報告題名：九孔生長基因之 cDNA 構築於轉殖用載體之相關生物技術

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出國地區：美國

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報告名稱:

九孔生長基因之cDAN構築於轉殖用載體之相關生物技術

主辦機關:

行政院農業委員會水產試驗所

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分類號/目: F9／漁業（養殖業）　F9／漁業（養殖業）

關鍵詞:　核醣核酸,互補去氧核醣核酸,載體,聚合酶連瑣反應

內容摘要: 此趟研習主要針對生長基因之cDNA構築於轉殖用載體之相關生物技術，研習內容整理出十一個項目：一、總核醣核酸之粹取 (Total RNA isolation) 二、互補DNA第一股的合成 (First-Strand cDNA Synthesis) 三、聚合酶連瑣反應 (Polymerase Chain Reaction; PCR) 四、PCR產物的回收 (Recover the PCR products) 五、限制酶的設計 (Restriction enzyme digestion) 六、載體的構築 (Plasmid vector restruction) 七、稱任細胞的製備 (Preparation of competent cells and CaCl2 transformation) 八、大腸桿菌的轉形 (Transfomation of E. coli) 九、PCR基因篩選 (PCR Screen) 十、基因定序 (Sequencing)十一、鱒魚基因轉殖之子代攜帶轉殖基因之鑑定。

本文電子檔已上傳至出國報告資訊網

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出國計畫/報告名稱：九孔生長基因之 cDNA 構築於轉殖用載體之相關生物技術

計畫主辦機關：行政院農業委員會水產試驗所

出國人員：楊鴻禧 副研究員 行政院農業委員會水產試驗所

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一、前言

本研習計畫在農委會所屬機關九十二年度派員出國計畫項下業奉行政院核定通過於九十二年八月十五日至九月十三日前往美國康迺迪克大學(University of Connecticut)分子及細胞生物研究所(Molecular and Cell Biology)陳鐵雄教授實驗室研習有關基因之選殖與構築轉殖用載體的技術。陳教授旅美已經有三十幾年，其專長在於分子生物學之研究，近幾年對於魚類生長及抗病基因的研究以及轉殖生長技術的研發有良好的結果，並常在國際刊物發表重要研究成果，其主持總編之國際期刊 Marine Biotechnology 是針對有關海洋生物之生物技術研究之研究成果，筆者親眼目睹他在審稿時之嚴僅與執著，因此，期刊中大部份發表的文獻對海洋生物技術的研究有關。陳教授對於臺灣水產生物技術的發展相當關心，他常常應國內學術單位邀請回臺參與學術研討，並也常在國內舉辦有關生物技術操作之研習營，國內有關海洋生物學術研究單位之研究人員大多數皆有參加過這種研習訓練，其對國內研究能力與水準的提升有很大的貢獻。

筆者因緣際會在十年前因參加過他的技術訓練班，也有機會常在國內之研討會上遇到他，故深知他的研究領域。此次有機會出國研習，同時也得到陳鐵雄教授之同意至其實驗室去研習，深感悻悻。

筆者於 92 年 8 月 14 日出發，抵達康迺迪克州哈特福（HARTFORD）國際機長已 8 月 16 日早上 2 點，此趟旅程倍感艱辛。旅程不順暢主因碰上美國東北大停電的影響，美國東北部幾個國際機場關閉，打亂了原有飛機航班時程，在層層的轉機下以及航班的故障與延誤，最後終抵達目的地，卻已是當地半夜三點。陳鐵雄教授事先已代預訂住宿的旅館，旅館位於大學城校園內，由此徒步至陳鐵雄教授實驗室僅約需 15 分鐘，每天往返堪稱方便。早上 10 點與陳鐵雄教授與夫人會面，之後前往實驗室介紹研究項目及設備。18 日（星期一）陳教授至旅館接送至實驗室並介紹研究人員。目前陳教授共有四位博士後研究人員，個個的研究經驗都很資深，其中林純民博士負責實驗室總管理其專長在於海藻（Seed weed）的研究；邱品文博士研究 Rainbow Trout 的基因轉殖研究；楊弼英博士研究有關 Rainbow Trout 新的生長賀爾蒙基因蛋白家族（Somatolactin-Like Protein）；Jenny Khoo 博士研究 Rainbow Trout 之基因轉殖魚之子代篩選。兩位技術研究員；一位是陳教授夫人(Maria Chin)，研究細胞癌症發生之機制；Jon

Larsen 博士研究魚類抗病基因。研究生有六位；研究之主題有魚的心臟血管形成之機制；環境 Xenobiotics 對鱒魚腦下垂體分泌生長賀爾蒙的影響；魚蝦貝轉殖抗病基因；鱒魚生長賀爾蒙基因家族 E-peptides 的研究，魚的癌細胞研究等項目。

此次能平安順利完成研習之旅在此誠摯感謝陳鐵雄教授的幫助。更感謝他兩夫婦熱心的接待；在此感謝廖育賢夫婦於半夜兩點前往接機，否則將夜宿機場矣。實驗室研究人員：林純民博士、楊弼英博士、邱品文博士、Jenny Khoo 博士、廖育賢君、羅宏傑君、Mr. Chang Zoon Chun、Mrs. Anitha Elango 、Jon Larsen 博士等之協助研習和提供資料及熱誠的對待與生活上的幫助，誠心的感謝他們。

二、研習項目與內容

茲將研習內容整理出十一個項目：

一、總核醣核酸之粹取 (Total RNA isolation)
二、互補 DNA 第一股的合成 (First-Strand cDNA Synthesis)
三、聚合鋂連瑣反應 (Polymerase Chain Reaction; PCR)
四、PCR 產物的回收 (Recover the PCR products)
五、限制鋂的設計 (Restriction enzyme digestion)
六、載體的構築 (Plasmid vector restruction)
七、稱任細胞的製備 (Preparation of competent cells and $CaCl_2$ transformation)
八、大腸桿菌的轉形 (Transfomation of E. coli)
九、PCR 基因篩選 (PCR Screen)
十、基因定序 (Sequencing)
十一、鱒魚基因轉殖之子代攜帶轉殖基因之鑑定

一、總核醣核酸之粹取（Total RNA isolation）

生物訊息的表現存在於轉訊 RNA (mRNA)上，轉訊 RNA 佔總 RNA (Total RNA) 約只 5%，因此欲得到轉訊 RNA 必先粹取總 RNA。由於組織結構的差異，欲得良好品質的核醣核酸，所採用粹取的方法也有不同。下述是適用不同組織細胞所應用之粹取方法：

Practice rainbow trout stock 的 total RNA spleen （0.9μl/λ）、kidney （2.54μl/λ）

A、RNA isolation with TRIZol Reagent

Reagents required:
Chloroform
Isopropyl alcohol
75% Ethanol (in DEPC – treated water)
Rnase – free water or 0.5% SDS solution

Method of RNA Isolation:

Homogenization

tissue: Homogenize tissue samples (with liquid nitrogen homogenize) in 1 ml of TRIzol. Reagent per 50 – 100 mg of tissue using a glass – Teflon or power homogenizer. The sample volume should not exceed 10% of the volume of TRIzol Reagent used for homogenization.

1. Adding 500ul Trizol
2. Dissolving Tissur Cell completely
3. Adding other 500ul Trizol, Stay in 5 minetes.
4. Adding 200 ul chloroform
5. Shaking tube 30 second
6. Standing for 2-3 minutes in RT
7. Centrifuging 15 minutes for 12000 rpm
8. Transferring RNA (Toppest) to a new tube.
9. Adding 500 ul isopropyl alcohol.
10. Inverting the tube up and down several times.
11. Standing for 10 minutes in RT.
12. Centrifuging 10 minutes for 12000 rpm.
13. Removing supernatant.
14. Washing pellet with 1 ml 75% Etoh.
15. Vortex.
16. Centrifuging for 8730 rpm for 5 minutes.
17. Pouring off liquid part (tube 傾斜, 吸掉剩餘透明液)

18. Centrifuging for 1200 rpm for 1 min.(再用 pipet 吸上層液丟棄)
19. Adding 50 ul DEPC 水, 用手彈或讓它自動溶解

B、RNA isolation with Guanidium Thiocynate Method for T-75 flak (prepare all the reagents and eauipments Rnase free)。

1. Lysis solution:

4M Guanidium Thiocynate	23.63g Guanidium Thiocynate
25mM Sodium Citrate	12.5ml 0.1M Sodium Citrate
0.5% Sarcosyl	2.5ml 10% Sarcosyl
1% β-mercaptoehanol	0.5ml

Add DEPC-H_2O to 49.5ml

2. DEPC-H_2O saturated phenol
3. 2M Sodium Acetate pH4.0
4. DEPC-H_2O

1. Pour out the medium
2. Wash the cells with 4ml of PBS
3. Add 3ml of lysis solution to the flask, mix gently to lyse the cells. Transfer the solution to the 15ml Blue Cape Tube.
4. Freeze tube at -80℃
5. Thaw tube on the ice
6. Use polytroneto gride up the tissue until it looks milk ～1-1.5min
   (10%H_2O_2 to clean polytroneto)
7. Add 300μl (0.1V) of 2M sodium acetate (pH4.0), mix well by inversion
8. Add 3000μl water saturated phenol. Mix well by vortex
9. Add 600μl (0.2V) of chloroform and vortex (we can repeat this step)
10. Chill on ice for 15min
11. Centrifuge 10,000rpm for 10min
12. Extract at least one time with $CHCl_3$ (1V:1V)
13. Centrifuge 10,000rpm for 10min
14. Transfer the aquaeous suspernatant to a new tube
15. Add 3000μl (1V) isopropanol, vortex and chill for o/n (over night) at -80℃
16. Centrifuge 10,000rpm for 20 min
17. Solubilize glycogen with 2ml LiCl (4M), vortex, use pipette to mix and break the pellet, wait 5min, vortex again

18. Spin for 12000rpm, 10min
19. Responded RNA（pellet） in 1ml H_2O, vortex again
20. Add 1ml （1V:1V） $CHCl_3$, vortex 20second
21. Spin for 12000rpm, 10min
22. Collect the upper solution to a new tube
23. Precipitate with 100μl 3M NaoAc（pH5.6） and 2ml isopropanol mix
24. Put in -20℃ over night
25. Spin for 10min
26. Wash pellet with 70% ethanol and spin for 5min
27. Dry pellet in the speedvac concentrator （10-15min）
28. Solubilize RNA in DEPC-H_2O （50μl）, determine the concentration by spectrophotometer

C、轉訊 RNA 的粹取 (mRNA Isolation)（Clonetech FsatTrack 2.0）

Item	Composition	Stock	100ml
Stock Buffer	200mM NaCl 200mM Tris, pH7.5 1.5mM $MgCl_2$ 2% SDS	5M 1M 25mM 10%	4ml 20ml 6ml 20ml 50mlH_2O
50x Protein/Rnase Degrader	10mg/ml protenase K	10mg/ml	
Binding Buffer	500mM NaCl 10mM Tris-Cl, pH7.5 in DEPC-treated water	5M 1M	10ml 1ml 89mlH_2O
Low Salt Wash Buffer	250mM NaCl 10mM Tris-Cl, pH7.5 in DEPC-treated water	5M 1M	5ml 1ml 94ml H_2O
Elution Buffer	10mM Tris-Cl, pH7.5 in DEPC-treated water	1M	1ml 99ml H_2O
2M Sodium Acetate	2M Sodium Acetate, pH5.2 in DEPC-treated water	2M	
5M NaCl	5M NaCl in DEPC-treated water	5M	
75mg Lyophilized oligo（dT） cellulose			

1. Prepare Lysis Buffer: 300μl of proteinase K（10mg/ml）to 15ml of Stock Buffer for

each intened isolation

2. Place ～1g tissue in a pre-weighed, sterile, 50ml centrifuge tube and weight. Add 15ml Lysis Buffer and quickly homogenize the tissue.

Isolation of mRNA

1. Incubate the 15 ml lysate at 45℃, 15-60minutes
2. Centrifuge at 4,000xg, 5minutes, room temperature.
3. Transfer the supernatant to a new tube.
4. Add 950μl 5 M NaCl stock solution and mix.
5. Shear DNA 3 to 4 time using an 18-21 gauge needle.
6. Add oligo（dT）cellulose to sample. Incubate for 2 minutes, room temperature.
7. Rock the tube gently at room temperature, 15 to 60minutes.
8. Centrifuge oligo（dT）at 3000xg, 5minutes, room temperature.
9. Remove the supernatant carefully from the oligo（dT）bed.

Washing Oligo（dT）cellulose

1. Resuspend oligo（dT）in 20ml Binding Buffer. Centrifuge at 3000xg, 5minutes, room temperature.
2. Resuspend oligo（dT） in 10ml Binding Buffer. Centrifuge at 3000xg, 5mintes, room temperature.
3. Resuspend oligo（dT） in 10ml Low Salt Wash Buffer. Centrifuge at 3000xg, 5mintes, room temperature.
4. Repeat Step3 until the buffer is no longer cloudy.
5. Resuspend the oligo（dT）in 800μl Low Salt Wash Buffer. Decant the liquid inside the microcentrifuge tube.
6. Centrifuge at 5,000xg, 10seconds, room temperature.

D、RNA isolation:（適用於 cell lysis 用）

1. 將 liver 研磨之後 Pour out the medium
2. Wash the cells with 1.5ml of PBS
3. Add 200μl of lysis solution to the dish, mix gently to lyse the cells. Transfer the solution to an eppendorff tube.
4. Add another 200μl of lysis solution to wash the ruins of cells in the dish. Transfer the solution to eppendorff tube.
5. Add 40μl（0.1volume）of 2M sodium acetate, PH4.0. Mix well by inversion
6. Add 400μl of water saturated phenol. Mix well by vortex.
7. Add 80μl （0.2volume）of chloroform and vortex.
8. Chill on ice for 15mins.

9. Centrifuge 10,000 rpm for 10mins.

10. Transfer the aqueous supernatant to a new tube. Add 400 μl（1volume）of isopropanol and chill for 1hr at -20℃.

11. Centrifuge 10,000 rpm for 20mins.

12. Pour out the supernatant. Suspend the RNA pellet in 100 μl of lysis solution.

13. Add 250 μl（2.5volume）of ethanol. Chill for 1hr at -20℃.

14. Centrifuge 10,000 rpm for 20mins.

15. Wash the pellet with 200 μl cold 70% ethanol, spin 10,000rpm 10min. Dry the pellet with speedvac concentrator.

16. Suspend the pellet in 25 μl H_2O（Heat for 10min at 65℃ if necessary）.

17. Take 2 μl to 98 μl H_2O to detect the concentration （read OD_{260}）

18. Take 2 μl for 1.2% agarose gel.

E、RNA isolation with Guanidium Thiocynate Method for 35 mm petri dish （小量 cell lysis 用）

※ Solution:

Stock solution:

1. **0.1M Sodium Citrate**
2. **10% Sarcosyl**

（將下列藥品溶入 DEPC-H_2O 總體積 49.5ml）

23.63g Guanidium Thiocynate （MW=118.16）

12.5ml **0.1M Sodium Citrate**

2.5ml **10% Sarcosyl**

DEPC-H_2O to 49.5ml

1. 配 Lysis solution 之濃度:
   4M Guanidium Thiocynate
   25mM Sodium Citrate
   0.5% Sarcosyl
   1% β-Mercaptoethanol （使用之前再加入）

2. 2M Sodium acetate pH4.0
3. DEPC-H_2O saturated phenol
4. DEPC-H_2O
5. 4M Licl

Reference:

Analytical Biochemistry, 162:156-159, 1987

F、With CTAB Total RNA Isolation （適用於含多醣類之貝類）

1. Pre heat water bath 60℃, get liquid nitrogen, dry ice, chill mortars and pestle
2. In chilled mortar and pestle, take from 0.5 – 1 g tissue, and grind thoroughly
3. Transfer to 10 ml CTAB solution, (add b-mercaptoehtanol to 0.2%, mix by inversion and incubate for 30 minutes
4. Add equal volumes phenol and Chloroform, mix by inversion.
5. Centrifuge for 10 min at 6000 rpm.
6. Transfer top (aqueous) phase to fresh tube, extract with chloroform, centrifuge for 6 min at 6000 rpm.
7. Transfer upper phase to new tube, with equal volume iso-propanol, mix by inversion, and sit for 15 – 30 minutes.
8. Pellet in centrifuge 30 min a 10000, re-suspend in small volume of TE.
9. Extract with phenol and then chloroform equal volumes each .
10. Prepcipitate with isopropanol.
11. Re-suspend in High TE.
12. Precipitated with 6 vol. Isopropanol, then wash with 70% ethanol.

G、CTAB Genomic DNA Isolation from Tissue （適用於含多醣類之貝類）

（J.J. Doyle and J.L. Doyle, BRL Focus 12:13-15）

1. Preheat 1 ml CTAB Isolation buffer (for each sample) in Eppendorf tube to 60℃ in water bath.
2. Grind 0.2 gm tissue to a powder in liquid nitrogen in a pre-chilled mortar and pestle.
3. Scrape powder directly into heated buffer and vortex gently to mix.
4. Incubate 30 min at 60℃ with occasional mixing by inversion.
5. Cool to room temperature. Extract with 500 ul of chloroform : isoamyl alcohol (24:1) by gentle but thorough vortexing.
6. Centrifuge at top speed for 2 min.
7. Carefully remove the upper aqueous phase with a blue tip with the end cut off. Pipet slowly and be very careful not to pick up any of the interface. Put in a clean Eppendorf tube.
8. Add equal volume of cold isopropanol and mix well by gentle but thorough vortexing. Centrifuge at top speed in microfuge for 15 min.
9. Pour off supernatant and add 500 ul TNE buffer and 2 ul of RNAse (from a 10 mg/ml stock). Incubate 10 min at 37℃.
10. After incubation, if pellet has not redissolved, resuspend pellet by gently flicking

tube or by gently vortexing. Add 0.55 ml Chloroform/Isoamyl alcohol (24:1) mix thoroughly by gentle inversion.

11. Spin 5 minutes at top speed in microfuge at 4 ℃.
12. Prepare a new tube containing 0.5 ml isopropanol and add the supernatant after centrifugation. Pipet slowly and be very careful not to pick up any of the interface. Mix thoroughly by gentle inversion.
13. Centrifuge at top speed in microfuge for 10 min. Care fully pour off supernatant and wash pellet of precipitated DNA with 80% ethanol.
14. Briefly dry pellet in Speed Vac. Resuspend in 50 ul TE.

CTAB Isolation Buffer
2% CTAB
1.4 M NaCl
20 mM EDTA
100 mM Tris-HCl, pH 8.0
(0.2% 2-mercaptoethanol, add just before use)

TNE Buffer
10 mM Tris – HCl, pH 8.0
0.1 M NaCl
1 mM EDTA
(0.2% 2-mercaptoethanol, add just before use)

從鯉魚肝臟粹取出之 **total RNA** 測 **OD** 值及跑膠照像實例操作
※RNA isolation: （tissue lysis 用）
1. 冰凍保存的組織（鯉魚的肝臟）或新鮮組織加液態氮在小型研磨碗內用研磨棒磨成粉狀，速度要快速。(2g 組織+液態氮)。

2. 將粉狀組織取出放入 50ml 的塑膠離心管內，加入 20ml 的 Lysis solution。以快速研磨機研磨。(研磨時會有泡泡產生主要 Lysis solution 含有 **Sarcosyl** 的關係)，研磨至顆粒狀消失為止。(2g+20ml Lysis solution)

3. 加入 0.1 倍體積 2M Sodium(2ml)混合均勻，再加入 1 倍體積的 phenol(20ml)用 vortex 混合，再加入 0.2 倍體積 chloroform 再用 vortex 混合。最終體積共 46ml。在冰塊內放置 15 分鐘。(2g +20ml Lysis solution+2ml 2M Sodium +20ml phenol +4ml chloroform =46ml)。

4. Chill 完後之溶液分別裝入 30ml 玻離圓底離心管（此轉移離心管視離心機之

roter 可調整)，以偶數分配（視量多少情況而定）並在天平稱上調整重量，以 chloroform 來做平衡調整，在離心機上以 10,000 rpm 離心 10 分鐘。

5. 用夾子取出離心管。液體在離心管內有三層，以吸管將底層吸出丟棄，留下上層及中層。再加入同體積之 chloroform mix 一下再平衡後在 10,000rpm 離心 10 分鐘。

6. 第 5 步驟可重複直至中間層消失為止。

7. 離心完後以夾子取出。此時液層只分兩層，用吸管將上層液吸取移至新管（不要吸到下層液)，加入同體積（1 倍體積）isopropanol 置入-80℃放置 1 小時。

8. 在-80℃取出（此時可見到雲霧狀的 RNA）在天平上以 isopropanol 添加平衡後在離心機 10,000rpm 離心 20min。

9. 以夾子取出離心管，將上層液倒出丟棄。加入 Licl（氯化鋰）mix 一下，放在粹冰內 5 分鐘，以 Licl 平衡後在離心（10,000rpm, 10-20 分鐘）。（洗沉澱的 RNA。在 tissue 粹取 RNA 時因含有大量酐醣，故以 Licl 將酐醣洗出，在 cell 粹取 RNA 時因無大量酐醣故無需以 Licl 洗酐醣的步驟）

10. 以夾子取出離心管，倒掉上層液。加入 2ml lysis soiltion，震盪一下以溶解 RNA，加入等體積 isopropanol 或 100% ethanol，震蕩一下，置放於-20℃1 小時或隔夜。

11. 平衡後在 10,000rpm, 離心 20 分鐘。

12. 倒掉液體，加入 5ml cold 70% ethanol 洗 pellet（因 isopropanol 有鹽類)，平衡後離心 10,000rpm 10min。用真空乾燥機乾燥 pellet（不能太乾燥，否則很難溶解於水)。

13. 加入 1.5ml H_2O。（如需要可在 65℃下加熱 10 分鐘)（水量的多寡以 OD 測值來決定加水量)

14. 取 2μl 加入 98μl 的水在 OD_{260} 下檢測濃度。

15. 取 1μl 或 2μl 在 1.2%跑膠。

16. 將膠片放入 UV box 內照像。

注意事項：(RNA 粹取時)

1、玻離器皿、試管、吸管必需在 180℃下烤 6 小時

2、飽和的 phenol 取底層黃色部份

3、從離心機取出離心管時要用夾子

4、離心管離心時不能蓋上蓋子

5. 研磨器以 H_2O_2 浸泡

二、互補 DNA 第一股的合成 (First-Strand cDNA Synthesis Reaction)

（Power Script™ Reverse Transcriptase） Protocol – at – a – Glance (PT3396-2)

以 mRNA 為模板，將訊息轉換成第一股 DNA，最後合成雙股 DNA，步驟如下述：

List of Components（store all at -20℃）

＊PowerScript™ Reverse Transcriptase

＊5X First-Strand Buffer

＊DTT（100mM）

Additional Materials Required

＊dNTP Mix （10mM each dATP, dCTP, dGTP, dTTP）

We recommend Advantag ；UltraPure PCR Deoxynucleotide Mis （＃4700-1）.

＊Oligo（dT）$_{12\text{-}18}$ primer（Amersham-Pharmacia Biotech., ＃27-2610-01）

Alternatively, random or gene-specific primers may be used.

＊Rnase-free H_2O

＊[Optional] 60 mM EDTA

First-Strand cDNA Synthesis Reaction

The 20-μl reaction described below is suitable for synthesizing first strand cDNA from50ng-1μg of poly A^+ RNA or 1-5μg of total RNA. To measure yield by monitoring the incorporation of [α-^{32}P] dCTP, see Ausubel et al., 1995

1. Add 500ng Oligo（dT）$_{12\text{-}18}$ to your RNA sample. Alternatively, you may use 50-250ng random primers or 2 pmol of a gene-specific. Add Rnase-free H_2O to 11μl final volume.
2. Heat mixture at 70℃for 10 min. Immediately cool on ice. Spin down and add the following:
   4μl 5X First-Strand Buffer
   2μl dNTP Mix （10mM each）
   2μl DTT （100mM）
3. Mix the contents of the tube by pipetting; add 1μl PowerScript RT, and mix by pipetting.
4. Incubate at 42℃for 50-90 min.
5. Terminate the reaction by heating at 70℃for 15 min or by the addition of 4μl of mM EDTA.
6. PCR
7. Running gel

Total RNA 在分光光度計上濃度及 OD 值

→70℃（10min）
→cool on ice

5x1st buffer	4μl x 9	36
0.1M DTT	2μl	18
10mM dnTP	1μl	9
Rnesin (40u/λ)	1μl	9

→42℃(2min)
→RT(1μl)
→42℃ (50min)
→72℃ (15min)
→(Rnase H)
→dissolved in 200 μl

互補 DNA 第一股的合成之記錄操作表

First-Strand cDNA Synthesis Exp. No.__________
Name:________Date:__________

First-Strand cDNA Synthesis---Preparation of RT-PCR
Use all Rnase-free stuffs.

1. Combine the following in separate 0.5-ml microcentrifuge tubes:

Sample	Conc. (μg/μl)	1μg total RNA (μl)	Oligo dT (0.5μg/λ)	H_2O (μl)	Total (μl)
kidney	1.1	2	1	8	11
spleen	0.9	2	1	8	11

☐ 2. Mix contents and spin the tube briefly in a microcentrifuge.
☐ 3. Incubate the tube at 70℃ for 2min; then cool the tube on ice for 2min.
☐ 4. Spin the tube briefly to collect the contents at the bottom.
5. Add the following to each reach reaction tube (already congaining 11μl):
- ☐ 4μl 5xFirst-Strand buffer (Clontch)
- ☐ 2μl DTT (100mM)
- ☐ 2μldNTP Mix (10mM each)
- ☐ 1μl PowerScript RT (200 units/μl, BRL)

20μl Total volume

☐ 6. Mix the contents and spin the tube briefly.

☐ 7. Incubate the tube at 42℃ for 1.5 hr in an air incubator.

☐ 8. Dilute the first-strand reaction product with 180 μl of Tricine-EDTA Buffer (pH8.5)

☐ 9. Heat tubes at 70℃ for 15 min.

☐ 10. Samples can be stored at -20℃.

PCR 雙股互補 DNA 實例及操作之記錄表

PCR cDNA 50

Exp. No.____________

Name:________Date:___________

Template

		Tag	venti			
H_2O	28.75μl	57.5	67			
10x Tag buffer w/o $MgCl_2$ (Promega)	5μl	10	10			
15 mM $MgCl_2$	5μl	10	10			
2mM dNTPs	5μl	10	10			
Tag DNA polymerse (Promega, 5U/λ)	0.25μl	0.5	0.5			
Template (1st cDNA)	(2μl)	4	4			
Forward primer (10 pmol/λ)	2μl	4	4			
Reverse primer (10 pmol/λ)	2μl	4	4			
Total	50μl	100	100			

PCRcondition:______________________

(Hot start: 94℃/____min, add______μl of______mM $MgCl_2$

94℃/____sec, T=____℃, G=____℃/____sec, 72℃/____min___sec x ______

72℃/5 min, 4℃/∞

Electrophoresis:__________% TAE-Agarose gel

practice rainbow trout cloning

Run PCR products：

Sample	5μl	
Loading dye	3μl	（loading dye 的濃渡要佔 6%）
Total	8μl	

＊Run 800bp 用 0.7%的膠

＊ EtBr 染 10 分鐘

＊跑完膠要用水洗才不會有雜點

三、聚合鋂連瑣反應 (Polymerase Chain Reaction; PCR)

此步驟主要應用聚合鋂連瑣反應技術將目的基因片段加以量化

This protocol is for amplifying a DNA fragment f**rom a purified plasmid DNA.**

Standard PCR amplification is carried out in a total volume of 100μl.

Reagents are add into a 200μl a 200μl thin-will PCR tube following the order:

H_2O	70.5μl
10x PCR buffer (w/$MgCl_2$)	10μl
1.25 mM dNTP mix	16μl
5'-primer (20 *p* mole/λ)	1μl
3'-primer (20 *p* mole/λ)	1μl
Template DNA (10-20ng/λ)	1μl
Taq polymerase (Promega, 5/λ)	0.5μl
Deep venti polymerase	

Instrument: Perkin-Elmer 2400 or Eppendorf Mastercycler

PCR condition: 94℃/1min

94℃/15", 55℃/15", 72℃/1min x35

72℃/5min

4℃/∞

For Eppendrof Mastercycler personal, use 50μl/tube.

【Note】

For setting up the PCR condition, check:

The Tm of the primers. (The GC/AT ratio of the primers and the numbers of bases in the primers)

The size of the PCR products. Start from 1Kb/min.

The concentration of $MgCl_2$ in the buffer.

The model of the instrument.

聚合鋂連瑣反應主要起始子(**primer**)的設計實例:

從 NCBI 網路去找 rainbow trout 的 CCAAT/enhancer binding protein β gene bank.

討論有關這段基因之 primer 的設計。

1、從網路去找相同基因的 gene bank

2、參考有關 plasmid 圖示公司之網站。

3、參考有關限制脢公司之書籍。

4、依照 primer 設計規範設計 5'----3' 及 3'-----5'兩個 primer.有關之資料在 paper 內。

※ Anneling 的溫度計算如 AT:GC=10:13 則 Anneling 的溫度為 10x2+13x4=20+52=72℃（一般 Anneling 的溫度在 55-60℃左右）

※primer 的長度在 18-20 之間 可向兩邊延伸在 23 or 24 bases。

四、**PCR 產物的回收** (Recover the PCR products)
應用沉澱和 venti 將 DNA 回收實例操作：

A、QIAGEN PCR Recover kit:
(使用 QIAquick, Gel Extraction kit 回收 DNA) Follow the protocol from the manufactory)

1. 將 4 管(venti 組) PCR production 合起來，共約 60 µl 分成兩次 loading.
2. 做大凹槽的膠片。
3. 跑膠 130v. 50min
4. 在 uv box 解讀並照像。
5. 在 uv box 下把含有 gene 的 band 切下。
6. 將切下之膠放入 tube 內，衣照膠的重量加入 kit 的 buffer QC 溶解膠。
   (此步驟依照說明書步驟進行)
7. 將溶解之膠在有 filter 之離心管內把 DNA 過濾在 filter 上。
8. 加入 QIA PE 750 µl 在 6000-9000 rpm, 30sec 將鹽類洗出。
9. 加入 QIA EB 30 µl 在 15000 rpm, 50 sec 將 DNA 洗出。
10. 在 OD_{260} 下測 DNA 濃度。以 QIA EB EB 做背景值。
    (稀釋 50 倍;2µl sample + 98µl H_2O;基因計量儀上選擇稀釋 50 倍的 program)

結果：
V（venti)（1,2,3,4）將四管合併跑膠，從膠內回收 DNA
沉澱回收和膠內回收之 DNA 以計量儀計量：結果未能檢測到

再次跑膠以檢測 PCR 產物

※1. kidney 2. spleen run PCR with Taq , then run the gel （the result for Ligation）
※ 繼上星期五用 venti 跑 PCR，回收之 DNA 在基因計量儀上無法測到。再次跑膠可跑出帶。因此繼續 Cycle Sequencing. （＊利用沉澱方法的 DNA 無法計量，跑膠亦未有帶狀出現，故丟棄）。

Sample: V（venti)（1,2,3,4）將四管合併跑膠，從膠內回收 DNA 繼續做定序
1、Cycle Sequencing（Sanger 定序法）

2. Big Dye 4µl （商品訂購）
3. PCR DNA 100ng
4. primer 3.2pmol （design 23bp；(sample 1: 5'----3')
   (sample 2: 3'----5')
5. ddH_2O

合計　　　　　　10μl

2、Mix

2、With GeneAmp PCR System 2400 之 PCR 設定：

1. set the volume to 10μl

2. 96℃ （10sec）, 50℃ （5sec）, 60℃（4min）, 4℃停留

3. run 25cycles

4、離心

5、沉澱（Ethanol precipitation of DNA）

Materials:

＊3 M sodium acetate pH5.2 or 5M ammonium acetate

＊DNA

＊100% ethanol

1. Measure the volume of the DNA sample.
2. Adjust the salt concentration by adding 1/10 volume of sodium acetate, pH5.2, or equal volume of 5M ammonium acetate. Mix well.
3. Add 2-2.5 volumes of cold 100% ethanol （calculated after salt addition）, mix well.
4. Place on ice or at -20℃ for 10min or（>20minutes）
5. Spin a maximum speed（12000rpm）in a microfuge 20-30min.
6. Carefully decant supernatant.
7. Add 1ml 70% ethanol. Mix.
8. Spin briefly （12000rpm, 10min）.
9. Carefully decant supernatant.
10. Air dry or briefly vacuum dry pellet.（此步驟完成即可送定序中心做定序）

pellet 1: 5'-----3'

pellet 2: 3'-----5'

6、送定序中心定序

11. Resuspend pellet in the appropriate volume of TE or water,做 sequencing.

＊kidney and spleen 之 RT-cDNA （first strand cDNA）做 PCR（RT-PCR; double strand cDNA）

＊PCR condition: 94℃/10sec, 57℃/10sec, 72℃/50sec, 40cycles, 72℃/5min, 4℃/∞

＊ Run gel

＊ PCR 產物準備做 ligation

B、Ethanol precipitation: T（Taq）(1,2,3,4）以 Ethanol 沉澱回收 DNA

1. Transfer the PCR product into a 1.5 ml tube.
2. Add $^{1/2}$ vol. of 7.5M ammonium acetate （NH_4Ac）(final conc. 2.5M)

(Use 1/10 vol. of 3M sodium acetate pH5.2, if the DNA will be used for phosphorylation 【kinase reaction】 or the PCR products are ≦ 100bp).

3. Add 2.5-fold of 【DNA+salt】 vol. of 100% EtOH. Mix well.
4. Keep the tube on ice for 15 min (or -20℃ o/n).
5. Spin, 12K rpm, at 4℃ for 20 min.
6. Discard the supernatant.
7. Add 200 μl of ice-cold 70% EtOH and vortex to wash the DNA pellet.
8. Spin, 12Krpm, 5min, at 4℃.
9. Discard the supernatant.
10. Dry the DNA. Use speed vac, or at 60℃ for 3min, or at r.t. for 10min.
11. Suspend the DNA in H_2O or 10mM Tris-Cl, pH8.5. (Usually use about half PCR vol., it depends on the yield of PCR products.)
12. Detect the concentration of recovered DNA-Read OD_{260} in a spectrophotometer.

五、限制酶的設計 (Restriction enzyme digestion)
限制酶的設計係在質體建構時將限制酶切點位置連接在質體上
限制酶的設計之記錄表格：
Exp. No.__________________
Name:__________________Date:_______-_______-________

DNA		
H_2O		
Buffer		
BSA		
Enzyme 1		
Enzyme 2		
Total		

Temperature:
Incubation time:
Electrophoresis:____________% TAE-agarose gel

操作實例：
以限制脢切 cDNA and plasmid 之後，再將 cDNA ligation 到 plasmid 上。
cDNA 濃度用 OD_{260} 去讀
plasmid 濃度用 OD_{260} 去讀

計算 1 molecule of vector : 2-5 molecules of insert DNA 之比率

ratio	vector	Insert DNA
	1μg/μl = 10kb	1μg/μl = 1kb
	1μg = x mole	10xmole
1:2	1μg	1/5μg = 0.2 = 200 ng
1:1	1μg	1/10μg = 0.1= 100 ng

Restriction enzyme digestion

	pcDNA（+）289ng/μl	C/EBP（kidney）115ng/μl	C/EBP （spleen）125ng/μl
DNA （4）	2μl	8μl	7μl
H_2O （1）	13μl	3.5μl	4.5μl
10x Buffer （3）	NE2 2μl	NE2 2μl	NE2 2μl
10x BSA （2）	2μl	2μl	2μl

Enzyme1 （5）	Hind III 0.5μl	Hind III 1μl	Hind III 1μl
Enzyme2 （5）	Xho I 0.5μl	Xho I 3.5μl	Xho I 3.5μl
Total	20μl	20μl	20μl

Temperature: 37℃

Incubation time: 2.5 hr

Electrophoresis: ___0.7_____% TAE-agrose gel（檢查 plasmid and insert 是否有切到）

六、載體的構築 (Plasmid vector restruction)

此步驟在將目的基因構築在載體上

Ligation: This protocol is for cloning restriction enzyme digested PCR fragments or recoved DNA fragments into a plasmid vector.

Vector (50-100ng/λ) 1μl
Insert DNA xμl
10x ligation buffer (USB) 1μl
T4 DNA ligase (USB, 1/λ) 1μl
H_2O xμl

Final 10μl

Incubate at room temperature for 3-5 hr or at 16℃ overnight

Note:

※The amount of insert DNA added depends on the size of DNA fragments.

※Usually, **1 molecule of vector : 2-5 molecules of insert DNA** is used for the cloning.

※You can add a small amount of restriction endonuclease to the ligation mixture and incubate the tube in optimal incubation temperature for 15 min to digest the self-ligated vector before using the DNA for transformation.

Sample:

Ligation

	kidney（C/EBP）	spleen（C/EBP）
Vector DNA	4μl（115.6ng）	4μl（115.6ng）
Insert DNA	1μl（46ng）	1μl（44ng）
10x ligation buffer (USB)	1μl	1μl
T4 DNA ligase (USB, 1/λ)	1μl	1μl
H_2O	3μl	3μl
Total	10μl	10μl

R.T. for over 5 hr

七、稱任細胞的製備 (preparation of competent cell and $CaCl_2$ transformation)
此步驟係將大腸桿菌製備成讓質體可穿透細胞膜達到轉形目的
Materials:
Autoclaved centrifuge tube（40ml）, 1.5ml microcentrifuge tubes and 250 ml flasks.

100mM $CaCl_2$（sterile, keep on ice）
LB（Luria-Beriani）medium：
1% trytone
0.5% yeast extract
0.5% Nacl
LB plates:
1.5% Bacto-Agar/LB
LB plates with antibiotics and/or supplements

Protocol:
※Preparation of competent cell
1. 從 Stock E.coli（儲存於-80℃）取 sample 畫上 plate（over night）。在 plate 上取 1 個 colony 接種到 1ml 的 LB。在 37℃, 250rpm shaking, over night.
2. Transfer 400μl of the culture into 40 ml LB （1:100 dilution）, Incubate the culture at 37℃ with shaking at 300rpm until the A_{600}=0.3-0.7.（一般 A_{600}=0.4）
3. Recover the cells by centrifugation at 6Kxg（7000rpm, JA20 rotor）for 10 min at 4 ℃
4. Decant the medium. Suspend the pellet in 1/2 volume of ice-cold 100mM $CaCl_2$ and incubate it on ice for 30min.
5. Recover the cells by centrifugation at 6Kxg for 10min at 4℃. Suspend the cells in 1/10 volum.
【Note】
1. The pellet should form a circle.
2. Do not vortex the cells after $CaCl_2$ treatment.
3. The amount of $CaCl_2$ added is depended on the OD of the culture. You may use less amount of $CaCl_2$
4. You can add glycerol to final 15%, and keep the aliquots （200μl/1.5ml tube） of competent cells at -80℃.

※ $CaCl_2$ transformation
6. Transfer 100μl of competent cells into a 1.5ml microcentrifuge tube; **mix the cells with 1-10μl（10-300ng）of DNA.** （The volume of DNA added should be less than

1/10 of that of the competent cells.)

7. Incubate the tube on ice for 30min.

8. Incubate the tube at 42℃ for 1min.

9. Add 200μl of LB into the tube and incubate it for 30min to 1hr at 37℃.

10. Dilute the cells and spread on the LB plate containing antibiotics and/or supplements and incubated overnight at 37℃.

(Usually, add 700μl of LB to the tube (final 1ml), and spread 100μl and 200μl on plates espectively).

八、大腸桿菌的轉形 (Transfomation of E.coli)

(from Micheal Koelle)

Materials:

BioRad Geme-Pulser II

0.1 or 0.2 cm electroporation cuvette for bacterium

Autoclaved 500 ml centrifuge bottles, centrifuge tubes (40 ml), 1.5 ml microcentrifuge tubes and two 1L flasks.

3L ice cold sterile H_2O

50 ml 10% glycerol, sterile

LB: 500 ml in 2 L flask, 2 flasks

LB plates with antibiotics and/or supplements

Protocol:

Preparation of frozen competent cells

1. From a fresh overnight culture grow 1L bacteria to OD600 of 0.5-0.6.
2. Cool on ice a few minutes.
3. Pellet cells 5K, 15 min, in 500 ml bottles.
4. Gently resuspend in 500 ml ice cold ddH_2O, and repellet.
5. Repeat step 4 twice more.
6. Resuspend in 20 ml 10% glycerol, transfer to 50 ml tube, and pellet 6.5K for 15 min.
7. Resuspend in 2 ml 10% glycerol.
8. Freeze 50 ul aliquots in liquid nitrogen and store at -70℃.

Electrotransformation

1. Set BioRad Gene-Pulser to

   for 0.2 cm cuvette: 2.5kV, 25uF, and set Pulse-Controller to 200 Z.

   for 0.1 cm cuvette: 1.5kV, 25uF, and set Pulse-Controller to 200Z.

2. Prepare DNA for transformation in a low ionic strength buffer, preferable TE or H_2O. If necessary, extract and precipitate DNA to get rid of unwanted salt.

3. Thaw cells at room temperature, transfer to ice when thawed.

4. Chill on ice one cuvette per transformation. (Between uses, wash the wuvettes well with H_2O and ethanol.)

5. Put DNA (in up to 5ul) into an electroporation cuvette, by pipetting the droplet onto the cuvette wall. Add 40 ul cells (or 20 ul for 0.1 cm cuvette) to the cuvette by pipetting over the DNA droplet, and shake the mixture to the bottom of the cuvette.

Cap the cuvette, and place on ice until ready to zip.

6. Place the cuvette in the transformation chamber and press both chargin/pulsing buttons simultaneously until the machine discharges.

7. Immediately add 100 ul BL to the cuvette.

8. Note the time constant, 4.5-5 is good (for 0.2 cm cuvette, 0.1 cm cuvette gives a lower time constant ~ 4.1- 4.5). Lower numbers indicate too much salt in the DNA/cell mixture.

9. Cap the cuvette, and incubate it at 37℃ for 30 min to 1 hr.

10. Plate appropriate dilutions on selective plates.

C. 將 Plasmid transform to E. coli （competent cell ）

1. Transfer 100μl of competent cells into a 1.5ml microcentrifuge tube; mix the cells with 1-10μl（10-300ng）of DNA. （The volume of DNA added should be less than 1/10 of that of the competent cells.）

2. Incubate the tube on ice for 30min.

3. Incubate the tube at 42℃ for 1min.

a flame), spread the solution over the entire surface of the plate. Incubate the plate at 37℃ until all of the fluid has disappeared. Because of the low volatility of dimethyformamide, this can take up to 3 or 4 hours if the plate is freshly made.

3. Inoculate the plate with the bacteria that are to be tested. This can be done by streaking with a bacterial loop or toothpicks or by spreading up to 100μl of a suspension of bacteria over the surface of the agar medium. After the inoculum has absorbed, incubate the plate in an inverted position for 12-16 hours at 37℃.

4. Store the plate at 4℃ for several hours. This allows the blue color to develop fully. Colonies that contain active β-galactosidase are pale blue in the center and dense blue at their periphery. White colonies occasionally show a faint blue spot in the center, but these are colorless at the periphery.

Identification of Bacterial Colonies That Contain Recombinant Plasmids by Insertional inactivation

1、用牙籤挑白點，點在圓盤上，圓盤底部有格線可編號，每點點一格---放入 37℃培養。

2、上述點完後再將同一牙籤之 DNA 溶入 1.5ml tube 之溶液內準備做 PCR。

1、genomic DNA 的抽取
2、PCR 及 primer 的設計
3、Southern probe 1. isotop 2.dig-p （or dig）---x-ray 去檢測。

九、PCR 基因篩選 (PCR Screen)

PCR 基因篩選係將轉形之大腸桿菌經過 genomic DNA 的抽取，再由 PCR 將目的基因擴殖。

實驗操作之記錄表格：

Exp.No.________________

Name:____________Date:________-________-_________

Template

Template				
H_2O	65.5μl			
10x Taq buffer w/o $MgCl_2$ (Promega)	10μl			
15mM $MgCl_2$	10μl			
2mM dNTPs	10μl			
Taq DNA polymerase (Promega, 5U/λ)	0.5μl			
Forward primer (10 pmol/λ)	2μl			
Reverse prime (10 pmol/λ)	2μl			
Total	100μl			

Prepare aliquots: 10μl/λ in 200μl PCR tube. Use toothpick to pick the bacteria from plate, add the bacteria into the PCR mixture.

PCR condition:_____________________________________

(Hot start: 94℃/_________add_________μl of __________mM $MgCl_2$.)

94℃/____sec, T=____℃, G=____℃/____sec, 72℃/_____min_____sec______

72℃/5 min, 4℃/∞

Electrophoresis:___________% TAE-Agarose gel

Sample: Kidney(E.coli colony)；Spleen(E.coli colony)

Template	Kidney(E.coli colony)	Spleen(E.coli colony)		
H_2O	67.5μl	67.5μl		
10x Taq buffer w/o $MgCl_2$ (Promega)	10μl	10μl		
15mM $MgCl_2$	10μl	10μl		
1.25mM dNTPs mix	10μl	10μl		
Taq DNA polymerase (Promega, 5U/λ)	0.5μl	0.5μl		

or Deep venti polymerase				
Forward primer (20 pmol/ λ)	1μl	1μl		
Reverse prime (20 pmol/ λ)	1μl	1μl		
Total	100μl	100μl		

十、基因定序 (Sequencing)

PCR Screen 出之 （＋）以 purification of plasmid DNA kit （QIAprep）抽 DNA.。DNA 以 Sanger 定序法在 PCR 儀器定序之後所得定序資料與， insert DNA 序列比對是否相同，以判定是否轉形成功。

十一、鱒魚基因轉殖後之子代攜帶轉殖基因之鑑定實例操作：
(PROTOCOLS FOR ANALYZING OSU TROUT SAMPLES)

Isolation of Genomic DNA from Rainbow Trout Fin Tissue

I-A. Dgestion Buffer （DB）

100mM NaCl

10mM Tris-HCl, pH8.0

0.5% Sarkosyl

0.1 mg/ml proteinase K (prepared freshly)

I-B. Porcedures:

1. Collect fin (dorsal fins) tissues in a 2-ml ependorf tube individually.
2. Add 0.5ml of DB (containing proteinase K) into each tube; 快速離心約 12000 rpm, 5min. 讓 sample 浸泡於 DB 中. Incubate the tubes at 50℃ 2-3 hrs or overnight.
3. Extract the samples with 0.5ml of phenol and chloroform (1:1) （以盒蓋壓住離心管上下顛倒搖晃約 30 次）; spin at highest speed （13000rpm）for 6 minutes at room temperature; transfer the aqueous phase (only 450μl) without disturbing the aqueous-organic interface to a fresh 1.5ml-ependorf tube.
4. Repeat **step 3**, but transfer only 400μl of the aqueous phase to a fresh tube.
5. Add 1/10 volume of 3M sodium acetate (pH7.0) and 2 volumes of 100% ethanol (400μl +40μl+800μl=1.24ml); mix gently by inverting the tubes several times; continue on to **step 6** immediately of store the tubes at -20℃ for a short period of time or overnight.
6. Spin the tubes for 12000 rpm, 4℃, 30 min; remove the supernatant and rinse the pellet with cold 70% ethanol twice （以 suction 裝置操作）; dry the pellet in a speed vac (do not overdry) without the heat on.
7. Re-suspend DNA in 50μl TE buffer; let the tubes sit for 30minutes at 37℃ or room temperature.
8. Measure DNA concentration at OD 260 and 280 nm and adjust the final concentration to 200ng/μl （有時介於 50-300 ng/μl 亦可用，不必調整濃度）by adding proper amount of TE. Store the DNA samples in the -30℃ freezer till PCR analysis.
9. Sample run the gel to see the quality. （此步驟亦可省略）

註：1. 拿新 tube（從購入之包裝）時，以夾子夾出。

2. 使用附有 filter 之 pipete 。

3. repeat pipette。

4. 混合時以搖晃方式，不能用震盪器。多樣品時以盒蓋壓住離心管上下顛倒搖晃 10-15 次，換邊再搖 10-15 次即可離心。
5. suction 裝置。

II. PCR Analysis

Note:

DNA template: (1) fish DNA samples at 200ng/μl

(2) two controls should be always included:

negative control ---H_2O

previously identified as PCR-positive fish

Additional positive controls: plasmid pc12 (1ng/μl)

Plasmid pc31 (1ng/μl)

PCR ANALYSIS OF OSU TROUT SAMPLES

Date PCR#

DNA samples:

Cycling parameters: 94℃ 3min; 40cycles of 94℃/15s, 55℃/10s, 72℃/1min;
72℃/2 min, 4℃/∞

Machine#:

	Final cone.	25μl	50μl	
10x Buffer	1x	2.5	5.0	
25mM $MgCl_2$	1.5mM	1.5	3.0	
5mM $dNTP_s$	0.2mM	1	2	
50μM TTC439	0.375μM	0.188	0.375	
50μM TTC440	0.375μM	0.188	0.375	
5U/μl Taq	1.25U	0.125	0.250	
ddH_2O		15.5	37.0	
DNA template	800ng DNA 2 ng plasmid	4	2.0	
Total	50μl	25μl	50	

III. Southern Hybridization

III-A. Blotting

1. Carry out electrophoresis of the PCR product on a 1 % agarose gel; use 5μl of the PCR product per sample lane; include 1kb DNA marker was an additional control for hybridization.
2. Transfer the separated DNA on the gel onto a nylon membrane by a "dry transfer" procedure. Let transfer go overnight.
3. Mark the position of wells with pencil before removing the gel from the nylon membrane. Fix the DNA on the membrane by UV.

III-B. Hybridization

1. Carry out prehybridization with 10ml of AHB at 38℃ while preparing for the probe.
2. Carry out hybridization with 10ml of AHB (containing ^{32}p-labelled oligomer probe, see below) at 38℃, overning.
3. Remove hybridization buffer; rinse the membrane briefly with WB once; wash the membrane with WB for 20 minutes three times; rinse the membrane briefly before subjecting to radiophotography or phosphoimage analysis.

Buffers:

Aqueous Hybridization Buffer (AHB)

Final conc			
1xSSC	20xSSC	1ml	2ml
1xDenhardt	50xDenhardt	400μl	800μl
0.5% SDS	10% SDS	1ml	2ml
	ddH_2O	17.6ml	35.2ml
	Total vol	20ml	40ml

Wash Buffer (WB): 6xSSC + 0.1% SDS

A. Preparation of ^{32}p-labelled oligomer probe: (end lable)

1. Assemble the reaction mixture on ice as follows:

Oligo* (10μM)	1.5μl
10x T4 PNK Buffer	3.0μl
T4 PNK (10u/μl)	1.0μl
DdH_2O	18.5μl
Total vol	24μl

2. Add 6μl of $\gamma\ ^{32}$-P-ATP into the mixture; incubate the mixture for 1hour at 37℃.

B. PCR product:

Probe 的製作有兩種方法：

1. Rendom priming
2. Nick translation

1. Rendom priming

Solutions	Components	Volumes
Solution O	1.25M Tris-HCl, pH8.0	250 μl
4℃	0.125 M $MgCl_2$	50 μl
	H_2O	100 μl
	Total	400 μl
Solution A	1 ml Solution O	400 μl
-20℃	18 μl 2-Mercaptoethaanol	7.2 μl
	5 μl dATP (0.1M, pH7.5, USB-PCR)	2 μl
	5 μl dGTP	2 μl
	5 μl dTTP	25 μl
Solution B	2M Hepes titrated to pH6.6 with 4M NaOH	
4℃		
Solution C	Hexadeoxyribonucleotides evenly suspend in TE	
	90 OD U/ml- Use Random hexamers 9U/mL	
OLB	Solution A (100μl)	20 μL
-20℃	Solution B (250μl)	50μl
(up to 3 months)	Solution C (150μl)	30μl
	Total (500μl)	100μl

Boil DNA (10-100 ng) for 7 min, quench on ice

Add: H_2O 12μl

OLB 10μl

10 mg/ml BSA 2μl

100ng DNA 20μl

α-[^{32}P] dCTP 5μl

E. coli DNA polymerase I – 2U 1μl

(NEB 3' – 5' exo-) E. coli DNA polymerase

Incubate at 37℃ for 1h., check incorporation and denature probe by boiling for 7 min and quenching on ice.

Check incorporation:

Spot 2 μl of a 1/10 dilution of the probe onto two DE81 Whatman discs; allow to dry

wash 1 disc in : 3 x 5 ml 0.4 M phosphate buffer

2 x 5 ml H_2O

2 x 5 ml 95% ethanol.

----------研習結束

三、結語與建議

未來九孔研究的方向：

1、目前對於九孔或貝類上是否有生長賀爾蒙基因尚無定論，有必要加以釐清。

2、於 1988 年在 nature 上有發表一篇有關在軟體動物（*Lymnaea stagnalis*）位於頭部的神經結有 insulin-related peptide 的先驅物（precursor），此先驅物是否與生長有關值得探討。

3、軟體動物有關生長基因的探討文獻不多，因此可供比對以設計起始子(primer)的訊息相對減少，加以有關貝類研究人員及設備不足，因此在貝類項目的研究進展有限，期盼將來有更多的人力及財力的加入研究行列。