行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

微陣列基因體醫學核心實驗室 I

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□赴國外出差或研習心得報告一份
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□出席國際學術會議心得報告及發表之論文各一份
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執行單位：國立台灣大學醫學院內科

中　華　民　國　92 年　5 月　15 日

中文摘要

關鍵詞: 人類基因體計劃，生物晶片，微陣列，晶片實驗服務

微陣列是發展及應用較為成熟的生物晶片技術之一，是後基因時代基因體醫學研究之重要平台，此晶片技術已廣泛的應用於各領域的基礎研究，亦將直接或間接的應用於疾病之診斷、分類、疾病之預後評估，並改善疾病之治療。由於基因晶片的設備及成本高昂，導致此技術無法廣為應用於各領域之研究。本計劃之目的為設立微陣列核心實驗室及基因庫，將以合理價格提供高品質之人類及老鼠基因微陣列晶片全套性且完整性實驗服務，包括核酸標記、雜交反應、呈色偵測、影像分析、數值擷取及叢集分析，另外並代為製作各式晶片。核心實驗室將依最大工作能量，提供參與基因體醫學國家型科技計畫之各學術及研究機構研究人員申請使用。

本核心實驗室已於台大醫學院附設醫院設置兩年有餘，製作超過千片的人類、老鼠及代製晶片，並提供醫學校區相關的研究諮詢與教育訓練。自 91 年度起受國科會補助成立「微陣列基因體醫學核心實驗室」，並立即提供全國性的微陣列服務，為目前國內正式且唯一能提供基因微陣列服務的核心實驗室。自從 91 年一月受國科會補助開始對外服務後，目前已提供台大、陽明、長庚、中山醫學等大學超過兩千片人類及老鼠及代製晶片，後續登記要求服務的更高達六百片，將依登記先後及實驗室製作容量提供服務。為提昇基因微陣列之教學及人員操作訓練，我們編撰基因微陣列操作儀使用手冊及基因微陣列資料分析實務手冊。提供使用者深入瞭解基因微陣列儀器之操作及資料分析。我們並建立基因體醫學核心實驗室網站〈http://microarray.mc.ntu.edu.tw〉，內含申請程序及作業流程，可供使用者上網申請並下載表格。研究同仁可直接上網申請服務，找尋資訊或查詢資料。

English abstract

Keywords: Human Genome Project, biological chip, microarray, service

Microarray technology is the central platform for functional genomics and translational research in the post-genomic era. Our main goal is to establish an up-to-date microarray core facility, dedicated to provide high quality microarrays, technical supports and services to researchers in genomic medicine nationwide. Access to the high quality microarrays will allow our researchers to be on the cutting edge of genetic research, focus on microarray-based gene expression profiling to explore the pathogenic mechanism of human diseases. The microarray technology is particularly important to clinical medicine in post-genomic era. It will provide high throughput and valuable insights into differences in an individual's disease as compared with constitutional mRNA expression. The microarray information can provide tissue-specific disease signatures and therefore, can improve disease diagnosis, disease classification, prognosis evaluation, identify biomarkers, individualized patient treatment and improve treatment outcome.

Although the cDNA microarray is extremely powerful, the current application is still limited. The major obstacles are its high cost and difficulty in maintaining good quality membrane and experiment. We had established the Core Lab and ESTs library to provide high quality service of membrane-based cDNA microarray under the reasonable price. The core facility also provide one-stop services including target labeling, microarray hybridization, colorimetric detection,

image digitization, and cluster analysis. In addition, the services of custom-made chips including membrane- and glass-format microarray are also provided. According to the work capacity, microarray core facility opens for the investigators of academic and research institution participated in National Research Program of Genomic Medicine to apply.

The core facility has been established in National Taiwan University Hospital, National Taiwan University College of Medicine for more than two years and manufactured more than one thousand of microarrays including human, mouse, and custom-made chips. Also, the Core Lab offered related research consultation and education training to medical campus. National Science Council supports our core facility since 2002 to set up the "Microarray Core Facility for Genomic Medicine", which offers nationwide microarray services right away. Our microarray core facility is the only one that can provide complete microarray services at present. Since Jan. 2002, we started to provide services for researchers granted by National Genome Project and other NSC Grants. We have provided the researchers of Taiwan University, Yang-Ming University, Chang-Gung University, and Chung-Shan Medical University for more than 2600 pieces of microarray membranes including human, mouse and custom-made chips. There are still about 600 pieces of chips waiting for preparation. We will provide services by the enrolled order and work capacity. The Web address of Microarray Core Facility is **http://microarray.mc.ntu.edu.tw** in which containing application procedure and working flow path, applicants can apply for services on-line and download the application form.

報告內容：請包括前言、研究目的、文獻探討、研究方法、結果與討論（含結論與建議）…等。若該計畫已有論文發表者，可以 A4 紙影印，作為成果報告內容或附錄，並請註明發表刊物名稱、卷期及出版日期。若有與執行本計畫相關之著作、專利、技術報告、或學生畢業論文等，請在參考文獻內註明之，俾可供進一步查考。

前言

為解開複雜的基因網絡以瞭解生物的各種生命現象，必須從許多基因的功能著手，這也意味著人類基因體計劃的結束將開啟另一個新紀元，即後基因組世紀(post-genome era)的來臨。功能基因分析(Functional genome)也被喻為繼人類基因體計劃後，二十一世紀最具潛力的研究主題之一。近年來幾項新發展且具前瞻性的生物晶片技術，如微陣列(Microarray)、基因晶片(DNA chip)、蛋白質晶片(Protein array)、組織晶片(Tissue array)及實驗室晶片(Lab-on-a-chip)等，正加速瞭解許多基因的功能，因此不難理解分子生物學的進展與新功能基因的尋求息息相關。微陣列是發展及應用較為成熟的生物晶片技術之一，此晶片技術已廣泛的應用於各領域的基礎研究，亦將直接或間接的應用於疾病之診斷、分類、疾病之預後評估，並改善疾病之治療。

研究目的

由於基因晶片的設備及成本高昂，導致此技術無法廣為應用於各領域之研究。本計劃之目的為設立微陣列核心實驗室及基因庫，將以合理價格提供薄膜式人類及老鼠晶片實驗服務，包括核酸標記、雜交反應、呈色偵測、影像分析、數值擷取及叢集分析，另外並代為製作各式晶片。

研究方法

Preparation of cDNA probes

The labeling reactions were performed during reverse transcription in the presence of 6 μM random primers or oligo-d$(T)_{20}$VN degenerate primers; 0.5 mM each dATP, dCTP, and dGTP; 40 μM dTTP; 40 μM biotin-16-dUTP (Roche Molecular Biochemicals; Mannheim, Germany); 1X reactio buffer; 10 mM DTT; 0.5 unit/μl Ribonuclease inhibitor (GIBCO-BRL; Gaithersburg, MD); and 200 units of MMLV reverse transcriptase (GIBCO-BRL; Gaithersburg, MD) in a 50-μl solution. The reaction mixture was incubated at room temperature for 10 minutes, then transferred to 42 °C for 90 minutes and was stopped by heating of the reaction mixture to 99 °C for 5 minutes. The RNA was degraded by adding of 5.5 μl of 3 M NaOH followed by a 30-min incubation at 50 °C. The labeled samples were neutralized by addition of 5.5 μl of.3 M acetic acid and then precipitated by adding of 50 μl of 7.5 M ammonium acetate, 20 μg of linear polyacrylamide as carrier, 375 μl of absolute alcohol, and water to make a total of 525 μl. The solution was mixed evenly and stood at –80 °C for 30 min, and then centrifuged at 14,000 rpm for 20 min to precipitate single stranded DNA probe. Pellet was washed with 1 ml of 70 % of ethanol and dried by speed vacuum, then dissolved in 5 μl or suitable volume of deionized water.

Gene microarray hybridization

The membrane carrying the double-stranded cDNA targets was pre-hybridized in 3 ml (option) hybridization buffer (5× SSC, 0.1 % N-lauroylsarcosine, 0.1% SDS, 1% blocking reagent made by Roche Molecular Biochemicals, and 50 μg/ml salmon sperm DNA) at 68°C for 1 hour before hybridization was carried out. cDNA probes (up to 5μg) were resuspended in 100 μl (option) hybridization buffer containing 200 μg/ml $d(A)_{10}$ and 300 ~ 400 μg/ml human COT-1 DNA (GIBCO-BRL) to prevent non-specific binding and were hybridized to the cDNA fragments on the membrane by Southern hybridization procedure. The 100-μl reaction mixture was sealed with the membrane in a hybridization bag or assembly (SureSeal, Hybaid, Middlesex, UK) attached to a weight and incubated at 95°C for 2 min (could be omitted) and then at 68°C for 12 hours. The membrane was then washed with 2× SSC containing 0.1% SDS for 5 min at room temperature followed by three washes with 0.1× SSC containing 0.1% SDS at 65 °C for 15 min each.

Colorimetry detection and image analysis.

After hybridization, the membrane was blocked by 1 ml (option) of 1 % blocking reagent (Roche Molecular Biochemicals) containing 2 % dextran sulphate at room temperature for 1 hour and then was rinsed with 1 X TBS buffer solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% BSA). To detect the spots on the membrane in single-color mode,

β-galactosidase-conjugated streptavidin (Strep-Gal, GIBCO-BRL) was employed. The membrane was incubated with a 3-ml (option) mixture containing 700 X diluted Strep-Gal (1.38 units/ml, enzyme activity), 4% polyethylene glycol 8000 (Sigma, St. Louis, MO), and 0.3% BSA in 1 X TBS buffer for 2 hours. The membrane was then washed with 1 X TBS buffer three times for 10 min each. The chromogen was generated by treating the membrane with X-gal substrate containing 1.2 mM X-gal, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ in 1 X TBS buffer for 30 ~ 60 minutes at 37 °C for β-galactosidase reaction. The color development reactions were then stopped by 1x PBS containing 20 mM EDTA. After color development the cDNA molecules labeled with biotin yield a blue chromogen. To measure the expression levels of the genes, the membrane was scanned or taken image by a flatbed scanner (PowerLook 3000). The scanner provided 3000 dpi resolution and was suitable for larger arrays such as arrays of 9600 elements.

Amplification of cRNA: *In Vitro* transcription

The RNA quantity derived from above extraction is very tiny. To achieve microarray analysis, RNA amplification should be performed (32,42). To synthesize full-length double stranded cDNA efficiently, "template-switching" effect was used in the synthesis of the second stranded cDNA. The template switch primer was designed according to literature and shown as follows: 5'-AAG CAG TGG TAT CAA CGC AGA GTA CGC rGrGrG-3' (43). Mixing 0.05-5 μg of total RNA (8 μl) and 1 μl of 1 μg /μl T7promoter- (T) 24 primer (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG TTT TTT TTT TTT TTT TTT TTT TTT-3'), and heated at 70 °C for 5 min, then put on ice. Four μl of 5X first strand buffer (GIBCO-BRL), 2 μl of 100 mM DTT, 1 μl of Rnase inhibitor (10 units/μl), 2 μl of 10 mM dNTPs, and 1 μl of template switch primer (1 μg/μl) were added in RNA/primer mix on ice and heated at 37 °C for 2 min, then added 1 μl of MMLV reverse transcriptase (200 units/μl) in reaction and incubated at 42 °C for 1 hr. Full length ds-cDNA was synthesized by adding 108 μl of DEPC-treated de-ion water, 15 μl of Advantage PCR buffer (Clontech), 3 μl of 10 mM dNTPs, 1 μl of RNase H (2 units), and 3 μl of Advantage cDNA polymerase (Clontech). The reaction mixture was incubated at 37 °C for 2 min (RNA digestion), 94 °C for 3 min (denaturation), 65 °C for 3 min (priming), and 30 min at 75 °C for extension. Reactions were terminated by incubation in 7.5 μl of 1 M NaOH with 2 mM EDTA at 65 °C for 10 min. cDNA was extracted with phenol/chloroform/ isoamylalcohol, then precipitated the pellet with ethanol in the presence of linear acrylamide, and finally resuspended in 16 μl of DEPC water. cDNA was passed through a Bio-6 chromatography column (Bio-Rad, Cambridge, MA) and washed three times with 700 μl DEPC-treated H_2O, and then were lyophilized to 16 μl. *In vitro* transcription was performed in the presence of 7.5 mM each ATP, TTP, CTP, and GTP; 1X reaction buffer; 10 mM DTT; and 2 μl of concentrated T7 RNA polymerase (AmpliScribe kits; EPICENTRE, Madison, USA) in a 20-μl solution. The reaction mixture was incubated at 37 °C for 4 hr, then added 10 units of RNase-free DNase I and stood at 37 °C for 15 minutes. The amplified RNA was extracted by phenol/chloroform and then precipitated by adding of 10 μl of 7.5 M ammonium acetate, 20 μg of linear polyacrylamide as carrier, and 80 μl of absolute alcohol. The solution was mixed evenly and stood at –80 °C for 30 min, and then centrifuged at 14,000 rpm for 20 min to precipitate RNA. Pellet was washed with 1 ml of 70 % of ethanol and dried by speed vacuum, then dissolved in 5 μl or suitable volume of

DEPC-treated deionized water. For the second round of amplification, the first stranded cDNA was synthesized as the same with above mention except 2 μg of random hexamer was used. Second stranded cDNA synthesis was initiated by 1 μg of T7promoter- (T) 24 primer in the conditions used in the first round. *In vitro* transcription of aRNA was carried out as for the first round. The procedures of biotinylated cDNA probe were the same with the above mentioned.

Amplification of signal: Catalyzed Reporter Deposition (CARD) method

To prevent no signal being observed, another amplification method, named CARD (44), is included in the detection procedures if no signal is obtained. After hybridization the membrane was washed with 2X SSC containing 0.1% SDS for 5 min at room temperature followed by three times washes with 0.1X SSC containing 0.1% SDS at 65°C for 15 min each. The membrane was blocked with 7% casein in PBS containing 0.05% Tween 20 (PBST) for 1 h at RT with gentle shaking and then incubated with streptavidin-HRP (1000X) diluted in PBST containing 1% BSA, 0.7% casein, and 4% polyethylene glycol 8000 (Sigma) at room temperature. The membrane was then washed with PBST bufer four times for 5 min each. For amplification, the membranes were incubated with BT at 15 mg/ml in 0.1 M borate buffer, 0.003% H2O2, pH 8.5 for 15 min at RT, and then washed with PBST. The membrane was incubated with streptavidin- -galactosidase (700X) diluted in PBST containing 1% BSA, 0.7% casein, and 4% polyethylene glycol 8000 (Sigma) at room temperature for 1 h. After washing with PBST the color were generated by treating the membrane with 1 ml X-gal substrate containing 1.2 mM X-gal, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ in 1X TBS buffer for 15-30 min at 37°C. The color development reactions were then stopped by 1X PBS containing 20 mM EDTA.

結果與討論

1. 提供晶片服務數量:

共提供 116 人次以及超過 2600 片的晶片代製及實驗代工，詳細資料如下。

基因體計畫申請者（91 年 10 月以後）	PI	Membrane
人類基因微陣列薄膜及實驗代工	4	30
代製微陣列薄膜	3	300
核醣核酸放大反應 (RNA amplification)	4	12

非基因體計畫所有申請者（91 年 2 月-12 月）	PI	Membrane
人類基因微陣列薄膜及實驗代工	41	903
老鼠基因微陣列薄膜及實驗代工	10	253
血管新生基因微陣列薄膜	6	198
癌轉移基因微陣列薄膜	2	25
代製微陣列薄膜	14	912

2. 收費標準:

經使用者委員會確認如下

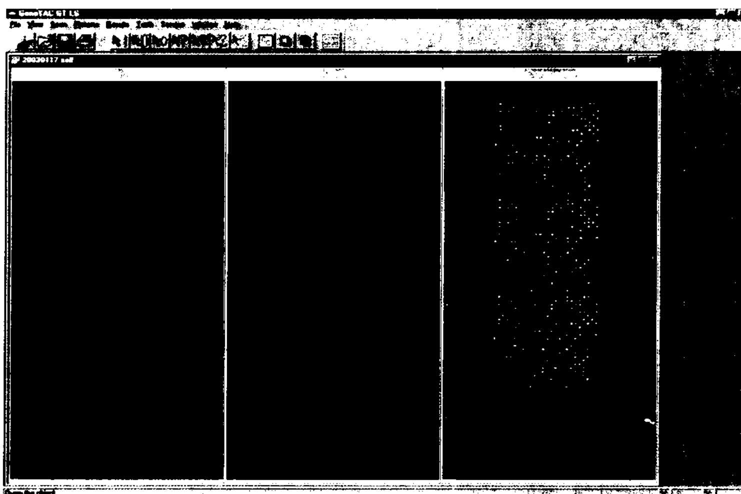
Human gene array	9600 ESTs	20x30 mm	NT 3000/8000

Human gene array	40,000 ESTs		
Mouse gene array	6144 ESTs	20x30 mm	NT 3000/8000
Angio-chip (Sequence verified)	345 ESTs	5x7.5 mm	NT 500/5500
Onco-chip *(Dr. TC Lee)* (Sequence verified)	768 ESTs	5x7.5 mm	NT 500/5500
Customized chip	Spacing 200 um	1 spot	NT 200
RNA amplification			Reaction/NT 3500

3. 晶片的品質控管

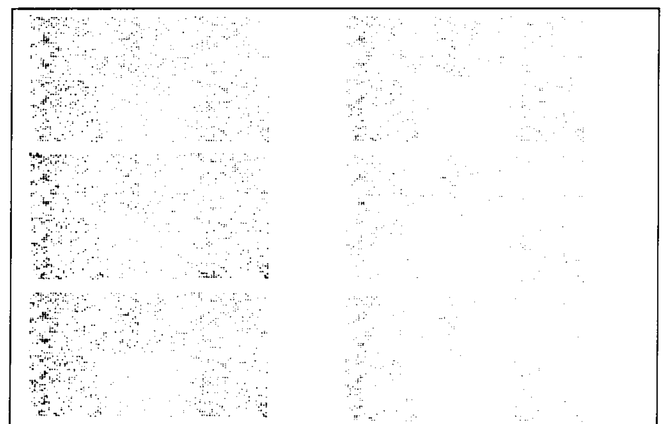
核心實驗室所製備的玻片式晶片和薄膜式晶片的品質舉例如下:

Glass Format



Cy3 label (test RNA) Cy5 label (test RNA) merged image

Membrane Format



One case of service data
(6 time points of time-course experiment)

且經使用者使用後調查，使用者對服務的品質及內容滿意度達 90%以上，顯示本核心實驗室的服務數量以及提供的服務品質，在台灣已居於領先的地位。

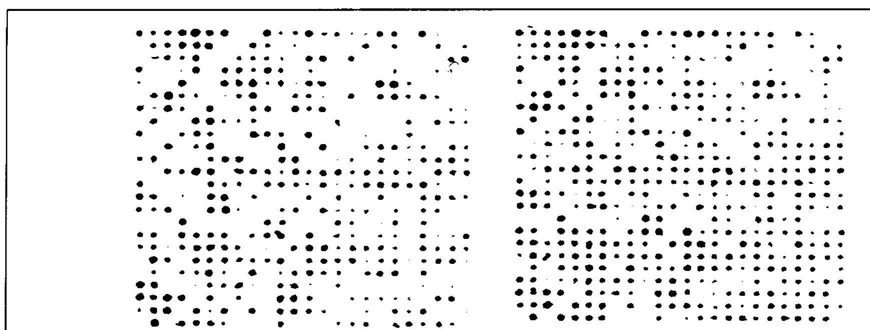
4. 微陣列晶片資料分析:

微陣列晶片資料分析分為 4 個步驟

1. Image Processing: 將微陣列晶片訊號以 flatted scanner (PowerLook 3000, UMAX)掃描並存成 TIFF 檔，在以 GenePix 3.0 (Axon) software 將影像量化為數值，存為 Excel 檔。
2. Rescaling 和 Log transformation: 在 Excel 下作 rescaling，消除晶片堅個別的差異，並作 log transformation。
3. Filtration and Normalization: 以 Cluster software 作差異較大的基因篩選，並將數據作標準化。
4. Clustering: 以 Genecluster (MIT software) 作叢集分析，可選擇 Hierarchical clustering 或 Self-Organizing Maps (SOMs) 分析，並從中選取使用者有興趣的基因表現型。

從 92 年度起並提供 Spotfire software (commercial software)供使用者選用。

5. RNA 放大技術的品質控管:
核心實驗室所執行的 RNA 放大技術的品質控管舉例如下:

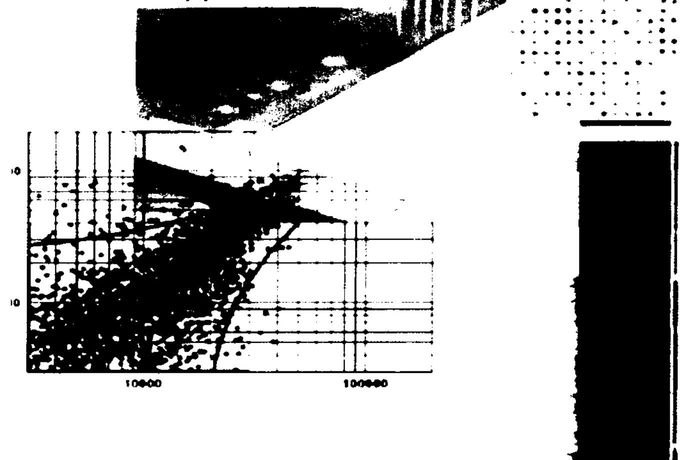


Normal total RNA 5ug　　Tumor total RNA 5ug

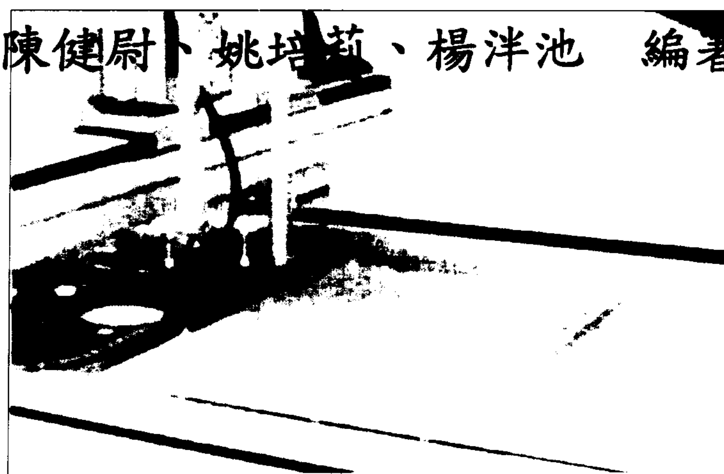
Normal 和 Tumor 檢體的 total RNA (5ug) 經 RNA 放大後的薄膜式晶片的品質，與使用 5ug mRNA 所獲得的訊號相當，顯示 RNA 放大技術適合使用於檢體量極少的研究上。

6. 教育訓練:
九十一年度已經個別為 8 個研究單位提供微陣列晶片操作技術的訓練，同時也為 2 個研究單位，提供核糖核酸放大技術的訓練，同時為推廣微陣列晶片於研究的應用，本實驗室匯集以往的經驗出版了兩本實驗手冊(附圖如下)，協助對微陣列晶片有興趣或有需要的研究人員，儘快地熟悉微陣列晶片的操作與分析。

基因微陣列資料分析實務手冊
第一版



基因微陣列操作儀使用手冊
第一版



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計畫成果自評

91 年度已完成的項目：

一、建立人類基因微陣列：購置序列確認的壹萬個基因供晶片製備，並提供服務。

二、建立老鼠基因微陣列：提供申請者使用於動物模式的基因體研究(約六千個基因)。

三、建立全基因組之人類基因微陣列：購置序列確認但未知功能的三萬個基因並於 92 年度供晶片製備，並提供服務。

四、成功發展玻片式螢光偵測微陣列：提供申請者另外的選擇與服務。

五、晶片代製服務：提供量身定做的晶片服務，申請者只需將有興趣的基因收集好，並以聚合酶鏈鎖反應複製、濃縮後，交由核心實驗室製備晶片。

六、核酸及訊號放大服務：便於申請者應用於少量樣本的基因體研究，如臨床應用。

七、微陣列實驗代工服務：申請者可將核酸樣本(RNA 或 mRNA)委託核心實驗室進行微陣列相關的實驗程序。

八、微陣列資料分析服務：核心實驗室將依委託實驗的類型，提供兩種基本的分析方法服務，即等級(Hierarchical)及自組型態(Self-Organizing Map)叢集分析法。

今年度已經達成 91 年度計劃中規劃的所有目標，達成率為 100%，本實驗室預期可以於三年內完成計劃中的目標。