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黃病毒多重螢光定量分子診斷系統建立

研 究 報 告

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研究主持人：舒佩芸

研究人員：黃智雄、廖采苓、張淑芬、郭郁中、蘇千玲、簡麗蓉、張云憶、游淳如、陳語潔、林鼎翔

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＊本研究報告僅供參考，不代表衛生署疾病管制局意見＊

目　　錄

中文摘要

黃熱病、登革熱、日本腦炎是台灣地區重要的法定傳染病，三者分別屬於黃病毒屬的黃熱病、登革熱及日本腦炎亞群。開發快速的分子診斷系統，監測台灣地區已知存在的黃病毒（登革病毒及日本腦炎病毒），及未來可能會侵入的黃病毒（如黃熱病毒、西尼羅腦炎病毒等）是十分重要的。目前本實驗室利用近年來開發的螢光定量 PCR 方法，已成功開發出 SYBR Green 即時螢光定量 RT-PCR 偵測病毒核酸，並已應用於黃病毒感染之例行性病毒核酸檢驗，不但降低了檢體污染的機率，而且大幅縮短檢驗的時程。為了再提升檢驗的靈敏度及專一性，本計畫開發出黃病毒多重螢光定量 PCR 偵測系統，利用 TaqMan Real-time RT-PCR 方法，配合自動化儀器，開發四色多重螢光檢驗技術，使能在單一試管中同時偵測四種黃病毒（包括登革熱、日本腦炎、黃熱病及西尼羅腦炎病毒等），或區分四種登革病毒血清型別。利用通報至疾管局之各種黃病毒感染的確定病例血清及疾管局歷年來所分離出之登革及日本腦炎病毒株，評估此黃病毒多重螢光定量 PCR 偵測系統之靈敏度及專一性。研究結果顯示此偵測系統具備快速、高靈敏度及高專一性等優點。由於能在單一試管中同時偵測到四種病毒核酸，將可大幅增加實驗室的檢驗能力，以因應登革熱流行時期大量檢體的篩檢，對黃病毒傳染病之防治工作極為重要。

中文關鍵詞

多重螢光定量 PCR 方法，黃病毒、登革病毒

英文摘要

ABSTRACT

Yellow fever, dengue fever, and Japanese encephalitis are three reportable infectious diseases in Taiwan belong to the yellow fever, dengue fever and Japanese encephalitis subgroups of flaviviruses, respectively. Development of rapid, molecular diagnostic system to monitor these already existing (dengue and Japanese encephalitis) and other potentially invading emerging infectious diseases (such as yellow fever, West Nile fever/encephalitis) is important. We had recently successfully developed a SYBR Green I-based quantitative one-step real-time RT-PCR assay for routine diagnosis of various flavivirus infections. This assay system had the advantages of rapidity, high sensitivity, high specificity and low contamination rate in the differential diagnosis of various flavivirus. To further improve the assay efficiency of real-time RT-PCR assay, a four colors multiplex real-time PCR system based on TaqMan amplification was developed to simultaneously detect and differentiate four different viruses in a single tube. Two sets of four colors multiplex real-time PCR systems were developed. The flavivirus-specific assay can detect and differentiate dengue virus, Japanese encephalitis virus, yellow fever virus, and West Nile virus, while dengue virus-specific assay can detect and differentiate four different dengue virus serotypes. Isolated flaviviruses and confirmed serum samples from flavivirus-infected patients reported to CDC, Taiwan were used to evaluate the specificity and sensitivity of these two multiplex real-time PCR assays. The results showed that the multiplex four colors real-time PCR assay constitutes a specific and sensitive alternative to SYBR Green I-based quantitative one-step real-time RT-PCR assay. Specific and sensitive results within 6 hours are important in a clinical setting, and therefore, this assay could

improve patient management by appropriate therapy following rapid diagnosis of a viral infection. The enhanced laboratory capacity to handle large amount of serum samples due to multiplex PCR assay in a single tube could greatly improved the assay efficiency in an outbreak and contribute significantly to the prevention and control measures.

Key words: Flavivirus, Dengue virus, Multiplex, Real-time RT-PCR

前言

由於國際間往來日益頻繁及全球溫室效應的影響，病媒性傳染病在全世界散佈情形正急速增加，其中又以蚊蟲（mosquito）及壁蝨（Tick）所媒介的黃病毒（Flavivirus）傳染病最為重要。為因應未來新興或再浮現傳染病之侵入，我們應建立一套完整的病媒性傳染病防疫體系，包括檢驗設備之硬體設施、病媒監測系統網、快速的檢驗技術與有效的防治策略等，否則將無法應付未來傳染病侵入所造成的危機。目前以實驗室為基礎的黃病毒快速診斷方法, 主要有二種, 一種為利用 ELISA 方法的血清學診斷技術, 另一種則是利用 PCR 技術為基礎的分子診斷方法。本計畫將利用近年來新開發的螢光定量 PCR 方法，建立一套完整的病媒性傳染病分子診斷系統，以快速的監測已知、新興與再浮現病媒性傳染病的侵入。

登革熱及日本腦炎是台灣地區兩種重要的法定傳染病，分別屬於黃病毒屬之登革及日本腦炎病毒亞群，其中日本腦炎是地方性疾病，而登革熱也有本土化的趨勢。日本腦炎亞群中各病毒在全球不同地區分別引起 Japanese encephalitis (JE), Murray Valley encephalitis (MVE), West Nile encephalitis (WN), Saint Louis encephalitis (SLE), Kunjin (KUN), Usutu (USU), Kokobera (KOK), Stratford (STR), Alfuy (ALF)等疾病。黃病毒家族 (Flavivirus family) 為小的、具外套膜的、單股正向 (single stranded,

positive-sense) 的 RNA 病毒。在對人類的致病源中，以黃熱(yellow fever, YF)病毒、登革(dengue)病毒、日本腦炎(Japanese encephalitis, JE)病毒、C 型肝炎病毒(hepatitis C virus)及 tick-borne encephalitis (TBE)病毒流行最為廣泛(Shope 1980；Monath, 1986)。登革病毒的流行區域包括了亞洲、美洲、非洲、大洋洲等熱帶、亞熱帶地區(Gubler, 1997)。隨著病媒蚊的擴散，病毒的種類、病情的嚴重程度、病例的數目和流行地區的分佈都大幅上升，例如 1981-1990 十年間的病例是 1956-1980 二十五年間的兩倍。雖然大部份的登革病毒感染引發的臨床症狀(Burke et al, 1988)為不明顯感染(inapparent infection)，症狀輕微者類似感冒的發燒(mild flu-like undifferentiated fever)或典型登革熱(dengue fever, DF)，但有少數的登革病毒感染者會導致嚴重致死的登革出血熱(dengue haemorrhagic fever, DHF)症狀(Nimmannitya et al, 1969, 1987; WHO, 1997)。登革出血熱在 1950 年代間只見於東南亞的菲律賓及泰國等地，但從 1980 到 1990 的十年間，卻在加勒比海周圍國家蔓延流行。1980 年代末期整個亞洲－太平洋地區也都受到波及，在中國大陸的廣東、海南島及印尼、斯里蘭卡等地紛傳疫情。根據世界衛生組織統計，全世界有超過二十億的人暴露在登革病毒的威脅之下，每年有一千萬以上的新病例發生，登革熱/登革出血熱已成為全世界最重要的蚊媒傳染病。在東南亞許多地方,包括台灣，是登革病毒與日本腦炎病毒重疊流行的地區。臺灣地區在日據時代曾有多次登革熱流行的記錄(Gubler, 1997)，但自民國

31 年的全島性大流行之後，直到民國 70 年才在屏東縣的小琉球再度爆發由第二型登革病毒造成的流行，估計全島近 80%的居民受到感染(謝維詮等，1981；吳盈昌，1986)。民國 76 年高雄屏東地區再度爆發了以第一型登革病毒為主的流行(報告病例 1,123 名，確定病例 527 名)，次年疫情繼續擴大，官方的統計共有 4,389 名確定病例，但實際感染人數估計可能超過五萬人。近年來，由於台灣社會環境的變遷，流行情況逐漸嚴重。而民國 90 年 10 月起在高雄市開始流行的第二型登革病毒，在成功越冬以後，91 年更逐漸擴大其流行範圍，造成高雄市、高雄縣、與屏東縣為主的南部地區大流行，確定病例數超過 5000 人，除了打破 76-77 年的高雄屏東地區官方記載確定病例數外，更創下台灣地區自二次大戰以來最大規模之流行。值得注意的是其中有 242 名病例出現登革出血熱的嚴重臨床症狀，並且有 21 人死亡。今年（93 年）屏東縣市及高雄市自七月起也開始流行的第一型及第四型登革病毒，至十二月累計確定病例已達 300 人以上，以目前的流行現況，顯示登革熱防治的急迫性，已成為臺灣公共衛生上的重要防疫問題。

黃病毒的實驗室診斷，主要靠病毒分離、反轉錄脢/聚合脢鏈鎖反應法(reverse-transcriptase polymerase chain reaction, RT-PCR)、及 Capture IgM and IgG ELISA 血清學方法。其中藉由細胞培養方法分離登革病毒，再以病毒特異性(virus-specific)之單株抗體做免疫螢光染色，仍是目前的 Gold

Standard，但已逐漸被 RT-PCR 方法取代，用以偵測急性期血清中的病毒核酸(RNA) 。而最近幾年快速發展的 real-time quantitative RT-PCR 方法，其靈敏度與傳統的 **nested RT**-PCR 相似，但可降低檢體污染率，檢驗時間可縮短為縮短為 6 小時，未來勢必取代傳統 RT-PCR 方法成為新的 Gold standard，有效提升診斷時效。

目前本實驗室利用近年來開發的螢光定量 PCR 方法，已成功開發出 SYBR Green 即時螢光定量 RT-PCR 偵測病毒核酸，並已應用於黃病毒感染之例行性病毒核酸檢驗，不但降低了檢體污染的機率，而且大幅縮短檢驗的時程。為了再提升檢驗的靈敏度及專一性，本計畫將開發黃病毒多重螢光定量 PCR 偵測系統，利用 TaqMan Real-time RT-PCR 方法，配合自動化儀器，開發四色多重螢光檢驗技術，使能在單一試管中同時偵測四種黃病毒（包括登革熱、日本腦炎、黃熱病及西尼羅腦炎病毒等），或區分四種登革病毒血清型別。利用通報自疾管局之各種黃病毒感染的確定病例血清（Den 1~4 型血清，共約 100 支檢體）及疾管局歷年來所分離出之登革及日本腦炎病毒株，評估此黃病毒多重螢光定量 PCR 偵測系統之靈敏度及專一性。研究結果顯示此偵測系統具備快速、高靈敏度及高專一性等優點。由於能在單一試管中同時偵測到四種病毒核酸，將可大幅增加實驗室的檢驗能力，以因應登革熱流行時期大量檢體的篩檢，對黃病毒傳染病之防治工作極為重要。本計畫的主要工作項目在利用 Stratagene 公司之 Mx 4000

定量 PCR 序列偵測儀及螢光定量 PCR 方法，開發病媒性傳染病臨床診斷的檢驗試劑。若配合傳統病毒學的分離及鑑定病毒、PCR 及核酸定序、血清學 ELISA 方法檢驗病毒之特異性抗原及抗體，則可建立一套完整的病媒性傳染病監測與診斷系統，有效的監測新興及再浮現傳染病的侵入。。

材料與方法

（1） **黃病毒培養**：病毒之體外細胞培養係利用 C6/36 或 Vero 細胞株生產各種黃病毒。經細胞培養大量生產後分裝保存於-70℃中。病毒的種類包括有登革病毒之四型參考病毒株(D1 Hawaii, D2 NGC, D3 H87, D4 H241)及衛生署疾病管制局病媒病毒實驗室自登革熱病人血清中分離出之病毒株(D1 157001, D1 8500600, D1 8500796, D2 454009, D2 454021, D2 466177, D3 8700829, D3 466322, D3 333137, D4 8700544, D4 8900508, D4 768346)。西尼羅病毒(West Nile virus, strain B 956, ATCC)及黃熱病毒(Yellow fever virus, strain 17D, ATCC)係採購自 ATCC。

（2） **病人血清檢體收集**：病人血清包括急性期(症狀出現後 0-7 天)與恢復期(症狀出現後 8-30 天)血清。病人血清收集後，將進行血清學、病毒學及分子生物學之實驗室檢驗，以確認感染源。經實驗室確診為陽性反應血清將加以分裝，儲存於 -70。C 冷凍櫃長久保存。

（3） **引子的設計與合成**：引子的設計可依不同的需要而定，其功能是在有效地擴增模版 DNA 序列。螢光探針則可與引子擴增出的 DNA 序列進行雜交作用，釋出螢光。引子與螢光探針的選擇可依據 Primer Express software (PE Applied Biosystems Inc., Foster City, CA)。螢光探針含有 5’端-reporter dye (6-FAM, Cy5, Texas Red-X, Hex 等)及 3’端-quencher dye (TAMRA 或 BHQ 等)。理論上螢光定量 PCR 的靈敏度可以到達 1~10 copies/Rx，可藉由核酸

引子之設計及純化、檢體核酸的萃取及純化、反應試劑之選擇、反應條件之修正等，改善系統之靈敏度及專一性。偵測黃病毒之引子列於表一。

（4）**抽取病毒核醣核酸 (RNA extraction)**：以 QIAamp Viral RNA Mini Kit (QIAGEN)，純化血清或細胞培養之病毒 RNA。詳細操作步驟見 Kit 之操作說明書。主要原理為利用裝有矽土-膠膜的離心圓柱，可以選擇性的與核醣核酸結合，再經過數次清洗步驟，進而達到純化的目的。其步驟如下：首先將病人血清檢體加入溶解液，分解蛋白質等雜質，同時將核醣核酸脢去活性，再將處理後的血清加到離心圓柱中，使核醣核酸與矽土膜結合，再經過離心及加入清洗液之重覆步驟清洗離心圓柱，最後以純水將核醣核酸洗脫下來。

（5）**TaqMan** 即時螢光定量反轉錄脢／聚合脢鍊鎖反應 (Real-time quantitative RT-PCR Reaction) ：使用 QuantiTect Probe RT-PCR Kit, QIAGEN 為反應試劑。依序加入以下試劑：25麵l 的 2x QuantiTect Probe RT-PCR Master Mix，RNase-free Water，核酸引子，TaqMan 探針，0.5 麵l QuantiTect Probe RT Mix，最後加入 10 麵l 檢體 RNA，反應最終體積為 50麵l。再進行 TaqMan one-step RT-PCR 反應：50°C RT 作用 30 分鐘，PCR 作用 95°C 15 分鐘，45 次循環之 94°C, 15 秒、60°C, 60 秒（讀取螢光值）。反應條件需依照實際實驗結果進行調整。

（6）在設計多重螢光定量 PCR 實驗前，各組引子及探針需先經過單獨測試，其

步驟如下：(1) 找出最適當的引子濃度，在濃度 50~900 nM 作序列稀釋，找出最佳濃度。(2) 找出最適當的探針濃度，在濃度 100~300 nM 作序列稀釋，找出最佳濃度。(3) 找出最適當的 Mg 離子濃度，選擇濃度在 3~8 mM 之間。進行多重螢光定量 PCR 時，先找出適合所有引子及探針的 Mg 離子濃度。(4) 多重螢光定量 PCR 反應開始時，先將病毒 RNA 混合作為 PCR 反應模版，在將各組引子及探針單獨或混合進行反應，找出最適化的引子及探針濃度。(5) 找出最適合的反應溫度及時間條件。

結果

(1) 利用 TaqMan 多重螢光定量 RT-PCR 方法偵測黃病毒：本研究所設計及使用之引子及探針的核酸序列如表一所示。以多重螢光定量 RT-PCR 方法，利用黃病毒共通引子及探針(MFU1-CFD2-R111)，可以偵測所有的黃病毒，除了可偵測四型參考病毒株(D1 Hawaii, D2 NGC, D3 H87, D4 H241)外，由疾病管制局所分離出之其餘 12 種病毒株（包括四型登革熱病毒）均可偵測出。此外，黃病毒屬之日本腦炎病毒、黃熱病毒及西尼羅病毒均可偵測出（表二）。登革病毒共通引子及探針(R117-R118-R119-R120) 可以偵測所有的四型登革病毒，但是不能測到日本腦炎病毒、黃熱病毒及西尼羅病毒，顯示對登革病毒具有專一性（表二）。

(2) 比較單一型與多重型螢光定量 RT-PCR 方法偵測黃病毒：所謂單一型是指每一試管內只放單一種探針，反之，多重型是指每一試管內放入至少兩種或兩種以上，標的不同螢光染劑之探針。圖一所示為單一型螢光定量 RT-PCR 方法所得之標準曲線圖。以四型參考病毒株(D1 Hawaii, D2 NGC, D3 H87, D4 H241)為模板，利用黃病毒共通引子及探針(MFU1-CFD2-R111)，對第一型至第四型登革病毒參考株的靈敏度分別為 0.32，0.30，0.21，0.19 pfu/ml。而利用登革病毒共通引子及探針

(R117-R118-R119-R120)，對第一型至第四型登革病毒參考株的靈敏度分別為 2.74，0.29，0.95，1.22 pfu/ml。圖二所示為多重螢光定量 RT-PCR 方法，以四型參考病毒株(D1 Hawaii, D2 NGC, D3 H87, D4 H241)為模板利用 MFU1-CFD2-R111 對第一型至第四型登革病毒參考株的靈敏度分別為 0.43，5.30，0.12，0.10 pfu/ml。而利用 R117-R118-R119-R120 對第一型至第四型登革病毒參考株的靈敏度分別為 1.52，1.21，1.07，2.52 pfu/ml。由圖一與圖二比較單一型與多重型螢光定量 RT-PCR 方法，利用黃病毒共通引子及探針，則多重型方法對第一型及第二型登革病毒參考株的靈敏度下降(第一型單一型 0.32 vs. 多重型 0.43；第二型單一型 0.30 vs. 多重型 5.3)，以對第二型登革病毒的靈敏度下降最多（下降約 18 倍）。反之，多重型方法對第三型及第四型登革病毒參考株的靈敏度較高(第三型單一型 0.21 vs. 多重型 0.12；第四型單一型 0.19 vs. 多重型 0.10)，但靈敏度的差異不大（約提高 2 倍）。利用登革病毒共通引子及探針，則多重型方法對第二型、第三型及第四型登革病毒參考株的靈敏度下降(第二型單一型 0.29 vs. 多重型 1.21；第三型單一型 0.95 vs. 多重型 1.07；第四型單一型 1.22 vs. 多重型 2.52)，以對第二型登革病毒的靈敏度下降最多（下降約 4 倍），反之，多重型方法對第一型登革病毒參考株的靈敏度較高(第一型單一型 2.74 vs. 多重型 1.52)，但靈敏度的差異不大。

(3) 以多重螢光定量 RT-PCR 方法區分四型登革病毒：表三所示為以多重螢光定量 RT-PCR 方法，利用登革病毒型別專一性引子及探針（NS5 region），分別可偵測不同型別之登革病毒。如 D1P3 探針只對第一型病毒具有專一性，D2P3，D3P3, D4P3 則分別對第二型至第四型病毒具有專一性。表四利用另一組登革病毒型別專一性引子及探針（Core region），也可得到與表三相類似的結果。比較單一型與多重型螢光定量 RT-PCR 方法，結果如圖三，利用登革病毒型別專一性引子及探針（NS5 region），單一型及多重螢光定量 RT-PCR 方法所得之標準曲線圖。單一型方法對第一型至第四型登革病毒參考株的靈敏度分別為 0.06，0.07，0.04，0.02 pfu/ml。多重型方法對第一型至第四型登革病毒參考株的靈敏度分別為 0.08，0.4，0.05，0.08 pfu/ml。多重型方法對第二型及第四型登革病毒參考株的靈敏度下降(第二型單一型 0.07 vs. 多重型 0.4；第四型單一型 0.02 vs. 多重型 0.08)，以對第二型登革病毒的靈敏度下降最多（下降約 5.7 倍）。對第一型及第三型登革病毒參考株的靈敏度，則單一型與多重型螢光定量 RT-PCR 方法之靈敏度的差異不大。

(4) 以多重螢光定量 RT-PCR 方法偵測登革熱病人血清檢體並與 SYBR Green 螢光定量 one-step RT-PCR 方法作比較：圖四所示為登革熱確定病例之急性期血清，以登革病毒型別專一性引子及探針（NS5 region），偵測登革病毒在病人血清中之含量。病毒含量在 10~107 pfu/ml.表五比

較 TaqMan 螢光定量 RT-PCR 方法與 SYBR Green 螢光定量 one-step RT-PCR 方法對病人血清的測試結果。由 Ct 值比較，兩種方法的靈敏度相當。

討論

目前黃病毒實驗室診斷，主要是以傳統之病毒分離、反轉錄脢/聚合脢鏈鎖反應法(reverse-transcriptase polymerase chain reaction, RT-PCR)及血清學方法。在急性期血清中，因抗體尚未大量產生，主要靠 RT-PCR 檢驗，因此我們建立了更快速、特異性更高的螢光定量 Real time one-step RT-PCR 方法，用來取代目前大部分實驗室所使用的 Nested RT-PCR 方法以檢測黃病毒特異性核酸序列。本計畫利用多重螢光定量 RT-PCR 方法，結合特殊的引子及探針以偵測及定量登革病毒。利用黃病毒共通引子及探針對第一型至第四型登革病毒參考株的靈敏度在 0.1~5.30 pfu/ml 之間，登革病毒共通引子及探針的靈敏度在 1.07~2.52 pfu/ml 之間，利用登革病毒型別專一性引子及探針之靈敏度則在 0.05~0.4 pfu/ml。

所謂多重螢光定量 PCR 方法即是在單一試管中可同時加入多種不同螢光標記的探針以偵測不同的核酸序列。利用 Mx 4000 儀器，可以偵測範圍在 400~700 nm 光波長的螢光，故在單一試管中若同時加入四種不同螢光標記(如 6-FAM, HEX, ROX, Cy5)的探針，則可同時偵測四種不同的核酸序列。但由於多重螢光定量 PCR 方法在單一試管中同時加入多條引子及探針，會因引子及探針彼此相互聯結及作用，影響 PCR 反應的靈敏度及專一性，故而引子及探針在設計及使用時均需考慮多重螢光定量 PCR 方法的複雜性。本研究比較單一型與多重型螢光定量 RT-PCR 方法偵測登革病毒。除了少數病毒用多重型方法靈敏度稍微降低外，大多數病毒用單一型或多重型方法所得的差異不大。

本研究利用 Multiplex TaqMan 螢光定量 PCR 方法偵測黃病毒，此種方法具備快速、高靈敏度及高專一性等優點，而且由於能在單一試管中同時偵測到四種病毒核酸，將可大幅增加實驗室的檢驗件數，以因應登革熱流行時期大量檢體的篩檢，對黃病毒傳染病之防治工作極為重要。不但靈敏度高，優於傳統 Nested RT-PCR 方法，而且步驟簡單、易操作，是非常適合病媒性病毒的分子診斷方法。

結論與建議

由於全球溫室效應影響，病媒性傳染病在世界各地散佈情形正急速增加，建立一套完整的病媒性傳染病診斷系統（病毒學、血清學及分子診斷），監測台灣地區已知存在的黃病毒（登革病毒及日本腦炎病毒）及未來可能會侵入的病媒性傳染病是十分重要的。本研究利用 Multiplex TaqMan 螢光定量 PCR 方法偵測黃病毒，此種方法具備快速、高靈敏度及高專一性等優點，而且由於能在單一試管中同時偵測到四種病毒核酸，將可大幅增加實驗室的檢驗件數，以因應登革熱流行時期大量檢體的篩檢，對黃病毒傳染病之防治工作極為重要。

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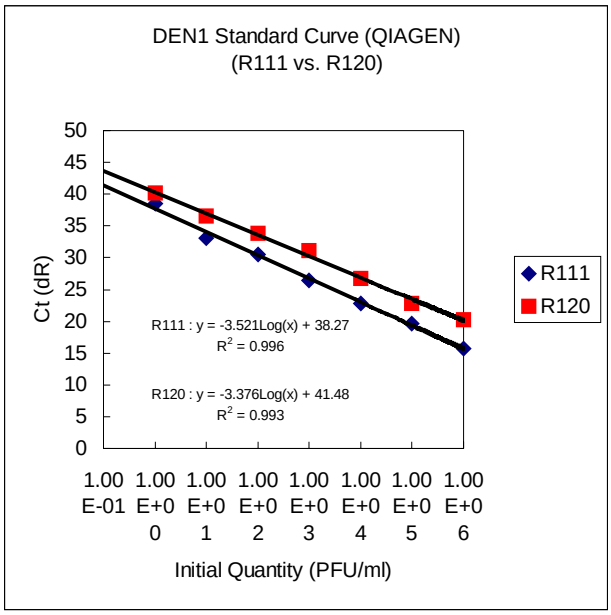
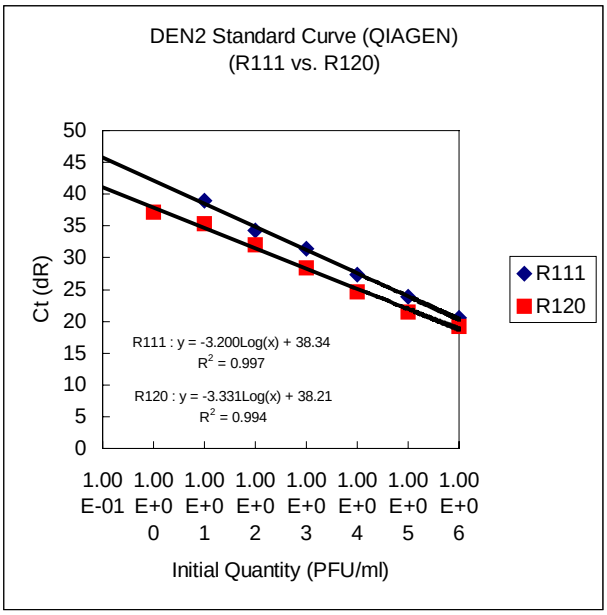
圖一. Standard curves of four dengue virus serotypes tested by TaqMan quantitative RT-PCR using flavivirus-consensus probe (R111) and-dengue virus group-specific probe (R120). Standard curves were generated from the amplification plots of each of the four dengue strains representing DEN-1 (Fig. 1A), DEN-2 (Fig. 1B), DEN-3 (Fig.1C), and DEN-4 (Fig. 1D). The starting viral titer (PFU/ml) was plotted against the Ct value of each dilution.

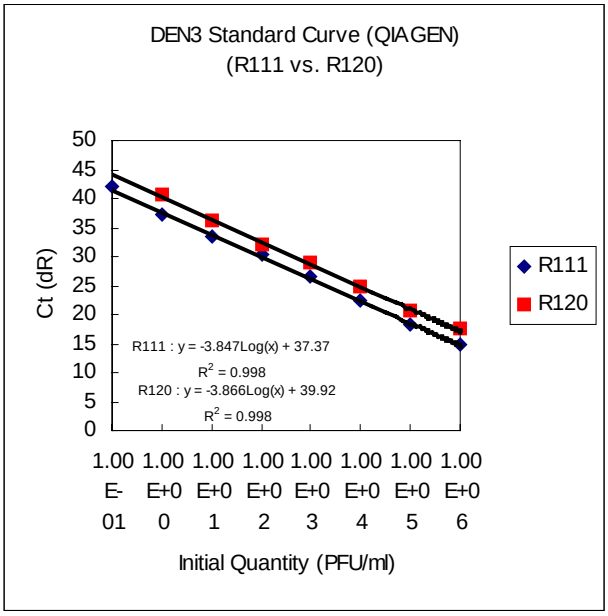
1A



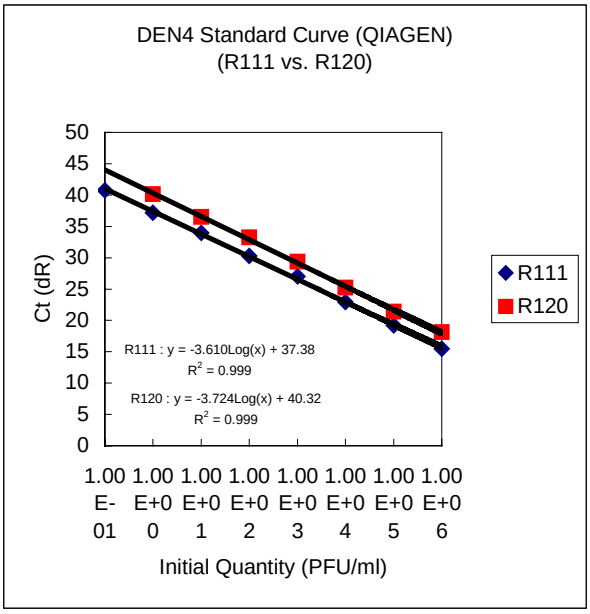
1B



1C

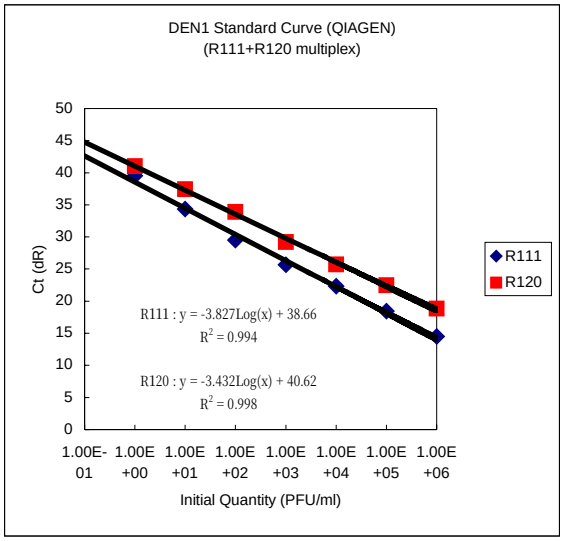


1D

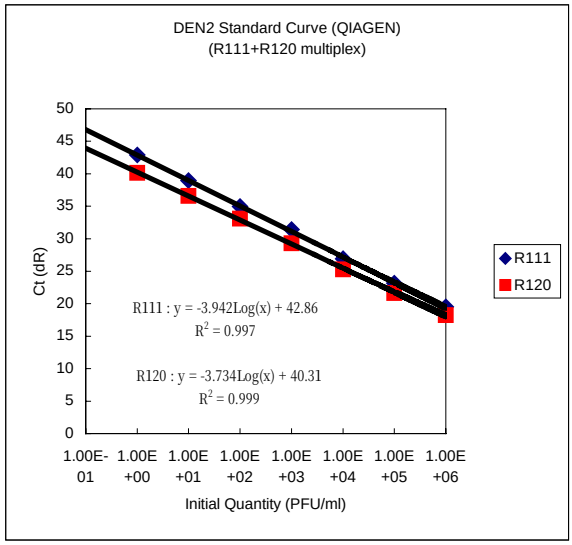


圖二. Standard curves of four dengue virus serotypes tested by multiplex TaqMan quantitative RT-PCR using flavivirus consensus probe (R111) and dengue virus group-specific probe (R120). Standard curves were generated from the amplification plots of each of the four dengue strains representing DEN-1 (Fig. 2A), DEN-2 (Fig. 2B), DEN-3 (Fig.2C), and DEN-4 (Fig. 2D). The starting viral titer (PFU/ml) was plotted against the Ct value of each dilution.

2A



2B



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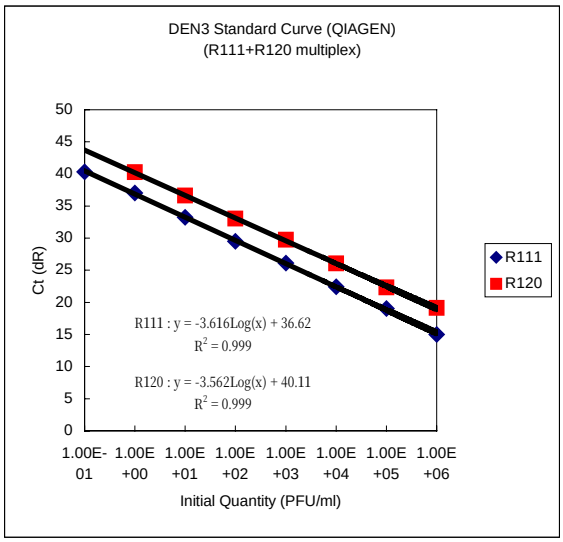
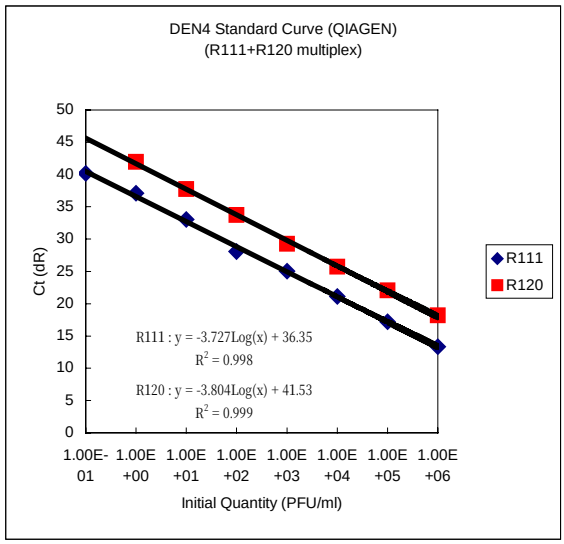
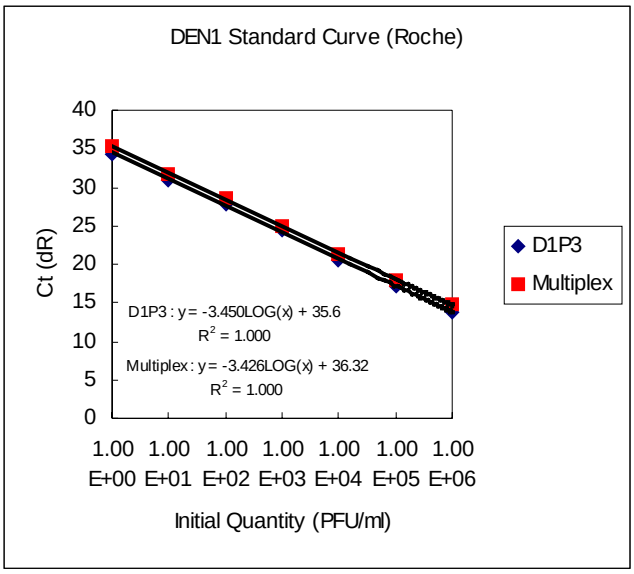


2D

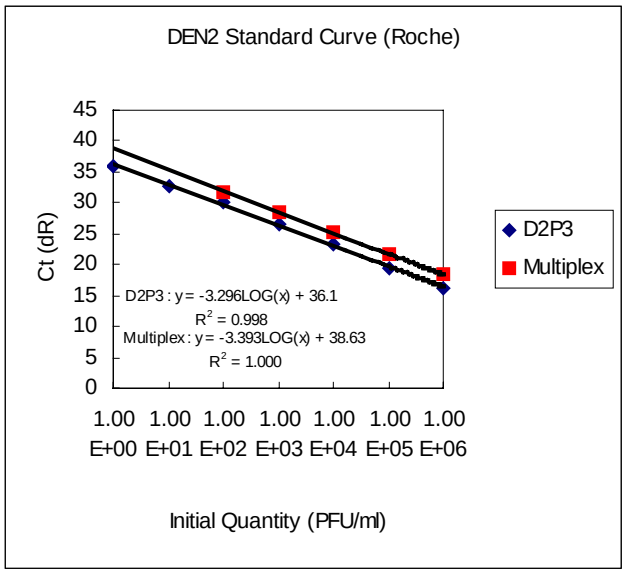


圖三 Standard curves of four dengue virus serotypes tested by simple (D1P3~D4P3)and multiplex (multiplex)TaqMan quantitative RT-PCR using dengue serotype specific probe. Standard curves were generated from the amplification plots of each of the four dengue strains representing DEN-1 (Fig. 3A), DEN-2 (Fig. 3B), DEN-3 (Fig. 3C), and DEN-4 (Fig. 3D). The starting viral titer (PFU/ml) was plotted against the Ct value of each dilution.

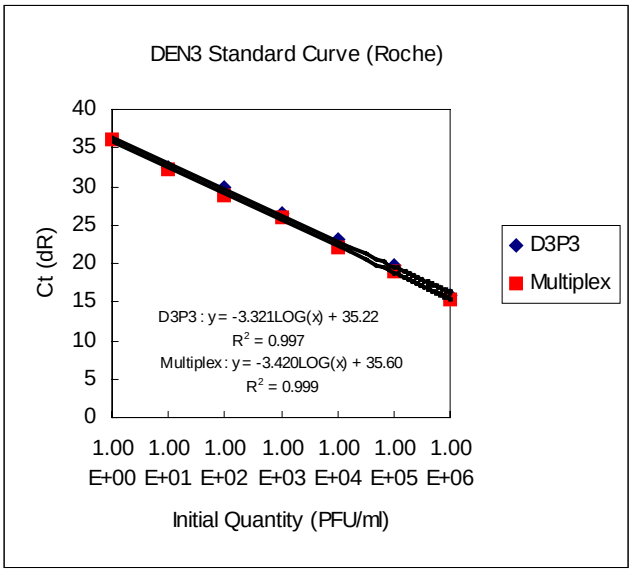
3A



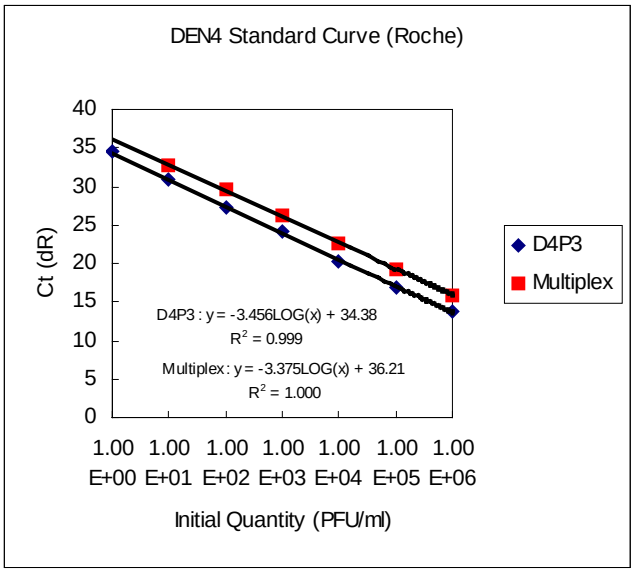
3B



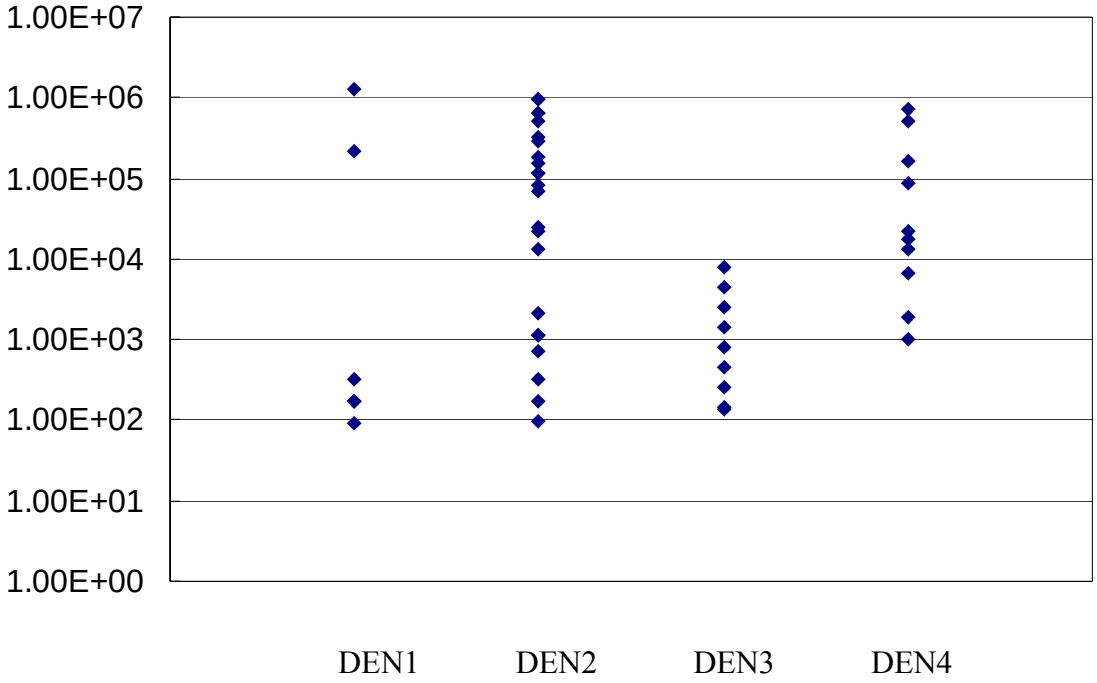
3C



3D



圖四、利用 Flavivirus consensus probe，以 TaqMan quantitative RT-PCR 方法偵測四型登革熱病人血清檢體之病毒含量，結果顯示登革病毒含量在 10~10^7 PFU/ml 之間。



表一、黃病毒 SYBR Green 與 TaqMan 螢光定量 RT-PCR 所使用之引子及探針

Primer/Probe	Sequence	Specificity	Reference
MFU1	TAC AAC ATG ATG GGA AAG CGA GAG AAA AA	Flavivirus	CDC/USA, Chang
CFU2	GTG TCC CAG CCG GCG GTG TCA TCA GC	Flavivirus	CDC/USA, Chang
R111	FAM-CTC CCA GCC ACA TGT ACC ATA TGG C-B	Flavivirus	CDC/Taiwan, Shu
D1-P3	FAM-TCA GAG ACA TAT CAA AGA TTC AGG GGG	DEN-1	CDC/USA, Chang
D2-P3	ROX-CAT TCC ATT TTC TGG CGT TC	DEN-2	CDC/USA, Chang
D3-P3	CY5-TGA GAG ATA TTT CCA AGA TAC CCG GAG GAG	DEN-3	CDC/USA, Chang
D4-P3	HEX-TGG AGG AGA TAG ACA AGA AGG ATG GAG ACC	DEN-4	CDC/USA, Chang
R36	CAA TAT GCT GAA ACG CGA GAG AAA	Dengue virus	CDC/Taiwan, J Clin. Microbiol. 41:2408
R115	CCC CAT CTA GCC AAA ATT CCT GCT	Dengue virus	CDC/Taiwan, J Clin. Microbiol. 41:2408
R116	CCC CAT CTC TTC AGA ATC CCT GCT	Dengue virus	CDC/Taiwan, J Clin. Microbiol. 41:2408
R125	ROX-CTA GAA ATC TTA GGA ATG CTA TRA AA	DEN-1	CDC/Taiwan, Shu
R122	FAM-TGT TAG GAA ACG AAG GAA CGC CAC CA	DEN-2	CDC/Taiwan, Shu
R107	HEX-TGA GGA AAG CTA TGA ACG CCA TA	DEN-3	CDC/Taiwan, Shu
R114	CY5-CCC TCA AGG GTT GGT GAA GAG A	DEN-4	CDC/Taiwan, Shu
R117	GGA TAG ACC AGA GAT CCT GCT GT	Dengue virus	Drosten et al. 2003
R118	CAT TCC ATT TTC TGG CGT TC	Dengue virus	Drosten et al. 2003

R119	CAA TCC ATC TTG CGG CGC TC	Dengue virus	Drosten et al. 2003
R120	HEX-CAG CAT CAT TCC AGG CAC AG	Dengue virus	Drosten et al. 2003

表二 Detection and differentiation of various flaviviruses by multiplex TaqMan quantitative using flavivirus consensus- and dengue group-specific primers and probes.

Strain	DEN Genotype	Taqman	
		MFU1-CFD2- R111 (flavi-consensus) (FAM)	R117-R118+R119-R120 (Dengue-consensus) (HEX)
DEN-1 Hawaii	I	+ (18.89)	+ (20.67)
DEN-1 157001	I	+ (20.22)	+ (20.77)
DEN-1 8500660	I	+ (17.18)	+ (17.42)
DEN-1 8600098	I	+ (19.15)	+ (20.61)
DEN-2 N.G.C	IA	+ (16.04)	+ (17.94)
DEN-2 466177	IB	+ (25.24)	+ (22.64)
DEN-2 454009	IC	+ (19.62)	+ (19.1)
DEN-2 454021	IC	+ (21.51)	+ (20.25)
DEN-3 8700829	I	+ (15.73)	+ (17.36)
DEN-3 H87	II	+ (15.94)	+ (18.7)
DEN-3 466322	II	+ (23.12)	+ (24.93)
DEN-3 333137	II	+ (19.44)	+ (21.69)
DEN-4 H241	I	+ (15.6)	+ (18.11)
DEN-4 768346	I	+ (19.31)	+ (19.89)
DEN-4 8900508	II	+ (21.07)	+ (28.01)
DEN-4 8700544	?	+ (18.75)	+ (24.39)
JEV (JaGAr)		+ (18.76)	- (no Ct)
JEV (PK1)		+ (22.35)	- (no Ct)
Yellow fever virus		+ (23)	- (no Ct)
West Nile virus		+ (19.08)	- (no Ct)
NTC		- (no Ct)	- (no Ct)

表三: Detection and differentiation of various flaviviruses by multiplex TaqMan quantitative RT-PCR using serotype-specific(D1P3, D2P3, D3P3, D4P3) probes.

Strain	D1P3-D2P3-D3P3-D4P3 (dengue type specific probe)			
	D1P3 (FAM)	D2P3 (ROX)	D3P3 (CY5)	D4P3 (HEX)
DEN-1 Hawaii	+ (15.02)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-1 157001	+ (13.91)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-1 8500660	+ (12.41)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-1 8600098	+ (16.01)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-2 N.G.C	- (no Ct)	+ (12.81)	- (no Ct)	- (no Ct)
DEN-2 466177	- (no Ct)	+ (19.1)	- (no Ct)	- (no Ct)
DEN-2 454009	- (no Ct)	+ (15.33)	- (no Ct)	- (no Ct)
DEN-2 454021	- (no Ct)	+ (16.04)	- (no Ct)	- (no Ct)
DEN-3 8700829	- (no Ct)	- (no Ct)	+ (13.81)	- (no Ct)
DEN-3 H87	- (no Ct)	- (no Ct)	+ (13.19)	- (no Ct)
DEN-3 466322	- (no Ct)	- (no Ct)	+ (19.49)	- (no Ct)
DEN-3 333137	- (no Ct)	- (no Ct)	+ (17.14)	- (no Ct)
DEN-4 H241	- (no Ct)	- (no Ct)	- (no Ct)	+ (13.73)
DEN-4 768346	- (no Ct)	- (no Ct)	- (no Ct)	+ (16.16)
DEN-4 8900508	- (no Ct)	- (no Ct)	- (no Ct)	+ (17.15)
DEN-4 8700544	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
JEV (JaGAr)	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
JEV (PK1)	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
Yellow fever virus	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
West Nile virus	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
NTC	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)

表四、Detection and differentiation of various flaviviruses by multiplex TaqMan quantitative RT-PCR using serotype-specific(R125, R122, R107, R114) probes.

DEN Genotype Strain	Taqman C (R36-R115-R116-R125-R122-R107-R114) (200-200-200-*400-300-300-300* nM)			
	R125 (ROX)	R122 (FAM)	R107 (HEX)	R114 (CY5)
DEN-1 Hawaii	+ (21.56)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-1 157001	+ (20.1)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-1 8500660	+ (20.01)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-1 8600098	+ (22.88)	- (no Ct)	- (no Ct)	- (no Ct)
DEN-2 N.G.C	- (no Ct)	+ (14.95)	- (no Ct)	- (no Ct)
DEN-2 466177	- (no Ct)	+ (19.79)	- (no Ct)	- (no Ct)
DEN-2 454009	- (no Ct)	+ (19.03)	- (no Ct)	- (no Ct)
DEN-2 454021	- (no Ct)	+ (18.57)	- (no Ct)	- (no Ct)
DEN-3 8700829	- (no Ct)	- (no Ct)	+ (17.33)	- (no Ct)
DEN-3 H87	- (no Ct)	- (no Ct)	+ (20.29)	- (no Ct)
DEN-3 466322	- (no Ct)	- (no Ct)	+ (24.45)	- (no Ct)
DEN-3 333137	- (no Ct)	- (no Ct)	+ (23.31)	- (no Ct)
DEN-4 H241	- (no Ct)	- (no Ct)	- (no Ct)	+ (16.21)
DEN-4 768346	- (no Ct)	- (no Ct)	- (no Ct)	+ (18.66)
DEN-4 8900508	- (no Ct)	- (no Ct)	- (no Ct)	+ (21.21)
DEN-4 8700544	- (no Ct)	- (no Ct)	- (no Ct)	+ (18.81)
JEV (JaGAr)	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
JEV (PK1)	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
Yellow fever virus	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
West Nile virus	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)
NTC	- (no Ct)	- (no Ct)	- (no Ct)	- (no Ct)

表五、SYBR Green 與 TaqMan 方法對病人血清測試結果

Patient's serum	SYBR Green* (Ct 值)	TaqMan** (Ct 值)
S9201814	20.14	19.15
S9201818	26.15	27.63
S9201839	15.59	19.66
S9201840	18.52	16.38
S9201842	36.97	No Ct
S9201850	21.37	19.4
S9201852	18.36	16.52
S9201733	22.44	18.36
S9201814	20.14	18.13
KSD8594	24.59	18.83
KSD8598	25.34	23.05
KSD6843	22.62	12.08
KSD6846	39.84	No Ct
KH1719	24.78	17.52
KSD10209	29.59	24.65
KH1667	27.16	27.59
KH1671	17.4	18.19
KSD10358	30.11	24.15
KSD10754	25.21	21.5
KSD10357	24.37	18.63
KSD7844	23.45	22.53
KSD7929	24.66	24.43
H9201821	23.31	16.98
H9201832	27.35	20.54
26568	38.3	32.69
26611	26.13	25.03

*SYBR Green 方法所使用之引子為 R36, R115, R116

**TaqMan 方法所使用之引子為 MFU1/CFU2/R111