摘要

為執行建立潛在及新興重大人畜共通傳染病監測及防禦體系國際合作計畫於99年10月30日至11月7日奉派赴美國疾病管制局進行技術研習，研習主題為蝙蝠麗沙病毒診斷與監測技術，順利完成前述新浮現與再浮現人畜共通傳染病診斷技術，包括反轉錄聚合酶鏈反應、快速螢光斑點抑制試驗及試驗用野生動物樣本採集等研習，返國後可應用於重大人畜共通傳染病診斷與監測，提供疫病篩檢與啓動早期預警系統。此次研習所用檢體來自行政院農業委員會家畜衛生試驗所99年度於國內採集之蝙蝠血清及腦組織，研習期間檢測蝙蝠血清抗體75例，初步完成2種麗沙病毒- Irkut virus及Khujand virus之抗體檢測，結果均呈陰性，其它麗沙病毒之抗體檢測將委由美國疾病管制局專家進行，此項檢測成果可供作臺灣為狂犬病非疫區佐證資料，期待臺美之間仍能持續針對蝙蝠等野生動物之新浮現與再浮現人畜共通傳染病，進行更深入的合作研究。

目的

本所（行政院農業委員會家畜衛生試驗所）肩負著國內動物傳染病防治技術研發與疾病檢驗診斷的重任，爲防範跨國界動物傳染病與新浮現人畜共通傳染病入侵，持續派遣診斷人員赴美、日、荷等國研習重要動物傳染病診斷技術，俾以強化相關疫病監測與診斷技能。近年來由於農業生產型態改變、人類活動增加及氣候變遷等，致使蝙蝠及浣熊等野生動物與人類或家畜接觸機會增加，促使新浮現與再浮現人畜共通傳染病遽增且傳播範圍擴大，而成爲重要議題。爲有效防堵新浮現與再浮現人畜共通傳染病入侵，除在國境內及國家邊境或疆界進行疫病診斷與監測，透過國際性或區域性合作進行防堵亦爲執行重點，行政院農業委員會家畜衛生試驗所爲執行野生動物新浮現疾病診斷與監測之臺美合作計畫，於99年10月30日至11月7日遴選1位研究人員，赴美國疾病管制局進行技術研習，研習主題爲蝙蝠麗沙病毒診斷與監測技術，順利完成前述新浮現與再浮現人畜共通傳染病診斷技術，包括反轉錄聚合酶鏈反應、快速螢光斑點抑制試驗及試驗用野生動物樣本採集等研習，返國後可應用於重大人畜共通傳染病診斷與監測，提供疫病篩檢與啓動早期預警系統。此次研習所用檢體來自行政院農業委員會家畜衛生試驗所99年度與台灣蝙蝠學會合作於國內採集之蝙蝠血清及腦組織，研習期間檢測蝙蝠血清抗體75例，初步完成

2種麗沙病毒- Irkut virus及Khujand virus之抗體檢測，結果均呈陰性，其它麗沙病毒之抗體檢測將委由美國疾病管制局專家進行，此項檢測成果可供作臺灣爲狂犬病非疫區佐證資料，期待臺美之間仍能持續針對蝙蝠等野生動物之新浮現與再浮現人畜共通傳染病，進行更深入的合作研究。

研習行程表

日期	研習內容
10/31(日)	搭機赴美
11/1(一)	進行” 快速螢光斑點抑制試驗(Rapid Fluorescent Focus Inhibition Test；RFFIT)”
11/2(二)	進行” 反轉錄聚合酶鏈反應(Reverse transcription polymerase chain reaction; RT-PCR) “
11/3(三)	進行” 快速螢光斑點抑制試驗(Rapid Fluorescent Focus Inhibition Test；RFFIT)”
11/4(四)	學習試驗用野生動物樣本採集技術
11/5(五)	進行” 快速螢光斑點抑制試驗(Rapid Fluorescent Focus Inhibition Test；RFFIT)”
11/6(六)~11/7(日)	搭機返國

研習行程

本次感謝本所（行政院農業委員會家畜衛生試驗所）李淑慧組長與長官們提供寶貴機會赴美國疾病管制局研習，亦承蒙狂犬病診斷實驗室主持人 Dr. Charles E. Rupprecht 同意赴其實驗室研習並與 Dr. Wun-Ju Shieh 熱心提供研習期程建議，此外 Pamela 女士協助繁複申請程序，Dr. Ivan V Kuzmin 指導蝙蝠麗沙病毒抗體檢測及反轉錄聚合酶鏈反應試驗，Dr. Sheiah 熱心協助研習事宜，楊淑珠女士等師長照料研習期間事宜，方能順利完成研習，不勝感激。茲將研習相關技術及實驗室檢驗行程概述於下

一、研習技術：

1、快速螢光斑點抑制試驗(Rapid Fluorescent Focus Inhibition Test；RFFIT)：本法爲現行中和抗體檢測技術之改良版，現行中和抗體檢測係使用 Teflon-coated chamber slide 進行待測血清與病毒感作、細胞培養及結果判讀，本法改用 Teflon-coated 孔洞玻片，僅須少量血清即可進行檢測(按檢測流程：檢測一種麗沙病毒抗體約須 3.5 μL 血清)，若以 Teflon-coated chamber slide 法進行一種麗沙病毒之抗體檢測，則檢測一種麗沙病毒之抗體，須使用 50 μL 血清，適用於人類、犬、貓、狐貍等動物檢測，蝙蝠因體型較小不易採集足量血清，故發展出使用

Teflon-coated 孔洞玻片的方法，檢測流程略述如下：血清樣本進行稀釋，滴入 Teflon-coated 玻片孔洞，加入 MEM 培養液混合，視欲檢測之 lyssavirus 種類，加入病毒液(病毒力價約 28~100 focus-forming units)感作約 90 分鐘，加入 MNA 細胞培養 20~44 小時(時間長短取決於病毒生長速度)，經丙酮固定及 Fluorescein isothiocyanate (FITC)標示之狂犬病抗體染色，在螢光顯微鏡下檢視，若血清樣本含有狂犬病病毒或某種 lyssavirus 之抗體，則於較低稀釋階，因病毒被抗體中和，MNA 細胞無綠色螢光，至血清稀適至某一倍數，因抗體濃度不足以中和病毒，則 MNA 因有病毒複製被染出綠色螢光，此一稀適階便爲抗體力價，進行結果判讀時，係以 20X 物鏡讀取 10 個視野，紀錄可看到綠色螢光的視野數，若出現綠色螢光的視野數小於 5 個，則判定具有中和抗體。進行血清樣本檢測時以相同稀釋倍數依據螢光有無或是否減少，篩檢出疑似具有抗體樣本，再以較細緻之稀釋階進一步決定其抗體力價。

2、反轉錄聚合酶鏈反應(Reverse transcription polymerase chain reaction; RT-PCR)：本試驗針對 Lyssavirus 病毒之核蛋白設計特異引子 N1F 及 N550B，及巢式反應用引子- N70F 及 N490B 進行檢體中 Lyssavirus 核酸之檢測，檢體進行增幅反應時須同時與陽性對照及陰性對照一同進行(引子序列如表 1)。本所（行政院農業委員會家畜衛

生試驗所）採集蝙蝠腦組織與 MEM 培養液混合研磨成 10%（W/V）乳劑，再利用 MagNA Pure Compact 自動核酸萃取儀進行核酸萃取，核酸可保存於冷凍或即時進行反轉錄聚合酶鏈反應及巢式聚合酶鏈反應。

Table1 . The primers used for detection of lyssavirus

primer	nucleotide(3’-5’)	target gene
N1F	ATG GAK TCW GAM AAS ATT GT	nucleocapsid of lyssavirus
N550B	GTR CTC CAR TTA GCR CAC AT	
N70F	GAYCAATATGARTATAARTA	nucleocapsid of lyssavirus
N490B	TCCATYCTRTCTGCWACATT	

3、試驗用野生動物樣本採集：本次研習亦學習紅狐及蝙蝠的保定與採樣技術。保定紅狐時由一位同仁負責用套圈或繫繩保圈住動物口吻部並固定動物頭部與身軀，另一位同仁以犬貓用鎮靜劑注射於動物，俟動物昏睡後進行外觀檢查後採取血液及拭子等樣本。進行蝙蝠保定時將蝙蝠捉握於左手掌內，露出些許頭部，姆指抵住其下顎上緣，右手拉出左側翼膜，另一人員以左手拉出尾膜，右手持注射針尖端輕輕刺破尾膜上 uropatagial vein，以毛細管收集血液後，以刀傷藥止血放回飼養籠。爲確保操作安全，操作前須穿戴防護衣、帽、工作

靴，並戴厚手套。

二、國內蝙蝠狂犬病及麗沙病毒監測樣本之檢測：

行政院農業委員會家畜衛生試驗所為進行國內蝙蝠狂犬病及麗沙病毒監測，與台灣蝙蝠學會合作進行族群調查與採樣監測，99 年於台灣地區採集蝙蝠血清及腦組織，計收集病弱或死亡蝙蝠之腦組織 59 例及外表健康蝙蝠之血清樣本 75 例供作狂犬病及麗沙病毒監測用，本次研習經由實驗室合作模式將蝙蝠血清樣本 75 例，送至美國疾病管制中心(Centers for Disease Control; CDC)之狂犬病診斷實驗室進行檢測，血清以快速螢光斑點抑制試驗 (RFFIT)檢測，研習期間完成 2 種 lyssavirus- Irkut virus, Khujand virus 之抗體檢測，結果均呈陰性，後續委由美國疾病管制管局專家進行其它 lyssavirus 之抗體檢測；期間亦實際操作 lyssavirus 之分子檢測，Dr. Ivan Kuzmin 係於 lyssavirus 核蛋白(nucleoprotein)基因之高度保留區域設計 2 組引子對- 外部引子、內部引子，以反轉錄聚合酶鏈反應(RT-PCR)搭配巢式聚合酶鏈反應(nest PCR)進行 lyssavirus 核酸檢測，本次研習係以 Lagos bat virus 及美國境內狂犬病病毒分離株作為測試樣本進行檢測，結果皆可得到預期的增幅產物。

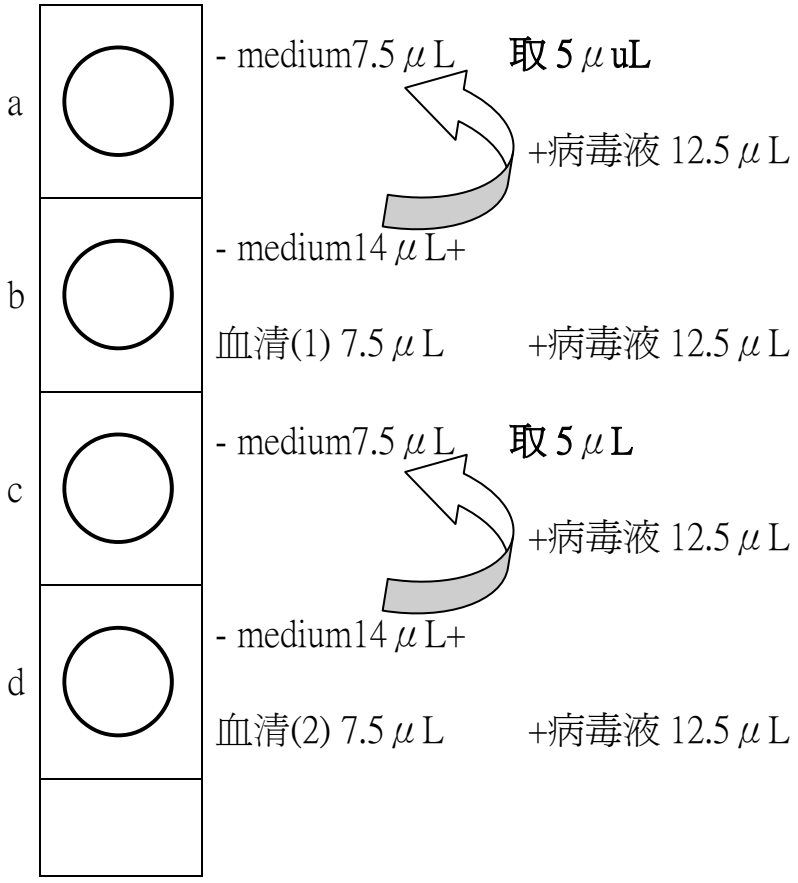
三、實驗室檢驗行程：

11 月 1 日（一）

上午由 Dr. Xian Fu Wu 至訪客中心帶領進入痘病毒與狂犬病分部門之狂犬病診斷實驗室，於實驗室與主持人 Dr. Charles E. Rupprecht 及 Dr. Ivan Kuzmin 與 Dr. Xian Fu Wu 討論本次實驗室研習與臺灣蝙蝠樣本檢驗規劃，受限於研習時間，決定以蝙蝠血清抗體檢測爲主軸，前半段時間(約 2 天半)進行 Irkut virus 抗體檢測，後半段時間則檢測 Khujand virus 抗體，空檔期間實際操作反轉錄聚合酶鏈反應，並觀摩試驗用野生動物樣本採集技術。

下午以國內蝙蝠血清樣本 75 例爲研習資材進行快速螢光斑點抑制試驗(Rapid Fluorescent Focus Inhibition Test；RFFIT)，檢測流程如下~血清離心後進行非働化，取 Teflon-coated 4 孔玻片(每例血清樣本須使用 2 孔，每片可檢測血清 2 例)，於孔洞 a、b、c、d 加入 medium7.5、14、7.5、及 14 μL，於孔洞 b、d 分別加入血清 1 及 2 各 7.5 μL，混合均勻後各吸取 5 μL 分別加入孔洞 a 及 c 混合均勻，於每孔中各加入 Irkut virus 病毒液 12.5 μL，混合均勻後置於 37℃ 、0.5%CO_2 培養 1 小時，加入 MNA 細胞 25 μL，混合均勻後置於 37℃ 、0.5%CO_2 培養 40 小時(圖一至圖四)。

Dr. Ivan Kuzmin 補充道進行血清抗體檢測時宜使用 2~3 天的 MNA 細胞，4 天以上的 cell 因老化，不適合用於細胞繼代。



(Teflon-coated 4 孔玻片)

11 月 2 日（二）

上午由 Dr. Ivan Kuzmin 指導進行反轉錄聚合酶鏈反應(RT-PCR); 本試驗針對 Lyssavirus 病毒之核蛋白設計特異引子 N1F 及 N550B，及巢式反應用引子- N70F 及 N490B 進行檢體中 Lyssavirus 核酸之檢測。操作流程如下：

(1)、反轉錄聚合酶鏈反應：正向引子（10 μM,）1 μL 混合檢體 RNA 5 μL，置於 PCR machine 於 94℃進行 denature 1 分鐘，移置冰上。加入 RT Mix14 μL，於 42℃進行反轉錄 90 分鐘。RT Mix： PCR Nucleotide mix(10 mM each dNTP) 150 μL 、 AMV(1000U, 22U/μL) 4 μL 、 RNasin(2000U, 40U/μL) 4 μL。

(2) 聚合酶鏈反應(Polymerase chain reaction; PCR)：

H_2O 750 μL、Tris Buffer 86 μL、正向引子（10 μM,）11 μL、反向引子（10 μM）14 μL、*Taq*（5U/μL）5.5 μL，混合後每管加入 89 μL。反應設定時間溫度： 94℃，1 分鐘；94℃，30 秒；37℃，30 秒；72℃，90 秒進行 40 個循環，72℃ ，8 分鐘。

(3) 巢式聚合酶鏈反應(nest PCR)：

H_2O 750 μL、Tris Buffer 86 μL、RT Mix140 μL、正向引子（10 μM,）14 μL、反向引子（10 μM）14 μL、*Taq*（5U/μL）5.5 μL，混合後每管加入 89 μL。

增幅的產物與 6×dye 混合後，放入 2%電泳膠片，以 100 伏特電壓進行電泳。電泳完成後，將膠片置於電泳膠片照相系統觀察(圖九)。

11 月 3 日（三）

上午重複 11 月 1 日操作之快速螢光斑點抑制試驗(RFFIT)前半段流程，進行 Khujand virus 抗體檢測。

延續 11 月 1 日操作之快速螢光斑點抑制試驗(RFFIT)，繼續進行 Irkut virus 抗體檢測與結果判讀；自培養箱取出 Teflon-coated 4 孔玻片，甩掉孔洞內液體，泡入 PBS(0.01 M，pH7.5)中，取出玻片浸入冰冷的 aceton 進行固定(至少 25 分鐘)，將 aceton 倒入廢液筒，取出玻片置於空氣中風乾，將玻片置於 wet chamber(鐵盤上鋪上濕潤的擦手紙，再以數個 pipet tip 盒蓋蓋在擦手紙上)，使用 1mL 針筒抽取狂犬病螢光標示抗體（FITC anti-rabies monoclonal globulin，FDI Inc），於每個孔洞加入 1~1.5 滴抗體(使抗體充滿每個孔洞，且液體表面呈圓形突起)，置於 37℃、0.5%CO_2 感作 40 分鐘，浸泡於 PBS 中 10 分鐘，取出玻片置於空氣中風乾 60 分鐘，置於螢光顯微鏡下以 20X 物鏡進行判讀(圖五至圖八)。

11 月 4 日（四）

狂犬病診斷實驗室同仁將試驗動物飼養於亞特蘭大郊外之試驗動物設施，本日在狂犬病實驗室 James A. Ellen 及同仁引導下見習紅狐貍(Red fox)及蝙蝠之飼養與檢體採集。

試驗用野生動物飼養室爲長形空間，中央有一走道，兩側各有一長排籠舍，James A. Ellen 表示如同紅狐貍飼養狀況，試驗動物飼養於不鏽鋼籠，每個籠舍分戶外區及室內區，亦爲長形籠子設計，中間具活動隔板，動物引入時由戶外區側入口送入，打開中間隔板，將動物驅趕至室內區，動物照護人員或實驗室同仁於室內區側餵飼或保定後進行體溫量測或血液等檢體採集；實驗室或動物照護人員進入動物舍前須穿戴防護衣物、面罩、口罩及防咬手套等設備，至少須兩位同仁方能進行操作，由一位同仁負責用套圈或繫繩保定動物，另一位同仁注射犬貓用鎮靜劑，俟動物昏睡後採取血液及拭子等樣本；據 James A. Ellen 表示因野生食肉目等動物犬齒銳利，若已接種病毒時操作時尤須注意安全。試驗動物舍除動物飼養室外有準備室，備有體溫計、體重計、採樣耗材及簡單醫療用品，另有動物解剖室，若人工感染動物發病或死亡，可就近進行解剖與採樣，再攜回亞特蘭大，動物屍體可儲存於冷凍庫等後處理。

試驗用蝙蝠係於野外捕獲，飼養於不鏽鋼長方形籠子，據 James 表示剛捕獲之蝙蝠須確認外表健康後方能進行試驗，此段時間爲一個月至數個月。試驗人員進入蝙蝠飼養室前亦須穿戴防護衣物、面罩、口罩及防咬手套等設備，至少須兩位同仁進行操作，由一位同仁負責保定蝙蝠，另一位同仁採取血液及拭子等檢體；保定時 James 以左手虎口握蝙蝠頸部，4 指握住身軀及翼膜，露出尾部及皮膜，另一位同仁以左手拉出尾膜，右手持注射針尖端(23G)輕輕刺破尾膜上 uropatagial vein，以毛細管或微量吸管尖(tip)吸取血液，以止血粉敷撒傷口，血液攜回實驗室離心分離血清，James 並補充道蝙蝠被保定時亦可一併採取其口腔拭子或沾取尿液(端視試驗目地及流程設計)，供檢測分析。返回實驗室後另與 Dr. Amy S. Turmelle 及 Dr. Ivan Kuzmin 等專家請教蝙蝠樣本採集及樣本運送等事宜，包含採材步驟、方式、盛放容器及採樣物品等，可供未來執行監測時修正野外調查時採樣流程之參考。

11 月 5 日（五）

延續 11 月 1 日操作之快速螢光斑點抑制試驗(RFFIT)，繼續進行 Khujand virus 抗體檢測與結果判讀。

四、研習機構簡介

本次研習實驗室爲CDC 位於亞特蘭大總部之National Center for Emerging and Zoonotic Infectious Diseases(NCEZID)轄下Division of High-Consequence Pathogens and Pathology(DHCPP)之痘病毒與狂犬病分部（Poxvirus and Rabies Branch）。CDC 職司傳染病之防治，在全球多次人類或人畜共通傳染病大規模爆發或發生不明原因傳染病時扮演相當重要的角色，例如：病毒性出血熱、立百病毒感染症、炭疽、西尼羅病毒腦炎、嚴重急性呼吸道症候群(SARS)等均能迅速確實地診斷，有效地遏阻傳染病之擴大趨勢，具有豐富之疫情處 理經驗與快速且正確的診斷技術，其診斷技術可謂全世界翹楚，故我國公共衛生與獸醫診斷機構歷年來曾多次派遣專家前往研習。

CDC 亞特蘭大總部約有6,000員工，與疾病診斷有關部門原本隸屬國家人畜共通傳染病及媒介性與腸道疾病中心（National Center for Zoonotic, Vector-Borne, and Enteric Disease; NCZVED），於2010年7月組織整合後納入更大的國家級機構- National Center for Emerging and Zoonotic Infectious Diseases(NCEZID)，NCEZID組織架構除了編制科學資源、全球變遷、健康照顧、新浮現傳染病準備等部門，如Division of Scientific Resources、Division of Global Migration and

Quarantine(DGMQ)、Division of Healthcare Quality Promotion(DHQP)、Division of Preparedness and Emerging Infections(DPEI)，尚有Division of Vector- Borne Diseases(DVBD)、Division of High-Consequence Pathogens and Pathology(DHCPP)、Division of Foodborne, Waterborne, and Environmental Diseases(DFWED)，其中DHCPP負責病毒性疾病、立克次體病及普里昂病診斷、預防與控制，其業務依疾病與病原種類等區分，由Bacterial Special Pathogens Branch(BSPB) 、Chronic Viral Diseases Branch (CVDB)、Infectious Diseases Pathology Branch (IDPB)、Poxvirus and Rabies Branch 、Viral Special Pathogens Branch(VSPB)等分部執行。本次研習之實驗室即屬於DHCPP之痘病毒與狂犬病分部(Poxvirus and Rabies Branch)，該部門又分爲痘病毒與狂犬病兩個研究單位，其中狂犬病研究實驗室由Dr. Charles E. Rupprecht領導，爲世界衛生組織(WHO)之狂犬病診斷合作實驗室及參考實驗室，對美國境內及國際衛生研究機構提供狂犬病監測、流行病學研究與疑似疫情診斷等協助、狂犬病診斷訓練與諮詢、狂犬病預防控制及治療等資訊，從事狂犬病診斷方法之研發、評估及改良等研究，並與美國境內及國際診斷研究機構合作研發診斷試劑與疫苗。研究人員例行性進行狂犬病直接免疫螢光抗體染色法(Direct Fluorescent Antibody test；dFA)、快速螢光斑點抑制試驗(RFFIT)及分子診斷等檢測。

感想及建議

本次於狂犬病診斷實驗室研習反轉錄聚合酶鏈反應(RT-PCR)及快速螢光斑點抑制試驗(RFFIT)等技術，期間與Dr. Ivan Kuzmin及Dr. Amy S. Turmelle等專家請教蝙蝠麗沙病毒檢測用引子設計及蝙蝠新浮現病原監測等事宜，Dr. Ivan Kuzmin除示範核苷酸比對軟體，亦熱心協助改良本所（行政院農業委員會家畜衛生試驗所）同仁自行設計之引子對，返國後可於實驗室內進行引子合成及測試，有助強化國內蝙蝠麗沙病毒診斷與監測技術。相關感想整理如下

重視新浮現疾病防堵與預防

美國公共衛生部門鑒於境內西尼羅病毒、新型 HIN1 流行性感冒等新浮現人畜共通疾病的疫情，且多數新浮現疾病源自野生動物，面對日趨複雜多變的疫病挑戰下在疾病防堵與預防上常需跨部門及跨學界間合作，爲有效整合相關部門人力與資源，美國公共衛生部門於 2010 年 7 月進行組織整合將 National Center for Preparedness, Detection, and Control of Infectious Diseases(DCPDCID)及 National Center for Zoonotic, Vector-Borne, and Enteric Diseases(NCZVED)兩個研究中心的部門與研究工作整併於 National Center for Emerging and Zoonotic

Infectious Diseases(NCEZID)，NCEZID 致力於常在性或新浮現或人畜共通疾病防治，透過強化防疫準備、建立公共衛生政策、公佈重大衛生資訊、建立跨部門合作關係等措施，期望達成人類、動物及環境全面健康即"One Health" strategy，隨著此波組織整併，狂犬病診斷實驗室所屬之 Division of Viral and Rickettsial(DVRD))，亦改編制成爲 Division of High-Consequence Pathogens and Pathology (DHCPP)，新部門 DHCPP 涵括細菌、病毒、prion 及傳染病等 branch，期望集合前述分部門的人員及設施等資源，以達到謀求全國及全球大眾的健康與安全。

重視人員控管及門禁管制

CDC隸屬於美國聯邦政府機構，911恐怖攻擊及炭疽郵包攻擊後特別加強進出人員之安全管制措施，國外訪客欲進入CDC，須在約2週至3週前提出申請，經審核通過後方得以訪客身份進入，且須有CDC實驗室同仁陪同，方能進入所申請研習之研究大樓與設施。本次研習期間原訂爲10天至12天，因CDC組織重整後，對國外訪客管制更加嚴格，按新制度規定研習期間超過7天以上訪客須在辦公室完成爲期2週的電腦安全測試與健康檢查，且須採取指紋建檔，方能進入實驗室從事研習或檢驗工作，慮及本次研習經費有限，經Dr. Charles E. Rupprecht與Dr. Shieh商量後建議將研習時間縮爲1週，方能於短期內順

利完成研習。由此可見CDC經組織重組後更加提昇外來訪客等人員管制措施，本次研習期間每日進入狂犬病診斷實驗室所在大樓均須由Dr. Ivan Kuzmin或Dr. Xian-Fu Wu陪同，進出研究大樓須出示訪客證、護照並簽名，且下班時須由實驗室同仁陪同方能離開研究大樓，CDC禁止外來訪客獨自於園區行走及拍照，嚴謹的安全檢查與對安全規定貫徹程度令人印象深刻。

注重防治技術研發

狂犬病研究實驗室爲世界衛生組織(WHO)之狂犬病診斷合作實驗室及參考實驗室，實驗室內專家除對球全提供狂犬病監測、流行病學調查與疑似疫情診斷等協助、狂犬病診斷訓練外，研發、評估及改良狂犬病診斷方法與研發診斷試劑與疫苗亦爲重點任務。本次研習有幸分享實驗室專家馬小姐現正研發新的抗體檢測技術，據瞭解現行病毒中和等抗體檢測技術，如快速螢光斑點抑制試驗(RFFIT)、螢光抗體病毒中和試驗(fluorescent antibody virus neutralization; FAVN) test 等，過程包含血清序列稀釋、病毒感作、細胞培養、細胞固定及螢光抗體染色等，最後於螢光顯微鏡下判讀，其操作繁複、須嫻熟病毒培養及細胞接種等技術，且因使用活病毒，須於高生物安全等級實驗室操作，而不適合供作大規模血清學調查，目前有商品化酵素連結免疫吸

附法等替代方法已取得 OIE 認可，可用於輸出入犬及貓的抗體檢測，此類 ELISA 法係使用狂犬病病毒表面醣蛋白作爲盤底塗佈之抗原，其餘 lyssavirus 表面醣蛋白差異較大，而不適用，此外進行 RFFIT 檢測時，野生動物血清有時具細胞毒性而會影響判讀，有鑑於前述困擾，狂犬病實驗室同仁已著手發展新的抗體檢測技術- Electro Chemical Luminescense，亦發表初步研究成果，目前顯示新技術檢測數據與 RFFIT 檢測結果相當，且敏感性更好，並可於短時間完成多量樣本檢測。

完備實驗室流程

狂犬病研究實驗室對於實驗室檢測技術流程管控，已建構完備之實驗室資訊，同仁只須自電腦搜尋即可列印出檢測流程，如本次研習 Dr. Ivan Kuzmin 立即自電腦系統中調出 RT-PCR 及 RFFIT 等流程及相關文獻，於討論中談及蝙蝠血液採集 Dr. Amy S. Turmelle 亦經電腦調閱試驗動物血液採集流程等，不僅便於檢驗業務執行，更利於人員訓練與檢驗流程標準化。除檢驗技術及動物試驗流程，實驗室專家對於檢驗相關試劑、耗材等資訊亦進行分類建檔，便於實驗室文件管理與資材設立。本次研習發現 CDC 同仁甚至須針對試驗動物血液採集設立採血體積與頻度等規定，據瞭解該份文件須送交 CDC 的動物照料

與使用委員會(Institutional Animal Care and Use Committee; IACUC)審核，確保試驗流程符合動物健康與福利要求後方能定稿執行，文件內容細分大鼠、小鼠、天竺鼠、倉鼠、兔、雪貂、犬等不同動物種類，規定每次採血時最大體積及相鄰二次採血間隔，研究人員若因試驗需求須增加採血數量，須提出科學證據向 IACUC 申請，審核通過方能採集較多血量，突顯美國 CDC 除重視公共衛生與疾病防治，在動物試驗方面亦非常注重動物福址，並將其精神放入動物血液採集等基本流程中。

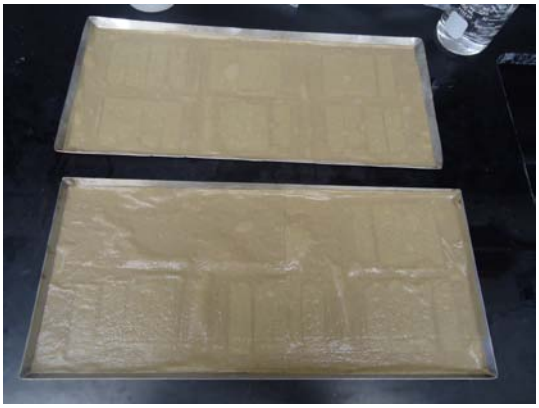
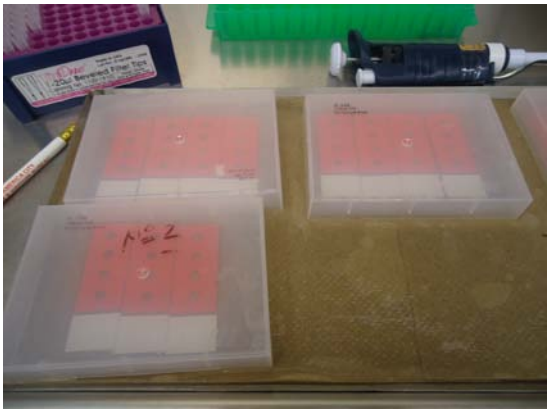
檢討與建議

目前已知大多數的新浮現傳染病爲人畜共通疾病，研究顯示在175種人類的新浮現傳染病中約75％ (132種)爲人畜共通疾病，而前述疾病如後天免疫缺乏症候群 (Acquired immune deficiency syndrome; AIDS)、流行性感冒、嚴重急性呼吸道症候群 (Severe acute respiratory syndrome; SARS)、麗沙病毒 (lyssaviruses)、立百病毒 (Nipah virus)及西尼羅病毒(West Nile virus)等疫病絕大多數與野生動物攜帶病原有關。近幾十年來由於農業生產型態改變、人類活動增加、動物棲地改變、全球化及氣候變遷等因素，致使野生動物與人類或家畜接觸機會增加，促使新浮現與再浮現人畜共通傳染病遽增且傳播範圍擴大，而成

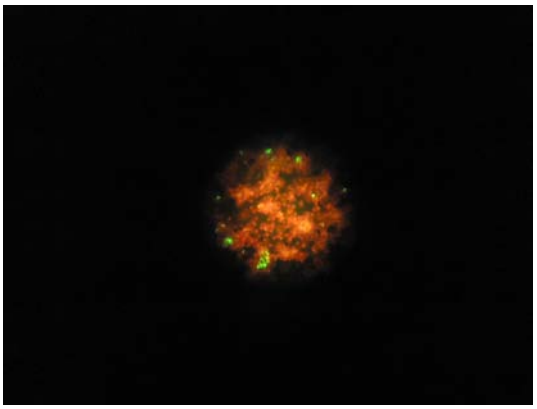
爲重要議題。爲有效防堵新浮現與再浮現人畜共通傳染病入侵，除在國境內及國家邊境或疆界進行疫病診斷與監測，透過國際性或區域性合作進行防堵亦爲執行重點。

本所歷來多次派遣診斷人員赴外研習重大傳染病診斷技術，返國後將習得技術應用於相關疫病監測與診斷，協助防堵重大動物傳染病及人畜共通傳染病入侵，並累積重要疫病監測資料。本次研習期間經由實驗室合作機制將蝙蝠樣本，送至美國 CDC 狂犬病診斷實驗室進行檢測，研習期間初步完成 2 種 lyssavirus- Irkut virus, Khujand virus 之抗體檢測，結果均呈陰性，後續委由美國疾管局專家 Dr. Ivan Kuzmin 進行其它 lyssavirus 之抗體檢測。此作法除可提昇研習效果，累積國內蝙蝠狂犬病血清學監測資料，更因與全球知名的狂犬病參考實驗室合作進行檢測，不僅能提昇狂犬病非疫區監測資料可信度，亦可供作臺美雙方野生動物新浮現人畜共通傳染病合作監測模式。鑑於重要疫病發生無疆界區分及新浮現及再浮現傳染病日益重要，未來仍須採派員研習、參與國際研討會或舉辦大型研討活動等措施培育疫病診斷防治人才，強化重要人畜共通傳染病診斷智能，加強國際合作等以爲因應。

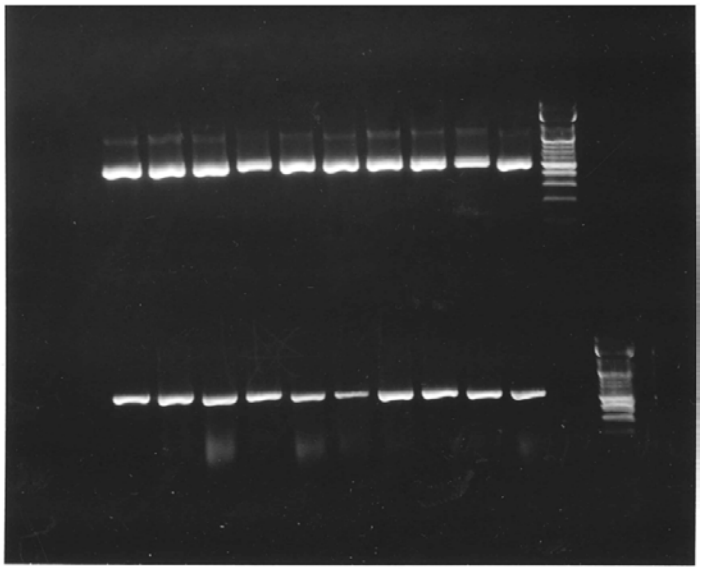
	
圖一、Dr. Ivan Kuzmin 指導進行 RFFIT，此爲準備病毒培養用 MNA cell	圖二、RFFIT 相關設備

	
圖三、以鋪上濕潤擦手紙之不銹鋼盤作爲 wet chamber 之容器	圖四、玻片於 wet chamber 中進行反應

	
圖五、經抗體染色後玻片浸於 PBS 中清洗	圖六、清洗後玻片置於空氣中風乾

	
圖七、Dr. Ivan Kuzmin 指導結果判讀	圖八、RFFIT 檢測結果：綠色螢光爲 MNA cell 內 lyssavirus 被螢光抗體染色，橙紅色爲未被螢光抗體染色之 MNA cell。

1 2 3 4 5 6 7 8 9 10

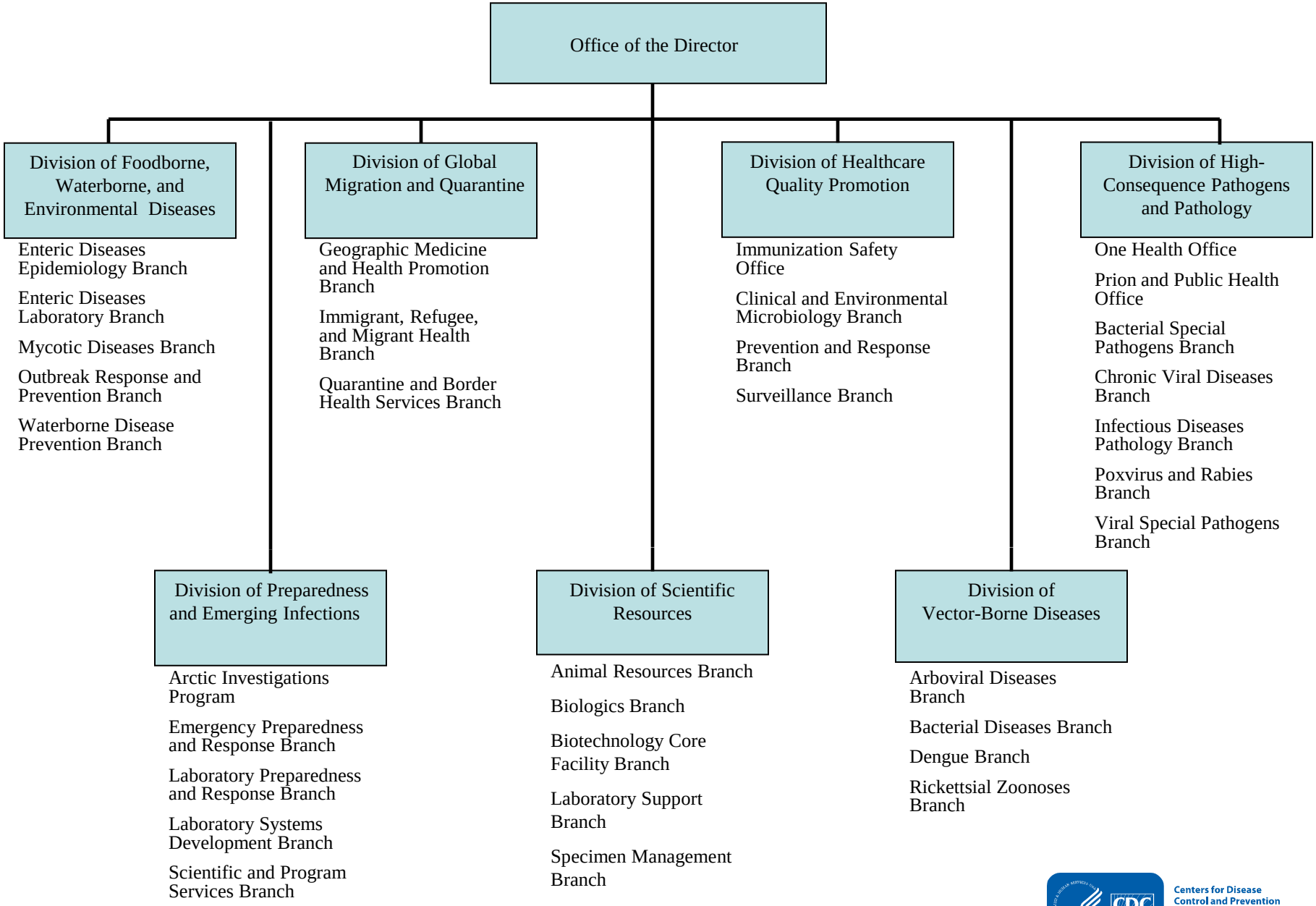


Lane 1, 5, 8: lagos bat virus
Lane 2, 3, 4, 6, 7, 9, 10: rabies virus variants

圖九、Lyssavirus 之 RT-PCR 及 nest PCR 增幅產物之電泳圖。
上排：nest PCR 增幅產物
下排：RT-PCR 增幅產物

附件一

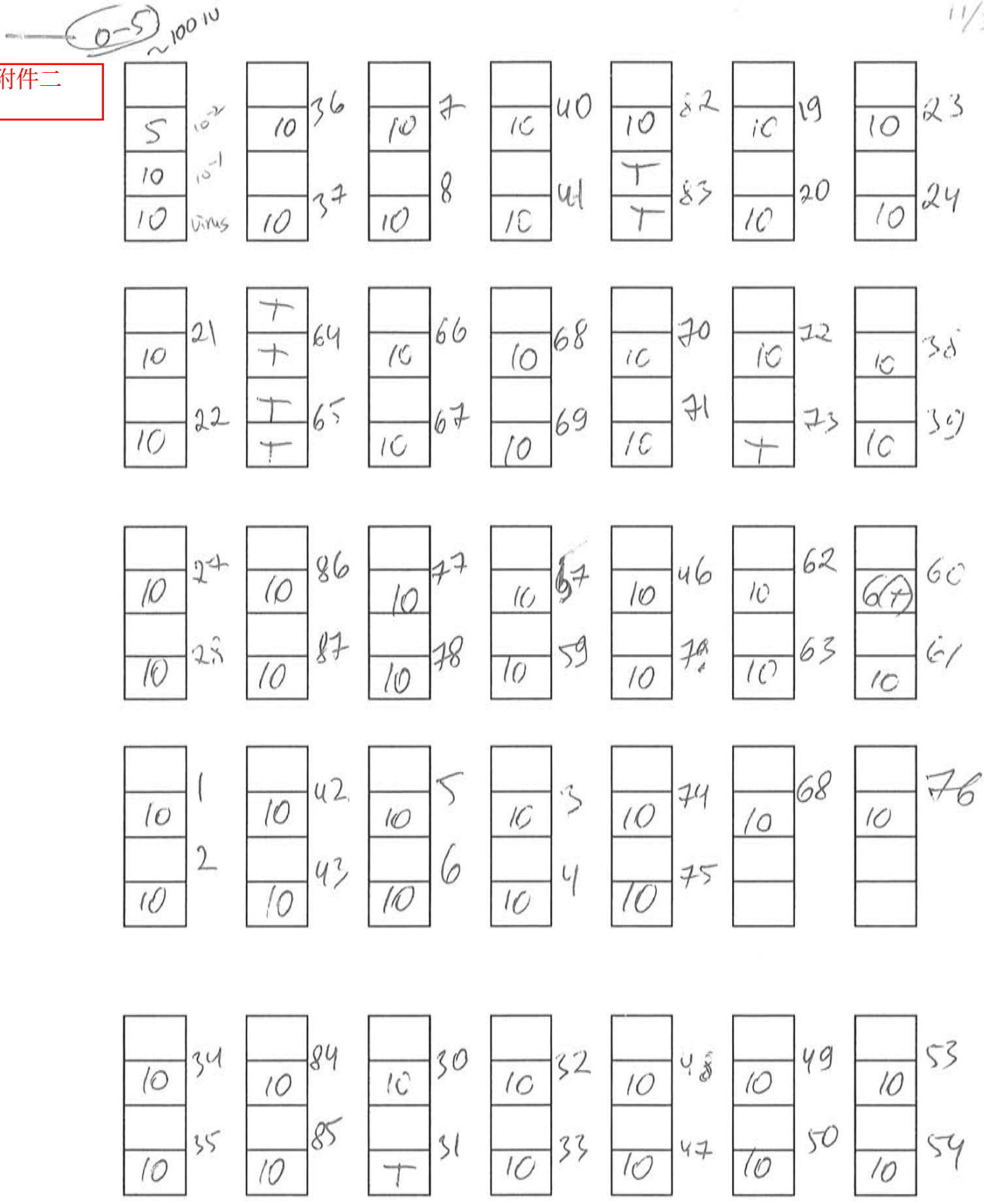
National Center for Emerging and Zoonotic Infectious Diseases



July 2010

Centers for Disease Control and Prevention
National Center for Emerging and Zoonotic Infectious Diseases

附件二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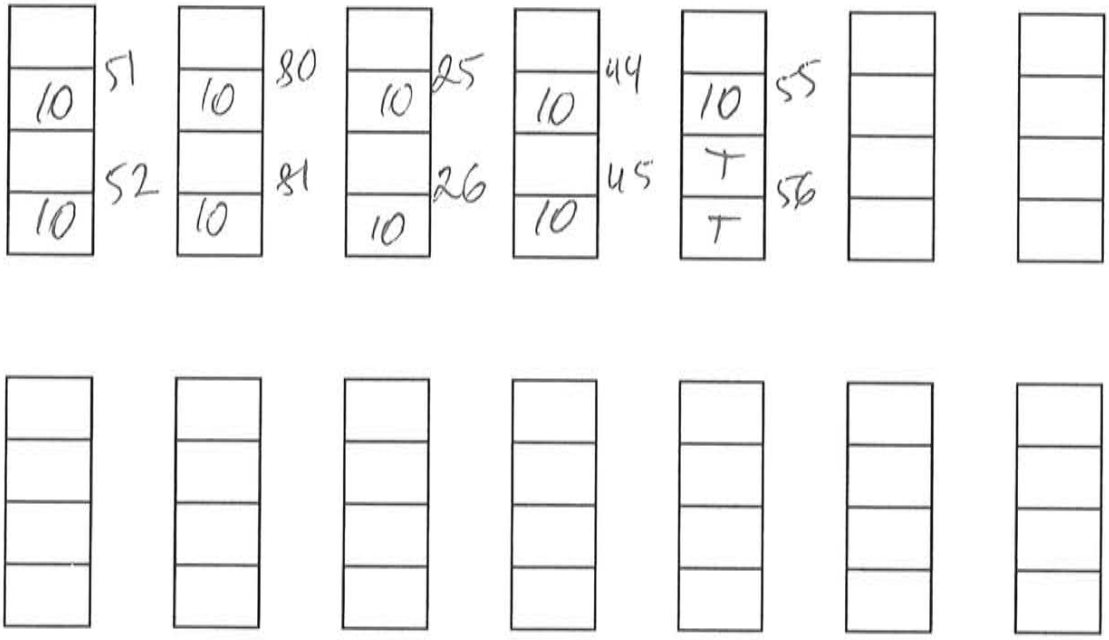
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Dose ~ 40 FFU

-2 / -1 / neat (Control)	23 / 24	19 / 20	80 / 81	57 / 59	42 / 43	38 / 39
			T	T		
2	10	10	T	T	10	10
10			T			
10	10	10	T	10	10	10

40 / 41	07 / 08	03 / 04	01 / 02	05 / 06	34 / 35	86 / 87
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44 / 45	21 / 22	70 / 71	66 / 67	68 / 69	78 / 79	60 / 61
			T	10	10	10
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10	10	10	10	10	T	10

55 / 56	64 / 65	62 / 63	27 / 28	32 / 33	30 / 31	82 / 83
	T					T
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RFFIT-KMUV.

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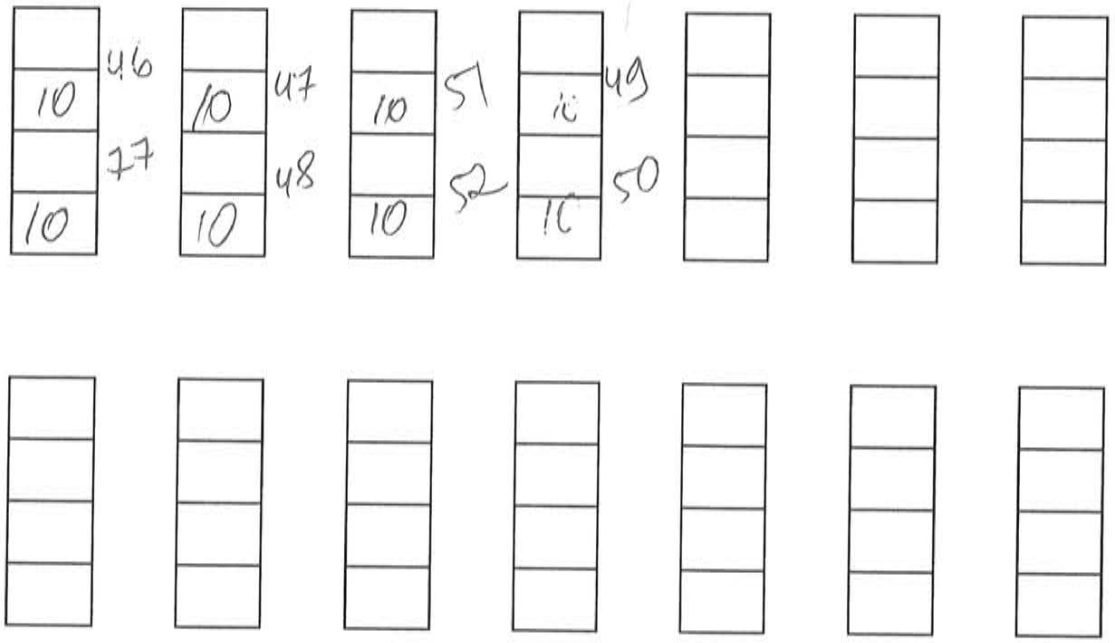
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附件三

A. Titers of challenge virus used in the RFFIT (Reed-Muench)

Challenge dose ($50_{,}FFD_{50}$/0.1 ml)	Number of fields with fluorescing foci / Total number of fields																								
	20/20 →																								
1:10 (-1)	10/20	11/20	12/20	13/20	14/20	15/20	16/20	17/20	18/20	19/20	20/20 →														
1:100 (-2)	0/20 →										0/20	1/20	2/20	3/20	4/20	5/20	6/20	7/20	8/20	9/20	10/20	11/20	12/20	13/20	14/20
FFD_{50}	10	15	15	17	19	25	26	26	27	30	32	32	35	40	43	47	51	59	68	80	100	150	150	170	190

Acceptable range

B. Acceptable range of fields with fluorescing foci for the control reference serum.
Standard rabies immune globulin; Lot R-3 (2 IU/ml):

Dilution	
1:5	0 of 20 infected fields
1:25	0 of 20 infected fields
1:125	4 to 8 infected fields of 20
1:625	20 of 20 infected fields

Appendix c.

50% Serum End-point Titers Corresponding to the Numbers of Fluorescing Foci (calculated by the Reed-Muench Method)

Number of Fields with Fluorescing Foci/Total fields

Serum dilution	0/20 20/20	1/20 20/20	2/20 20/20	3/20 20/20	4/20 20/20	5/20 20/20	6/20 20/20	7/20 20/20	8/20 20/20	9/20 20/20	10/20 20/20	11/20 20/20	12/20 20/20	13/20 20/20	14/20 20/20	15/20 20/20	16/20 20/20	17/20 20/20	18/20 20/20	19/20 20/20	20/20 20/20
1:5 1:25	11	11	10	9	9	8	7	7	6	5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	>5
1:25 1:125	56	54	50	50	45	42	40	36	33	29	25	21	19	17	16	14	13	13	13	12	--
1:125 1:625	280	270	250	250	230	210	200	180	170	145	125	110	95	85	75	70	70	65	60	60	--
1:625 1:3125	1400	1300	1300	1200	1100	1100	1000	900	800	700	625	540	480	440	390	360	340	320	320	290	--
1:3125 1:15625	7000	6800	6300	6000	5700	5400	5100	4600	4200	3600	3125	2700	2400	2200	1900	1800	1600	1600	1500	1500	--
1:15625 1:78125	34800	33489	31950	30313	28571	26718	24747	22652	20432	18086	15625	13500	12000	10000	10000	9500	8500	8000	7500	7500	--
1:78125 1:390625	174692	167448	159750	151565	142857	133591	123735	113263	102160	90434	78124	67491	59744	53887	49326	45687	42724	40269	38206	36450	--

C. **Calculations**

Using the control slide and test serum values from the previous example, the test serum end-point titre and international units can be calculated.

1. **Determination of 50% end-point titres of serum**

The serum neutralization end-point titer is defined as the dilution factor of the highest serum dilution in which there is a 50% reduction in the number of fluorescing foci.

The 50% end-point titre of serum can be made by determining the number of fluorescing foci at each dilution, and then use the cumulative totals in the Reed-Meunch formula:

a. Calculate the percentage of fields containing infected cells

Serum Dilution	No. of fields containing infected cells	Fields containing infected cells	Fields containing no infected cells	Percentage of fields containing infected cells
1:5	0/20	0	88	0/88=0
1:25	0/20	0	68	0/68=0
1:125	0/20	0	48	0/48=0
1:625	0/20	0	28	0/28=0
1:3125	12/20	12	8	12/20=60
1:15625	20/20	32	0	32/32=100
1:79125	20/20	52	0	52/52=100
1:390625	20/20	72	0	52/52=100

b. Using the method of Reed & Muench, calculate the difference between the logarithm of the starting dilution and the logarithm of the 50% end-point dilution (difference of logarithms) from the formula:

$$\frac{50\% - (\text{infectivity next below } 50\%)}{(\text{infectivity next above } 50\%) - (\text{infectivity next below } 50\%)} \times \text{logarithm of dilution factor}$$

In this example the starting point dilution (the dilution showing an infectivity next below 50%) is 625, the dilution factor is 5. Hence, the difference in logarithms is:

$$\frac{50-0}{60-0} \times 0.69897 = 0.582475$$

c. Since the infectivity is increasing as the dilution increases, the 50% end-point dilution is higher than the starting point dilution and is calculated by adding the difference of logarithms as follows:

log (reciprocal of the starting point dilution) + difference of logarithms

= 2.79588 + 0.582475 = 3.38 (approx.)

Hence, log (50% end-point dilution) = -3.38 and the 50% end-point dilution = $10^{-3.38}$ = (1:2399).

2. **Determination of the potency of test serum in international units (IU) per ml**

   The results of the RFFIT can be expressed as a serum titer or in International Units (IU) of antibody. In either case, a reference serum of known titer is required. When the results are to be reported as a titer, the reference serum is used as a control to insure the sensitivity of the test. It should demonstrate approximately the same titer determined in previous tests.

   When the test serum results are expressed in IU, the calculation is dependent on the number of IU in the reference serum. The reference serum is diluted to contain 2 IU/ml and titered along with the test serum. The ED_{50} titers of the reference serum and the test serum are then related in the following formula for calculation of IU/ml in the test serum:

$$\text{Number of IU/ml} = \frac{\text{End-point titer of the test serum}}{\text{End-point titer of the reference}} \times \text{2 IU/ml in the reference serum}$$

Example from above:

Test serum titer = 2399

Reference serum titer = 200

$$\text{Number of IU/ml in the test serum} = \frac{2399}{200} \times 2 \text{ IU/ml}$$

$$= 12 \times 2 \text{ IU/ml}$$

$$= 24 \text{ IU/ml}$$

Example 1.

Titration of stock virus CVS-11 for RFFIT

Titration volume (0.1 ml)	Virus dilution	# of fields with fluorescing foci / total # of fields	Percent of infected cells per single field
	-5 (1:100,000)	0/20	0 infected cells
Dilution with 10 of 20 infected fields ←	-4 (1:10,000)	10/20	0 to 1 infected cells/field
	-3 (1:1000)	20/20	1 to 5 infected cells/field
	-2 (1:100)	20/20	10 to 50 infected cells/field
	-1 (1:10)	20/20	

The –4 dilution of virus contains at least one focus of infected cells in 50% of the observed microscope field (160-200x) = 1 FFD_{50} /0.1ml

The stock virus suspension should be diluted $10^{-2.3}$ to obtain a challenge dose of 50 FFD_{50} in 0.1 ml.

4.0 log of dilution with 0 to 1 infected cells per field
\- **1.7** log of 50
= **2.3** dilution of virus required to achieve 50 FFD_{50} in 0.1 ml.

$10^{2.3}$ = 1:200

To calculate the amount of challenge virus suspension needed for each test:
Each slide will receive 0.8 ml virus (100ul per well, 8 wells per slide).
Calculate using 1.0 ml virus per slide. If the test run is 16 slides, need 16 ml of virus suspension.
Add 80 μl of stock virus to 16 ml MEM-10 (1:200 dilution)
Each well receives 100ul virus suspension.

附件四

Detection of rabies virus nucleic acid in human saliva and neck biopsy samples by heminested and nested reverse-transcription polymerase chain reaction (RT-PCR)

TABLE OF CONTENTS

1. Introduction

Rabies is an acute progressive encephalitis, caused by negative-stranded, non-segmented RNA viruses in the Genus *Lyssavirus*. This zoonosis has the highest case fatality rate of any infectious disease. Given the virulence of the disease, and the need for public health consultations regarding human post-exposure prophylaxis management, accurate laboratory diagnosis is a priority. Among the findings of a U.S. National Working Group on Rabies Prevention and Control was a need for a sensitive, specific and rapid confirmatory method as an adjunct to the standard direct fluorescent antibody (DFA) test for the post-mortem diagnosis of rabies in animals, and either post- and ante-mortem diagnosis in humans. The available confirmatory methods for the DFA test include rabies virus isolation, and molecular methods, such as reverse transcriptase polymerase chain reaction (RT-PCR). Current RT-PCR techniques are extremely sensitive-specific procedures and may be reliable for rabies diagnosis provided that the test conditions are optimal. Among the variables are the sample template, RNA extraction method, RT-PCR parameters, primers selected, interpretation of the results, and confirmation of a positive result by sequence analysis. To this end, a national working group was formed to develop a protocol for the use of RT-PCR in the confirmatory diagnosis of rabies. The recommendations provided in this document should be closely followed to ensure a test of robust sensitivity and specificity. Modifications in procedures without validation may lead to false results and non-specific or non-interpretable reactions.

2. Safety

All persons involved in rabies diagnosis should receive pre-exposure immunization with regular serologic surveillance and booster immunizations as necessary (9). Unimmunized individuals should not enter laboratories where rabies work is conducted. Besides vaccination, adequate training and personal protection equipment (PPE) are needed. All tissues processed in an infectious disease laboratory must be disposed of as medical waste and all activities related to the handling of animals and samples for rabies diagnosis should be performed using appropriate biosafety practices to avoid direct contact with potentially infected tissues or fluids (CDC and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories, 5th edition, U.S. Government Printing Office, 2007 – currently available on-line at http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm). Personnel working in the rabies laboratory are at risk of exposure through accidental injection or contamination of mucous membranes with rabies virus contaminated material and by exposure to aerosols of rabies virus-infected material. All manipulations of tissues should be conducted in a manner that does not aerosolize liquids or produce airborne particles. Biological safety cabinets are required for the homogenization of brain tissues for RNA extraction and virus isolation. After the initial suspension and processing in the TRizol reagent for 5 minutes at room temperature, infectious virus has been inactivated and remaining RNA extraction steps may be performed on the bench if necessary. Fume hoods are required for aliquoting of organic solvents and other hazardous chemicals. Laboratorians performing RNA extraction and RT-PCR should be familiar with the MSDS sheets for the chemicals used. Glass chips and sharp objects are also a potential source of exposure

to rabies virus. Polypropylene tubes, plates and containers should be used to store sample aliquots, perform extraction steps, and collect chemical waste. Many of the chemicals are hazardous. Hazardous waste should be discarded appropriately by consulting the MSDS sheets for the chemical and institutional guidelines for the discard of chemical waste. Individual waste chemicals should be kept separately and not combined with other chemicals. Care should be taken to protect eyes by wearing safety glasses, goggles or face shields in addition to routine PPE during manipulation of chemicals during all steps of RNA extraction, RT-PCR, gel electrophoresis, bench clean up, and disposal of chemical waste. Appropriate PPE should be used when ultraviolet (UV) light is utilized for viewing amplicons in agarose gels containing ethidium bromide to avoid exposure to eyes and skin. The use of a certified UV face-shield in addition to safety glasses, lab coats (which cover wrists), and latex or nitrile gloves are recommended. Additional information concerning chemical and environmental hazards, PPE, emergency response to chemical spills, Material Safety Data Sheets (MSDS) and other related topics may be found at the CDC National Institute for Occupational Safety and Health Website (www.cdc.gov/niosh/topics/chemical-safety).

3. Principle of RT-PCR for rabies diagnosis

Rabies virus is a ribonucleic acid (RNA) virus and thus replicates in cytoplasm of infected neurons, abundant rabies viral proteins and rabies virus RNA (genomic RNA, m-RNA and full transcripts) can be found in the cytoplasm. Of all the tests for rabies diagnosis, the RT-PCR is considered the most sensitive. However, all aspects of the test must be optimal for the above statement to be true, and these would include adequate sampling of tissues in good condition, extraction methods to harvest minute amounts of RNA, primers which are complementary to the target sequences, optimized RT-PCR cycle parameters, and laboratory procedures to eliminate cross-contamination between samples.

3.1. RNA Extraction

The first step in using RT-PCR for detection of rabies nucleic acid is the extraction of total RNA from brain tissues (brain stem and cerebellum), frozen saliva or skin biopsy of suspected rabid humans. The RNA extraction process using TRIzol™ reagent (figure 1) involves the lysis of the cytoplasmic membranes, emulsifying of lipids with chloroform, separation of the total RNA from cellular protein and DNA, precipitation of the RNA by isopropanol, removal of residual salts by washing with 75% ethanol, and reconstitution or rehydration of the RNA with RNase–free water.

3.2. Reverse Transcription (RT)

PCR is an in vitro method for amplification of DNA. RNA can not serve as a template for PCR, so it must first be reverse transcribed into cDNA by using an enzyme reverse transcriptase which is a RNA dependent DNA polymerase. Avian myeloblastosis virus DNA deoxynucleotidyltransferase is used in the rabies virus protocol. (4) This enzyme will catalyse the extension of a complementary primer (synthetic oligonucleotide)

sequence to the template RNA to produce the opposite strand of cDNA. If the primer sequence contains mismatches with respect the target sequence, there will be no extension and no cDNA produced. Other limitations of RT are the RNA template concentration, RNA may be limited or degraded in less than optimal samples, also the concentration of MgCl2 and deoxynucleotides (dNTPs) bases necessary to make the cDNA. Reverse transcription (RT) is coupled with the next step, PCR for amplification of a specific gene or part of a gene resulting in a cDNA product.

3.3. Polymerase Chain Reaction (PCR)

This method uses a heat-stable enzyme derived from a bacteria *Thermus aquaticus* (Taq) which allows for as many as 40 cycles of amplification including high temperature denaturing steps at 94° C. Each of the PCR cycles has 3 steps, the first step denaturation, is heating of the cDNA to high temperature (94° C) which reduces cDNA to single linear strands without any secondary structure. The next step annealing is cooling to a temperature (usually a minimum of 5 ° C below the primer melting temperature) to bind the complementary primer to template DNA. The rabies virus PCR uses a low annealing temperature of 37 ° C for 30 seconds, which increases the sensitivity of the PCR. The 3rd, final step of each cycle is the extension, synthesis of the complimentary DNA strand at 72 ° C for 90 seconds resulting in double-stranded (ds) cDNA. As demonstrated in figure 2, both strands of the cDNA then become template for amplification, and after 40 cycles of PCR may amplify10^{12} times a single copy, which makes this under optimal conditions, one of the most sensitive protocols for rabies diagnostics.

3.4. Nested PCR

In cases when rabies virus RNA is limited, using a hemi-nested PCR protocol may further amplify the cDNA to a detectable level. From studies performed at CDC the sensitivity of RT-PCR may be increase 10-100 times by performing nested or hemi-nested RT-PCR using primers targeting regions internal to the primer locations used for primary PCR amplification (for hemi-nested, only one primer is internal and the other paired primer is identical to the one used in the primary PCR). In the nested and heminested procedures 1/10 of the primary PCR product is subjected to additional 40 cycles of PCR under the same PCR conditions with primers as described.

3.5. Detection of amplicons

The detection is performed by visualization of amplicons in agarose gels containing ethidium bromide which binds to DNA and fluoresces when exposed to UV irradiation. Amplicons are subjected to electrophoresis, a process by which cDNA molecules are separated into distinct bands according to size. Migration of cDNA toward positive pole occurs with small sized molecules first. Confirmation of size of the specific amplicons is made by comparison to the molecular weight standards, and controls. All bands of the correct molecular weight should be sequenced to confirm specificity. Detailed protocols for RT-PCR testing for detection of rabies virus RNA will be provided in this manual.

4. Equipment and Reagents

4.1. Equipment and Supplies

4.1.1. Dissection instruments should be of sufficient quantity for 1 set per sample to prevent cross transfer of infected tissue between samples. Chemical disinfection is not adequate method to decontaminate instruments used on samples for RT-PCR. All instruments used during necropsy and dissection must be thoroughly disinfected by boiling 30 minutes or autoclaving at 121 °C for 60 minutes and then receive a thorough washing and sterilization before reuse. The instruments not in use should be kept in closed storage. Accidental contamination of a sample at necropsy or processing of tissues by the standard DFA test will not be detected by routine quality control or meticulous handling of the RT-PCR samples. Always cut internal pieces of tissue for RT-PCR. These samples are less likely to be contaminated than on the surface. Sterile wood applicator sticks are recommended for making brain, saliva and neck biopsy suspensions.

4.1.2. Sterile wood applicators can be prepared by autoclaving in tubes or foil packages at 121 °C, 15 lbs pressure for 60 minutes. An alternative is to use commercially available wood applicator portion of sterile swabs (Fisher Cat# 14-959-85).

4.1.3. Autoclave. All instruments should be cleaned and sterilized before reuse. Autoclave specifications for decontamination of instruments and other materials are at 121 °C for 60 minutes. A sterilization indicator should be included in each load to insure that sterilization conditions have been reached. It is recommended to check on autoclave operation monthly with spore indicators such as SPS Medical Cat# CS100 (Fisher NC9828662) or Thermo Scientific B/T Sure biological Indicator Cat# AY759X3 (also available from Fisher Scientific).

4.1.4. Specimen storage containers for aliquots of saliva and neck biopsy tissues for RT-PCR polypropylene tubes, sample containers, or ointment tins should be used. Glass vials and tubes are unacceptable for specimen storage because of the risk of breakage.

4.1.5. Refrigerated storage. Saliva, neck biopsy and long-term storage of RNA samples require a freezer at -70°C or below. Dry ice is suitable for sample transportation. Frost-free freezers should **not** be used. The heat cycles in frost-free freezers may denature proteins in specimens and may compromise test results. TRizol™ and lysis buffer 1 should be stored at 4°C. However, most enzymes used for RT-PCR are best stored at -20°C. Please check the manufacturer's recommendation concerning storage temperatures of reagents for RNA extraction and RT-PCR.

4.1.6. Sterile polypropylene micro-centrifuge tubes (2.0 ml) with external screw caps with rubber gaskets are recommended for RNA extraction and long term RNA storage. (Such as Sarstedt Cat# 72.694.006). External screw caps with rubber gasket enclosures are an added safety feature during centrifugation, vortexing and handling, and reduce potential contamination problems associated with snap-capped enclosures.

4.1.7. Individually Packaged Sterile gauze pads (4 inch x 4 inch) are excellent for blotting tubes during the RNA extraction protocol. These are available commercially (Fisherbrand Cat# 22-415-469), and provide clean sterile blotting surface for tubes during the RNA extraction process.

4.1.8. Micropipettors and sterile aerosol-free (RNase-DNase free) pipette tips are required for RNA extraction, RT-PCR and gel electrophoresis. Separate sets of pipettors should be used for each of these steps (reagent preparation, RNA extraction, RT-PCR and gel electrophoresis.) Pipettors calibrated to accurately deliver 1-20 µl, 20-200 µl and 200-1000 µl are needed. Prior to each use the surface of each pipettor should be decontaminated with 3% hydrogen peroxide or RNase away (Invitrogen Cat# 10328-011, MBP Cat# 7003, 7002 or Research Products International Corp Cat# 1470001) which destroys RNases and DNA.

4.1.9. RT-PCR reaction tubes and plates should be polypropylene and certified as RNase and DNase-free. Snap-capped 0.2 ml, 0.5 ml and 0.65 ml tubes are preferred over microtiter plates for rabies diagnosis, to avoid cross-contamination.

4.1.10. Micro-centrifuges are needed for RNA extraction and RT-PCR.
Specifications require that the centrifuge head and adapters contain 2.0 ml screw-capped microfuge tubes, 1.5 ml tubes and 0.5 ml tubes. RNA extraction requires centrifugation speeds at 12,000 x G, and 7,500 x G at 4 °C. Alternately, if a refrigerated centrifuge is unavailable, a non-refrigerated micro-centrifuge may be moved to a cold room, or chromatography refrigerator. In addition to RNA extraction, a micro-centrifuge is required for RT-PCR. Reaction tubes for RT-PCR should be centrifuged briefly before uncapping to avoid cross-contamination.
Care should be taken when opening and closing tubes to avoid cross-contamination, and gloves should be changed immediately if contamination is suspected.

4.1.11. Thermal Cyclers are required for RT-PCR amplification. Current thermal cyclers possess heated tops which prevent evaporation, condensation, and potential contamination problems. These do not require the use of mineral oil.

4.2. Reagents

4.2.1. Lysis Buffer 1 (not commercially available) may be prepared by adding 100 µl of 1M Tris HCL pH 7.5, 333 µl of 5M NaCl, 33 µl of 0.5 M MgCl, 65 µl NP40 and add quantity sufficient (qs) of molecular grade water to bring the volume to 10.0 ml. The buffer aids in the homogenization of brain tissues and the hypotonic lysis of the cells to free cytoplasmic RNA. (11)
4.2.2. TRIzol Reagent (Invitrogen, Life Technology15596-026) is a commercially available RNA extraction buffer which is a mixture of phenol, guanidine isothiocyanate (GITC) red dye and other proprietary components. The reagent is used to isolate total RNA. The TRI in the name stands for Total RNA Isolation. Care should be taken to avoid contact with skin and to use the reagent in a well ventilated area or fume hood. TRIzol and the more concentrated version TRIzol LS which was specially developed for liquid

samples (LS) have worked well for isolation of total RNA in rabies virus-positive samples. If solid samples such as brain tissues are tested with LS, there will be less yield of RNA unless a suspension is made. It is recommended that all solid samples be suspended first in lysis buffer 1 which seems to aid in disruption of the cytoplasmic membranes over direct suspension in the TRIzol reagents. Other options suggested for suspension include cell culture media and RNase/DNase free water. Tissues homogenized and suspended in the TRIzol reagents can be stored for at least 1 year at -70 °C or below. TRIzol tissue suspensions should set at room temperature at least for 5 minutes (or overnight at 4 °C) before proceeding to chloroform separation steps.

4.2.3. Chloroform (molecular reagent grade without isoamyl alcohol added) is commercially available from Sigma# C7559, Thermo Scientific Acros # 364321000, MP Biomedicals #19400725.) The addition of isoamyl alcohol to the chloroform is not necessary for the TRIzol extraction protocol. According to the manufacturer, BCP (1-bromo-2chloropropane) can be substituted for chloroform (however, validation of this substitution has not been made for rabies virus RNA extraction).

4.2.4. Isopropanol (2-propanol or Isopropyl alcohol) molecular grade may be obtained commercially from Sigma Chemical #I9516, Fluka # 59304, and Thermo Scientific Acros# 36440-00010 (1L) or 3644001000 (100 ml) and other sources. Isopropanol is used to precipitate RNA. To avoid cross-contamination of diagnostic samples, it is best to aliquot isopropanol (0.5 ml) into sterile external screw-cap micro-centrifuge tubes in a clean reagent preparation area for each reaction before use.

4.2.5. Ethanol (Ethyl alcohol) 200 proof–absolute, molecular grade may be obtained from Aldrich# E7023, Fluka# 51976 or other sources. Absolute ethanol is required to make 75% ethanol solution prepare with molecular grade RNase/DNase-free or DEPC-treated water. The 75% ethanol solution is used to wash the RNA pellet. For diagnostic specimens, it is best to dilute absolute alcohol and prepare a fresh aliquot sufficient for each run of the diagnostic samples daily. Samples should not be stored in 75 % ethanol.

4.2.6. Water (molecular grade, RNase/DNase-free) obtained commercially (e.g., Sigma# W4502, Promega# P1195, ATTC# 602450, Fisher Bioreagents# BP561-1). The molecular grade water should be used to reconstitute RNA pellets and prepare all reagents used in RNA extraction and RT-PCR.

4.2.7. Reverse transcription reaction buffer (RTRX) should be prepared in advance, and may be stored in convenient aliquots without reverse transcriptase and RNase inhibitor, for at least 6 months at -20 °C in a non-frost-free freezer. Before using the buffer, the reverse transcriptase and the protector RNase inhibitor are added to the RTRX buffer (Table 2). The RTRX buffer is prepared by adding 1568.0 µl molecular grade water, 864.0 µl Roche 5x Reverse Transcription Buffer (1465 368 provided free with purchase Reverse Transcriptase #1019118001), and Roche PCR nucleotide mix (11581295001) 432.0 µl. Aliquots of 71.6µl of RTRX are sufficient for 5.4 RT reactions. Just before use, the working buffer is prepared by adding 2.0 µl of Reverse Transcriptase (Roche #1019118001) and 2.0 ul Protector RNase Inhibitor (Roche# 3335402001) to

71.6 µl of the RTRX. For each reverse transcription reaction, 14 µl of RTRX working buffer is added to each tube containing the RT primer and RNA.

4.2.8. PCR Pre-mix Buffer (for 5.4 reactions) is prepared by adding 372.6 µl molecular grade water, 43.2 µl of Tris pH 8.3, 2.7 µl (5 U/µl) Taq polymerase (Amplitaq™ PE Biosytems# N880153), 5.4 ul of 20µM specific or 40µM degenerate Primer 1 (RT primer) and 6.75 µl of 20µM specific or 40µM degenerate Primer 2 (Table 3)

4.2.9. Tris pH 8.3 is prepared by adding Tris base 74.28 g, Tris hydrochloride 60.97 g, and add sufficient molecular grade water to bring volume to 1000 ml.

4.2.10. Synthetic Oligonucleotides (Primers)

Primer selection is an important component of any RT-PCR test, but especially diagnostic tests for rabies virus. No single primer is universal because a single point mutation in a critical priming position will affect the ability to produce a specific amplicon. Rabies diagnostic RT-PCRs have focused on the nucleoprotein (N) gene because it is one of the most readily conserved genes within the *Lyssavirus* genus, it has the most abundant sequence database for primer design and comparative information for sequence analysis, and because the N gene is abundant in infected cells. More information needs to be gained before other rabies virus genes (P, M, G, L) are considered for routine nucleic acid detection by RT-PCR. Therefore, it is essential to use multiple, broadly reactive, specific or degenerate primers, and to test multiple regions simultaneously concentrating on the N gene as a target of RT-PCR. To maximize sensitivity of the RT-PCR if RNA is limited or degraded, and increase the probability of a primer match, confirmatory diagnostic RT-PCR tests should include reactions with a minimum of 3 primers sets to target 3 different regions of the N gene. Recommended primer sets for the primary RT-PCR include: set 1) Lys001 or JW12 with 1066 deg B, set 2) 550F or JW6 deg C with 304 or N8 deg C, and set 3) 1066 deg F with 304 or N8 B deg C. Hemi-nested or nested primers include: for set 1) Lys001 or JW12 with 550 B; set 2) 550F or JW6C with 1066 deg B; and set 3) 1087i deg F with 304 or N8 B deg C, except samples from California, which require 1087s deg with 304 or N8 B deg because of a mutation noted in the rabies N gene of California skunks; and 504s with 304 or N8B alternatevely. However, primer selection needs to be predicated upon tissue type, condition of the sample, and local rabies virus epidemiology. Although the focus of this protocol is a confirmatory diagnostic procedure for brain tissue samples, in testing of non-CNS tissues, samples in less than optimal condition and RNA content, or weakly positive samples, shorter amplicons and/or nested RT-PCR may be required to produce a detectable amplicon. Local epidemiological considerations are also important. For example, broadly reactive primers used to detect rabies virus variants in the USA, may not be successful in producing amplicons from weak RNA samples from Asia, and primers designed specifically for these viruses may be necessary for RT-PCR. A table of commonly used primers for application is presented in the appendix.

5. Sample

Information concerning sample collection, shipment and handling is taken from the protocol for post-mortem diagnosis of rabies in animals by direct fluorescent antibody testing (8).

Rationale for sample collection

Rabies virus reaches the central nervus system where it replicates abundantly, causing encephalitis, and thereafter spreads along nerves into various peripheral organs. Salivary glands are the specific organs where rabies virus is excreted through saliva, once the virus reached the CNS. At this stage during the infection, the virus is often present in the nerves innervating hair follicles. Thorough examination of saliva and skin biopsy samples by using nested or heminested RT-PCR is required for antemorten rabies diagnosis in humans. When available, the brain biopsy is the sample of choice. As the central nervous tissue is the only tissue where rabies virus may be detected unequivocally, this tissue (specifically, brainstem and cerebellum) are used for rabies diagnosis in postmortem samples.

Saliva samples should be taken by using a sterile eyedropper pipette, collect saliva and place in a small sterile container which can be sealed securely. No preservatives or additional material should be added, but specimens should be immediately frozen at -70 °C or lower. Tracheal aspirates and sputum are not suitable for rabies tests.

Neck Biopsy. Should be a section of skin 5 to 6 mm in diameter taken from the posterior region of the neck at the hairline. The biopsy specimen should contain a minimum of 10 hair follicles and be of sufficient depth to include the cutaneous nerves at the base of the follicle and fat. Place the specimen on a piece of sterile gauze moistened with sterile water and place it in a sealed container and get it immediately frozen at -70 °C or lower. Do not add preservatives or additional fluids.

Brain bipopsy. Should be a section of available brain tissue, approximately 4x4x4 mm.

Brain tissue taken post-mortem. A cross-section through the brainstem will sample maximum ascending and descending nerve tracts. If antigen indistinguishable from rabies was observed upon examination by the standard DFA in another regions of the brain (hippocampus, cerebrum), these should be also be examined by RT-PCR. See section 4.1.1. for the necropsy instrument requirements. The size of brain section, suitable for RT-PCR, is approximately 4x4x4 mm.

5.1. **Shipment of samples** for primary rabies diagnosis by DFA are shipped and transported at refrigeration temperatures of approximately 4 °C. However, samples should be frozen if stored for greater than 72 hours. Samples (brain tissues and RNA samples) specifically for confirmatory testing by RT-PCR should be shipped by overnight carrier on dry ice. Bio-containment during specimen transport is critical, to prevent both contamination of the outside of the package and cross-contamination between samples within the package.

5.2. **Unacceptable deterioration or decomposition of a sample** is a qualitative assessment of a sample's condition upon receipt in the lab. Unacceptable samples may demonstrate altered color (e.g. green, black, brown), desiccation, liquidness,

or unrecognizable gross anatomy. RT-PCR should not be used to rule-out rabies on a sample demonstrating the above characteristics or if it is unsatisfactory for testing by the standard DFA test (neck biopsy).

5.3. **Laboratory handling of samples.** All necropsy and tissue processing must include proper identification of each sample and avoidance of any practice that could lead to cross contamination of samples. Each specimen should be handled on a clean work surface with new disposable gloves. Collection of samples for RT-PCR should be performed using sterile disposable instruments (scalpels and forceps). Internal pieces of tissue should be collected to avoid potential surface contamination.

6. Controls

Controls must be introduced at the step of RNA extraction, and used in the RT-PCR reaction along with the sample tested.

6.1. **Positive controls** must contain the rabies virus RNA. The strong positive control is represented by a positive brain tissue or a cell culture homogenate infected with the rabies virus. The weak positive control will be represented by a tiny piece of the strong rabies positive brain tissue diluted 1:1000 W/V with a rabies negative saliva. These controls are used to demonstrate the test is able to detect rabies virus in both sides of the spectrum concentration wise.

6.2. **Inhibitors control** for this purpose, the near-threshold dilution of positive sample (brain suspension or cell culture supernatant) is added to the second aliquot of the sample tested ("spiked aliquot"). To ensure that potential inhibitors is not an issue in the ensuing diagnostic procedures, positive controls should be handled after the test samples, negative controls, and reagents controls in a segregated bench area. Gloves should be changed immediately after handling positive RNA samples. Similarly, reagents should be added in the same order.

6.3. **Negative controls** will be tissues of non-rabid animals (e. g., saliva and skin biopsy from healthy individuals).

6.4. **Reagent control or Blank** the entire procedure applied to the sample tested to do RNA extraction should be run simultaneously by using reagents only. At the end of the RNA extraction protocol this tube will be added with the same amount of water used to rehydrate the RNA pellet in the sample tested. This will be a RNA extraction blank, which should be used as another sample for the RT-PCR. If multiple diagnostic samples are extracted, it is best to include one reagent control first, and one last.

Negative controls and reagent controls determine the test has been free of any cross contamination during sample processing and that the reagents have not been contaminated with rabies RNA at any step during the process.

6.5. **Template control reactions** should also be set up for the diagnostic and confirmatory testing of RNA samples. Failure to amplify ribosomal RNA, B-

actin, or glycose 6-phosphate dehydrogenase mRNA from the sample would indicate sample RNA degradation and the potential for false negative results.

6. Processing and Testing for Rabies Virus Nucleic Acid

7.1. RNA Extraction

7.1.1. Clean the biological safety cabinet (BSC) and bench surfaces with 3 % hydrogen peroxide or RNase Away prior to starting. Only equipment for the current sample should be in the BSC during an extraction. Frozen samples should be thawed just prior to testing, and reagents and samples should always be kept cold on ice.

7.1.2. In a clean area label sterile micro-fuge tubes and aliquot in the tubes the following reagents: lysis buffer 0.1 ml, Trizol 1.0 ml, chloroform 0.2 ml, isopropanol 0.5 ml. RNA extraction should be performed with one diagnostic sample, followed by clean up before starting another diagnostic sample.

7.1.3. Remove the fat tissue from the bottom of the skin biopsy using sterile scalpel or applicator stick and homogenize the specimen into small fine pieces in a sterile petri dish.

For saliva samples use pipetter with droplet-free barrier tips to transfer 100 to 200 microliters aliquot.

7.1.4. Transfer the homogenized tissue or 100-200 µl of saliva to the screw cap micro-fuge tube containing 100 µl lysis buffer and homogenize using two sterile applicators or sterile micro-tissue grinder sticks as a "mortar".

7.1.5. Add by decanting 1.0 ml of TRIzol reagent into the tube containing the specimen and lysis buffer suspension, homogenize thoroughly using two sterile applicators again, cap the tubes tight and mix approximately 30 seconds by shaking vigorously and allow to sit 5 minutes at room temperature.

7.1.6. Add 0.2 ml chloroform to these tubes. Vigorously shake approximately 30 seconds by hand, and allow to settle 2-3 minutes. Upon mixing, the suspension will become opaque as chloroform emulsifies the cellular lipids.

7.1.7. Centrifuge at 12,000 x G at 4 ^{o}C for 10 minutes to separate phases (aqueous phase in the clear upper layer contains RNA, middle white layer contains protein, and the bottom red layer contains phenol and DNA).

7.1.8. Carefully collect by pipeting the upper aqueous phase and add it to the tube containing 0.5 ml isopropanol. Mix by shaking or vortexing briefly. Allow RNA to precipitate for 10-15 minutes at room temperature.

7.1.9. Centrifuge the RNA/isopropanol tube for 10 minutes at 12,000 x G at 4 °C.

7.1.10. Carefully pour off the isopropanol and discard into chemical waste.

7.1.11. Add 1.0 ml 75% ethanol, and centrifuge 7,500 x G for 5 minutes.

7.1.12. Carefully pour off ethanol from sample tube, discard into the chemical waste, and blot the sample tube on a sterile gauze pad.

7.1.13. Rehydrate the RNA by adding 100 µl molecular grade DEPC water to the tube. Some labs prefer to use RNA secure ™.

7.1.14. Vortex on low to medium speed for 2 minutes and spin it down during 5 seconds, then incubate at 56 °C for 10 minutes in a water bath to dissolve the RNA pellet. Do not keep in water bath longer than 10 minutes as this can lead to RNA degradation. Use immediately or freeze at -70 °C or below. RNA prepared in this way is stable for several years

7.2. RT-PCR Protocol Outline

7.2.1. Reverse Transcription

7.2.1.1. Prepare RTRX working buffer: For each set of five samples, thaw one tube RTRX mix (71.6 µl). Add 2.0 µl (approximately 50 units) reverse transcriptase and 2.0 µl (approx. 80 units) protector RNase inhibitor to make the working RTRX buffer. Remember to keep reagents and samples on ice while setting up the RTRX reactions. RNA and enzymes are heat labile. Briefly spin* all RNA sample tubes in a microcentrifuge tube to avoid cross-contamination.

7.2.1.2. Prepare working dilutions of the RT primer using molecular grade water. RT primer: 10 µM (if degenerate) from 40 uM stock, or 5 µM (if specific primer) from 20 µM stock.

7.2.1.3. Add 2 µl of the RT primer (10 µM degenerate or 5 µM specific) to each labeled PCR reaction tube (snap-cap size 0.5 ml, 0.2 ml). Set up multiple tubes for each sample with different primer sets, and label with sample identification and primer information on the tube or accompanying information sheet.

7.2.1.4. Briefly vortex and centrifuge the RNA sample tubes, and add 5 µl RNA to each reaction tube containing the RT primer. Briefly centrifuge the reaction tubes.

7.2.1.5. Denature the sample and primer by heating the tube to 94°C for 1 min, and then cool on ice for approximately 3-5 minutes.

7.2.1.6. Add 14 µl RTRX Buffer to each reaction tube (RTRX buffer with reverse transcriptase and RNase inhibitor) briefly vortex and centrifuge.

7.2.1.7. Preheat block to 42°C before placing the tubes in the thermocycler. Incubate tubes at 42°C for 90 min with 4°C hold. Although shorter intervals are suitable depending on the strand size, the 90 minute incubation may increase the sensitivity for the diagnostic RT-PCR. *Important: The heat block should not exceed 42 °C or the RT enzyme will be degraded or inactivated.*

7.2.2. Polymerase Chain Reaction (PCR)

7.2.2.1. In a separate clean room prepare the PCR Pre-mix Buffer (as previous described for every 5.4 reactions) by adding 372.6 µl molecular grade water, 43.2 µl of Tris buffer pH 8.3, 2.7 µl (5 U/ µl) Taq polymerase (Amplitaq™ PE Biosytems# N880153), 5.4 µl of 20uM specific or 40uM degenerate Primer 1 (RT or forward primer) and 6.75 µl of 20uM specific or 40uM degenerate Primer 2 (reverse), and label appropriately.

7.2.2.2. Remove the RT reaction tubes from the thermal cycler, centrifuge the tubes briefly, and place them on ice.

7.2.2.3. Add 80 µl PCR premix buffer to each of sample reaction tubes. Mix briefly by vortexing and centrifuge briefly.

7.2.2.4. Place tubes in thermocycler preheated to 94 °C and run 40 cycles of PCR (1 minute at 94°C followed by 40 cycles of 94 °C for 30 seconds, 37 °C for 30 seconds, and 72 °C for 90 seconds; followed by 7 minute extension at 72 °C).

7.2.3. Nested PCR (Reamplification of PCR reactions with primers internal to the sets used for gene fragment amplified during the primary PCR or hemi-nested using only one internal primer and one of primers used for the primary RT-PCR)

7.2.3.1. If nested or hemi-nested RT-PCR is to be performed, prepare the nested PCR buffer premix in advance by adding (for every 5.4 reactions tubes) 372.6 µl molecular grade water, 43.2 µl l M Tris buffer 8.3, 2.7 µl Taq polymerase (Amplitaq), 71.6 µl of RTRX buffer (without enzymes and protector RNase inhibitor), 6.75 µl of forward nested (or hemi-nested) primer and 6.75 µl of primer reverse nested (or hemi-nested) primer in the same concentrations as for the primary RT-PCR, e.g. 20uM for specific and 40uM for degenerated primers.

7.2.3.2. Remove 10 µl of the primary RT-PCR product from each tube in a designated area where cDNA may be handled, and add it to a tube containing 90 µl of nested PCR buffer Pre-mix.

7.2.3.3. Run the nested (or hemi-nested) reaction with the same cycle parameters that were used for the primary RT-PCR.

7.2.4. Gel Electrophoresis and Detection of Amplicons

Gel Electrphoresis for detection of amplicons should be done in a separte laboratory for handling cDNA samples. PCR products should never be opened in an area where RNA extraction or RT is performed.

NuSieve 3:1 (4% agarose) is an excellent medium for high resolution separation and visualization of short amplicons of 400 bp or less required for diagnostic RT-PCR. Molecular weight markers and products of 50 base-pairs (bp) are distinctly visualized. RT-PCR cDNA products of 600-1500 bp are easily visualized with clarity using 2% general electrophoresis grade agarose (See TableX below). To prepare add the appropriate amount of NuSieve 3:1 agarose or general purpose electrophoresis agarose to a polypropylene flask, and mix by swirling. Using a microwave, melt the agarose completely. Cool to approximately 45 °C by running tap water along the outside wall of the flask until just warm to touch. Add 1.5 µl of ethidium bromide to 30 ml of the agarose, swirl to mix, and add aqgarose solution to a mold. Place a gel comb into the agarose. Allow the gel to solidify approximately 30 minutes at room temperature.

7.2.4.1. Add 1x TBE to the surface of the solidified gel. Place the gel into an electrophoresis chamber.

7.2.4.2. Mix 4 µL of (6x) loading dye with 20 µL of DNA 100 bp ladder (molecular weight markers) and add 10 ul of the molecular weight marker mixture to the first and last wells of the agarose gel.

7.2.4.3. Mix 4 µL of (6x) loading dye with 20 µL of each RT-PCR, nested or hemi-nested PCR product, and add the mixture to the remaining wells of the gel.

7.2.4.4. After all samples have been loaded, subject the gel to electrophoresis at 150 V for 15-20 minutes

7.2.4.5. Check for specific amplicons by exposing the gel to a high wave UV light source. Document the results by photographing.

8. PCR Test interpretation

Experience is necessary to interpret the PCR results correctly. First, it is necessary to determine if the test is valid. To consider a test valid, all of the controls implemented in the test must perform as expected:

8.1. Negative controls: A band *must not* be present in any of the lanes run for negative controls. If this occurs (i.e., false positive), sample contamination may have occurred.

8.2. Reagent control: A band *must not* be present in any of the lanes run for reagent controls. Again, if this occurs (i.e., false positive), sample contamination may have occurred.

8.3. Positive controls: Bands *must* be present in all lanes run for positive control samples.

8.4. Each sample *must* have a band corresponding to the positive template control (e.g., band present for B-actin or other gene tested). Failure to amplify the template control could indicate inhibition of the PCR or degradation of the sample.

8.5. If criteria 1-4 above are not met, results cannot be reported. The run *must* be repeated.

8.6. A sample is considered positive ("Nucleic Acid Detected") if criteria 1-4 above are met and a band of the expected size is present in the lane run for the sample. Nucleic acid sequencing is required to confirm that the band represents cDNA from rabies virus.

8.7. A sample is considered negative ("Nucleic Acid Not Detected") if criteria 1-4 above are met and there is no band present in the lane run for the sample.

8.8. If a sample is negative for the template control on two consecutive tests, report as "specimen unsatisfactory for testing".

8.9. All positive and suspected results must be confirmed by nucleotide sequencing, and the sequence obtained must be identified via comparison with sequences available in the laboratory and public domain (GenBank).

TABLE 1: SUGGESTED PRIMERS FOR RT-PCR FOR RABIES DIAGNOSIS

Designation ID	Broadly Reactive or Degenerate Primers			
	Orientation	Genome position [1]	Sequence	Reference
Lys001	Forward	1-16	ACGCTTAACGAMAAA	Markotter et al., 2006
JW12	Forward	55-73	ATGTAACACCICTACAATG	Heaton et al., 1997
550 F*	Forward	647-666	ATGTGYGCTAAYTGGAGYAC	*Markotter et al., 2006
550B	Reverse	647-666	GTRCTCCARTTAGCRCACAT	Markotter et al., 2006
JW6degC*	Forward	641-660	CAYAARATGTGYGCIAAYTG	*Heaton et al., 1997
1087Sdeg	Forward	1157-1173	GAGAARGAACTTCARGA	Orciari et al., 2007
1087ideg	Forward	1157-1176	GAGAARGAACTTCARGAITA	Mcquiston et al., 2001
1066 deg F	Forward	1136-1155	GARAGAAGATTCTTCAGRGA	Orciari et al., 2007
1066deg B*	Reverse	1136-1155	TCYCTGAAGAATCTTCTYTC	*Orciari et al, 2007
1312Bdeg C	Reverse	1382-1401	TTGTTTARAAAYTCIGCRAA	Mcquiston et al., 2001
†304	Reverse	1514-1533	TTGACGAAGATCTTGCTCAT	Smith, 1995
N8 B deg C	Reverse	1567-1584	GTYTCTTCIGCCATYTCT	Bourhy et al., 1993
†504S	Forward	1296-1312	TCATGATGAATGGAGGT	Orciari et al., 2007

[1]According to the full genome sequence of the fixed rabies virus strain, SAD B19 (Conzelmann, K.K., et al 1990. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology. 175:485-499.

† Non-degenerate primers

*Reverse complement of a published primer

9. Sensitivity and specificity

Sensitivity and specificity of RT-PCR was compared to those of the direct fluorescent antibody (DFA) test (Dean et al., 1996). When implemented for fresh brain tissue samples of rabid animals, DFA is considered as the "gold standard" for rabies diagnosis. When performed according to the CDC SOP (http://www.cdc.gov/rabies/docs/standard_dfa_protocol_rabies.pdf), DFA was never reported to fail. The situations when DFA test may give inconclusive results include highly deteriorated field specimens. In addition, DFA test can not be used for saliva and other liquids, as only tissue impressions or sections may be tested.

For randomly collected 75 field samples of animal brain, sensitivity of RT-PCR compared to DFA was 97% (Durr et al., 2008). The discrepancies between DFA and RT-PCR results were observed for several deteriorated, dry specimens, collected from carcasses of animals that died several days ago. In 3% of such samples DFA gave positive results when RT-PCR was negative however, in other 8% of such samples RT-PCR provided positive results (confirmed by further sequencing of viral genome), whereas DFA results were negative. For fresh brain and skin biopsy samples tested routinely at CDC (n>200), sensitivity of RT-PCR compared to DFA test was 100%.

Specificity of RT-PCR is 100% as long as the reaction results are confirmed by nucleotide sequencing. Host RNA of various animal species may be occasionally amplified by primers specific for rabies virus genome. In most of such cases the length of the amplified product is different from the length of positive control. However, in other cases nucleotide sequencing is the only available way to confirm the specificity of the RT-PCR result.

10. Precision

Precision of RT-PCR was assessed during over 10 years of routine diagnostic and experimental work of Rabies Program, CDC. Specifically, if positive and negative control samples were run daily for 25 days, no unexpected results were detected. The same concordance was obtained when samples (total n umber over 1600) were tested 2-3 times per week. When monitoring of viral RNA load in human saliva was implemented via titration of the specimens by two independent investigators (4 samples x 4 runs x 2 investigators), no discrepancies between the investigator results and between run results were documented.

11. Analytical sensitivity

Analytical sensitivity of RT-PCR was examined for serial dilutions of viral inoculum, in comparison with mouse intracerebral lethal doses ($MICLD_{50}$). The values depend on the sample condition, and nucleotide sequences of viral genomes, which provide comprehensive or partial matching of the RT-PCR primers. Primers provided in the Table 1 were shown to be able to amplify all major rabies virus variants circulating worldwide in different animal species. For fresh viral suspensions the analytic sensitivity of RT-PCR usually vary between 1 to 10 $MICLD_{50}$/0.03 µl. However, when nested RT-PCR is used, the sensitivity increases up to 0.01-0.1 $MICLD_{50}$/0.03 µl. When samples are deteriorated,

infectious titers degrade faster than the ability of RT-PCR to detect partial fragments of viral genome. When the most abundant N gene of rabies virus is used as target, and short nucleotide chains are amplified (100-500 nuc), the nested RT-PCR is the most sensitive diagnostic method known to date.

12. Analytical specificity

The potential presence of substances that interfere with the performance of RT-PCR is assessed by spiking of the second aliquote of the test sample by positive control sample, taken in concentration close to the limit of detection (routinely 0.1 ml of 0.1% infected brain suspension per 0.1 ml of the test sample). The spiking is performed immediately before the RNA extraction. The spiked aliquote is processed along with the non-spiked aliquote and with a clean positive control sample.

In turn, the presence of substances that may cause non-specific amplification is detected at the step of nucleotide sequencing of the RT-PCR product. The sequencing is performed whenever the size (nucleotide length) of the RT-PCR product is indistinguishable from the size of the positive control sample. Therefore, a potential cross-reactivity and false positive results are verified and eliminated. In addition, sequencing provided identification of the virus variant.

13. Acceptability of reagents

The verification of diagnostic reagent performance is undertaken for each new lot of reagents. At least one known positive and one known negative samples are tested concurrently using reagents from the old lot and from a new lot. In addition, the reagent control is implemented into each reaction run, to ensure the absence of non-specific amplification caused by reagents in the absence of the specific nucleotide sequence.

14. Calibration and control procedures

Calibration of pipets and thermal cyclers used for RT-PCR is performed once per year via standard procurement provided by CDC. Proficiency testing is performed once per year via blind testing of two negative, two weak positive and two strong positive samples by at least two diagnosticians, using independently prepared reaction mixes.

15. Laboratory design and practices

Unidirectional workflow is maintained for the RT-PCR procedures. Three physically separate areas are used for sample preparation, preamplification and postamplification procedures. Separate ventilation systems are provided for these areas. Negative pressure is maintained in all laboratory facilities. Prior to the sample preparation and the reaction all laboratory surfaces and equipment are cleaned by 3% hydrogen peroxide solution.

Sample preparation is performed in a class II biological safety cabinet. After mixing with TRIzol reagent and incubation for 5 min at room temperature, the sample is considered non-infectious and may be transferred to a bench of the room designated for

RNA extraction and RT-PCR amplification. Gloves are changed between each reaction step, after handling the positive samples, and additionally as often as needed if a possibility of contamination is suspected. Only aerosol-free tips are used for the reaction. After completion of the reaction, the tubes are transferred to the postamplification room, where they may be opened and subjected to gel electrophoresis. After amlification the reaction tubes may never be open in the room where preamplification steps and the amplification were carried out. In addition, separate long-sleeved laboratory coats are used for pre- and postamplification areas, the equipment, reagents and tube racks used in the postamplification area may never be transferred back to the preamplification area.

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附件五

Centers for Disease Control and Prevention
Institutional Animal Care and Use Committee (CDC-Atlanta) **Policy 026**

TITLE: Blood Collection Volumes	**APPROVED:** 03/01/2010
IACUC POLICY: 026 **REVISION:** 2	**REVISED:** 02/22/2010
SCOPE: This policy applies to any blood collection from commonly used laboratory animals in survival protocols.	
PURPOSE: To describe the maximum blood volume that may be collected in a single sample or multiple samples over a specified time period for commonly used laboratory animals.	
KEYWORDS: blood, collection, volume, serial sampling	

1. **BACKGROUND:**
The Animal Welfare Act Regulations [9 CFR §2.33(b)(2)], the *PHS Policy on Humane Care and Use of Laboratory Animals* (IV.C.1.e), and the *Guide for the Care and Use of Laboratory Animals* (p. 55-66) all require the provision of adequate veterinary care for the diagnosis, control, and treatment of diseases and injuries.

2. **POLICY:**
Investigators are responsible for designing animal use protocols in which blood collection volumes are not detrimental to the health and well being of the animal. Collection volumes in excess of these guidelines require scientific justification in order to receive approval of the CDC-Atlanta IACUC. It is the responsibility of the Attending Veterinarian to determine whether the age and general health of a particular animal may affect the allowable blood withdrawal volumes. The Attending Veterinarian may also restrict blood withdrawal during scheduled repeat bleeds if the health of the animal is jeopardized as determined under the Animal Resources Branch Standard Operating Procedures.

A. Guidelines for a single blood sample collection:

(1) Limit a single sampling to 10% of Total Blood Volume (TBV) assuming 1 ml of blood weighs 1 gm. A single sampling exceeding 10% of the blood volume may be feasible in an animal in prime health when scientifically justified and when adequate replacement fluids are prescribed and administered under veterinary supervision. Under no circumstances will a single sampling exceeding 20% of blood volume be approved. Table 1 gives the total blood volume of some common laboratory animals. The mean value listed for blood volumes should be used to determine maximum blood collection volumes, not the range.

(2) Single samplings that exceed 10% of estimated total blood volume require a 30 day resting period before another sample may be collected.

(3) Blood volume as a percentage of body weight varies with the size of the animal. In general, larger animals have a lower blood volume per unit weight than do smaller animals. If the blood volume for a species is not listed below and not known, a value

equal to 6% of the body weight will be used. This estimated volume should be used to determine maximum allowable single sample or serial sample blood collections.

B. Guidelines for repeated (serial) sampling

(1) Weekly blood collection volume should be limited to 7.5 % of total blood volume (TBV).

(2) For repeated bleeds at shorter intervals, a maximum of 1.0% of an animal's TBV can be removed every 24 hours.

(3) Repeated (serial) sampling will not exceed 7.5 % TBV within a seven (7) day period.

(4) Amount of blood samples required on serial bleeding as per protocol will be adjusted based on the actual weight of the animal assigned to the protocol.

(5) When performing serial bleeds, 24 hours of rest should be allowed for each 1% of body weight sampled. Protocols requiring serial bleeds in amounts that do not meet the guideline on rest, and are scientifically justified, may be approved by the IACUC, but may not exceed the 7 day or 30 day maximums.

(6) The total blood volume withdrawal for macaques should not exceed 6.6 ml/kg/month.

Table 1. Blood volumes of common laboratory animals

Species	Mean Vol. (ml/kg)	Range (ml/kg)	Body Weight	Est. Total Blood Volume (ml)
Rat	64	58-70	300 g	19.2
Mouse	79	68-84	30 g	2.4
Hamster	78	66-80	100 g	7.8
Guinea Pig	75	67-92	700 g	52.5
Rabbit	56	44-70	4 kg	224
Ferret	75	60-80	1kg	75
Dog	86	79-90	12kg	1032
Rhesus	54	44-67	7 kg	378
Cynomolgus	50	44-61	5 kg	250

3. RESPONSIBILITIES:

A. CDC-Atlanta IACUC - review protocols involving blood collection to ensure consistency with the provisions of this policy.

B. Animal Care and Use Program Office - provide resources and guidance to the IACUC, animal research investigators, and care staff on current regulatory and laboratory animal standards in blood collection.

C. Animal research investigators and care staff - ensure that blood collection regimens in proposed and approved animal studies meets the provisions described in this policy to minimize animal pain and distress.

4. REFERENCES:

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E. Ageyama, N., H. Shibata, H. Narita, K Hanari, A. Kohno, F. Ono, Y. Yoshikawa, and K. Terao. 2001. Specific gravity of whole blood in Cynomolgus monkeys (*Macaca fascicularis*), squirrel monkeys (*Saimiri sciureus*), and tamarins (*Saguinus labiatus*) and total blood volume in Cynomolgus monkeys. *Contemporary Topics*, 40:33-35

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G. McGill, M.W., and Rowan, A. N., 1989. Biological effects of blood loss: implications for sampling volumes and techniques, *ILAR News*, 31(4):5-20.

H. National Centre for the Replacement, Refinement, and Reduction in Laboratory Animal Research. Blood Sampling Microsite http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=313

5. REVISION HISTORY:

Revision Number	Summary of Revisions	Revision Date
1	Reformatted into new standardized policy template.	08/16/07
2	Adjusted language to match ARB SOP and AV recommendations and added references.	08/04/09
3	Addition of ferrets and dogs to Table 1.	2/9/10

6. PERIODIC REVIEW:

Review Date	Reviewer

08/16/07	CDC-Atlanta IACUC Policy Subcommittee
08/04/09	CDC-Attending Veterinarian Review
09/14/09	CDC-Atlanta IACUC
03/01/10	CDC-Atlanta IACUC