0}C in three early egg developmental stages-one-cell stage, two-cell stage and four-cell stage. During newly fertilized until first cleavage, formation of germinal disc is still thin and normally orients up on the upper petridisc. Chorion is soft and not too glutinous. Therefore, microinjection in this stage could be easily performed. At two-cell and four-cell stages,

Table 4 DNA fragment of pXGH 5 when cut with restriction endonuclease

Restriction enzymes	Number of fragments	Expected size from restriction map (kb)
Hind III or Kpn I or Bgl II	1	6.7
Hind III and Bgl II	2	3.4, 3.3
Hind III and Kpn I	2	5.6, 1.1
BamH I and Bgl II	2	3.4, 3.3
EcoR I	2	4.1, 2.6

Table 5 Duration time taken and flow rate of microinjection at various amount of TE buffer at nitrogen gas pressure 2000 hpa

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Amount of TE buffer (µl)	Time taken (min)	Flow rate pl/sec
0.5	35	278.78
1.0	77	216.45
1.5	105	238.10
2.0	140	228.31
		average= 240.41

formation of germinal disc enlarges, chorion was more glutinous and make it difficult to do the microinjection. The developmental time during one cell stage was longer time than developmental stages at the two cell and the four cell. After injection, eggs were allowed to development until hatching.

Result of hatching rate of the microinjected egg and the control is shown in Table 6. The number of microinjected eggs at one-cell, two-cell and four-cell respectively reduced, while hatching rates respectively increased. For statistical analysis, hatching rate of the three groups of microinjected eggs were significant lower than the control (uninjected egg) ($P<0.05$).

Survival rate of fries at one month age

The fries, which were microinjected at the three stages and the control were separately cultured and maintained for 1 month. Survival rate of control fries, and fries derived from one-cell, two-cell and four-cell microinjected eggs were respectively reduced, (Table 6). However, there was no significant difference ($P>0.05$).

Comparing of growth performance

The microinjected groups and the control were separately reared for comparing the weight gain. Maintaining and feeding were done routinely. The fish growth of the same spawner varied greatly.

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The linear regression analysis estimated growth rate of microinjected fish at one-cell, two-cell, four-cell and that of the control was shown in Figure 16, 17 and 18, respectively. The estimated slope of growth rate of microinjected fish were shown in Table 7. The growth rates (b) of microinjected fish at one cell and four cell were higher than the control fish. The growth rate of the microinjected fish at four cell and the control was similar. However, only the microinjected fish at one-cell stage showed a significance higher growth rate than that of the control ($P<0.05$).

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Table 6 The hatching rate of eggs and the survival rate of fries

Stage of Microinjection	Hatching of eggs		Survival of fries at one month	
	Number of eggs	% Hatching rate	Number of frys	% Survival rate
one-cell	776	$25.91^{b} \pm 23.00$	148	52.02 ± 19.81
two-cell	242	$29.71^{b} \pm 29.45$	50	46.67 ± 30.49
four-cell	182	$30.94^{b} \pm 30.22$	38	35.51 ± 34.13
control	1200	$40.67^{a} \pm 20.76$	383	53.61 ± 22.30

Different superscript indicated the significant difference ($P<0.05$) in the column

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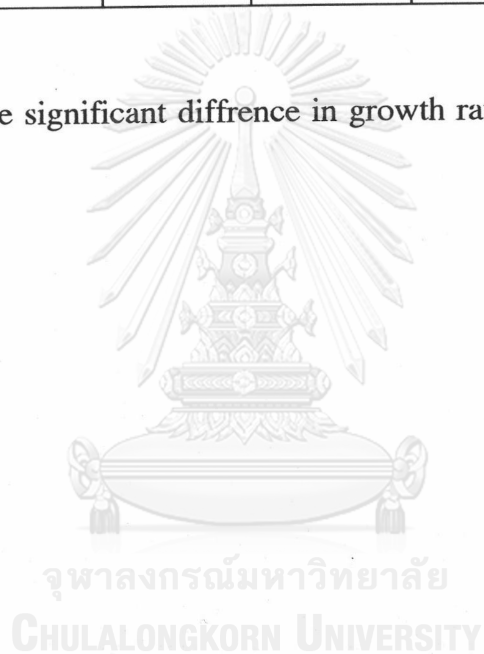
Table 7 The estimated slope of growth rate of microinjected fish and control

Developmental stage of egg	Microinjection		Control		Confidental limit (α)
	Slope (b)	intercep	Slope (b)	intercep	
One-cell	0.28*	-10.58	0.18*	-6.47	0.03
Two-cell	0.22	-8.66	0.15	-5.41	0.09
Four-cell	0.09	-0.67	0.11	-2.31	0.99

* indicated the significant diffrence in growth rate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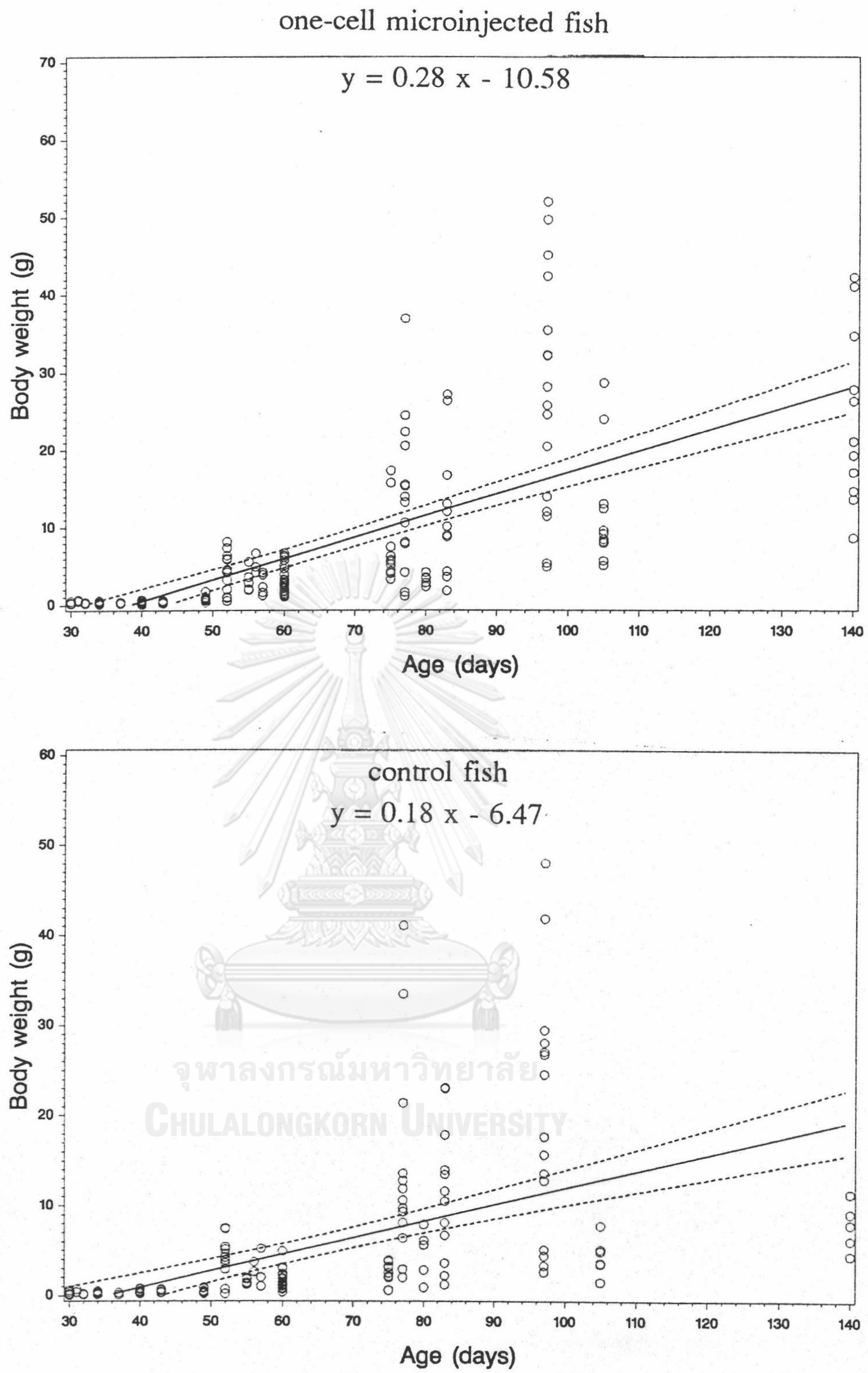

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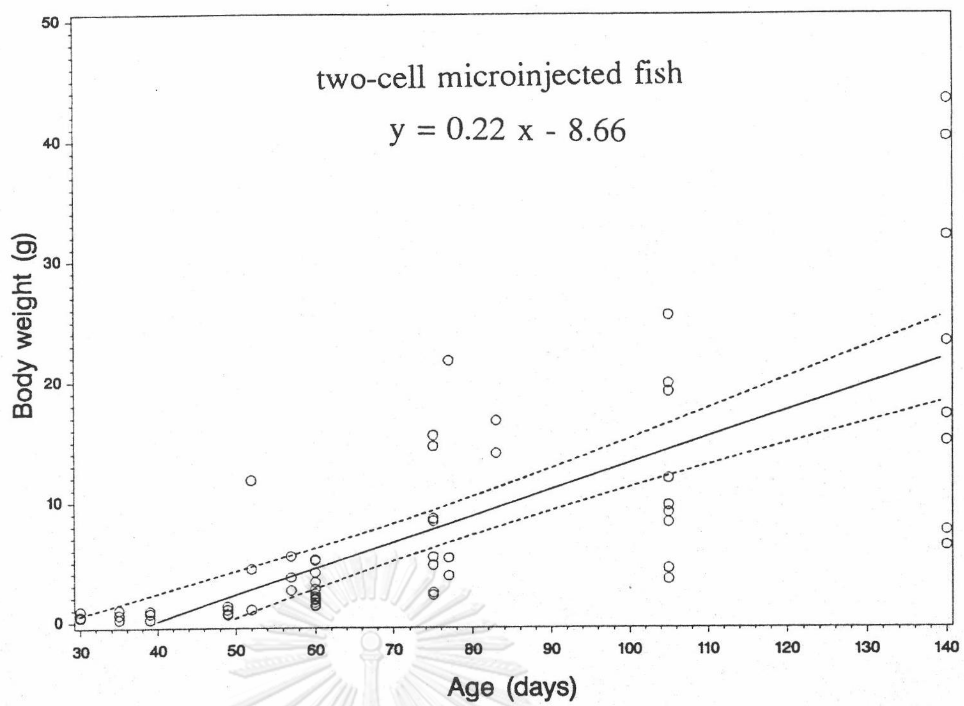
Figure 16 The linear regression of estimated growth rate of microinjected fish at one cell and control



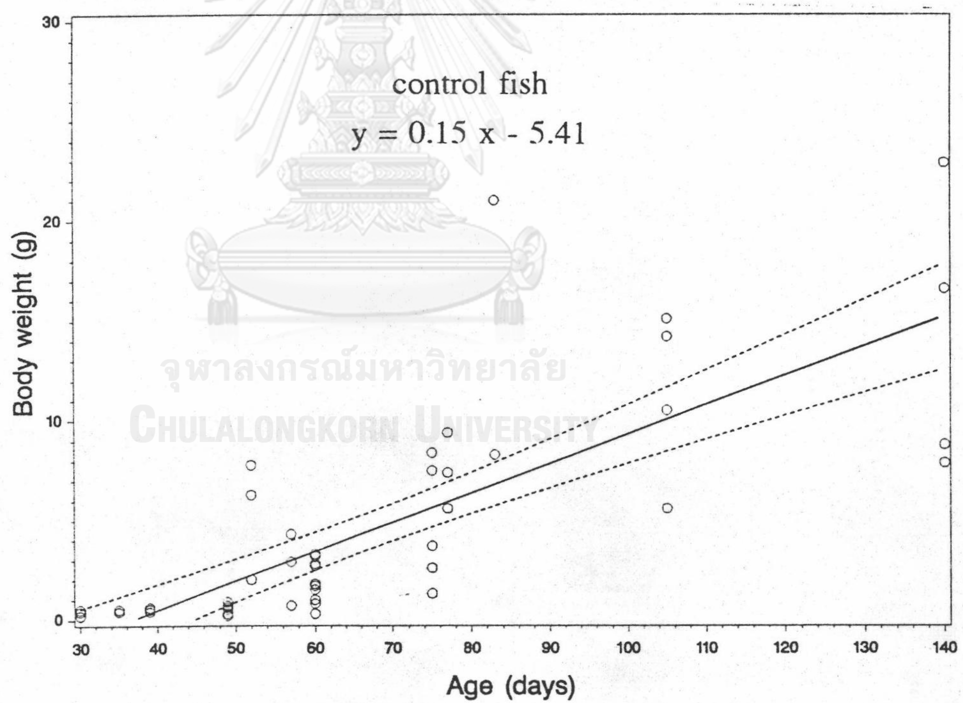


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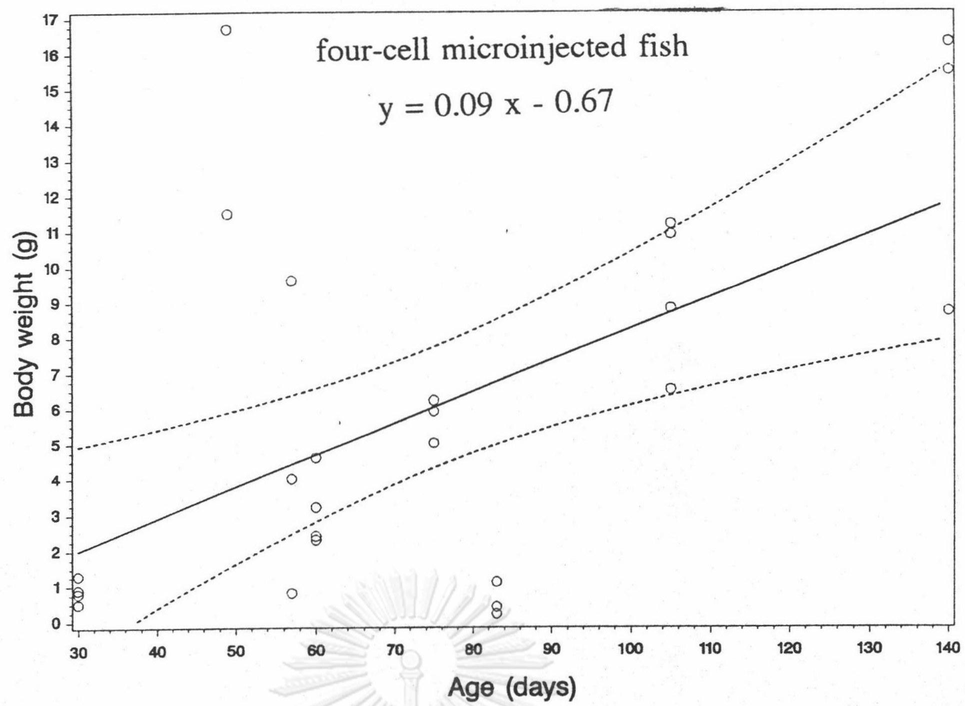
Figure 17 The linear regression of estimated growth rate of microinjected fish at two cell and control



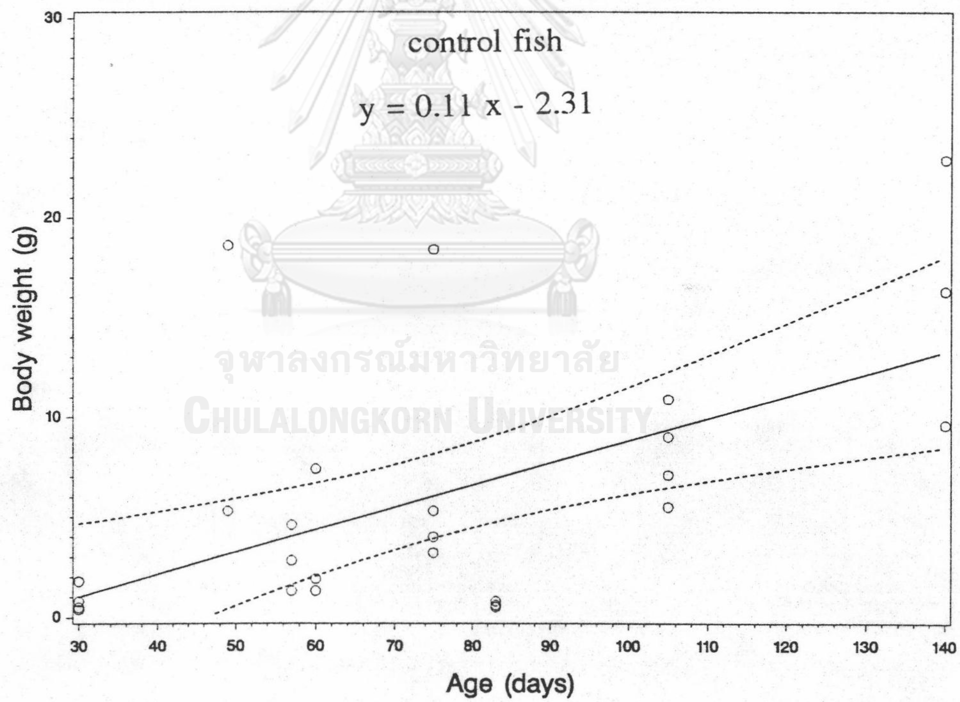


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Figure 18 The linear regression of estimated growth rate of microinjected fish at four cell and control

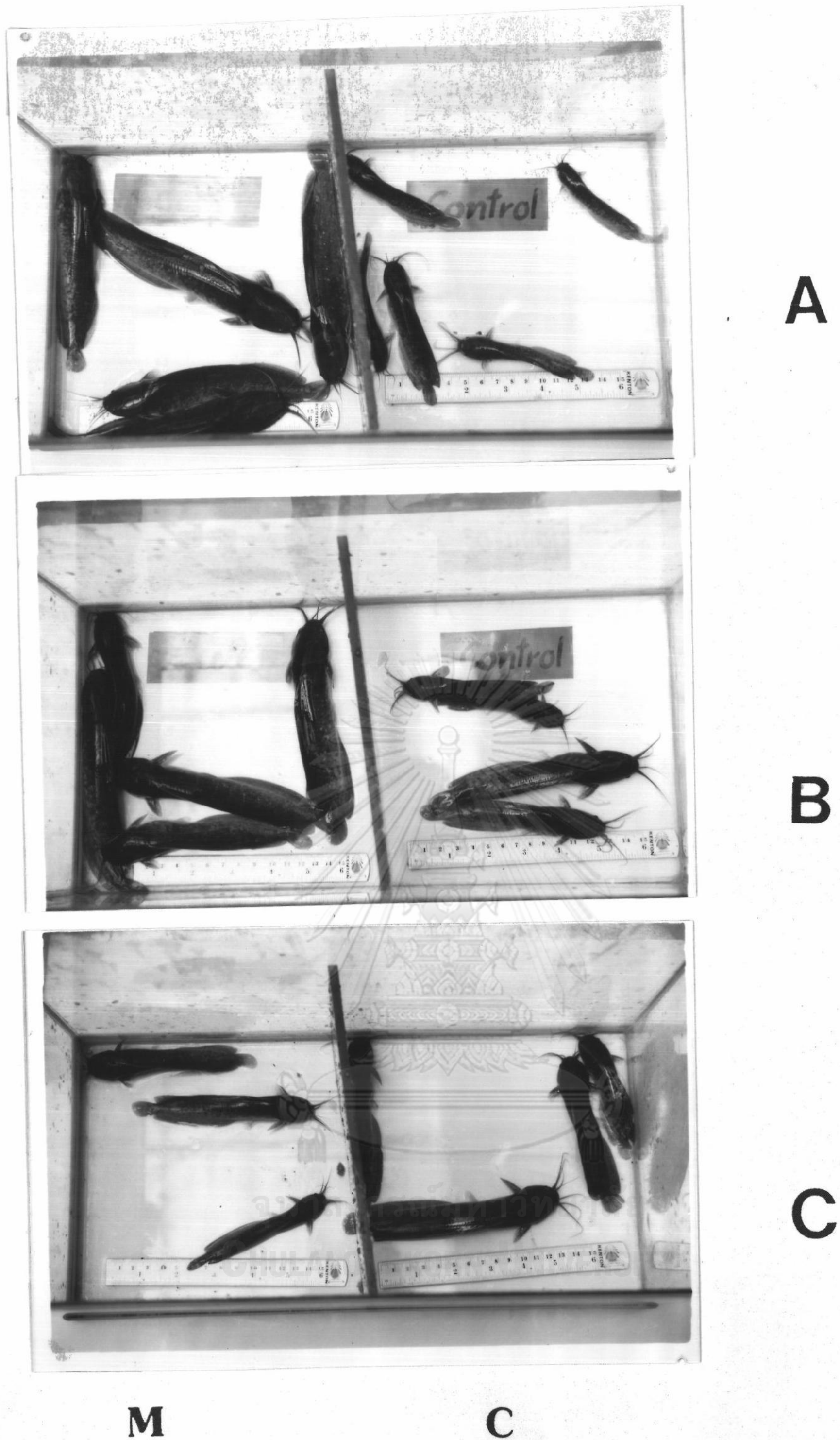

Figure 19 Picture of catfish at age 105 days

M = microinjected fish   C = control (uninjected fish)

A) Microinjection of hGH gene at one-cell stage

B) Microinjection of hGH gene at two-cells stage

C) Microinjection of hGH gene at four-cells stage

Preliminary amplification of 186 bp from plasmid pXGH 5 by PCR

The sequences and annealing positions of the suitable PCR primers designation was shown in Figure 6. Calculating of melting temperature (Tm) of the 5' and 3' primers was shown in Appendix 3, and equal to 58 ^{0}C. Predicted size of the PCR product amplified by the primers was 186 bp.

Preliminary PCR reaction was performed with varying amount of pXGH 5 plasmid template as 1, 5, 10, 100 pg and 1, 10 ng, annealing temperature as 50 and 55 ^{0}C by using the preliminary conditions. PCR product was shown in Figure 20. The PCR product began to visible at amount of template as 5 pg and intensity increment would relate to amount of template. At annealing temperature 50 ^{0}C, the PCR product was intensively visible as 186 bp and also indistinctly visible a bigger fragment band approximately size of 400 bp. At annealing temperature 55 ^{0}C, the PCR product was visible as a single 186 bp at 5, 10, 100 pg and 1 ng of template, but 10 ng of template a bigger fragment band approximately 400 bp was also indistinctly visible. In negative control reaction, primer-dimer was visible as lower band approximately size of 40 bp. At low amount of template, primer-dimer was also distinctly visible than at high amount of template. From this experiment, the amount of template 5 pg was chosen as positive control for further work.

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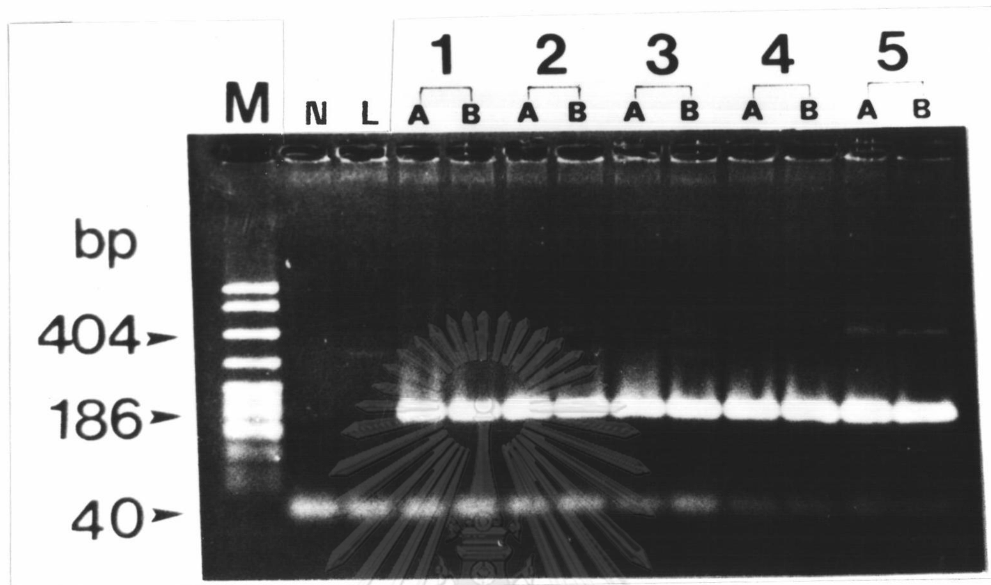

Figure 20 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 186 bp PCR amplification product from the designed primers

M = pBR322 Msp I size marker

A = At annealing temperature 55 ^{0}c

B = At annealing temperature 50 ^{0}c

N = Negative control, (no DNA template)

L = Plasmid template as 1 pg

1, 2, 3, 4, 5 = Plasmid template as 5, 10, 100 pg and 1, 10 ng, respectively.

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Blood cell preparation for PCR reaction

Blood cells were lysed with 0.001 % SDS and 10 mM NaOH using three procedures. Result of DNA pattern was shown in Figure 21. Both lysis with 0.001 % SDS and 10 mM NaOH gave the similar patterns of DNA. DNA patterns (Lane 1,2) from the first procedure were not consistently obtained. DNA patterns (Lane 3,4) from the second procedure were consistently obtained, but after heating blood cell lysis was packed, thereby, only DNA arround of packed cell were dissolved, while the inside DNA was not dissolved. The last procedure, DNA pattern was consistently obtained, and DNA was dissolved in sterile distilled water before packed by heating. From this experiment, blood cell lysis of the last procedure was choosen for blood preparation prior to PCR amplification.

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Blood cell lysis solution for reaction

The volume of blood varied from 1, 2.5, 5 and 10 μl were used as template and comparing between lysis with 0.001 % SDS and 10 mM NaOH, five picograms of pXGH 5 was added for positive control. Results are shown in Figure 22 a and b. In absolute blood, PCR product in some lanes were indistinctly visible as non-specific band approximately 180 bp in size and closely lower band. While in pXGH 5 adding blood, 186 bp of PCR product was intensively visible. Intensities of DNA smear patterns was due to volume of blood. For using the lab-produced taq polymerase activity, PCR product in positive NaOH blood lysis was seem to more intensities than in positive SDS

blood lysis. Thus, 10 mM NaOH was chosen for blood cell lysis for detection of introduced gene by PCR detection, and 2.5 µl of blood was used for further optimizing PCR condition.

Optimization of some conditions for detection the introduced gene in fish blood

Some factors had been tried to optimize the conditions for detection the introduced gene in fish blood, such as annealing temperature and annealing time, extension time and primer concentration.

Annealing temperature and primer concentration

2.5 µl of absolute fish blood and plasmid adding blood were performed for PCR reaction. At annealing temperature 50 ^{0}C, result was shown in Figure 23. Positive control with plasmid template clearly obtained the expected 186 bp band, but negative control was only obtained the primer-dimer band of PCR products. Using primers concentration at 1 µmole, in absolute fish blood, PCR products were obtained as many non specific bigger bands, and also visible band just like 186 bp and the closely lower band. In blood with plasmid adding obtained the intensities of expected 186 bp band and also many non-specific bigger-bands in PCR products. When using 0.1 µmole of primers concentration, in absolute blood, some of non specific bigger band of PCR product diminishing, and the indistinct band just like 186 bp and lower band were still visible. In fish blood added plasmid template, non specific bigger

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band of PCR product was diminished, the intensities of expected 186 bp was visible. At 1 μmole of primers concentration, primer-dimer was more intensively visible than at 0.1 μmole of primers.

At annealing temperature 55 ^{0}C, result was shown in Figure 24. PCR product of positive control and negative control were obtained as same as previous annealing temperature 50 ^{0}C results. Using primer concentration at 1 μmole, in absolute blood and in blood with plasmid adding, PCR product was similarly obtained as previous result with diminishing of non specific band. When using primer concentration at 0.1 μmole, in absolute blood, the non specific band were not visible, the primer-dimer were visible as lower band. In blood with plasmid adding, PCR product obtained an expected band as 186 bp, the primer dimer was also visible as lower band.

Annealing time, extension time with concentration of primer

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2.5 μl of absolute fish blood and plasmid adding blood were performed for PCR reaction with using annealing at 55 ^{0}C for 15 sec and extension time at 72 ^{0}C for 15 sec. Figure 25 showed the PCR product with using primer concentration 1 μmole. There was intensively visible of expected band 186 bp and also indistinctly nonspecific bigger band in plasmid adding blood. However, in absolute blood, the non specific band just like 186 bp and the lower band was indistinctly visible. The primer dimer of all reactions was visible. PCR product of positive control and negative control were obtained as same as previous result.

Figure 26 showed the PCR product with using primer concentration 0.1 μmole. In plasmid added blood, PCR products were intensively visible of expected band as 186 bp. while in absolute blood, did not obtained any non specific PCR product. The result of positive and negative control were obtained as same as previous result.

From this experiment, obtaining the optimal conditions as following; denaturation at 95 ^{0}C for 1 min, annealing temperature at 55 ^{0}C for 15 sec, extension temperature at 72 ^{0}C for 15 second, for 30 cycles, primer concentration as 0.1 μmole, were used for detection of introduced gene in fish blood.

Heparins effect

Effect of heparinized fish blood to the amplification of the DNA target by PCR was tested by adding plasmid template as positive control tested. Figure 27 showed that the expected 186 bp band was obtained as strong signal. From this experiment, heparins 1 I.U./ml blood was usable for blood sampling without inhibition of PCR amplification.

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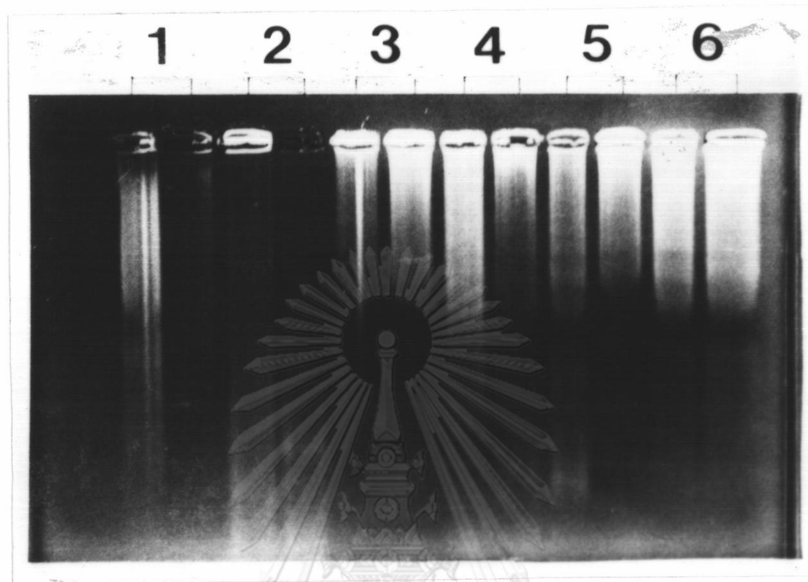

Figure 21 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of DNA patterns from blood cell preparations

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1, 2 = Blood cell lysis by NaOH and SDS, respectively from first procedure

3, 4 = Blood cell lysis by NaOH and SDS, respectively from second procedure

5, 6 = Blood cell lysis by NaOH and SDS, respectively from third procedure

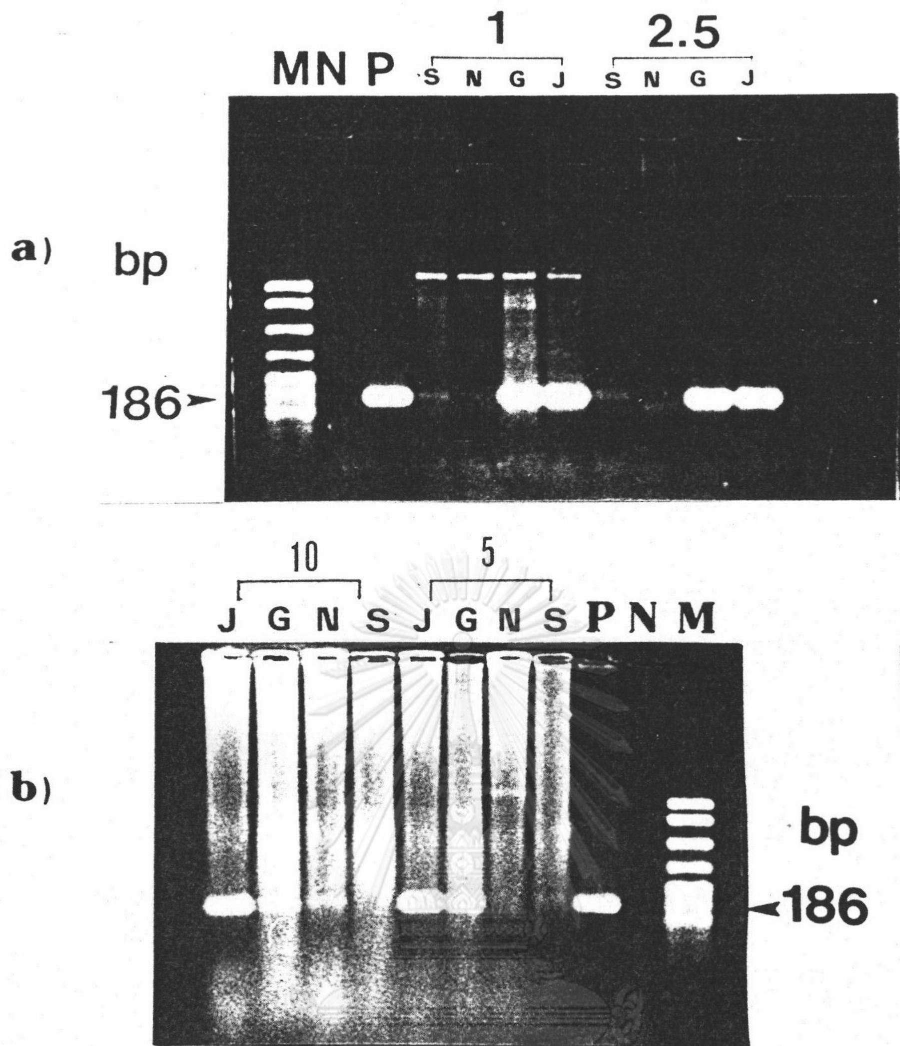

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Figure 22 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 186 bp PCR procuct amplified in various volume of blood and lysis solution

a) 1 and 2.5 μl of blood volume b) 5 and 10 μl of blood volume

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

S and G = 0.001 % SDS and its positive control

N and J = 10 mM NaOH and its positive control

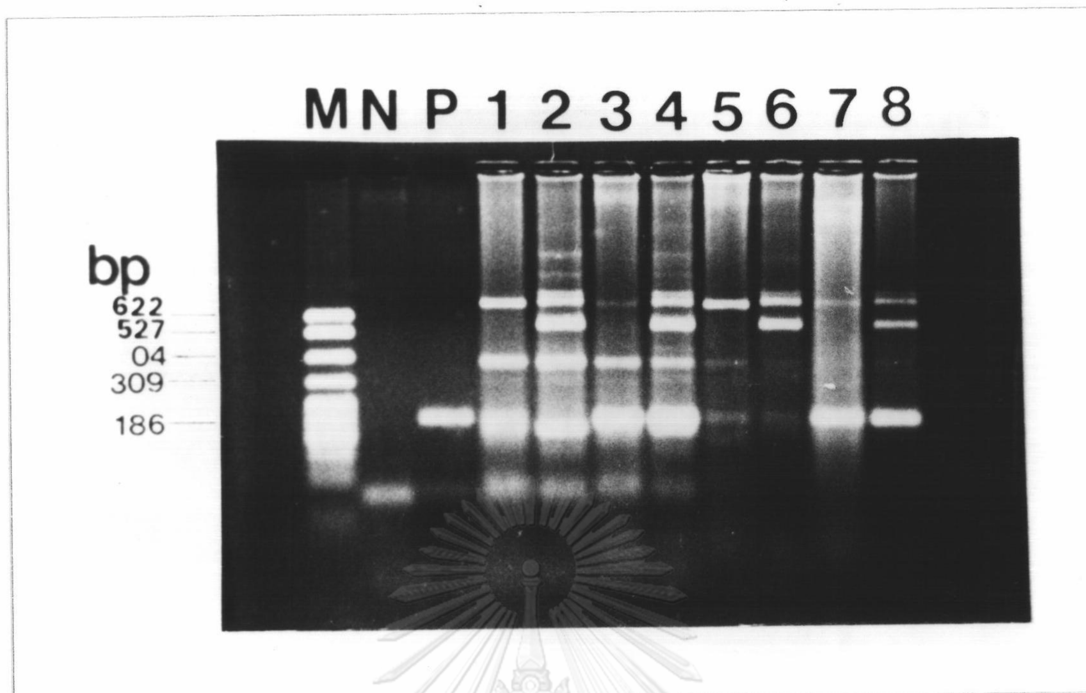

Figure 23 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 186 bp PCR product amplified in various primer concentrations ; at annealing temperature 50 ^{0}C

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

At 1 μmole primers concentration

1, 2 = fish blood 2.5 μl

3,4 = fish blood with adding plasmid 5 pg

At 0.1 μmole primers concentration

5,6 = fish blood 2.5 μl

7,8 = fish blood with adding plasmid 5 pg

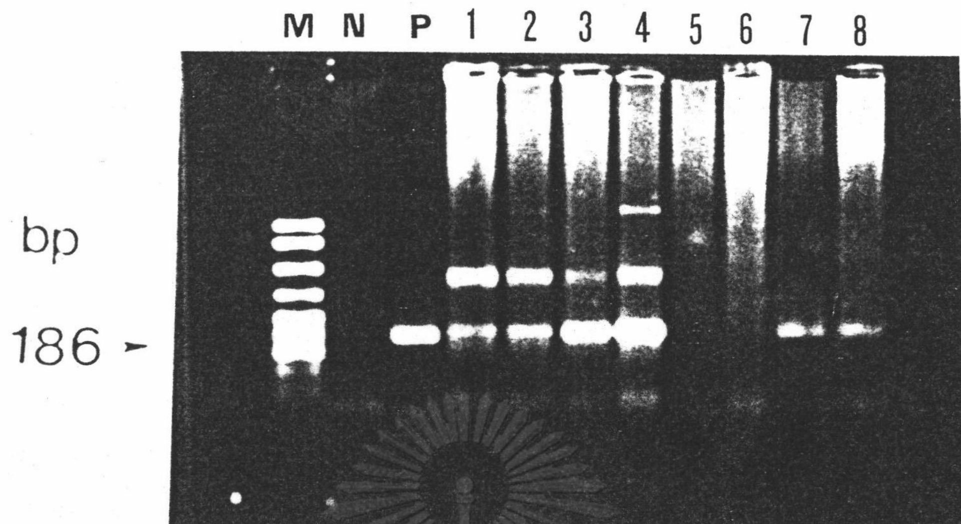

Figure 24 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 186 bp PCR procuct amplified in various primer concentrations ; at annealing temperature 55 ^{0}C

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

At 1 μmole primers concentration

1, 2 = fish blood 2.5 μl

3,4 = fish blood with adding plasmid 5 pg

At 0.1 μmole primers concentration

5,6 = fish blood 2.5 μl

7,8 = fish blood with adding plasmid 5 pg

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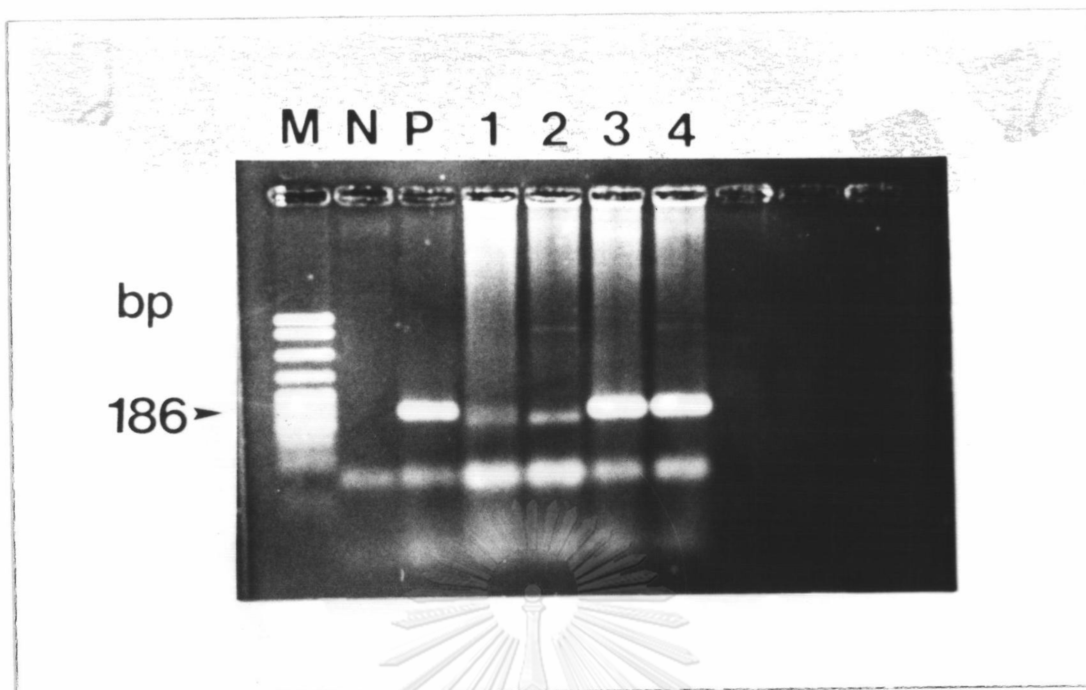

Figure 25 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 186 bp PCR procuct amplified in various primer concentrations, annealing temperature at 55 ^{0}C for 15 sec, extension temperature at 72 ^{0}C for 15 sec ; and 1 μmole primers concentration

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

1, 2 = fish blood 2.5 μl

3,4 = fish blood with adding plasmid 5 pg

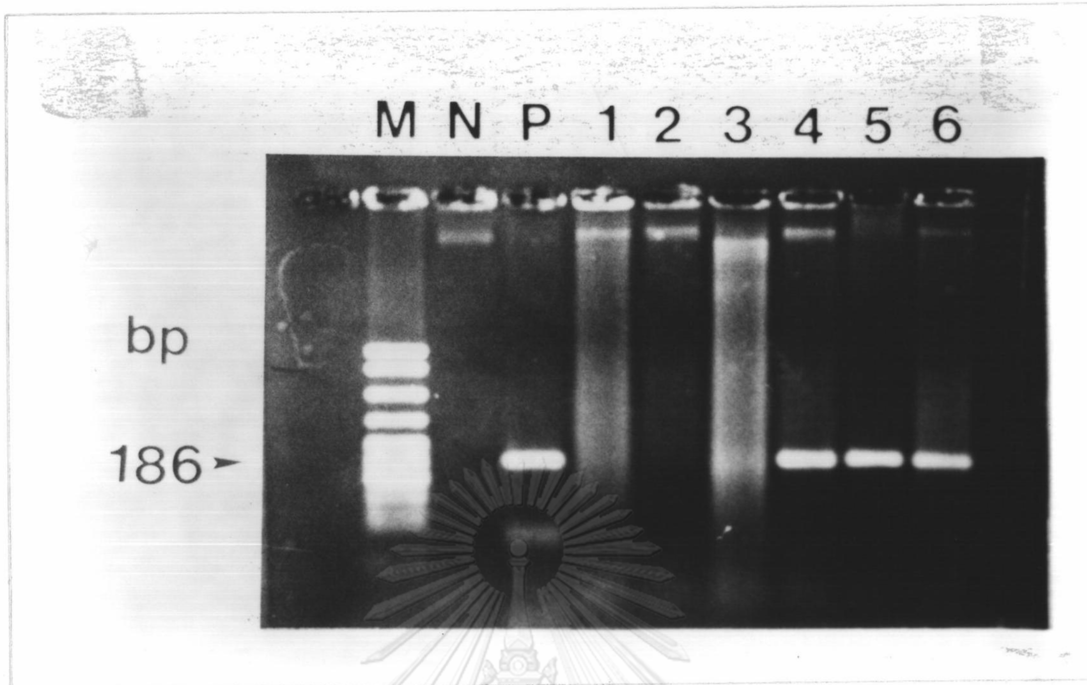

Figure 26 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 186 bp PCR product amplified in various primer concentrations annealing temperature at 55 ^{0}C for 15 sec, extension temperature at 72 ^{0}C for 15 sec ; and 0.1 μmole primers concentration

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

1, 2, 3 = fish blood 2.5 μl

4, 5, 6 = fish blood with adding plasmid 5 pg

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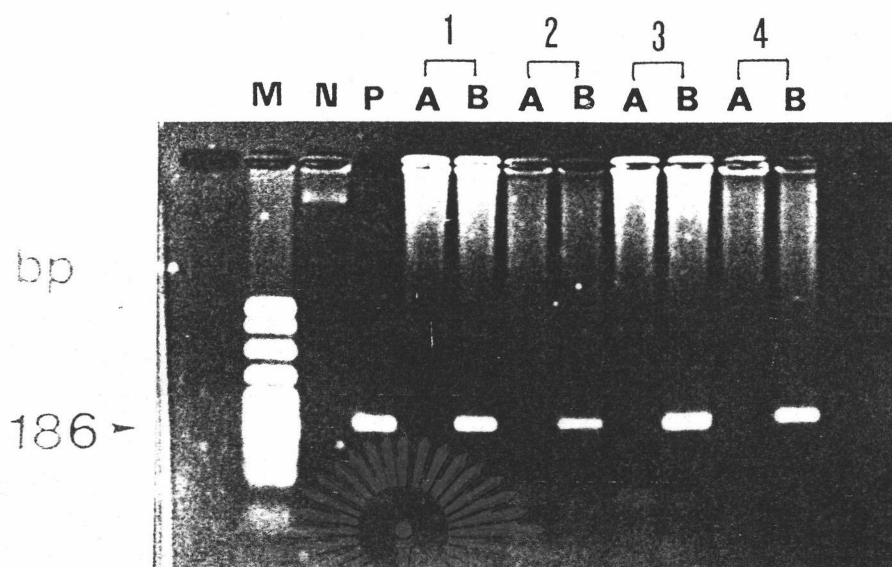

Figure 27 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of heparins effect on PCR detection

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

1, 2, 3, 4 A = fish blood 2.5 μl

1, 2, 3, 4 B = fish blood with adding plasmid 5 pg

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Amplification of 186 bp in human blood

The amount of heparinized human blood 5, 7 and 10 μl were amplified by using the obtaining optimization condition. The expected band of 186 bp was obtained with more intensity in higher amount of blood sample (Figure 28).

The amount of heparinized blood as 1.5, 3.5 and 5 μl were also amplified. Southern blotting and hybridization with a dioxigenin labelled pXGH 5 plasmid probe was confirmed for specificity of the expected 186 bp band. The result was shown in Figure 29. The expected 186 bp band of PCR product in human blood could hybridized with plasmid probe, and in less intensively visible could improve to raise visible, and invisible expected band were capable to visible.

Amplification of 150 bp in fish blood

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The sequence of *P. sutchi* growth hormone gene, cDNA, and annealing positions of the suitable PCR primers was designed in Figure 6. Calculation of melting temperature (Tm) of the sense and antisense primers shown in Appendix 3, and equal to 54 and 56, respectively. Predicted size of the PCR product amplified by this set of primers is 150 bp.

2.5 μl of *P. sutchi* and *C.* macrocephalus blood were lysed with 10 mM NaOH and amplified by using the obtaining optimization of conditions but annealing temperature were settled as 50 ^{0}C. Figure 30 showed the expected

150 bp band of PCR product was obtained from both fish blood. With this result, 2.5 μl of fish blood would be used to amplify DNA fragment from genome. This volume was used for detecting the introduced gene in genome.

Detection of introduced gene in microinjected fish blood by PCR

Blood sampling in the presence of heparins 1 I.U/ml blood was taken from microinjected fish and control. 2.5 μl of blood samples of microinjected fish at one-cell stage (99 samples), two-cell stage (31 samples), four-cell stage (12 samples) and controls were detected the introduced gene by using PCR method under the optimization conditions. PCR products were shown in Figures 31, 32, and 33, respectively. Positive control, 5 pg of plasmid template given intensively visible of the fragment 186 bp. All of PCR products in blood samples of microinjected fish and those controls were not visible the expected 186 bp band.

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10 μl of PCR products of microinjected fish blood were dot bloted, and hybridized with a dioxigenin labelled pXGH 5 plasmid probe for detection of the unvisible 186 bp fragment. Figure 34 shown eight of PCR products of fish blood gave low intensity of hybridization signals. These were PCR product from blood of microinjected fish number 116, 217, 326, 327, 314, 618, 721 and 821. While PCR products of control fish blood did not give any signal.

Southern blot and hybridization with a dioxiginin labelled pXGH 5 plasmid probe had been confirmed for size of the positive signals from dot

blotting. Figure 35 shown the size of positive signals were 186 bp fragment as same size as the expected band.

The result showed the obtaining successful transferring and genomic integration of pXGH 5 plasmid in fish blood by microinjection method. Table 8 shown a label of the stage of microinjection, group experiment of fish, which were found the introduced gene in blood by PCR method.

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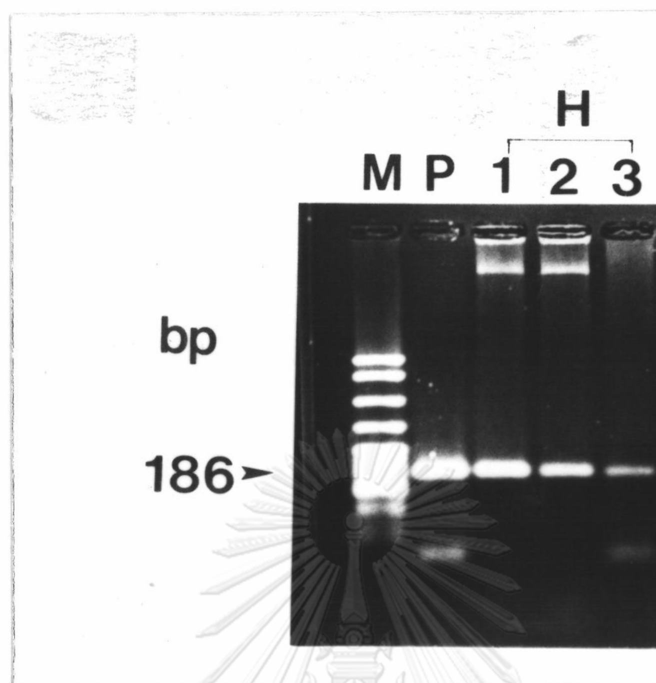

Figure 28 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of amplification in heparinized human blood by using hGH gene primers

M = pBR 322 Msp I size marker

N = Negative control, no DNA template

P = Positive control, plasmid template 5 pg

1 = 10 μl of heparinized human blood

2 = 7 μl of heparinized human blood

3 = 5 μl of heparinized human blood

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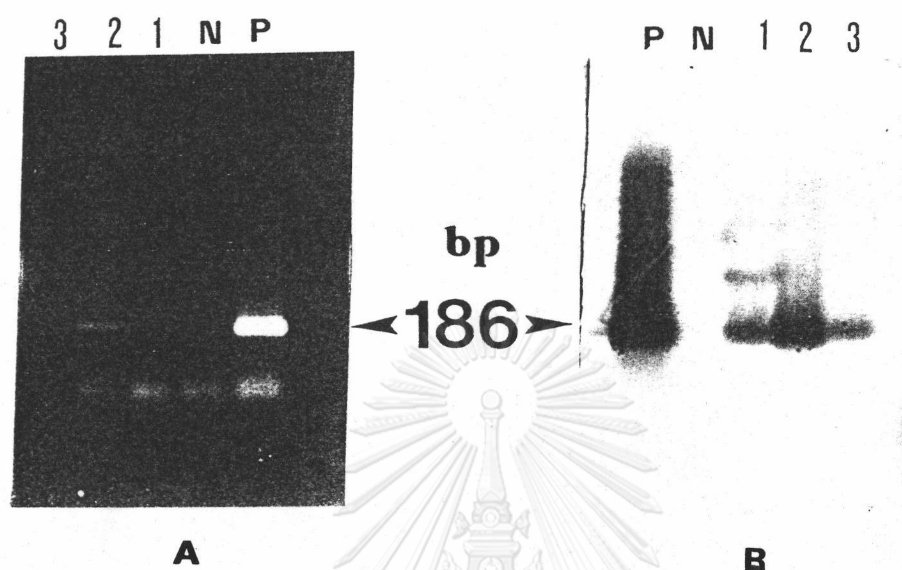

Figure 29 The fragment of 186 bp from human blood amplification

P = Positive control, plasmid template 5 pg

N = Negative control, no DNA template

1 = 3.5 μl of heparinized human blood

2 = 5 μl of heparinized human blood

3 = 1.5 μl of heparinized human blood

A = Ethidium bromide staining of 1.5 % agarose gel electrophoresis

B = Southern blot hybridization from gel A with digoxigenin labelled pXGH 5

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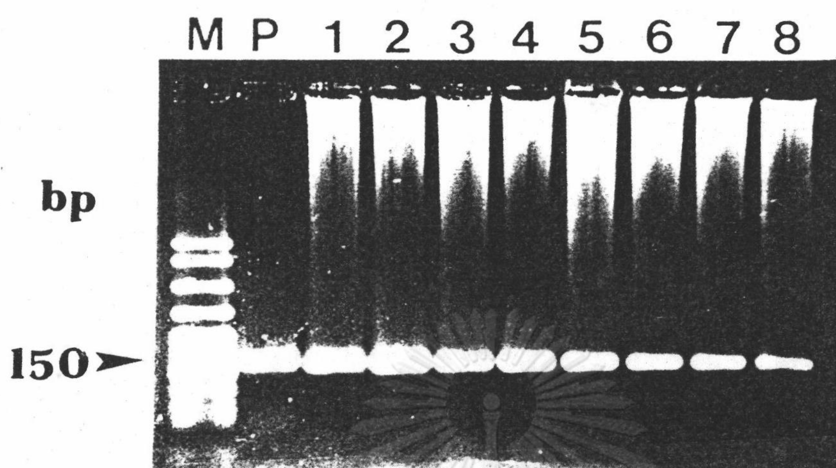

Figure 30 Ethidium bromide staining of 2.5 % agarose gel electrophoresis of 150 bp PCR product amplified from heparinized fish blood by using *P. sutchi* growth hormone gene primers

M = pBR 322 Msp I size marker

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P = DNA fragment as 158 bp size marker

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1, 2, 3, 4 = 2.5 μl of heparinized *P. sutchi* blood

5, 6, 7, 8 = 2.5 μl of heparinized *C. macrocephalus* blood

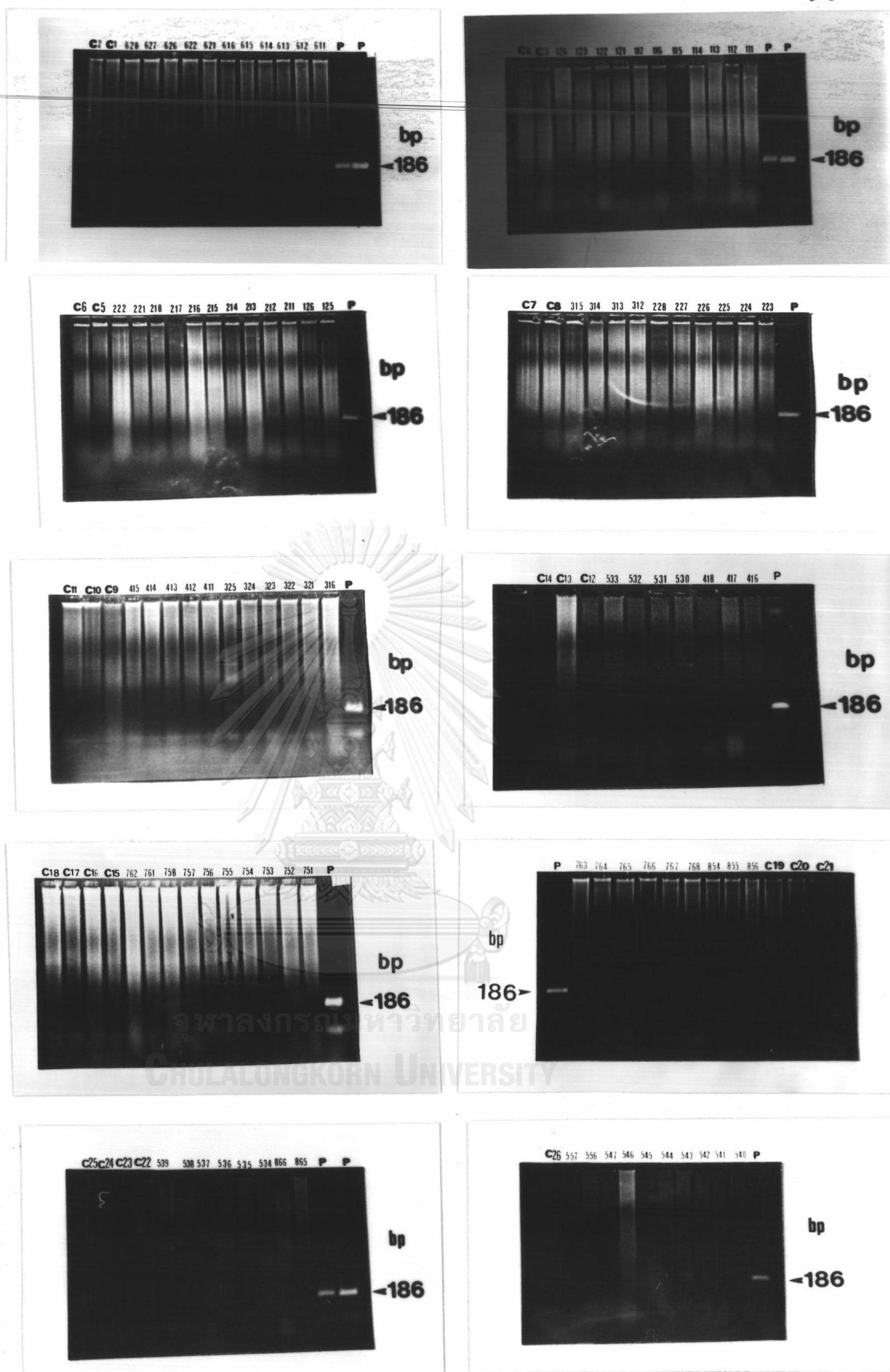

Figure 31 Ethidium bromide staining of 2.5 % agarose gel electrophoresis for detection of the introduced gene in one-cell stage microinjected fish blood by PCR

P = Positive control, plasmid template 5 pg

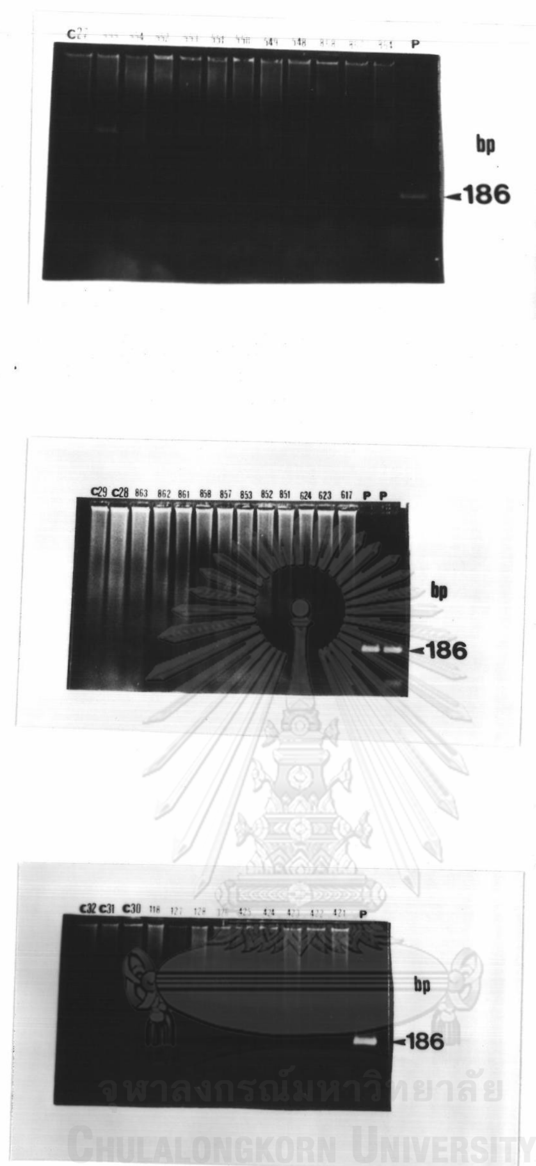

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Figure 32 Ethidium bromide staining of 2.5 % agarose gel electrophoresis for detection of the introduced gene in two-cell stage microinjected fish blood by PCR

P = Positive control, plasmid template 5 pg

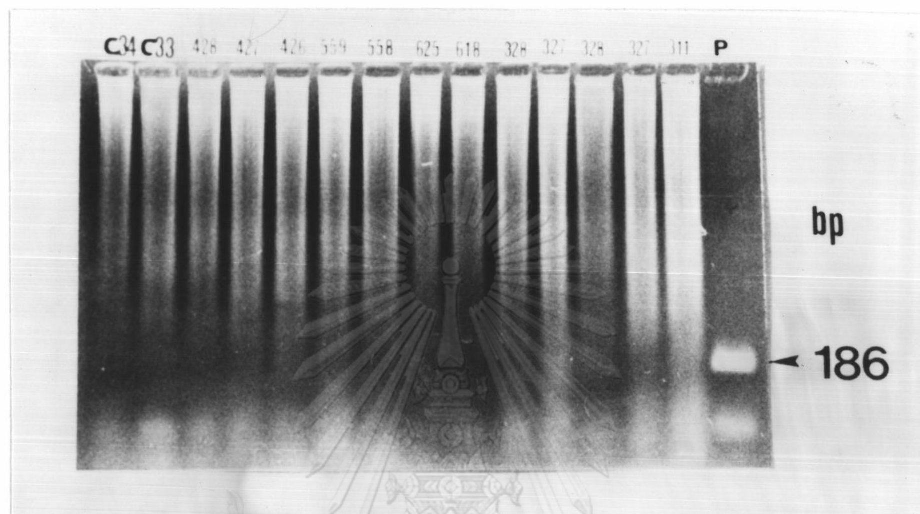

Figure 33 Ethidium bromide staining of 2.5 % agarose gel electrophoresis for detection of the introduced gene in four-cell stage microinjected fish blood by PCR

P = Positive control, plasmid template 5 pg

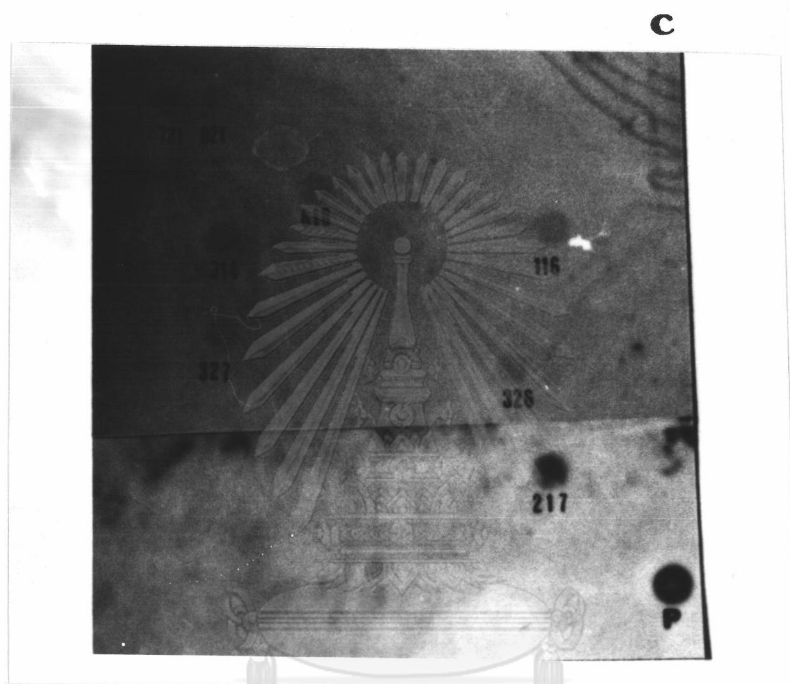

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Figure 34 Dot blot and hybridization of PCR product from microinjected fish blood with digoxigenin labelled pXGH 5 plasmid

P = PCR product from plasmid pXGH 5

C = PCR product from control fish

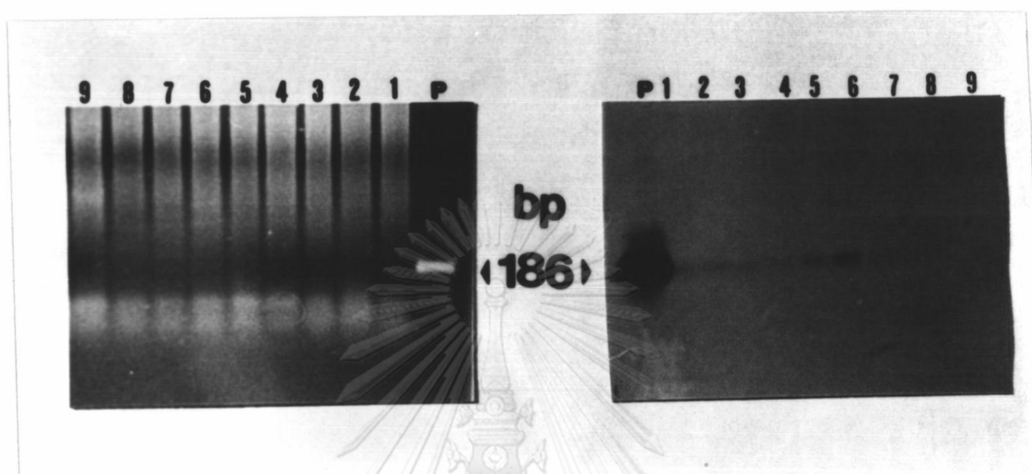

Figure 35 PCR products from detection of fish blood

P = PCR product from plasmid pXGH 5

1 = PCR product from fish no. 721

2 = PCR product from fish no. 821

3 = PCR product from fish no. 116

4 = PCR product from fish no. 326

5 = PCR product from fish no. 314

6 = PCR product from fish no. 217

7, 8, 9 = PCR product from control fish

A = Ethidium bromide staining of 1.5 % agarose gel electrophoresis

B = Southern blot hybridization from gel A with digoxigenin labelled pXGH 5 plasmid

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Table 8 Stage of microinjected egg obtaining the successful of gene transfer

Fish no.	stage of microinjection
217	one-cell
618	one-cell
314	one-cell
721	one-cell
116	one-cell
326	two-cell
821	two-cell
327	four-cell

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Detection of the introduced gene in genomic DNA by hybridization

Genomic DNA extraction from fish blood

Fish blood, which was positive detection by PCR, were extracted the high molecular genomic DNA. Figure 36 shown the genomic DNA pattern was higher molecular weight with approximate size as 23 kb.

Dot blot of genomic DNA and hybridization

For detecting the introduced gene in genomic DNA, 5 μg of extracted genomic DNA was blotted and hybridized with a digoxigenin labelled pXGH 5 plasmid probe. The result are shown in Figure 37, eight dot of genomic DNA of fish blood gave positive hybridization signals above background. Four positive signals found in fish number 326, 327, 821 and 217 with higher intensity visible than that of fish number 618, 721, 314 and 116. The control fish were not obtained any signals.

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Detection of the introduced gene in tissues

Genomic DNA extraction from fish tissues

Two alive fish, which positive and negative detection of the introduced gene in blood, were sacrificed and some tissues taken such as liver, brain, muscle, stomach, kidney, fin and gonad. Genomic DNA was extracted

via the tissue. Pattern of extracted DNA were analysed by 0.7 % agarose gel electrophoresis. The results were shown in Figure 38. Some of the suffered fish were also tissues taken and genomic DNA extracted. Pattern of extraced DNA was shown in Figure 39. Extracted genomic DNA was seem to obtained greater smear pattern than that of the alive fish.

Dot blot of genomic DNA from tissues

20 μg of extracted genomic DNA form tissues were blotted and hybridized with a digoxigenin labelled pXGH 5 plasmid probe, for detection of the introduced gene in various tissues of microinjected fish. Results of detection of introduced gene in various tissues of some microinjected fish was shown in Table 11 and Figure 40. Genomic DNA from control fish did not give any signal. The one-cell stage microinjected fish did not give hybridization signals above background in all tissues, but more tissues showed hybridization signals than the two-cell stage microinjected fish. The microinjection at four-cell stage did not show any hybridization signal. The result indicated the mosaicism in the transgenesis fish by microinjection at all stage of early development.

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Figure 36 Ethidium bromide staining of 0.7 % agarose gel electrophoresis of high molecular weight DNA patterns from fish blood

R = λ Hind III DNA size marker

1 = fish no. 116	6 = fish no. 821
2 = fish no. 721	7 = fish no. 327
3 = fish no. 314	8 = fish no. 326
4 = fish no. 618	9 = control fish
5 = fish no. 217	10 = control fish

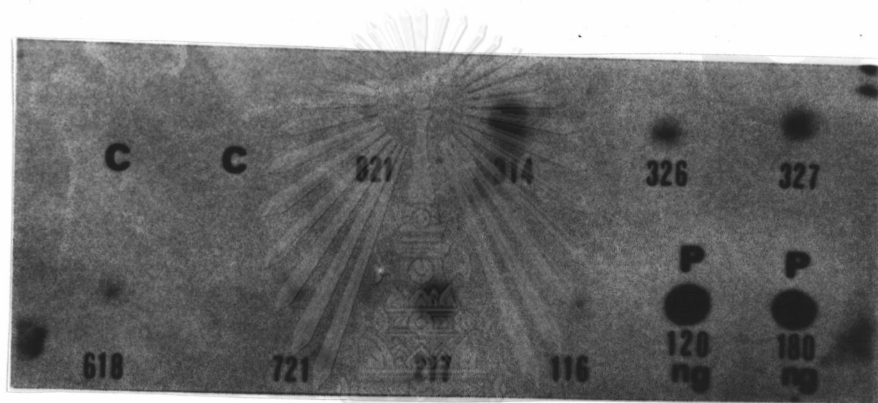

Figure 37 Dot blot and hybridization of genomic DNA from fish blood with digoxigenin labelled pXGH 5 plasmid

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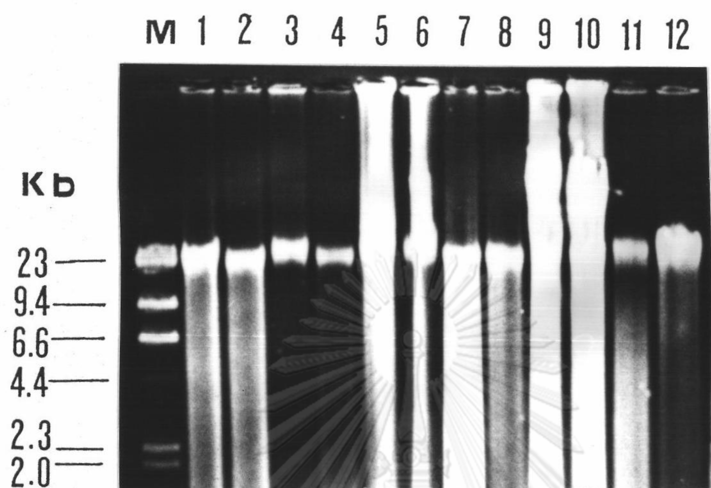

Figure 38 Ethidium bromide staining of 0.7 % agarose gel electrophoresis of genomic DNA patterns from various tissues of alive fish

M = λ Hind III DNA size marker

Fish no. 217	Fish no. 311
1 = Fin	2 = Fin
3 = Brain	4 = Brain
5 = Gonad	6 = Gonad
7 = Stomach	8 = Stomach
9 = Liver	10 = Liver
11 = Kidney	12 = Kidney

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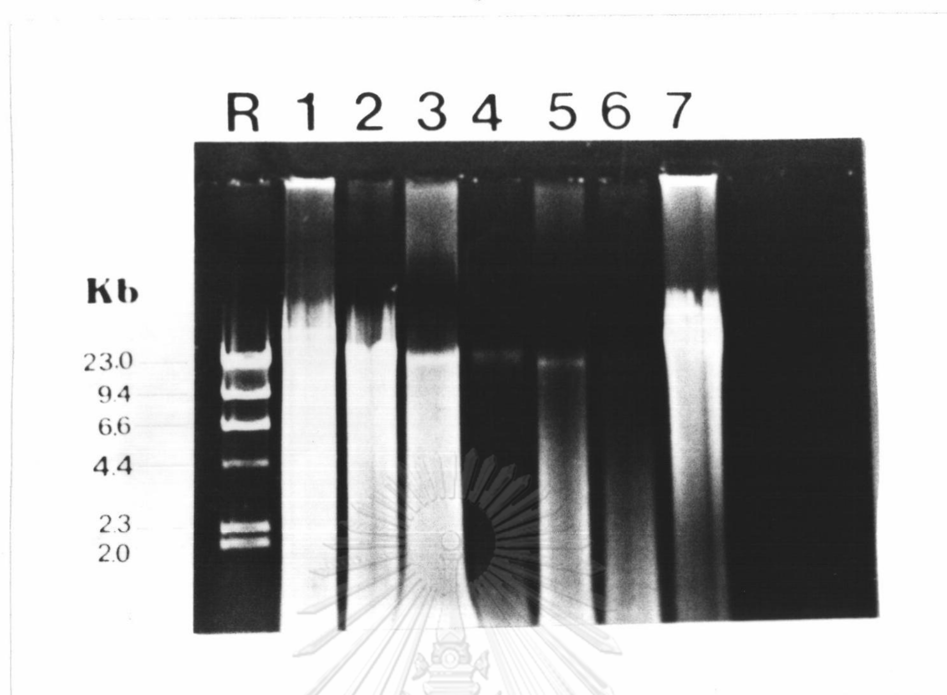

Figure 39 Ethidium bromide staining of 0.7 % agarose gel electrophoresis of genomic DNA patterns from various tissues of died fish

R = λ Hind III DNA size marker

Fish no. 339

1 = Fin

2 = Brain

3 = Gonad

4 = Stomach

5 = Liver

6 = Kidney

7 = Muscle

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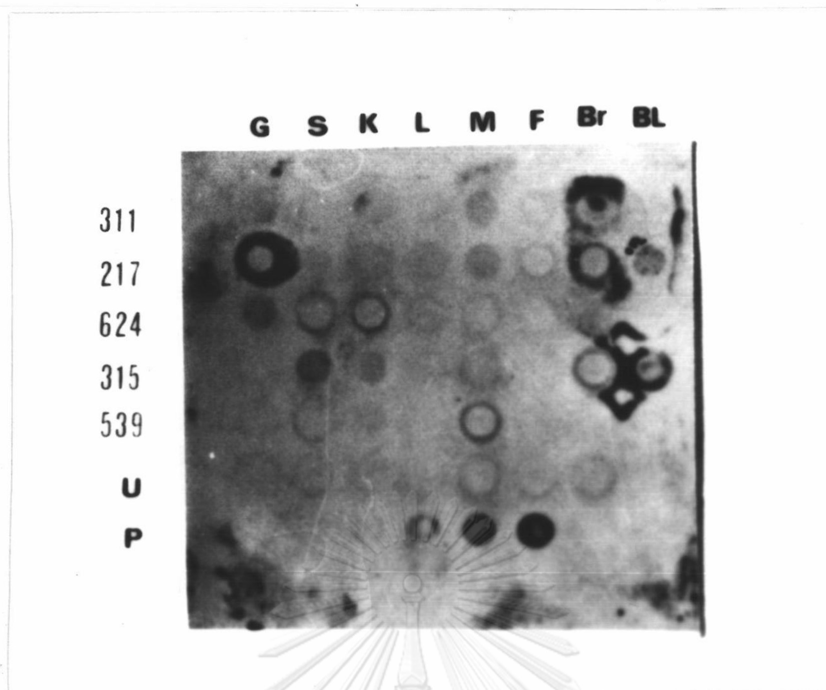

Figure 40 Dot blot and hybridization with digoxigenin labelled pXGH 5 plasmid of genomic DNA from various tissues

U = Control fish (uninjected fish)

P = Positive control (plasmid pXGH 5)

G = Gonad

S = Stomach

K = Kidney

L = Liver

M = Muscle

F = Fin

Br = Brain

Bl = Blood

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Table 9 Detection of the introduced gene in various tissues of fish, which was microinjected at various stages of development

Fish no.	Stage at microinjection	Gonad	Stomach	Kidney	Liver	Muscle	Fin	Brain	Blood
U	Control	-	-	-	-	-	-	-	-
311	1*	-	-	-	-	+	-	-	-
217	1*	-	+	+	+	+	-	-	+
315	1	-	+	+	-	-	-	-	-
624	2	+	-	-	-	-	-	-	-
539	4	-	-	-	-	-	-	+	-

* alive fish

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