出國報告（出國類別：研究）

赴澳洲動物衛生實驗室研習
「狂犬病診斷及特性」

服務機關：　行政院農業委員會家畜衛生試驗所

姓名職稱：　張仁杰 助理研究員

　　許偉誠 助理研究員

派赴國家：　澳洲

出國期間：　099 年 11 月 06 日至 099 年 11 月 21 日

報告日期：　100 年 02 月 17 日

摘要

爲執行「野生動物新浮現疾病監測體系及防疫策略研究」，加強臺澳農業國際合作，於99年11月6日至21日奉派至澳洲，赴澳洲動物衛生實驗室 (Australian Animal Health Laboratory, AAHL)，參加「狂犬病之診斷及特性 (Diagnosis and Characterisation of Rabies)」訓練班，透過在AAHL狂犬病診斷實驗室與其研究人員共同進行各項實驗室診斷，從收件、處理檢體、實際操作犬、貓及蝙蝠等野生動物採樣並進行麗莎病毒 (Lyssavirus) 與狂犬病病毒各項檢驗，包含動物剖檢採樣、血清中抗體檢測 (Rapid Fluorescent Focus Inhibition Test, RFFIT；Fluorescent Antibody Virus Neutralization, FAVN)、病毒分離、分子生物學檢測 (RT-PCR)、澳洲蝙蝠麗莎病毒 (Australian Bat Lyssavirus, ABLV) 螢光抗體染色 (Direct Fluorescent Antibody Test, FAT)、電子顯微鏡學檢查等，瞭解澳洲狂犬病診斷、監測及防檢疫體系、實驗室操作實務及生物安全控管，頗值得本所狂犬病診斷實驗室借鏡，並可強化我國針對野生動物新浮現疾病監測及防檢疫體系，有助於相關策略研擬。

目 次

目的

近年來野生動物在新浮現人畜共通傳染病的重要性日漸增加，其中又以蝙蝠之狂犬病及麗莎病毒 (Lyssavirus) 感染症等疾病對我國社會民心之影響最劇。有鑑於澳洲在蝙蝠麗莎病毒 (Australian Bat Lyssavirus, ABLV) 之研究經驗豐富，居世界領先地位，本所又是全國唯一動物衛生試驗研究專責機構，肩負著國內動物傳染病防治科技研發與疾病檢驗診斷的重責大任，對防範新浮現人畜共通傳染病之入侵責無旁貸。爲強化新浮現人畜共通傳染病診斷與監測體系，以及培育動物疾病診斷人才，藉由派兩名研究人員赴澳研習野生動物新浮現人畜共通傳染病之診斷與監測技術，初步選定蝙蝠之狂犬病、Lyssavirus感染症等疾病，建立蝙蝠新浮現人畜共通傳染病之診斷與監測技術，經由研習或參加國際研討會等模式，觀摩收集澳洲野生動物新浮現人畜共通傳染病之標準診斷作業程序與疾病監測制度資料。

研習機構介紹

澳洲動物衛生實驗室 (Australian Animal Health Laboratory, AAHL) 位於澳洲東南部維多利亞省的第二大城Geelong，距離墨爾本約1小時車程。AAHL隸屬於澳洲科學與工業研究組織 (Commonwealth Scientific and Industrial Research Organization, CSIRO)，爲澳洲國家級動物疾病診斷中心，主要負責動物疫病爆發時之快速診斷，是澳洲防止新浮現動物疾病入侵，維持漁、牧產業貿易競爭力，及維護人畜健康之重要機構。AAHL具備全世界規模最大的生物安全設施，避免家畜疾病及野生動物疾病病原外洩，提供安全、快速且準確的疾病診斷服務；另具備生物安全第四等級 (BSL-4) 實驗室，讓研究人員能夠安全地進行各種具高度傳染性及致病性人畜共通病原的診斷與研究。同時AAHL與國內外實驗室合作，建構疾病診斷網絡及跨國合作平台，獲得多項國內及國際組織認證，包括：(1)世界動物衛生組織 (OIE) 之禽流感、立百病毒與亨德拉病毒感染症、新城病、藍舌病之參考實驗室；(2)OIE新浮現疾病合作實驗室；(3) OIE獸醫實驗室建構能力合作實驗室；(4)世界衛生組織 (WHO) SARS病毒合作實驗室；(5)聯合國糧食及農業組織 (FAO) 區域參考實驗室；(6)狂犬病及布氏桿菌症之國家參考實驗室…等。AAHL的任務除了疾病診斷及研究外，同時也提供國內外實驗室人員重要疾病診斷技術的培訓，因此每位AAHL的研究人員都很會教學且十分有耐心，可稱得上是澳洲動物疾病診斷的教學中心。AAHL員工總人數約300人，其中科學家約150人，技術人員約50人，負責機電技師及維修人員約100人。AAHL每年維持運作之相關經費相當可觀，總計達4,500萬澳幣 (約新臺幣13億5千萬)，其中80%經費來自於澳洲政府，10%來自國際組織，如FAO、OIE等，10%來自與民間企業或公司之研究合作或委託試驗補助經費。

圖一、澳洲動物衛生實驗室 AAHL。

研習課程表

日期	星期	研習內容
11/ 6	六	由桃園國際機場搭機前往澳洲
11/ 7	日	經曼谷轉機抵達澳洲維多利亞州墨爾本機場，搭接駁車至 Geelong 住宿處
11/ 8	一	實驗室介紹與生物安全防護訓練
11/ 9	二	螢光抗體染色 FAT 之實作
		生物安全第四等級實驗室及設備維護部門參訪
11/10	三	狂犬病疑似病例剖檢
		病毒分離之實作
11/11	四	中和抗體檢測 RFFIT 之實作
		狂犬病病例切片判讀
11/12	五	免疫組織化學染色 IHC 之實作
		中和抗體檢測 RFFIT 之結果判讀
11/13	六	假日
11/14	日	假日
11/15	一	中和抗體檢測 FAVN 之實作
11/16	二	即時反轉錄聚合酶連鎖反應之實作
11/17	三	中和抗體檢測 FAVN 之結果判讀
11/18	四	病毒分離之結果判讀
11/19	五	電子顯微鏡實驗室及實驗動物房參訪
		綜合討論
11/20	六	搭機啓程返國
11/21	日	返抵臺灣桃園國際機場

研習行程

為執行 99 年度自辦科技計畫「野生動物新浮現疾病監測體系及防疫策略研究」(99 農科-4.1.1-衛-H1)，經與澳洲動物衛生實驗室 (Australian Animal Health Laboratory, AAHL) 取得聯繫，自 3 月開始提出申請，至 8 月中旬通過審核同意，隨即辦理澳洲簽證相關事宜，終於在 11 月 6 日得以順利啓程。

本次研習承蒙 AAHL 獸醫病理學專家 Dr. John Bingham 及獸醫部門主任 Dr. John Allen 百忙之中為我國與尼泊爾共 3 名研究人員規劃辦理為期兩週之「狂犬病之診斷及特性」訓練班，以及 Mrs. Nicole Mclure 等人協助行前申請及電子郵件聯繫。研習期間受到澳洲病毒學專家 Dr. Andrea Certoma 在工作及日常生活上熱心接待，安排課程（狂犬病訓練計畫表、附件一）與悉心指導，及病理學、病毒學及血清學實驗室研究同仁的指導，使得本次研習得以順利完成並收穫豐碩。

一、11 月 8 日 (星期一)：

(一) 生物安全與環境介紹

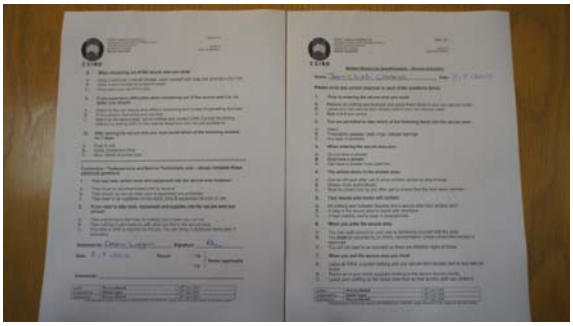
AAHL 基於生物安全的考量，將行政區 (non- secure area) 與實驗區 (secure area) 做完全的區隔，研究人員在 non- secure area 處理文件與一般事務，但如果要做實驗則須進入另一棟建築，稱為 secure area。AAHL 的 secure area 據稱是目前全世界規模最大的生物安全設施，整棟建築皆採高生物安全等級的負壓實驗室設計，所有人員進入 secure area 前要將所有私人衣物、飾品在外部更衣室換下，接著通過第一道氣密門進入淋浴間，進入 secure area 時不需淋浴，直接通過第二道氣密門進入內部更衣室，在更衣室內備有一套經滅菌消毒的乾淨內衣褲、鞋襪、工作服及門禁管制卡，換上之後才能進到 secure area。離開的方式與進入相同，但是必須要洗澡後才能離開，為防止同仁偷懶沒有淋浴就直接出去，淋浴室氣密門還有計時裝置，進入淋浴間要等 3 分鐘才能打開另一側的氣密門。研究人員在 secure area 內有處理文書的辦公室，但所有物品嚴禁攜出，文件只能以掃描方式存成電子檔，紙本則視為醫療廢棄物丟棄。其他任何實驗耗材、儀器、甚至食物、飲料等，在 secure area 內也都是只進不出。進入實驗室還要再套上一件防護服及乳膠手套才能進行實驗。

(二) Secure area 測驗

訪客與新進員工要進入 secure area 前，需接受 AAHL 生物安全介紹課程，並進行如何進出高生物安全等級實驗室區域 secure area 簡介與測驗，測驗通過之訪客方能進入實驗室觀摩與實務操作，除狂犬病診斷，還有藍舌病、日本腦炎、立百病毒等傳染病實驗室診斷，還有魚病檢驗實驗室 (主要針對進口魚類檢疫)。特別的是 AAHL 擁有 BSL-4 的實驗室，可利用來進行立百病毒 (Nipah virus)、亨德拉病毒 (Hendra virus)、伊波拉病毒 (Ebola virus)等新浮現人畜共通傳染病之試驗研究。secure area 測驗重點：

1. Secure area 內，所有物品只進不出。唯一例外的是眼鏡，離開前眼鏡要泡消毒水至少 2 分鐘，然後一起跟著淋浴沖洗後才可攜出。
2. 進入 secure area 不需洗澡，出來才要洗澡。
3. 從 secure area 出來前，指甲縫必須刷洗乾淨。
4. 進出 secure area 後，一週內不可接觸家畜。
5. Secure area 內禁止拍照，實驗過程有需要拍照可由講師利用實驗室內的相機拍攝後，將檔案透過內部網路傳輸至行政區域辦公室電腦。
6. 所有筆記只能用 scan 方式攜出。
7. Secure area 內也有 clean zone 與 non-clean zone 的區別，分別備有藍色手術衣及深綠手術衣做爲防護衣。
8. 人員依工作性質給予不同權限的門禁管制卡 (badge)，每經過一個管制門都要感應，若前一道門忘記感應，下一道門就不能開而且回不去 。
9. 訪客在 secure area 內隨時都要有員工陪同，訪客的 badge 在 secure area 所有實驗室都需要員工先行感應才能通過，不能單獨留訪客在實驗室。
10. 進出 BSL-3 實驗室要填進出紀錄簿。

圖二、生物安全介紹課程。


圖三、 Secure area 測驗 。

二、11 月 9 日 (星期二)：

(一) 設備維護樓層參訪

由 Mr. Warren Mitchell 介紹維持 AAHL 運作重要機電設施，如廢水處理 (secure area 廢水經過兩道處理)、空氣過濾 (進氣端有一道初級濾網及一道高效率過濾網箱體 3 μm 孔徑可濾過 99.97 %以上，可防止回流造成病原外洩；排氣端有兩道高效率過濾網箱體確保病原不外洩)、焚化爐 (每週進行焚化處理一次)；AAHL 編制有大約 40 名機電設施維護人員，24 小時輪班負責前述例行性業務。另有 5 名人員負責輪值中央監控中心，遇到機電異常或突發狀況時可立即透過對講機聯繫維護人員處理。

Secure area 整棟建築爲一地上 3 層、地下 2 層的 5 層樓建築，其中實驗室只有 1 層，由其他 4 層機械設備維持正常運作。各樓層配置如下：

Level 5	抽風機、冷熱空調設施
Level 4	高效空氣濾網 (Hepa) 設備
Level 3	負壓實驗室
----------地平面----------	
Level 2	廢水儲存槽
Level 1	廢水消毒槽

圖四、樓層配置。

圖五、機電設施樓層配置模型 。

實驗室廢水處理流程：實驗室水槽→儲存槽→第一消毒槽→第二消毒槽→冷卻管→外部環境。廢水經水管管線，緩緩流至 level 2 儲存， 廢水儲存到達一定的量後送至消毒槽。消毒槽內有感應器，當廢水半滿即自動關閉，會將水導引到另一槽。第一消毒操的滅菌條件爲 125℃、30 分鐘，滅菌完成後再排到第二消毒槽。第二消毒槽的滅菌條件則爲 100℃、20 分鐘。所有實驗室排出的廢液會經過二次消毒後，才會排放到一般水溝。冷卻管裝有溫度感應器，若偵測到廢水溫度低於 100℃時，會把廢水引入第二消毒槽再次加熱滅菌。

(二) BSL-4 訓練實驗室

Secure area 在 level 2 建構一間 BSL-4 訓練實驗室，提供內部人員及澳

洲其他實驗室訓練用，必須穿上類似太空服的高生物安全防護服 (頭盔、雨鞋、手套一體成型)，訓練如何進出 BSL-4 實驗室及操作試驗。防護服腰際處有空氣連接環，可連結空氣設備管；頭盔上有排氣孔，排出多餘的空氣。

BSL-4 實驗室配置 (四道氣密門)：

1. 外部緩衝室

2. 更衣室：在此處脫光衣物，先穿上橘色工作服，再穿上防護服

3. 化學消毒室 (chemical shower)

4. BSL-4 實驗室

進入更衣室後，每個房間都有多套不同來源的空氣供給設備固定於天花板，以確保人員安全 (至少 4 組供給設備)。因空氣供給設備爲固定式，人員移動時需拔掉空氣管，朝下一個供給設備處前進，空氣管拔掉後防護衣內的氧氣還可維持數分鐘，要盡快找到可使用的空氣管。除練習進出實驗室、體驗穿著笨重的防護衣操作實驗外，最重要的是可進行各種緊急情況的模擬，訓練研究人員的應變能力。

BSL-4 實驗室安全規範簡介：

1. 每次進入需 2 人爲一組，同進同出以確保發生意外時有另一人可對外求援；若非不得已只有 1 人進入時，會由單位主管從實驗室外部每 10 分鐘與人員以無線電通話一次確認狀況。
2. 人員須經 10 小時以上安全訓練，包括穿著防護衣的實際操作及緊急狀況演練等，經單位主管督導與測驗，通過後始可進入實驗室。
3. 手會 double glove (內層戴一般乳膠手套，再穿上一體成形的防護衣) 。
4. 人員離開時要先對衣服實施 chemical shower 後，再脫掉高生物安全防護服洗澡。
5. 需 BSL-4 實驗室才能操作的病原：Hendra virus、Nipah virus、Ebola virus …。Hendra 要在 BSL-4 的主要原因在 pathogen 的未知性，有報告指出有人的病例感染 5 年後才發病。

(三) AAHL 狂犬病診斷實驗室

AAHL 的狂犬病診斷實驗室接受來自全國各地具攻擊傾向的動物樣本及其他實驗室有疑義的病例，主要檢測對象爲蝙蝠，而輸入犬、貓進行邊境

管制 (檢疫期間死亡才做檢測)。澳洲各地偶爾會發生蝙蝠攻擊人類的事件，尤其是氣候溫暖的昆士蘭省發生頻率更高。當某處發生蝙蝠攻擊事件，地方政府會派人赴現場捕捉蝙蝠，通常具攻擊傾向的生病蝙蝠都會滯留在原地，遇到有人或動物經過時再次發動攻擊，因此很容易被捕捉到。捉到的蝙蝠經符合生物安全規範的嚴密包裝後會立即被送到 AAHL。蝙蝠樣本直接在狂犬病實驗室的生物安全操作台內進行採樣，主要以腦部爲主。AAHL 的狂犬病實驗室爲一間 BSL-3 等級的實驗室，所有進入該實驗室的人員都需施打狂犬病疫苗，並經過狂犬病中和抗體檢測，抗體力價達 0.5 IU/ ml 以上，具足夠的保護效力始可進入以確保安全。腦組織取出後沿正中線做縱切，一半放入 10%中性福馬林固定，至少 24 小時、待狂犬病病毒不活化後送至病理室進行組織切片製作及 IHC 染色；另一半則進行 FAT、RT-PCR 及病毒分離。目前僅在蝙蝠檢出屬於麗莎病毒 (Lyssavirus) 第 7 基因型的 Australian Bat Lyssavirus (ABLV)，犬隻檢體皆爲陰性。抗體檢測部分除輸出入犬貓等動物血清檢測外 (澳洲犬貓平時不打疫苗，欲輸出至國外才打，且狂犬病抗體力價需大於 0.5 IU/ ml 才准許輸出)，AAHL 還接受人類血清檢測抗體力價。AAHL 近年檢測成果：

2007 年— ABLV 陽性 10 例，但檢體來自同一區域。

2008 年— ABLV 陽性 0 例。

2009 年— ABLV 陽性 3 例。

2010 年— ABLV 陽性 1 例。

(四) 螢光抗體染色

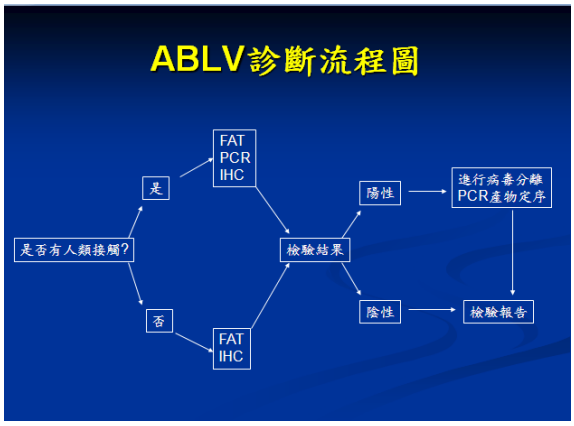
螢光抗體染色 (Direct Fluorescent Antibody Test, FAT) 爲狂犬病及 Lyssavirus 感染症診斷之標準方法之一，原理爲利用單株抗體標定的特異性螢光物質，來偵測組織或細胞中是否含有病毒的方法。具高敏感性及快速診斷的優點，在 AAHL 一個疑似病例從收件到做完 FAT 檢測僅需約 2 小時。詳細試驗操作流程如附件二、PROCEDURES FOR RABIES AND LYSSAVIRUS DIAGNOSIS INCLUDING RABIES FLUORESCENT ANTIBODY TEST (FAT) AND VIRUS ISOLATION。

由病毒學專家 Dr. Andrea Certoma 示範及指導，進行澳洲蝙蝠麗莎病毒

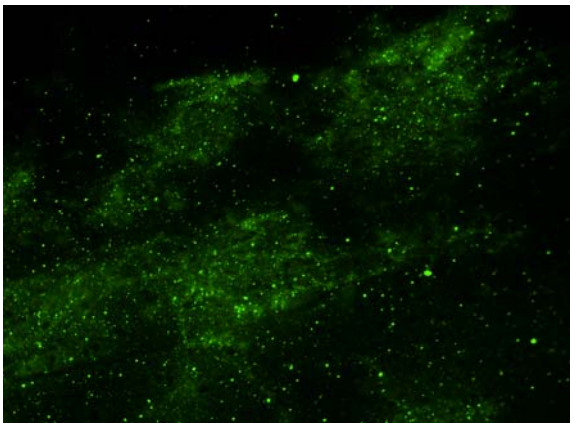
螢光抗體染色 (ABLV FAT)、判讀及結果拍照，實習檢體計有 pteropid positive, pteropid negative 及 insectivorous positive 三組。首先進行陽性對照及陰性對照蝙蝠腦壓片，包括玻片放置絕對酒精內浸泡後擦乾，按壓片製作 (左手食指與拇指固定標的按壓片，用拋棄式刀片取適量腦組織塗抹並移去多餘組織，右手握住另一玻片讓兩玻片垂直交叉約略呈直角對齊邊線，用拇指力道按壓另一玻片上的腦組織塗開抹勻後，分別向左右兩邊拉出，兩片皆可留做進行 FAT 使用)，以及 acetone 固定 30 分鐘 (免用紙巾壓乾稍作風乾可放置進 acetone 液槽，陽性、陰性對照、不同區域採樣材料製成之壓片須考量交叉污染可能性，要使用不同槽擺置)，放置-20°C 冷凍保存，可放置數個月之久；acetone 液槽使用後會加入消毒劑，隔夜後 (具揮發性) 倒入水槽內，進入廢水處理系統。AAHL 狂犬病實驗室使用的消毒劑主要有以下兩種： (1) 戊二醛 (Glutaraldehyde)，一種常用於醫療設備消毒的有機化合物，爲強效消毒劑，具有毒性且對眼、鼻、喉嚨及肺有強烈刺激性。(2) 衛可 (Virkon S)，係一平衡穩定的過氧化物、表面作用劑、有機酸及一種無機緩衝系統的混合物，較戊二醛溫和且安全性高。FAT 在我國亦爲狂犬病標準診斷方法，臺灣與澳洲 FAT 操作方式有以下差異：

	AAHL	畜衛所
玻片保存	絕對酒精	玻片盒
腦壓片	厚塗抹片	按壓抹片 (Impression smear)
玻片標示	製作壓片前	製作壓片後
Acetone 固定時間	30 分鐘	overnight
conjugate 稀釋倍數	5 倍	40 倍
conjugate 稀釋液	DW	even blue 混合其他試劑
加 conjugate	3 c.c.針筒加 filter	微量分注器
Incubation 溫度	35℃	37℃
Wet chamber	無	有
Wash buffer	PBSABC (CaCl2 及 MgCl2)	PBS
PBS wash	潤濕 5-10 秒、2 次	浸泡 10 分鐘、3 次

	AAHL	畜衛所
封片膠	含 DAPI 染劑	不含 DAPI
廢液處理	與消毒劑混合浸泡 overnight 後倒入水槽	滅菌後當作醫療廢棄物處理
廢棄物處理	加消毒劑後滅菌，再當作醫療廢棄物處理	滅菌後當作醫療廢棄物處理
試驗紀錄	詳細，包含試劑的編號	僅記錄結果
生物安全	物品拿出操作台前用消毒劑擦拭	物品直接拿出操作台
檢體保存	陰性-20℃保存 3 個月；陽性-80℃永久保存	陰性-20℃保存 1 年；尚無陽性檢體留存



圖六、ABLV 診斷流程圖。



圖七、蝙蝠腦壓片之陽性對照 。

三、11 月 10 日 (星期三)：

(一) 狂犬病疑似病例剖檢

上午由病理學專家 Dr. John Bingham 介紹 AAHL 動物解剖房設施、流程及生物安全注意事項，並實際進行動物剖檢，示範狂犬病動物之採樣標的及方式，包含海馬腳 (hippocampus)、大腦、小腦、延髓、脊髓、腦神經 (cranial nerves)、唾液腺、丘腦 (thalamus) 等。實習動物爲來自流浪動物收容所之健康犬、貓。

AAHL 解剖房只有經許可的特定人員才能進入，一般實驗人員則無進入權限。依不同病原採用不同等級防護措施，(1) BSL-4 病原：人員穿高生物

安全防護衣，動物不進解剖房，直接在繫留室剖檢，採樣完畢將屍體分切，裝到鐵桶內滅菌後，才送進焚化爐，繫留室則以福馬林薰蒸消毒。(2) BSL-3 病原：人員穿 Tyvek 防護衣，動物送至解剖房。屍體一樣要分切滅菌，空間一樣要燻蒸消毒。(3) BSL-2 以下病原：人員穿工作服，動物送至解剖房。解剖房動物繫留室有獨立負壓設施。廢棄物每週焚化處理一次。

解剖時右手 (慣用手) 戴乳膠手套，左手戴雙層手套 (乳膠手套及防切手套) 以防切傷，除部分大型器械 (解剖刀、金屬剪刀、骨剪及鋸子) 外，其他器械都滅菌後使用。開腦特殊器械：金屬剪刀 (Tin Snip)。專門剪金屬的剪刀，可於一般五金行購得，刀面不銳利但很耐用，有兩段式關節的省力設計。優點：1. 降低氣霧 (aerosol)，減少吸入病原、粉塵而感染的可能 (相較於使用電鋸及線鋸)。2. 可保持全腦的完整性 (相較於使用柴刀劈開)。狂犬病疑似病例剖檢流程：

(1) 沿頸部作一環切，將皮膚向兩邊掀開。

(2) 採集唾液腺 (通常採體積最大的顎下腺)。

(3) 清理頸部肌肉與軟組織，把頭向後折，將頭顱卸下。

(4) 將頭皮完全掀開。

(5) 將頭骨表面肌肉清除 (如同一般用線鋸開腦的前處理)。

(6) 用金屬剪沿腦幹部向前一片片剪開，至腦組織完全露出為止。

金屬剪刀開腦及採樣重點：

(1) 一次剪下一小片頭骨，勿採旋轉及拉扯方式。

(2) 順著頭骨邊緣，由後往前剪、由內往外剪。

(3) 海馬腳採樣：將腦取出後，由側面觀之，自大腦的後三分之一處做橫切，當切面看見白色條狀組織時，即為海馬腳。

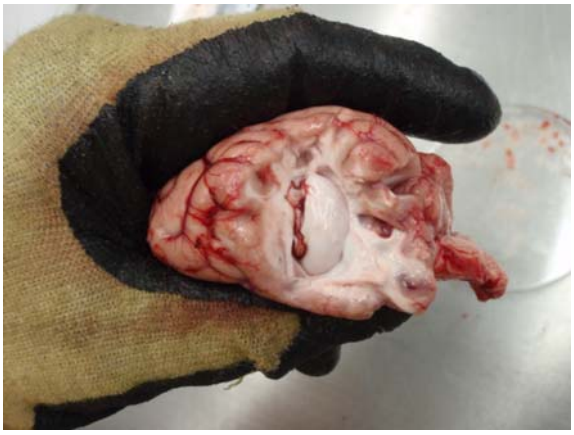
(4) 丘腦採樣：同上述位置，繼續將大腦做橫切，橫切面處底部有一白色圓形組織。

(5) 腦神經採樣：取出腦組織後，由上方俯視頭蓋骨內側，可見到白色粟狀或條狀組織在凹陷的小窩中。

(6) 唾腺採樣：頭皮完全掀開後，自側面觀察頭部組織，約在頭頸交接處可見數個白色、大小約 2-5 公分團塊，即為唾腺 (淋巴結大小僅 1-2 公分)。

(7) 疑似病例的完整採樣應包括：唾腺、腦幹、大腦皮質、小腦、海馬腳、丘腦、腦神經與脊髓等 8 個部位。

圖八、狂犬病疑似病例剖檢及採樣。



圖九、腦組織採樣_海馬腳。

各部位重要性：小腦、海馬腳—在 H&E 染色下最易看到 Negri body (IHC 陽性率最高)。腦幹、丘腦—抗原量最多，爲發病早期可做診斷的部位 (FAT 陽性率最高)。脊髓、腦神經、唾腺—當其他部位無法取得時之替代部位 (如一開始未將 rabies 列入考量，之後才懷疑而需補採樣時可利用，抗原量雖不如其他部位，但較易取得)。

腦組織固定時不預先橫切，以避免組織變形，直接浸泡於 10%中性福馬林 1 週到 1 個月可固定完全。要縮短固定時間有以下方法：(1) 改用 20%福馬林固定；(2) 檢體置於震盪器搖晃；(3)浸泡一週後換液。

(二) 蝙蝠檢體處理

實習過程 Dr. Andrea 準備一蝙蝠疑似病例， AAHL 處理流程簡述如下：檢體爲一隻小型的 insectivorous bat，因咬傷 2 位民眾，經民眾通報後地方政府派員捕捉後送來。

解剖流程：

(1) 以酒精擦拭毛髮作消毒。

(2) 把頭切下。

(3) 將頭對半縱切，一半直接泡福馬林 24 小後送病理室；另一半留做病毒 (FAT、RT-PCR 及病毒分離)。

(4) 取全腦及唾腺，腦做壓片，唾腺做 stamp smear。

(6) 腦取約 0.5 g 與 4.5 ml 的 PBSA buffer 混合做成乳劑 (使用針頭及針筒

進行均質化)，供病毒分離用。

(7) 取 100 µl 乳劑加入 Buffer，供 PCR 檢測使用。

(8) 剩餘的腦及唾腺分別裝在小管子中，保存於-80℃冰箱。

※此類疑似病例會在 2 小時內完成 FAT 並出報告，其他檢測則在完成後再陸續出報告。

AAHL 依物種、檢體差異採不同措施：

(1) 整隻犬貓或頭顱：由病理室剖，一半做病理、一半依部位分裝送病毒室 (virology lab)。

(2) 整隻蝙蝠：病毒室收，在生物安全操作台內剖檢。一半分給病理室，一半做 FAT，乳劑 (PCR、病毒分離)。

(3) 動物腦組織：同(2)，但不送病理室。

(三) 病毒分離（附件二、PROCEDURES FOR RABIES AND LYSSAVIRUS DIAGNOSIS INCLUDING RABIES FLUORESCENT ANTIBODY TEST (FAT) AND VIRUS ISOLATION）

下午 Dr. Andrea 介紹組織培養及病毒分離，並進行實務操作，將繼代 50 代生長 5 天之神經胚胎細胞株 (Neuroblastoma 2a) 一分爲三，接種澳洲蝙蝠 (pteropid 及 insectivorous 分離株) Lyssavirus 病毒陽性及陰性對照，一週後進行收集， 病毒分離前處理：

(1) 用刀片取約 0.5 g sample，與 4.5 ml PBS buffer 混合， PBS 與 sample 的比例約 9:1。

(2) 3 c.c.針筒裝上磨平 18 G 針頭以"快拉慢放" (可減少 bubble 產生) 的方式將 sample 與 PBS 充分混合。

(3) 抽 100µl 到 1.5 ml 的微量離心管 (供 PCR 檢測用)，將管子放入-80℃冰箱備用。

病毒分離操作流程：

(1) 神經胚胎細胞株：好微酸環境 (pH＜7)，圓形，貼附性普通，有時會聚集成一小坨。優點 (vs BHK cell)：1.耐酸性環境，數天不換液也不會死掉。2.對 ABLV 很敏感。

(2) 顯微鏡觀察細胞生長：觀察細胞是否長滿一盤？是否健康?

(3) 泡 reagent

1. typsin：商品化包裝，直接加入 10 ml PBS 即可。用途—消化細胞。

2. HMEM 加胎牛血清。用途—供細胞生長所需營養。

3. HMEM。用途—讓病毒進入細胞 (加胎牛血清會阻擋病毒之進入)。

(4) 加 trypsin 消化：加 5ml trypsin 至 flask 內，放入 37℃培養箱約 2 分鐘。

(5) 用力拍打 flask，使細胞與管壁分離，可用顯微鏡觀察是否消化完全。

(6) 加 PBS 至 flask：終止 trypsin 的作用及潤濕細胞。

(7) 倒入標示"cell"的 50 c.c.離心管。

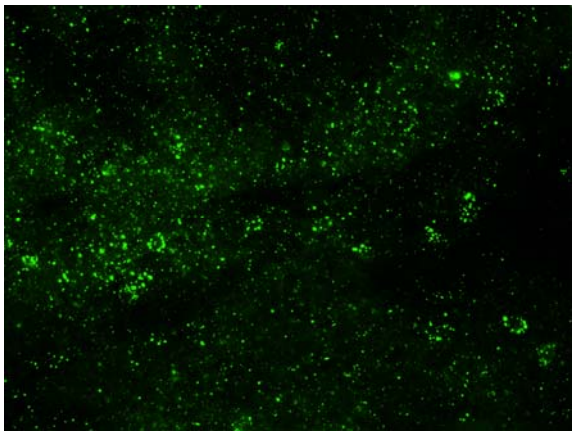
(8) 用 PBS 把殘留在 flask 上的細胞沖下來

(9) 以 2000 rpm 轉速離心 5 分鐘，使細胞集中於離心管底部。

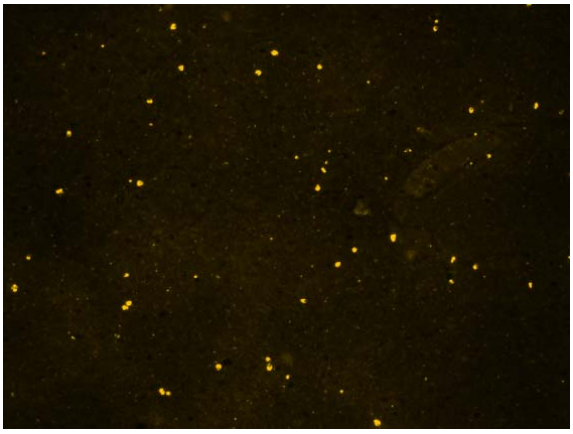
(10) 倒掉上清液，用 HMEM 讓細胞再次懸浮。

(11) 加入 200μl 的腦乳劑上清液，用 3 c.c.針筒充分混合，同時沖洗管壁把剩餘細胞沖下來。

(12) 把混合液放入新的 flask，37℃、培養 7 天。



圖十、澳洲蝙蝠 pteropid 分離株陽性對照。



圖十一、澳洲蝙蝠 pteropid 分離株陰性對照。

四、11 月 11 日 (星期四)：

(一) RFFIT (Flurescent focus inhibition test)

上午由 Mr. Dyren 介紹狂犬病快速螢光抑制試驗 (Rabies Rapid Fluorescent Focus Inhibition Test, RFFIT)，並進行實務操作，本日進行病毒中和，將病毒與樣本及國際標準血清感作後加入細胞 (BHK 21 cell line) 培養 22 小時後再進行判讀，操作流程詳如附件三、RABIES RAPID

FLUORESCENT FOCUS INHIBITION TEST (RFFIT) FOR THE MEASUREMENT OF SERUM NEUTRALISING ANTIBODY TO RABIES VIRUS (SEROTYPE 1 LYSSAVIRUS)，RIFFT 所用抗原盤製備圖示如下。

11+100+100V 1:20 (Sample1+BME+V)	11+100+100V 1:200 (Row1+BME+V)
11+100+100V 1:20 (Sample2+BME+V)	11+100+100V 1:200 (Row2+BME+V)
11+100+100V 1:20 (Sample3+BME+V)	11+100+100V 1:200 (Row3+BME+V)
11+100+100V 1:20 (Sample4+BME+V)	11+100+100V 1:200 (Row4+BME+V)
1_Sample slide	

100+300 (1:4)+100V (Nat+BME+V)	100+100V 1:8 (Row1+V)
200+200 (1:8) +100V (Row1+BME+V)	100+100V 1:16 (Row2+V)
200+200 (1:16) +100V (Row2+BME+V)	100+100V 1:32 (Row3+V)
200+200 (1:32) +100V (Row3+BME+V)	100+100V 1:64 (Row4 +V)
3_National standard sera	

11+100+100V 1:20 (Sample5+BME)	11+100+100V 1:200 (Row1+BME)
11+100+100V 1:20 (Sample6+BME)	11+100+100V 1:200 (Row1+BME)
11+100+100V 1:20 (Sample7+BME)	11+100+100V 1:200 (Row1+BME)
11+100+100V 1:20 (Negative+BME)	11+100+100V 1:200 (Row1+BME)
2_Sample slide	

100+100 1:2 (Neat Virus+BME)	100+100 1:2 (Neat Virus+BME)
100+100 1:20 (Virus_1:10+BME)	100+100 1:20 (Virus_1:10+BME)
100+100 1:200 (Virus_1:100+BME)	100+100 1:200 (Virus_1:100+BME)
200 (BME_No virus)	200 (BME_No virus)
4_Virus Back titration 30ul virus+9,000ul BME(1:300 Neat) 50ul Neat+450ulBME_1:10 50ul (1:10)+450ul BME _1:100	

測抗體用的標準血清 (international standard serum) 購自英國。

步驟：

(1) 將 BHK 細胞株加至 8 孔玻片型檢測盤，一盤可做 4 個 sample。另外還需準備兩盤，一盤爲標準血清、一盤爲病毒 (virus back titration)。

1. Sample 檢測盤配置：每個 well 加 100μl BHK 細胞株。
2. 標準血清檢測盤配置：加入之 BHK 細胞量如下所示。

300μl、0μl

200μl、0μl

200μl、0μl

200μl、0μl

3. virus back titration 檢測盤配置：

100μl、100μl (1:1)

100μl、100μl (1:10)

100μl、100μl (1:100)

200μl、200μl (no virus)

(2) 加待測血清：11 μl / well。

(3) 加標準血清：100μl / well。

-------以上步驟在一般實驗室 (BSL-2) 進行 ---------

(4) 加病毒：

1. 病毒液製備：

1:1 — 30μl CVS strain + 9 ml BME mixed with 10% FCS

1:10—上述病毒液(1:1) 50μl + 450μl BME mixed with 10% FCS

1:100—上述病毒液(1:10) 50μl + 450μl BME mixed with 10% FCS

2. 分別加 100μl 病毒液至相對應 well 中。

3. 於 35℃培養箱感作 90 分鐘 (讓病毒進入細胞中)。

(5) 加細胞株：200μl/ well，作用 22 小時。

----------以上在狂犬病實驗室 (BSL-3) 進行----------

※最後各檢測盤稀釋倍率：

1. sample 有 2 個稀釋階：1:20、1:200。

2. 標準血清有 4 個稀釋階：1:8、1:16、1:32、1:64。

3. back titration 有 4 個稀釋階：1:1、1:10、1:100、0 (no virus)。

(二) Pathology 實驗室參訪

下午 Dr. John Bingham 帶領我們參觀病理實驗室，指導蝙蝠狂犬病與 Lyssavirus 感染症之組織病理學檢查要領；另由日本訪問學者 Dr. Manabu Yamada 介紹腦組織修片並與其討論狂犬病病理學檢查，Dr. Manabu 對動物神經系統病毒性疾病診斷及研究相當有興趣及豐富的經驗，例如狂犬病、馬

西尼羅腦炎等。

AAHL 病理室的任務包括疾病診斷病例以及與其他部門合作之研究病例。病理切片主要進行的染色方法包括 H&E 染色及免疫組織化學(Immunohistochemistry, IHC) 染色，但並未建立原位雜交染色法(*in situ* hybridization, ISH)，因 ISH 染色成本太貴且 IHC 染色運作良好，足夠應付目前的診斷及研究工作需求。目前已建立多種重要疾病之 IHC 染色技術，包括：Rabies virus、Lyssavirus、AI、West Nile virus、TSE、Ebola virus。病理室共有 4 名成員：一位病理學家、兩位技術人員，另還有一位來自日本的 visit researcher。

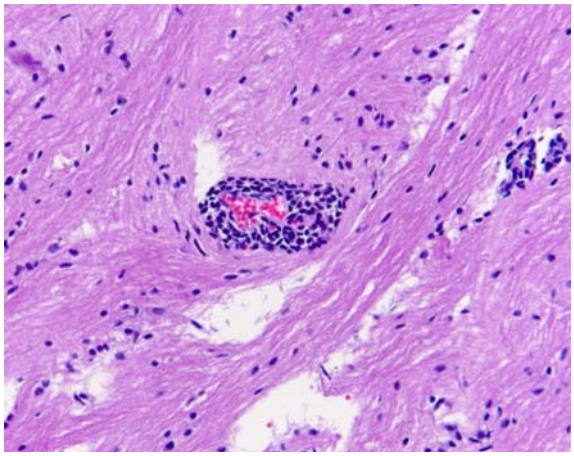
腦組織 trimming 示範 (以澳洲之大型狐蝠 Greyhead flying fox 爲例)：分別取 (1) 大腦後 1/3、(2) 小腦及 (3) 腦幹三部分做橫切，放入 casste。其中 (1) 包含大腦、海馬角與丘腦；(2) 包含小腦與延腦。若懷疑細菌性疾病時，左右兩邊皆修整；若懷疑病毒性疾病時，只修單側。

日本專家 Dr. Manabu 經驗分享：(1) 最易見到 Negri body 的部位會依動物別略有不同，如犬主在小腦與海馬腳；蝙蝠則是丘腦及腦幹最多。Nergri body 不像一般包含體很均質，內部經常還有一個核狀結構。(2) 犬及蝙蝠狂犬病：幾乎都不會有肉眼病變，僅有非常輕微的組織病變 (有時可見到 Negri body，但大部分 case 什麼都看不到)。(3) 山羊、馬的狂犬病：肉眼即可見到腦出血。所以 rabies 的診斷要靠 FAT 及 IHC，一般 H&E 染色不可靠 (視病程而定，若一有症狀就被安樂死的幾乎沒什麼病變，但發病好幾天、自然死亡的組織病變就很明顯)。

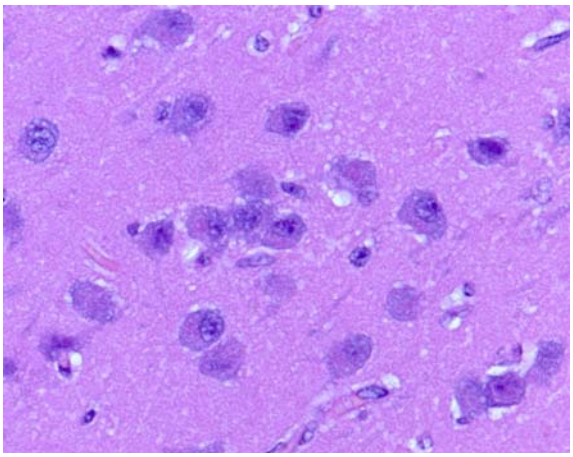
切片判讀 (Slide reading)：IHC 陰性對照切片是由與 sample 同一個蠟塊切出來的，只是沒加 monoclonal antibodies (通常會切 3 片：(1) for H&E stain；(2) for IHC stain；(3) for IHC (-) control)，好處爲易於對照尋找病毒所在。

Rabies case：來自南非的乳牛病例，可能有經過冷凍，所以組織與組織間有空隙。(1) H&E stain：perivascular cuffing 明顯，疑似有 gliosis 及 Negeri body (不易判定所以 rabies 診斷需仰賴 IHC 染色)。(2) IHC：小腦顆粒層旁邊的 neuron 有陽性訊號，腦幹附近的 neuron 及 axon 有陽性訊號。

Lyssavirus case：很多病例，幾乎都是蝙蝠，橫切—大腦、小腦、腦幹、丘腦；縱切—海馬腳。(1) H&E 幾乎沒有病變 (very mild)，Nergri body 更是少見，因此更凸顯出 IHC 染色在疾病診斷上的重要性。(2) 並非所有動物感染 rabies 都會有攻擊性，非洲有些野生動物感染後的臨床症狀是"變笨"，會誤闖民宅或跑去咬刺蝟。



圖十二、牛狂犬病病例，H&E 染色。



圖十三、蝙蝠 ABLV 病例，H&E 染色。

五、11 月 12 日 (星期五)：

(一) 組織免疫化學染色 (附件四、IHC protocol)

由 Ms. Jean 介紹及操作蝙蝠狂犬病之 IHC 染色，並判讀犬之狂犬病及 ABLV 陽性對照與陰性樣本，IHC 陽性呈紅色，背景染色可將細胞核染成藍色。IHC 染色爲狂犬病診斷的標準方法之一，染色過程中最重要的就是 wash 和保持玻片的濕潤，若操作不當容易有非特異性產生，造成判讀的困擾。使用的 rabies 單株抗體分讓自加拿大。

步驟：

(1)脫蠟：3 缸 xylane (同濃度，需要 3 缸的原因爲減少雜質)、3 缸酒精 (濃度分別爲 100%、90%、70%)，脫完蠟的空白切片若不立即染色可浸泡在 DW 中保存。

(2) H_2O_2

(3) PBS

(4) CK treatment

(5) PBS

(6) monoclone Ab

(7) marked polyclonal Ab

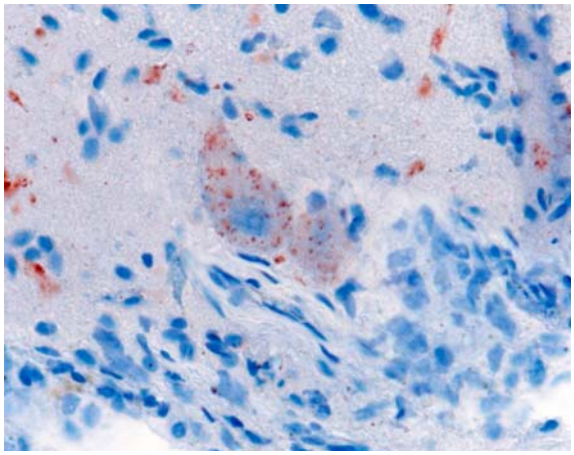
(8) PBS

(9) ACE

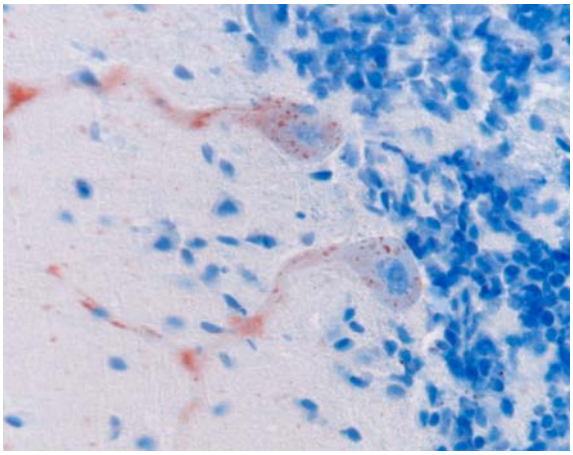
(10) PBS

(11) Hematoxylin 染色 30 秒，水洗。

(12) DAPI 封片：背景爲藍色，抗原爲紅色 (分布於 neuron 細胞質、細胞與細胞間)。



圖十四、牛狂犬病病例，IHC 染色。



圖十五、牛狂犬病病例，IHC 染色。

(二) RFFIT 結果判讀

下午與 Mr. Dyren 進行 RFFIT 後半段的操作步驟：

(1) 倒掉培養液。

(2) Wash

(3) 把塑膠 well 拆掉 (只剩一個塑膠 slide)。

(4) acetone 固定 30 分鐘。

(5) 加 conjugate：50μl/ well，再用 pipette 塗開。

(6) 放入 35℃培養箱、30 分鐘。

(7) 用 pipette 把 conjugate 吸掉。

(8) 以 PBS 沖掉剩餘的 conjugate。

(7) 封片：DAPE

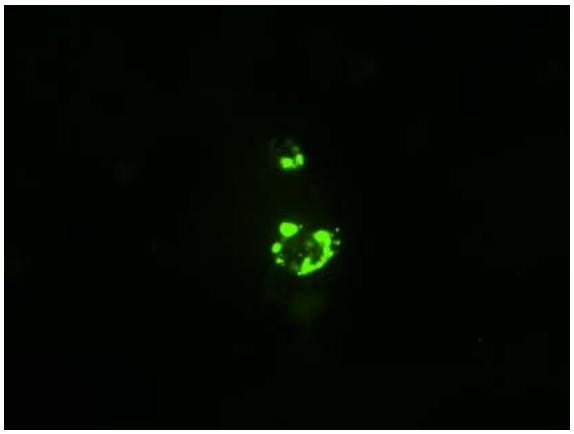
(8) 讀片：

1. 先看標準血清和 virus back titration (BT)，標準血清的最高稀釋階 1:64 和 BT 的最低稀釋階 1:1 都要 20/ 20 (20 個視野中有 20 個可見到至少一個細胞被感染而發出螢光)，此爲 RFFIT 的 quality control。

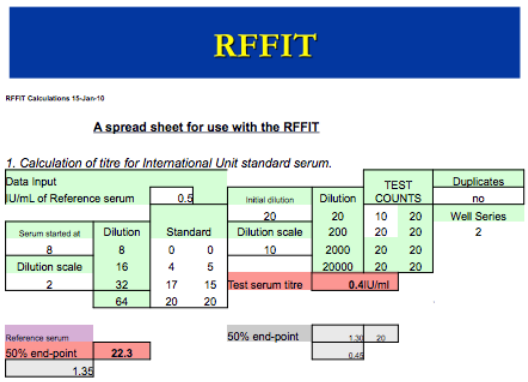
2. 看 sample：看 20 個視野，看有幾個視野有螢光

3. 記錄：?/20

抗體力價換算：藉由判讀後的數字計算除值 (樣本 50%力價/國際標準血清 50%力價)，可進行與國際標準血清抗體力價 (0.5 IU/ ml) 比較強弱，除值爲 1 代表相當換算後爲 0.5 IU/ ml，除值爲 2 代表樣本比國際標準血清力價強 2 倍換算後爲 1 IU/ ml，依此類推，若除值小於 1 代表樣本比國際標準血清力價弱換算後爲小於 0.5 IU/ ml。



圖十六、RIFFIT 陽性可在顯微鏡視野下觀察到螢光呈現。



圖十七、RFFIT 抗體力價換算。

六、11 月 15 日 (星期一)：

(一) FAVN (Fluorescent antibody virus neutralization)

由血清學專家 Mr. Di Antonio 介紹 FAVN 及 RFFIT 的原理及差異 （詳如附件五、Rabies Serology at AAHL – Assessment of the Fluorescent Antibody Virus Neutralisation (FAVN)），FAVN 優點是可以利用微盤作更多稀釋倍數，可以求抗體力價 end point。AAHL 正逐步把 RFFIT 停掉，全部改成 FAVN (兩者皆爲 OIE 認可方法)。兩種血清中和試驗比較：

	RFFIT	FAVN
耗材	玻片	96 孔盤
檢測費用	貴	便宜
檢測時間	2 天	3 天
穩定性	判定標準因人而異	不同人做結果相同 (易比較)

本日進行病毒中和，將病毒與樣本及國際標準血清感作後加入 BHK 細胞株培養 48 小時後再進行判讀，用 96 孔盤，一次至少兩盤 (一盤 sample、一盤 control)。一盤可測 12 case (1 個 case 用 8 well：4 個稀釋階 x 2 重複) control 盤包括：virus back titration、negative control 及 international standard sera。操作流程 (詳如附件六、FLUORESCENT ANTIBODY VIRUS NEUTRALISATION TEST (FAVN) RABIES VIRUS (SEROTYPE 1 LYSSAVIRUS))及 FAVN 所用抗原盤製備及圖示如後：

(1) 加 BME 100 µl/ well

(2) 加血清 50µl/ well

(3) 加病毒 50µl/ well，35℃、感作 1 小時 (一般是用 CVS strain，但做研究時會改用 ABLV 的 pteropid 與 insectivorous 病毒株)。

(4) 加 BHK 細胞株 50µl/ well，35℃、培養 48 小時。

P1	Sample 1		Sample 3		Sample 5		Sample 7		Sample 9		Sample 11	
	1	2	3	4	5	6	7	8	9	10	11	12
A 1:3	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100
B 1:9	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100	50A 100
C 1:27	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100	50B 100
D 1:81	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100	50C 100
E 1:3	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100	50S 100
F 1:9	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100	50E 100
G 1:27	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100	50F 100
H 1:81	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100	50G 100
	Sample 2		Sample 4		Sample 6		Sample 8		Sample 10		Sample 12	

P2	Virus Back Titration						Negative Control		International Unit Titration			
	1	2	3	4	5	6	7	8	9	10	11	12
A 1	50V	50V	50V	50V	50V	50V	50 100	50 100	50Std 100	50Std 100	50Std 100	50Std 100
B 1:10	33.3V 300	50(B1)	50(B1)	50(B1)	50(B1)	50(B1)	50 100	50 100	50A 100	50A 100	50A 100	50A 100
C $1:10^2$	33.3B 300	50(C1)	50(C1)	50(C1)	50(C1)	50(C1)	50 100	50 100	50B 100	50B 100	50B 100	50B 100
D $1:10^3$	33.3C 300	50(D1)	50(D1)	50(D1)	50(D1)	50(D1)	50 100	50 100	50C 100	50C 100	50C 100	50C 100
E $1:10^4$	33.3D 300	50(E1)	50(E1)	50(E1)	50(E1)	50(E1)			50D 100	50D 100	50D 100	50D 100
F $1:10^5$	33.3E 300	50(F1)	50(F1)	50(F1)	50(F1)	50(F1)			50E 100	50E 100	50E 100	50E 100
G $1:10^6$	33.3F 300	50(G1)	50(G1)	50(G1)	50(G1)	50(G1)			50F 100	50F 100	50F 100	50F 100
H Neg	Neg 300	50(H1)	50(H1)	50(H1)	50(H1)	50(H1)			50G 100	50G 100	50G 100	50G 100

(二) 尼泊爾狂犬病疫情現況

尼泊爾國內目前狂犬病疫情非常嚴重，有非常多人類病例發生，在動物方面主要爲犬，另外豬、羊等家畜被犬咬傷後發病的事件也時有所聞。尼泊爾狂犬病診斷做：(1) 抗原快速診斷套組；(2) FAT； (3) virus isolation。但不做 RT-PCR。抗原快速診斷套組爲韓國製的商品化套組，只要將腦乳劑滴在 KIT 上，數分鐘內即可判讀，據尼泊爾專家說該診斷套組敏感性還不錯，與 FAT 檢測結果很接近。每個 test 約需$10 美金。

七、11 月 16 日 (星期二)：

(一) Real-time PCR：

Dr Ian 介紹 ABLV 及其分子生物學診斷 (比較傳統 RT-PCR、nest PCR 及 real-time PCR，分子診斷流程詳如附件七、Molecular Diagnosis at AAHL，簡報如附件八、Molecular Detection of Lyssaviruses)，並進行 ABLV real-time PCR 實務操作，樣本是上週準備，核酸萃取係利用 Qiagen RNA 萃取套組，real-time PCR 儀器系統係使用 AB 7500 。境外 (人) 疑似病例 (採血清、腦脊髓液、腦幹 punich biopsy)：做 conventional PCR；all genotype of Lyssavirus 境外 (動物) 疑似病例：做 real-time PCR；two isolated ABLV (pteropid、

insectivorous)，其他血清型尚未建構完成。

Real-time PCR 原理：其實與 conventional PCR 很像，只是在步驟 4 加 primer/ probe pre-mixed (P/P pre-mixed) 時，probe 有 level 螢光，會被 real-time meachtion 記錄，所以從反應的第 1 秒起便會持續被偵測 copy number，而能隨時瞭解反應情況，故稱做"即時"PCR。 Real-time PCR 與 conventional PCR 的比較如下表：

	Real-time PCR	Conventional PCR
檢測時間	2 小時	24 小時
特異性	特異性高	
定量	可	無法定量
花費	貴	便宜

步驟：

(1) 核酸萃取：使用商品化的套組操作核酸萃取，每件成本約澳幣 10 元。

1. 把乳劑放在有 filter 的管子 (過濾雜質)。
2. 離心取底部濾液。
3. 與酒精 1:1 混合，加入另一個 filter (把 RNA 凝固聚集)。
4. 離心把濾液倒掉。
5. 重複步驟 3~步驟 4。
6. 用 RNA buffer 把 RNA 離心沖下來。

(2) 加 DNTP water

(3) 加 Buffer

(4)加 P/P pre-mixed 37.5µl

(5) 加 18s RNA P/P pre-mixed 0.375µl

(6) 加 enzyme

(7) 加 sample 2µl

(8) 離心

(9) 上機：約 2 小時

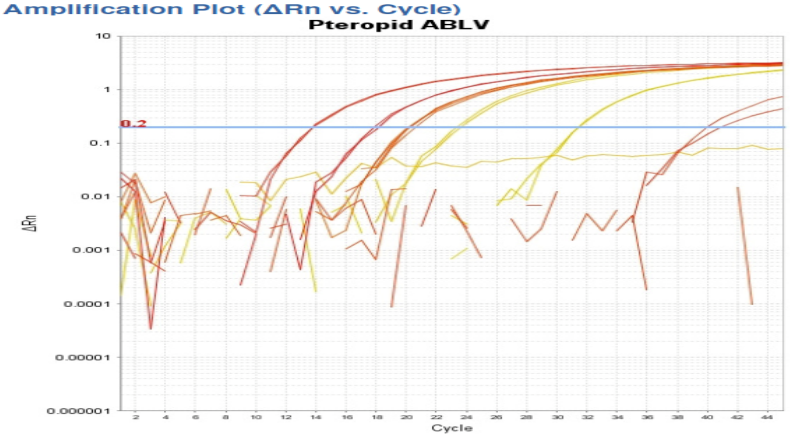
重點提示：(2)~(7) 加 reagent 時都不要觸底及 pipette-mix，待最後(8)時會離心把 all reagent 甩下去 (避免汙染與 reagent 流失)。18s RNA 是爲了 quality control，會與病毒 RNA 競爭 enzyme，所以不能加太多 (only 0.375/

well)。但若 sample 沒病毒時，會與其他任何 RNA 作用，可印證 RT-PCR 確實有進行。

檢驗盤配置:(做2重複) 96孔盤 (A~H;1~12),第一排放sample 1~sample 6 (A1~A12)、第二排依序放 Negative control (B1~B2)、strong positive (B3~B4)、weak positive (B5~B6)。縮寫 sample = S,Negative control = N,strong positive = SP，weak positive = WP。每個 plate 成本約澳幣 10 元。SP 及 WP 皆來自陽性檢體，WP 可能是經過稀釋的檢體，所以需要較多的閥值 (copy threshold, CT) Case 較少時也可用微量離心管進行。

Real-time PCR 圖形：上機後，配合的軟體會"即時"把"定量"的 copy number 顯示出來,會製出一幅以時間爲橫軸、以 CT 爲縱軸的圖形。Threshold 決定陽性與陰性,例如 copy 數大於 40 判定爲陽性,37~40 判爲疑陽性,copy 數小於 36 判定爲陰性。SP 會最先跑出來，WP 則較慢。陽性病例的圖形會介於兩者之間。陰性病例的圖形上升緩慢且 copy 數低於閥值。判讀前先 check SP、WP 及 N 是否正確 working，然後判讀 sample。另外要 check 18s RNA，通常陰性 sample 一定要跑出 curve，因爲代表沒有病毒核酸存在，enzyme 都被 18s RNA 用來做 RT-PCR (陽性 sample 可能也有或是只有微弱的反應)。

操作分三區，且各區用不同的 pipette，減少汙染的機會。(1) clean zone (生物安全操作台 A)：做 step (2)~(6)的乾淨工作。(2) contamination zone (生物安全操作台 B)：做抽核酸等可能含汙染物的工作。(3) outside (一般)：做 step (7)，把所有 reagent 加入 plate 的工作。



圖十八、 Real-time PCR 檢測結果之圖形 。

八、11 月 17 日 (星期三)：

(一) FAVN 判讀：

FAVN 加入 conjugate 呈色後進行判讀，步驟爲：

(1) 把 incubate 48 小時的 plate 拿出 incubator。

(2) PBSABC wash：400 μl/ well。

(3) 甩掉

(4) PBSABC 200μl/ well → 甩掉

(5) 4 %福馬林固定 1 小時。

(6) 機器 wash (with PBSABC) 3 次

(7) 加 conjugate (20 倍稀釋)，30 min ~ 1 小時、35℃。

(8) 機器 wash → 甩乾

(9) 加 PBSABC 200μl/ well (防止乾燥)

(10) 讀片

讀片前要先用光學顯微鏡 check 細胞生長情況，有些細胞可能會有"serum toxicity"(血清中含有某些物質會抑制細胞生長，當血清放太久時很常見)，鏡下看起來細胞生長緩慢、甚至出現空洞。若有，依嚴重程度註記爲"tt" (very toxicity) 或是"t" (mild toxicity)。

讀片時先 check plate 2 (BT、Negative、IS)：看 control 有沒有問題。用 10x 接物鏡看。Back titration 最上面一排 (A1~A6) 應爲(+)，Negative 全部 (A7~A8、B7~B8、C7~C8、D7~D8)應爲 (+)，international standard 最上面一排 (A9~A12)應爲 (-)。Control 都沒問題之後看 plate 1 (sample)：

(1) 稀釋階由濃度高的往濃度低的看 (由上往下)，看到出現 (+)就可不必往下看了，因爲理論上血清濃度越低越無法中和病毒，故高濃度出現(+)就不避往低濃度看了。例如：1:1 爲 (-)、1:10 爲 (+) 時，1:100 及 1:1000 可直接判定爲 (+)。

(2) 若鏡下 even blue 的紅色看不清楚時，可以開燈判讀。因有光線時 well 的邊緣可以看得很清楚。

(3) check 細胞生長：用 4 x

1. 長得滿滿的—OK；

2. 幾乎空的—tt；

3. 有細胞但是有小空洞—t

只要整個 well 中有一顆細胞被感染，即判定爲陽性。 Standard serum (SI) 與 sample serum 高低息息相關，因爲公式如下：抗體力價等於血清力價除以 SI 乘以 0.5 (單位：IU)。所以 SI 判讀時要很仔細，不要 miss。BT 判讀目的在確認病毒濃度適中。勘誤：Excel 中"BT"的"number of well show nutralization"應該爲"number of well have virus"。只要一顆細胞 (+) 即爲 (+)，特別注意不要 miss "weak positive"：整個 well 只有一兩顆 (+) 細胞的)。

FAVN 算 titer 要知道以下幾個數值：

(1) BT：

1. have virus：11 wells

2. replication：6 replications

3. 稀釋階：10 (1:10→1:100…)

(2) SI：

1. show neutalization：10 wells

2. replication：4 replications

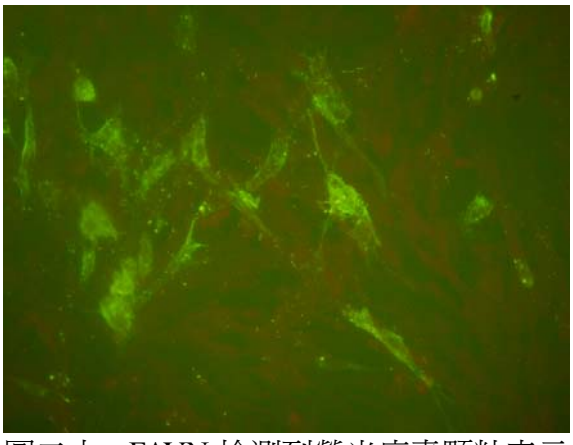
3. 稀釋階：3 (1:3→1:9…)

(3) sample：

1. show neutralization：6 wells

2. replication：2 replications

圖十九、FAVN 檢測結果判讀示意圖。



圖二十、FAVN 檢測到螢光病毒顆粒表示無足夠之中和抗體存在。

3. 稀釋階：3

(二) 狂犬病診斷、監測及研究座談會議

下午與Dr. Ross及中國大陸農業科學院長春獸醫研究所 (位於吉林省長春市) 流行病學研究室扈榮良研究員、Di Antonio、Mrs. King共同討論狂犬病診斷、監測及研究議題，扈研究員之實驗室大約有30位同仁負責狂犬病相關試驗及研究，監測犬貓血清量每年約5,000件，多數來自於大城市，如上海、北京、武漢、深圳等，監測結果顯示家犬施打狂犬病疫苗後具保護抗體力價之覆蓋率皆可達70%以上，但流浪犬問題相當嚴重，中國大陸發生人類感染狂犬病導致死亡病例僅次於印度，動物病例亦可見於家畜如牛、豬等，野生動物黃鼠狼、鼬獾 (ferret badger)，該研究室近年亦進行蝙蝠Lyssavirus監測，目前結果仍爲 陰性，其血清學試驗採用FAVN (認爲FAVN比RFFIT準確)，狂犬病FAT試驗使用自行開發之單株抗體，結合兩種不同的單株抗體可資檢測不同的狂犬病分離株，疫苗研發除一般家犬使用之狂犬病疫苗，其研究室開發口服狂犬病疫苗給流浪犬食用透過粘膜免疫機制達到免疫效果，企圖增加流浪犬免疫保護覆蓋率，扈研究員指出，因爲中國大陸係狂犬病疫區，該研究室進行診斷及相關研究時，僅使用 BSL-2 實驗室並在生物安全操作櫃內進行操作。

中國狂犬病病例檢測做FAT、FAVN、PCR、virus isolation。FAVN一年約3,000病例 (人醫體系做RFFIT)。人的死亡病例每年約2,300例 (僅次於印度的每年3,000例) 最近亦開始做蝙蝠：(1) 尚無發現Lyssavirus；(2) 但有蝙蝠咬人，造成人過度流涎且蝙蝠腦乳劑打裸鼠造成死亡的未知病例。境內只有type 1 Lyssavirus (即classic rabies virus)，主要宿主爲犬，但亦會感染家畜如：豬、牛、羊及野動 (鼬獾及黃鼠狼)。蝙蝠目前監測結果皆爲陰性。研發口服的活毒疫苗 (for dog) 有自製的monoclonal Ab及polyclonal Ab。Bat FAVN：有測到其他Lyssavirus抗體 (非第一型)。Target：腦幹 (Ag)；血清、唾液 (Ab) 與AAHL同樣認爲測Ab的ELISA準確度差，使用FAVN的準確性較高。採樣：用特殊的tube由腦幹插入眼，取腦幹 (medulla)、小腦、海馬腳。

九、11 月 18 日 (星期四)：

(一) 病毒分離結果判讀：（結果如圖九、十）

於 37℃培養箱中培養 5 至 7 天後取出，進行下列步驟：

(1) 拍打 flask，用吸管把細胞沖下 (不需使用 trypsin 消化細胞，因爲 neuro-2a 細胞貼附性不佳)。

(2) 將 media 倒至 15 c.c.離心管

(3) 離心：2,500-3,000 rpm，2-3 分鐘。

(4) 上清液倒回 flask (若有需要可做繼代)，留 0.5 mL 左右即可。

(5) 用 50 μl pipette wash，塗抹於玻片上。

(6) air dry (一定要完全乾燥後才能放入 acetone，不然細胞會被沖走)。

(7) acetone fix 30 min (-20℃)。

(8) add conjugate

(9) 37 ℃、incubate 30 分鐘。

(10) PBS wash：

1. 甩掉 conjugate
2. 把 slide 放在架子上
3. 陰性 control 先 wash，然後才 wash sample 與陽性 control。

(11) 封片、判讀：

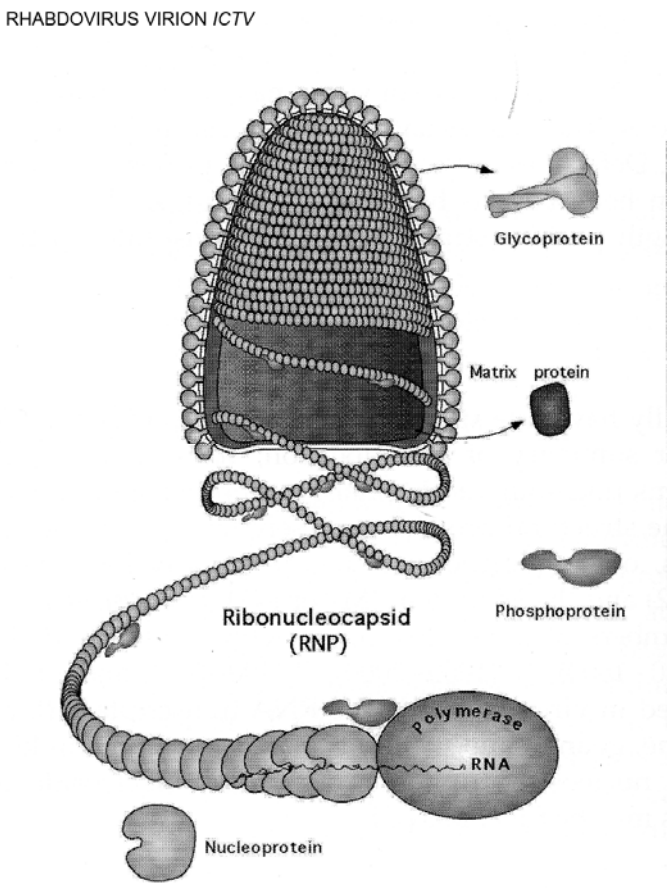
1. 要仔細看，有時 only one cell (+)，有時 cell 會在不同平面。
2. 用螢光顯微鏡以 10 倍接物鏡觀察並判讀。

十、11 月 19 日 (星期五)：

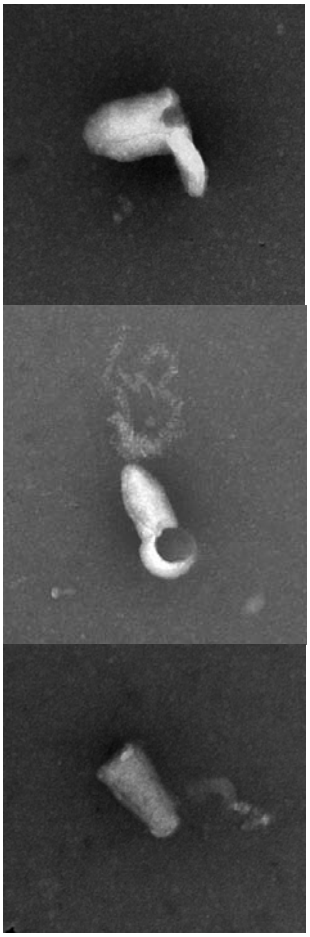
(一) 電子顯微鏡診斷實驗室參訪

上午在電子顯微鏡診斷實驗室進行棒狀病毒科 (*Rhabdoviridae*) 介紹（簡報如附件九、*Rhabdoviridae*）並利用負染色法觀察並拍攝病毒顆粒。Rabdovirade (棒狀病毒科) 家族包含 Lyssavirus、BEV (牛流行熱病毒屬)…等重要疾病。大小多變 (100~400 nm)，且有各種型態。AAHL 的 EM 實驗室有超薄切片、負染色及病原顆粒斷層掃描儀器 (雲台可以調整厚度，擷取各截面後重組成 3D 立體圖樣。優點：不易因爲病毒被固定的角度不好而 miss)。EM 使用時機：(以 Lyssavirus 爲例)，動物 (Bat or dog) 具侵略性、

攻擊人，但 FAT 陰性，PCR 也陰性，而裸鼠腦內注射乳劑會死亡，表是必定有某種原因或 unknown 病原存在。Sample：至少要 10^{-6}/ ml 才易檢出，因此以病毒分離的 suspension 最適合。



圖二十一、*Rhabdovirus* 病毒顆粒示意圖。



圖二十二、電子顯微鏡下的 *Rhabdovirirus* 病毒顆粒。

(二) Animal house 參訪

下午與 Dr. John Bingham 至大動物 (感染動物) 實驗動物房參訪。BSL-2、BSL-3、BSL-4 病原都有，依病原危險性消毒方式略有不同，BSL-3 以上 (zonosis) 實驗動物房使用福馬林燻蒸全室，BSL-2 只需潑灑消毒劑。配置與解剖房相似，更衣室分一間男用一間女用，進入後會到達"緩衝區－人員走道"，此走道也用於實驗動物從外面移入各動物房 (此時動物尚未攻毒，很安全)，所以地面有時可見動物排泄物。動物房另一側爲動物走道，爲動物實驗結束後犧牲動物運輸用的走道，走道的盡頭就是解剖房。BSL-4 動物就地解剖 (在該實驗動物房內)，剖完切成小塊高溫高壓滅菌 (autoclave)

後才能出去；BSL-3 區域進解剖房，穿 Tyvek 防護衣，一樣切塊 autoclave 後才送至焚化爐銷燬。相機：water-proof 的傻瓜相機，用完潑浸衛可消毒劑，沖清水後才可移出動物房。

(三) Dr. John Bingham 狂犬病專題演講（附件十、Rabies and Lyssaviruses）

澳洲自 1996 年檢出第一例 ABLV 起，歷年來陸續發現到至少 180 例 ABLV，其中以 Pteropid strain (macro bat) 佔大多數，insectivorous (micro bat) 少。Dr. Bingham 認爲 insectivorous ABLV 早已存在，但不具致病性，可能在 1995 年間，跨越 species barrier 到 pteropid Bat 才會造成疾病爆發。從基因樹可明顯看出各病毒間有地域性區分，故 Dr. Bingham 認爲若臺灣蝙蝠若有 Lyssavirus 幾乎可肯定爲 new strain，對我國現行狂犬病監測有以下兩點建議：(1) 若 target 爲 dog rabies virus，準備單一種 conjugate 就足以做診斷，因犬幾乎 99%都是感染 Lyssavirus 第一型 (即 rabies virus)，而此型 Lyssavirus 非常穩定。(2) 若 target 爲 Bat Lyssavirus 則至少要有兩種以上 conjugate 才容易檢出 (因只用 fugirabio 偵測可能會 miss 掉)。利用分子生物學方法如：PCR 等做蝙蝠 Lyssavirus 診斷亦不甚理想，因爲只要病毒一變異就會測不到。自行研發 polyclonal Ab 才是未來可努力的目標 (AAHL 正在研發中)。Dr. Bingham 認爲血清學也沒太大意義，測到 Ab 不能代表什麼。監測生病或死亡蝙蝠是正確的方向，但要做到能追蹤蝙蝠原始的來源。

研習期間觀察與心得

本次出國研習目的爲瞭解澳洲對野生動物之狂犬病診斷、監測及防檢疫體系、實驗室操作實務及生物安全控管，並進行學術交流座談及相關實驗室實務研習，俾利應用於執行狂犬病診斷業務及蝙蝠新浮現疾病監測，相關之觀察與心得如後：

一、重視人員安全與疾病管控

AAHL是澳洲官方家畜疾病以及人畜共通疾病之診斷機構，爲保障研究人員的安全及防堵疾病的散播，統一將所有實驗室建構於一棟全負壓之建築物中，據了解AAHL是目前全世界規模最大的高生物安全等級實驗室，包含BSL-3, BSL-4。每位研究人員有專屬之通行證，依工作性質給予進入不同等級實驗室之權限，減少意外事件發生。另外人員進出每間實驗室都必須刷卡，電腦系統可隨時掌握人員所在位置，若發生意外事件時能在第一時間掌握人員動向；當員工超過下班時間仍待在實驗室時，亦會以電話聯繫確認人員安全，對長時間處於高風險環境之研究人員提供安全之工作環境。另設有專責人員約40人負責實驗設施之維護，確保負壓設備與廢水滅菌設施運作正常，研究人員可專心於研究，無需分神於設備檢修。本所之主要任務雖與AAHL相仿，但對人員安全、疾病控管及設施維護上仍有許多部分可再加強，尤其是狂犬病診斷實驗室執行全國動物狂犬病診斷後送檢驗及監測任務，但礙於經費僅在BSL-2 plus等級之實驗室實施檢驗及相關研究，於生物安全防護上略顯不足。

二、疑似病例之病理學診斷

狂犬病已有人類病例被證實是經由氣霧(aerosol)感染，在動物試驗上亦發現空氣中的病原濃度夠高時，可以造成動物的發病。因此在處理狂犬病疑似病例，需剖檢採樣時，應該儘可能選擇最不會產生氣霧的方式進行。本病理室常規使用的線鋸以及電鋸在開腦的過程中會產生大量的粉塵，極易造成人員的危害。本次赴澳研習，經AAHL獸醫病理學專家Dr. John Bingham的指導，學到兩種較安全的做法，第一種須準備稱爲「金屬剪刀」(Tin Snip)的特殊器械，此器械具有堅固及省力的優點，剖檢時從腦幹部爲起點，以金屬剪刀一片片把頭骨剪開，而不會產生任何粉塵及氣霧。另一種做法適用於野外或是在防護設備不足的情況下使用，只需要一把刀把頭骨敲開一個小洞，即可取得部分腦組織進行檢驗。

研習課程還包括蝙蝠Lyssavirus陽性病例切片判讀，陽性病例切片在H＆E染色下幾乎沒有病變或僅有輕微之非化膿性腦炎（非具有特徵性診斷價值），教科書上描述的特徵性病變Negri body更是少見，因此僅憑H＆E染色診斷狂犬病及Lyssavirus感染症是很困難且容易誤診的。狂犬病及Lyssavirus感染症的病理診斷必須依賴免疫組織化學染色(IHC)，陽性病例可在神經元與神經纖維見到大量狂犬病病毒抗原。本所經美國疾病管制局(CDC)專家指導，已建立狂犬病之IHC染色技術，本次赴澳研習期間，實際操作蝙蝠Lyssavirus之IHC染色及判讀技術，對本所在蝙蝠Lyssavirus之病理學診斷能力之提升有很大幫助。

三、出國研習有助於促進國際交流

此次赴澳洲AAHL研習，了解到澳洲這個國家以外來移民人口居多，AAHL研究人員來自世界各國，研習期間曾與阿根廷、南非、中國大陸、愛爾蘭、義大利等國家的研究人員一起進行實驗或討論交流；亦遇到其他國際參訪或研究學者或學員，包括日本、中國、尼泊爾，無論從生活上交談及研究學術上的經驗交流，皆有助於促進國際交流並增進臺灣在國際上的能見度。

圖二十三、與 Dr. John Bingham 合照。

圖二十四、與獸醫部門主任 Dr. John Allen 合照。

四、瞭解並可學習各國狂犬病實驗室診斷及監測方法

臺灣、澳洲、美國狂犬病實驗室診斷及監測

診斷及監測方法	臺灣	澳洲	美國
直接螢光抗體染色法	✓	✓	✓
病毒分離	–	✓	✓
聚合酶連鎖反應	✓	✓	✓
病理學檢查	✓	✓	✓
免疫組織化學染色	✓	✓	✓
抗體檢測	ELISA	RFFIT (FAVN)	RFFIT
監測對象	犬隻為主 蝙蝠	蝙蝠為主 檢疫動物	野生動物為主 浣熊、臭鼬、蝙蝠 狐狸等

圖二十五、我國與澳洲、美國狂犬病實驗室在診斷及監測比較表。

檢討與建議

本出國計畫目的在強化人畜共通傳染病診斷實驗室與動物健康防疫檢疫體系，就研習期間觀察，以動物疾病檢診與研究及動物健康防檢疫體系兩個面向重新歸納出以下重點提供參考：

一、對我國現行狂犬病監測方式之建議

(一) 持續犬隻狂犬病監測

雖然目前臺灣爲狂犬病非疫區，但我國鄰近之中國、東南亞、韓國等地，其狂犬病疫情有日趨嚴重之傾向，且近年來國際交通往來密切，非法走私動物及其產品之行爲頻繁防不勝防，而我國與中國大陸兩岸開放小三通之後，交通更是日趨頻繁，使狂犬病入侵我國之風險大爲提高，根據世界衛生組織之資料，大陸地區每年均有數百人死於狂犬病，近五年狂犬病感染人數持續上升；據中國衛生部公佈，近年中國大陸之狂犬病疫情逐年上升，病例數高居全球第2位，僅次於印度，2004年至2006年各通報2,651、2,537和3,279例，3年總計8,403例死亡，占總死亡數的30.1%，高居37種法定傳染病之首。2007年截至10月底共通報2,717例狂犬病，其中2,532例死亡，較前年同期（2,653例）上升2.41%，總計共23個省發生病例，其中以廣西、貴州、四川、湖南和廣東省之疫情最爲嚴重。因此除積極地防範非法走私動物及其產品之行爲以防止狂犬病入侵外，仍應藉由宣導與教育強化全民對此疾病之認識，呼籲定期對自己飼養之家犬注射狂犬病疫苗，嚴格控管流浪狗且減少其族群之擴張，降低狂犬病入侵之風險。透過辦理狂犬病預防與控制國際研討會，可集結各國優秀研究人員及狂犬病診斷與監測之專家，除進行公開演講宣傳推廣狂犬病之重要性，亦可給予我國相關單位針對狂犬病監測之建議，進一步建立狂犬病診斷監測技術平台，並邁向國際化。

犬隻狂犬病監測一直以來爲我國狂犬病監測計畫中最重要的一環，每年針對各縣市防治所收容之流浪犬隻進行檢測，近年來更將檢測樣本數提高至一年1,500例以上。本次研習詢問澳方專家，發現澳洲對境內犬隻幾乎完全不進行監測，僅在發生犬隻攻擊人類事件時，對該犬進行嚴密觀察，若犬隻出現神經症狀或死亡時才會進行狂犬病病原檢測。但是對於檢疫犬隻則採取

嚴格控管，國內的犬隻如欲輸出至其他國家，必須先施打狂犬病疫苗 (澳洲境內犬隻皆未施打狂犬病疫苗)，然後將血清樣本送至AAHL進行狂犬病中和抗體力價檢測，結果需高於0.5 IU/ ml才可出口，否則須補強至抗體力價足夠為止。而澳洲輸出犬隻若要再輸入回國，除了隔離檢疫以外，同樣要確定血清中和抗體力價足夠後才准予輸入。檢疫期間，所有死亡的犬隻，無論死因為何皆須採樣進行狂犬病病原檢測。雖然臺灣與澳洲主客觀條件不盡相同，但我們也許可以擷取其中部分方法來強化我國之預防策略與監測體系。

(二) 持續蝙蝠Lyssavirus感染症監測

我國於1961年後便無動物感染病例，並自1999年開始進行本病之監測，另美、澳及歐洲等國家鑑於蝙蝠可傳播Lyssavirus、亨德拉病毒和立百病毒等疫病，均已將其納入監測及防疫重點。國內雖無前述疾病報告，但蝙蝠保有病原之相關文獻較少，且蝙蝠可經由遷徙將疾病帶入國內，故建議對境內蝙蝠進行Lyssavirus、西尼羅病毒等重要人畜共通傳染病原之監測，以強化人畜共通傳染病監測體系並提供後續研究參考。現階段建議針對蝙蝠之狂犬病病毒或其他Lyssavirus持續進行監測。

澳洲對蝙蝠之監測分為主動與被動兩種，其中主動監測部分為依蝙蝠種別 (species) 進行採樣，每種採數十例進行檢測。被動監測則是當有民眾通報遭蝙蝠抓傷或咬傷時，政府會派員至該區域捕捉，整隻蝙蝠立即送至AAHL進行檢測，遭攻擊之民眾則送至醫院進行狂犬病暴露後處置(Post-Exposure Prophylaxis)。蝙蝠檢體會同時進行FAT、IHC及PCR檢測，通常這種病例Lyssavirus檢出率很高。本所自97年起開始與蝙蝠協會合作，對國內蝙蝠進行狂犬病及Lyssavirus感染症之主動監測，檢驗結果皆為陰性。自今年起改為被動監測，由蝙蝠協會接受民眾送來受傷或死亡之蝙蝠，蒐集一定數量後寄送至本所進行檢驗。目前面臨到的問題為蝙蝠樣本因存放時間太久，導致病材狀態不佳。今後應加強與蝙蝠協會之溝通，縮短樣本存放時間，避免因病材腐敗影響檢測結果。

二、建構高生物安全等級 (BSL-3, BSL-4) 實驗室以及動物房

考量任務導向，建議可籌措經費於本所興建高生物安全等級 (BSL-3)

實驗室以及動物房，並建立相關診斷及檢驗技術，例如病毒分離、病毒中和試驗，如狂犬病快速螢光抑制試驗 (Rabies Rapid Fluorescent Focus Inhibition Test, RFFIT) 及 FAVN 。

三、培訓優秀疾病診斷技術人才及建立國際化狂犬病診斷監測技術平台

(一) 積極培訓疾病診斷技術人才：

1. 應持續編列相關經費積極培訓疾病診斷技術人才，利用病理學及相關實驗室檢驗技術進行新浮現人畜共通傳染病診斷，並建立我國及跨國新浮現人畜共通傳染病診斷及監測技術平台。
2. 持續辦理新浮現人畜共通傳染病國際會議或實驗室訓練課程：透過持續辦理新浮現人畜共通傳染病國際會議或相關訓練課程，除考慮邀請美、澳、印尼、菲律賓、日本等國，亦可邀請對岸中國大陸狂犬病或其他新浮現人畜共通傳染病診斷監測及相關研究之專家來臺與會，進行兩岸學術交流，促進瞭解鄰近國家對狂犬病監測及研究現況。

(二) 加強民眾對狂犬病及蝙蝠宣導

1. 嚴格管控輸入野生動物及動物檢疫：目前臺灣爲狂犬病非疫區，但我國鄰近之中國、東南亞、韓國等地，其狂犬病疫情有日趨嚴重之傾向，且近年來國際交通往來密切，專家建議臺灣應加強宣導與教育，請民眾勿走私或輸入野生動物，政府也應強加把關， 嚴格管控輸入野生動物，尤其是自疫區輸入野生動物，狂犬病潛伏期可達數年之久，臺灣不應該冒此風險，至於動物檢疫把關更應嚴格執行。
2. 認識狂犬病及宣導蝙蝠可能帶有人畜共通傳染病：建議疾病管制局、行政院農業委員會動植物防疫檢疫局及相關局處署應加強狂犬病及蝙蝠可能帶有人畜共通傳染病之教育宣導，讓民眾不輕易接觸蝙蝠，對狂犬病及蝙蝠有正確的認知。
3. 犬及蝙蝠咬傷處理與通報機制建立：讓民眾及醫師重視犬及蝙蝠咬傷處理，教育民眾減少與蝙蝠接觸，OIE 規定進行狂犬病研究或診斷以及接觸或暴露在蝙蝠環境中實施 PREP (Pre-exposure Prophylaxis) ，甚至遭蝙蝠咬傷後應額外施打免疫球蛋白。

(三) 建立國際化狂犬病診斷監測技術平台：

1. 持續推動此類計畫可資強化國際交流合作、狂犬病宣導及國際化狂犬病診斷監測技術平台，且有助於我國針對境內蝙蝠等野生動物進行狂犬病監測。
2. 加入世界狂犬病控制聯盟 (Global Alliance for Rabies Control)或與其他狂犬病診斷參考實驗室合作交流：世界狂犬病控制聯盟是非政府組織(NGO)，大力邀請臺灣從事狂犬病防治的專家們加入該聯盟共同為世界狂犬病控制來努力，倘若得以順利加入，相信對建立國際化狂犬病診斷監測技術平台相當有幫助，也可對全球狂犬病控制貢獻臺灣一份心力。

四、持續蝙蝠 Lyssavirus 監測

1. 依照澳洲經驗，同為狂犬病非疫區，澳洲於蝙蝠族群檢測到 Australian Bat Lyssavirus (ABLV) ，蝙蝠有類似狂犬病病毒感染之相同症狀，也曾有蝙蝠抓傷人類而造成類似狂犬病感染之情形，但 ABLV 與狂犬病病毒雖同屬 Lyssavirus 但不同基因型，專家建議臺灣應持續進行蝙蝠 Lyssavirus 監測，就算偵測到 Lyssavirus，也得進一步分離病毒並分析，有助於瞭解臺灣蝙蝠是否感染 Lyssavirus，並非是增加宣告臺灣為狂犬病疫區的機會。

2. 蝙蝠飛行距離甚遠，依據澳洲的研究報告，蝙蝠可以從澳洲大陸飛行 1,600 公里到達紐西蘭，然而亞洲地區不論是中國大陸、菲律賓、印尼等國家皆有狂犬病疫情傳出，且蝙蝠 Lyssavirus 調查及相關研究資料不足，臺灣於此方面之研究走在亞洲各國前面，爾後更應持續蝙蝠 Lyssavirus 甚至其他新浮現人畜共通傳染病之監測。

3. 監測目標對象：專家建議監測不可非漫無目標或僅追求特定數量以達到統計上有顯著意義，初步應就生病或死亡蝙蝠進行監測，比較容易獲得陽性結果，也不會造成無謂的資源浪費。

五、規劃蝙蝠疾病監測國家型計畫

專家建議蝙蝠並非只帶有 Lyssavirus，還可帶有其他新浮現人畜共通傳染病，例如立百病毒、伊波拉病毒、冠狀病毒 (SARS)甚至還有細菌及黴菌

性病原等，倘若要進行蝙蝠疾病監測國家型計畫，進一步瞭解臺灣地區蝙蝠新浮現人畜共通傳染病，美國 CDC 願意分享其執行相關研究計畫之成果，並提供臺灣相關研究調查上的協助。

六、研發及生產狂犬病疫苗 (人用及動物用) 因應可能之疫情

1. 疫苗是控制狂犬病最佳處理方式：專家一再提醒當狂犬病疫情或人類感染事件發生，疫苗使用就是控制狂犬病最重要也是最佳的處理方式，然而儲備多少量的疫苗才夠，就是項困難的決定，無論如何，當疫情發生，可以預想到國際間對狂犬病疫苗的需求會大增，臺灣政府應居安思危，思考並面對此一嚴肅的問題，未來應具備開發及生產安全且足夠量的狂犬病疫苗 (人用及動物用)。
2. 中國大陸及鄰近國家時有狂犬病疫情傳出，市場需求量大，如果我國可以量產狂犬病疫苗，亦可考慮規劃輸出鄰近國家，解決疫苗產銷問題。

結論：

臺灣自民國88年起對犬隻進行狂犬病監測，迄今已邁入第12個年頭，檢測超過5,000例犬隻腦組織病例，所有樣本經直接螢光抗體染色法(FAT)及反轉錄聚合酶連鎖反應(RT-PCR)檢測，結果皆爲陰性。此一監測成果爲我國維持「狂犬病非疫區國家」提供強而有力的科學證據。然而近年來，野生動物新浮現人畜共通傳染病的重要性日漸增加，其中又以蝙蝠之狂犬病及Lyssavirus等疾病對我國社會民心之影響最劇。澳洲在蝙蝠Lyssavirus之研究經驗豐富，居世界領先地位。經多次聯繫溝通，於11月8日派遣兩位研究人員赴澳洲動物衛生國家實驗室研習兩週，學習蝙蝠狂犬病及Lyssavirus感染症等疾病之診斷及監測技術，觀摩澳洲蝙蝠之Lyssavirus檢驗流程及實驗室生物安全防護，並在澳方專家指導下，實際操作蝙蝠之Lyssavirus陽性檢體，研習抗原檢測方法：直接螢光抗體染色法、病毒分離、即時定量聚合酶連鎖反應、組織免疫化學染色；以及血清中和抗體檢測方法：Rapid Fluorescent Focus Inhibition test (RFFIT)、Fluorescent Antibody Virus Neutralisation test (FAVN)。本次研習對強化我國蝙蝠新浮現人畜共通傳染病之診斷監測技術有極大助益，並可促進臺澳日後之雙邊合作。

附件一

RABIES TRAINING PLAN													
	Week 1							Week 2					
	Monday	Tuesday	Wednesday	Thursday	Friday	Sat	Sun	Monday	Tuesday	Wednesday	Thursday	Friday	
	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	
Introduction, induction, etc	all day												
Biosecurity/Biosafety (can join into this training scheduled for AAHL staff)-OPTIONAL IF TIME PERMITS													
Primary containment & BSCS - Boardroom & filter test lab			10-11am										
Incident & Spills Awareness Training - Secure Training lab									10-11.30am				
Identification of Hazards at PC3&4 - Secure Training lab										10-11am			
Tour of secure area(Warren Mitchell)		9-10am											
SEROLOGY													
RIFFT				9:00-12:00	12:00-16:30								
FAVIN								9:00-12:00		11:00-16:30			
ANIMAL DISSECTION			9:00-12:30										
VIROLOGY													
PREPARATION OF HISTOLOGY MATERIAL			9:00-12:30										
PREPARATION OF PCR SAMPLES		13:30-16:30											
ABLV FAT		10:30-12:30						13:00-16:00					
VIRUS ISOLATION			13:00-16:00								9:00-12:00		
HISTOLOGY				12:00-16:00	9:00-12:00								
EM-Paul Monahan												9:30-11:30	
MOLECULAR PCR									ALL DAY				
LECTURES/SEMINARS		15:30-16:30		16:00-17:00							16:00-17:00		
SOCIAL EVENTS											DINNER		
		Introduction, induction etc											
		Biosecurity/Biosafety training		(These are currently scheduled for AAHL staff - can the visitors fit into these)									
		Serology (RIFIT/ FAVIN tests?)											
		Histology											
		Virology											
		Molecular (RT-PCR)											
		Social event											
		John Bingham-animal dissection,Rabies Lecture											
		EM-Paul Monahan											

PROCEDURES FOR RABIES AND LYSSAVIRUS DIAGNOSIS INCLUDING RABIES FLUORESCENT ANTIBODY TEST (FAT) AND VIRUS ISOLATION.

AUSTRALIAN ANIMAL HEALTH LABORATORY

DISEASE DIAGNOSIS PROJECT

REVISION PAGE

Versions

**Main Author is defined as the principle writer / contributor to the document and who takes responsibility for its technical content.*

Version	*Main *(Responsible)* Author	Authorised by	Authorisation Date (dd/mm/yy)
1	Paul Selleck	A J Forman	December 1992
2	Paul Selleck	Mike Johnson	04/11/2005
3	Paul Selleck	Paul Selleck	11/04/2008
4	Andrea Certoma	Ina Smith	24/06/2009
5	Andrea Certoma	Wilna Vosloo	10/9/10

Document Details: Creation / Modifications (Details of changes)

**Modification Author is defined as a writer / contributor who makes changes to the document but does not take on the Main Author responsibility.*

Version	Modification Author/s	Date	Modification Details
1	Paul Selleck	December 1992	Location of electronic text object found in AAHL LAN at O:\DIAG\DXPROT\rabiesdx
2	Ross Lunt	4 November 2005	Principles from previous version unchanged. Methodology similar. No technical modifications of consequence. Detail added to previous version.
3	Paul Selleck	April 2008	Reviewed with no further modifications identified. Tracked changes which had not been accepted since the last review were all accepted at this review.
4	Andrea Certoma	June 2009	Minor technical and PCR requirements changes made
5	Andrea Certoma	September 2010	Updated technical and Laboratory location

Synopsis of electronic components within this document

Explain all object linkages / formats imbedded, and any linkages to web sites that have been imbedded within this document. This information is important for the current and future management (i.e. readability and preservation) of this electronic document.

Page	Details of electronic object imbedding or linking
4	http://www.cdc.gov/ncidod/dvrd/rabies/professional/publications/DFA_diagnosis/DFA_protocol-b.htm#III_A_5 - Minimum Standard for Rabies Diagnosis in the United States
4	SCAHLS Web Page links to http://www.scahls.org.au/ - home page of Sub-Committee on Animal Health Laboratory Standards
9	CDC Rabies DFA Technique links to http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm#V_A
6, 11, &12	Links within this document

1 INTRODUCTION

1.1 Disease

Rabies is an infectious zoonotic disease characterised by encephalitis that may be associated with paralysis, behavioural changes and other neurological manifestations prior to an ultimately fatal sequela. Wildlife reservoirs of rabies virus are maintained in a number of species and the virus from particular reservoirs is genetically identifiable as originating from that population. In Australia a rabies related virus, Australian bat lyssavirus, has been isolated from both mega- and microchiroptera bats and the isolates to date appear representative of a clade of the virus associated with, and distinguishable according to each of these groups. While Australia has no terrestrial form of rabies virus, there are two known instances of spread of the virus to humans. The possibility must exist for other animals also to be infected with the bat variant of this virus. Additionally, trans-border passage of infected animals or humans remains a potential source of rabies cases in Australia.

The lyssavirus investigations at AAHL are most commonly used for:

- Suspect lyssavirus infection of animals (mostly bats)
- Regulatory exclusion testing for imported animal death whilst in quarantine
- Investigations of suspect acute human rabies infection

1.2 Assay

The **scope of this protocol** covers detection of rabies and lyssavirus antigen either directly in infected animal tissues or following inoculation of cell cultures. The direct fluorescent antibody test (FAT) using a specific fluorescein labelled monoclonal antibody is used to identify virus antigen both in tissue samples and cell culture. Detection of lyssavirus in cell cultures is dependent on FAT staining, as cytopathic effects (CPE) are not usually evident. At CSIRO AAHL rabies and lyssavirus antigen can also be detected in infected tissues by immunohistochemistry (IHC) staining and polymerase chain reaction (PCR). These tests are carried out using test specimens generally supplied from the rabies laboratory.

Lyssaviruses are classified for work at biosafety level 3z.

Only appropriately vaccinated staff can open and process fresh material for Rabies and Lyssavirus investigations.

1.3 Results

Results may be expressed as an evaluation for the presence of fluorescence consistent with the presence of lyssavirus antigen. Specific staining by the conjugated antibody in any tissue is positive for rabies infection.

1.4 Assay validation

Rabies antigen detection and virus isolation testings are based on the use of internationally recognised standard protocols. The FAT test has been validated at this laboratory through on-going parallel testing using different test systems (FAT, IHC, PCR and virus isolation) and comparison of results. In general, all brain specimens are separately tested for antigen by both FAT and IHC staining. Virus isolation is also conducted for antigen positive samples. Polymerase Chain Reaction (PCR) is carried out on all category 3 (potential human exposure/disease) samples and on other submissions for which an antigen positive result has been obtained. Analysis of data from 2001-2004 on Bat ABLV submissions showed complete agreement between real time PCR and FAT for 103 submissions of which 20 determined to be positive.

1.5 References

Laboratory Techniques in Rabies. 4th edition. Edited by F.-X. Meslin, M.M Kaplan and H. Koprowski (1996) WHO.
Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing
A Minimum Standard for Rabies Diagnosis in the United States.
http://www.cdc.gov/ncidod/dvrd/rabies/professional/publications/DFA_diagnosis/DFA_protocol-b.htm#III_A_5

McColl KA and Lunt RA (2003) Australian and New Zealand Standard Diagnostic Procedures Australian Bat Lyssavirus SCAHLS Web Page

Fujirebio product insert: FITC Anti-Rabies Monoclonal Globin

2 EQUIPMENT

Petri dish, 90mm
Petri dish, 50 mm
Scalpel with handle (disposable no:11)
Sterile tip, large white
Glass Slides- frosted end (Alcohol cleaned).
Coverslips 50mm x 22mm
Glass slides plain
Slide rack
Polypropylene Coplin slide jar
Yellow capped 50 ml specimen container.
7ml sterile polypropelene tubes
10ml centrifuge tube
10 & 3 ml syringes
18g 1½" drawing up needle without bevel BD Ref 300204
Burn bin
Pipettes – currently verified for use
Sterile plugged 200µl & 1ml tips
Wooden tongue depressor sticks (for impression smear technique only)
Kimwipes
Cell scrapers-optional
Sterile individually wrapped transfer pipettes
Sterile dissecting instruments

Should be available in the room approved for this testing

Approved and monitored

- Freezers/Refrigerators

–80°C Freezer (for reagent storage),

–20°C Freezer (for acetone fixation step),-modified to eliminate risk of explosion due to acetone vapour build up

4°CRefrigerator (for reagent storage)

- Incubators

CO_2 Incubator (for cell growth) approved and monitored

- Centrifuge

Able to spin at 2000rpm,containing pots with biocontaiment lids.

3. REAGENTS

3.1 Chemicals

3.1.1 Pyroneg detergent – Store item 2786 (for use in bread box & Sharps Biocan.)
3.1.2 Aidal Plus (21g/L glutaraldehyde) for general decontamination(Store item)
3.1.3 Acetone (Store Item) .
3.1.4 Glycerol, AnalR Store Item 2571.
3.1.5 MagMax buffer obtained from Molecular diagnosis group
3.1.6 Evans Blue eg Koch Light Laboratories (England)
3.1.7 Buffered formalin from Histology laboratory

3.2 Biologicals

3.2.1 Lyssavirus FAT positive control slides (see 9.5)
3.2.2 Neuro 2a cells (Obtain from Cell Culture Laboratory)
3.2.3 HMEM – from Store or Invitrogen
3.2.4 Glutamine – from Store or Invitrogen
3.2.5 NEAA – from Store
3.2.6 Pen/ Strep 100units/mL+100µg/mL – from Store
3.2.7 Fungizone – from Store
3.2.8 PBS A – from Store
3.2.9 PBS ABC – from Store
3.2.10 PBSA + 10% BSA – from Store
3.2.11 0.5% Evans Blue in DD H_20 (see 9.4)
3.2.12 Fujirebio (Centocor) Anti Rabies Monoclonal FITC Conjugate (1:5 Dilution) see 9.6
3.2.13 Conjugate diluent (see 9.2)
3.2.14 Buffered Glycerol Mounting Medium pH 8.5 (see 9.3) or commercially available FITC mounting medium

4. PREPARATION

4.1 Preparation of Samples

4.1.1 **Receipt.** Samples received by laboratory staff may include fresh brain material from human, cat, dog, bat and occasionally other animals. Salivary gland material is also frequently submitted. Cerebro-spinal fluid (CSF) and skin biopsy samples may also be received in the investigation of acute human rabies.

4.1.2 **Check Paperwork.** On delivery to the laboratory, samples are matched with the corresponding Job Number from the Specimen Advice Notice (SAN) or other paperwork by reference to sample number/bar code stickers supplied in Sample Accessions and accompanying sample container(s). The sample paperwork is examined to identify the nature and required testing for the submitted sample. Note: all paperwork accompanying samples may be viewed from Sample Manager

4.1.3 **Opening.** Only to be performed by rabies vaccinated staff. Sealed tins containing samples **for disease investigation** should be opened in a Class II biological safety cabinet in the Rabies laboratory or another approved laboratory .

4.1.4 **Sample Acceptance/Rejection Criteria.** Examine the condition of the sample and record on the Specimen Advice Notice (SAN) or other submission paperwork any sample leakage, poor quality or condition that may indicate a discrepancy between the received sample and the material identified in the accompanying paperwork Samples should not be tested without recourse to the Laboratory Supervisor if there is cause to believe that the sample quality or identification is deficient or compromised. Sample acceptance will be based on complete confidence that the submitted material is correctly identified, is appropriate and remains fit for testing having accounted for transport and storage conditions. Any concerns regarding the quality of the sample must be noted on the sample paperwork and as a qualifying remark added to the Analysis Comment section of the Sample Manager Report. Report any discrepancies to the laboratory supervisor.

4.1.5 **Numbering and storage.** The identification on each sample should be compared with the accompanying paperwork. Mark by highlighter pen on the paperwork any identifying numbers corresponding sample labelling. Other unique identifying numbers etc may be handwritten on to the front of the SAN. Label each sample with the Job Number. Disease investigation and virus samples may require storage at –80°C if testing is not to be carried out on the day of receipt.

4.1.6 **Assignment of Tests.**

Please check with the Duty Vet and Sample Manager for the testing that is required. The following tests and priorities are a general guideline only:

Table 1

Case	**Sample**	**Tests**	**Priorities**
Dog/cat death in quarantine	Brain	FAT, Isolation, IHC	Category 2
Dog/cat death in quarantine	Salivary gland	FAT, IHC	Category 2
Bat Whole	Remove brain and salivary gland. Process and assign priorities as for bat tissue below. Remove lung, liver, spleen and kidney for exclusion of other non-lyssaviruses.		
Bat, no human exposure, no disease	Brain, salivary gland	FAT, IHC	Category 1
Bat, no human exposure, disease	Brain, salivary gland	FAT, IHC	Category 2
Bat human exposure	Brain, salivary gland	FAT, IHC, PCR on brain.	Category 3
Bat, lyssavirus positive	Brain, salivary gland	Virus isolation, IHC, PCR, sequencing	As already assigned
Other animal with suspect rabies or lyssavirus	Brain, salivary gland	Virus isolation, IHC, PCR, sequencing	Category 2 or 3 if human exposure.
Human encephalitis	CSF, Serum	PCR (on CSF), virus isolation*, serology*	Category 3 (unless otherwise indicated by duty vet). **PCR testing has priority**
Human encephalitis	Biopsy or scraping, Saliva	PCR, FAT*, IHC*, virus isolation *, serology*	Category 3 (unless otherwise indicated by duty vet). **PCR testing has priority**
Human encephalitis	Post mortem tissue	PCR, FAT*, IHC*, virus isolation *, serology*	Category 3 (unless otherwise indicated by duty vet). **PCR testing has priority**

* These tests are carried out only if sufficient material is available. Consult with duty vet.

4.2 Preparation of Staff

4.2.1 General

4.2.1.1 Staff working with rabies, rabies related viruses and other lyssaviruses must be familiar with the relevant sections of the Microbiological Security and Safety Manual, in particular, "Standard Operating Practices: Biosecurity Level 3(Z) Rabies" (Section 5.18 Special Conditions for Rabies, Rabies-like viruses and Australian Bat Lyssavirus).

4.2.1.2 Staff working who may be exposed to rabies or related virus must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months for laboratory staff who regularly test for the virus. Staff should be aware that rabies vaccination does not confer immune protection for all lyssaviruses.

4.2.1.3 Staff who undertake the determination of antibody in human serum are recommended to have been vaccinated against hepatitis B virus.

4.2.1.4 Work is generally conducted in Rabies Laboratory Room 8 (3L3-130) .

4.2.1.5 If work is conducted in another approved laboratory only vaccinated staff should be present during active manipulation of potentially infectious materials. The amber warning light or appropriate signage should be used to restrict access.

4.2.1.6 All potentially infectious work must be carried out in a biological safety cabinet.

4.2.2 Staff Competence

4.2.2.1 All staff competent to carry out lyssavirus diagnosis must have a documented training record in QM 9-4 (Staff Training) and be identified in the appropriate Health, Safety and Environment Assessment of Work documentation.

4.2.2.2 Staff will be judged as competent by the laboratory supervisor (or delegate) based on knowledge of the operators experience with this and other related assays and techniques.

4.2.2.3 A minimum training period of six months shall apply to all staff new to this procedure regardless of prior training experience. At least 6 supervised FAT tests shall be conducted over this period; more may be required at the discretion of the supervisor.

4.2.2.4 A minimum retraining of staff who have not performed this assay in the previous five years will include two supervised FAT tests

4.2.2.5 Competency of staff under training will be measured by observation of test performance and/or by testing an in-house panel of "test" samples.

4.2.2.6 At the completion of the training period, the laboratory supervisor will advise the technical manager that updates to the appropriate QM sections can be made.

4.2.2.7 On-going staff competence will be maintained by ensuring that all competent staff have the opportunity annually to carry out the test procedure.

4.2.2.8 On-going staff competence will be assessed annually by participation in proficiency testing either by an external provider or by testing an in-house panel of "test samples".

5. PERFORMANCE OF THE TESTS

5.1 Fluorescent Antibody Test (FAT) for Rabies and Lyssavirus Antigen

5.1.1 Generate a Sample manager worksheet or commence Rabies/Lyssavirus Test Record and Results Form (located in O:general\ISO9000\Forms\Virology).

5.1.2 Five alcohol cleaned glass slides slide will be required and should be labelled in pencil with the AAHL accession number and SG, CC, H, C, BS according to the tissue that will be smeared to each (viz. salivary gland – SG, cerebral cortex – CC, hippocampus – H, brain stem – BS). Label a yellow capped specimen container with the accession number and fill with Neutral buffered formalin . Add 5ml sterile PBS to a plastic 7ml sterile tube and label a centrifuge tube with the accession number, date and "10% brain homog.". Ensure that there is clean acetone in a plastic slide jar at –20°C.

5.1.3 Unpack specimen in biosafety cabinet and discard packing materials for autoclaving. Note the samples received, the labelling of the sample container and general condition of samples.

5.1.4 Whole animals/ heads will need to be dissected by an experienced person. It is advisable to obtain assistance as necessary from the laboratory supervisor, duty veterinarian or LAF staff. Extensive training is required for competency in dissecting Flying Foxes, dogs or cats heads to be approved.

5.1.5 When brain (BR) and salivary gland (SG) have been supplied, prepare smears of salivary gland tissue before working with the brain tissue. Place SG in a clean Petri dish.

5.1.6 Working with a clean scalpel and pipette tip, remove a section of SG tissue, about match head size to the surface of the SG labelled slide. Using the scalpel blade, drag the tissue over the lower half of the slide to produce a smear of adherent cells. Ideally the tissue should not be overly moist, as this will prevent a good smear of cells.

5.1.7 Remove about $1cm^3$ of SG to buffered formalin and return remaining SG to the original container.

5.1.8 Place the brain in a 90mm or 50mm Petri dish as appropriate (larger brains can be worked with on a large clean plastic bag). Identify brain stem, cerebellum, hippocampus and cerebral cortex. (See CDC Rabies DFA technique for descriptive images). Remove portions (about of two match heads in size) of each of the nominated regions to a clean Petri dish, cutting with a handled scalpel blade and manipulating with a sterile plastic pipette tip. If it is not possible to identify these regions due to the nature or condition of the material supplied then remove tissue from four relatively disparate sites.

5.1.9 Bisect each portion, removing one piece of each to the centre of the Petri and finely chop with the scalpel. Place this in a 7ml plastic centrifuge tube with 5ml of PBS. Using a 3ml syringe equipped with an 18g blunt drawing up needle, aspirate and expel the homogenate several times to produce a fine tissue suspension. Use a vigorous uptake of the homogenate into the syringe and slow expulsion. NOTE: DO NOT USE EXCESS FORCE TO EXPEL TISSUE HOMOGENATE AS THIS CAN CREATE INFECTIOUS AEROSOL. If the needle becomes blocked, attempt to aspirate then re-expel. If this does not clear the blockage, replace the drawing up needle.

5.1.10 NOTE: THIS TECHNIQUE FOR HOMOGENATE PREPARATION IS PARTICULARLY SUITABLE FOR SMALL ANIMAL BRAIN TISSUE. TISSUE FROM LARGER ANIMAL BRAINS IS MORE APPROPRIATELY HOMOGENISED USING A MORTOR AND PESTLE.

5.1.11 Centrifuge at 2000rpm for three minutes in sealed centrifuge pots.

5.1.12 In this time, smears can be prepared for the remaining four pieces of brain tissue in the order CC, H, C, and BS.

5.1.13 Preparation of Brain Smear – Slip smear technique

This technique is useful for preparation of thin uniform smears, particularly when the material is soft and unlikely to produce good touch smears.

5.1.13.1 Mince the tissue with the scalpel and remove a small amount (eg ½ match head size) to the surface (just below half way) of an appropriately labelled slide. Underlie this slide with a clean glass slide to provide additional support against breakage during the preparation of the smear.

5.1.13.2 Hold the paired slides with thumb and index finger, and provide support from underneath with the middle finger. A thin film smear of the brain tissue is prepared by gently pressing down on the tissue with a clean glass slide, carrying out a single circular motion to spread the tissue, then sliding the glass slide down the lower portion of the slide. Care should be taken not to contaminate gloves with brain tissue.

5.1.13.3 CAUTION: IT IS VERY IMPORTANT THAT CORRECT TECHNIQUE IS USED TO PREPARE THE SMEAR AND THE OPERATOR SHOULD BE WELL TRAINED BY WORKING WITH NORMAL BRAIN TISSUE PRIOR TO CARRYING OUT THIS PROCEDURE. IMPORTANTLY SLIDES MUST NOT BE SUBJECTED TO UNSUPPORTED PRESSURE. THE PRESSURE FROM THE OVERLYING SLIDE IS DELIVERED BY PLACING THE THUMB ON THE SLIDE DIRECTLY ABOVE THE TISSUE TO BE SMEARED. FAILURE TO DO THIS WILL RESULT IN UNSUPPORTED STRESS TO THE OVERLYING SLIDE AND RISK OF THIS SLIDE SNAPPING.

5.1.13.4 Discard the overlying slide used to prepare the smear, place the smeared slide in the rack to dry and proceed with the next piece of tissue.

5.1.14 Preparation of Brain Smear – Impression (touch) smear technique

This technique should be used for larger (eg than flying fox) brain material, particularly in which the tissue is fresh and firm. Soft autolyzed tissue will not give good results by this method.

5.1.14.1 Place a cube of CC brain tissue (about length 3mm X width 3mm X thickness 2mm) on one end of a wooden tongue depressor stick.

5.1.14.2 Holding the stick in one hand, lightly touch a slide labelled appropriately for that tissue to the cut surface of the cube. Do this 3 to 4 times to form that number of touch smears.

5.1.14.3 Place the slide in a rack and allow drying.

5.1.14.4 Repeat the process for additional brain sites H, C and BS.

5.1.15 Open the centrifuged pots in the cabinet. Complete preparation of sample for PCR testing by adding 100µl of centrifuged brain homogenate to 260µLMagMax buffer Mix vigorously. Wipe tube with Aidal, remove from the cabinet, label with the accession number and place upright in a plastic specimen container. Inform PCR staff that the treated sample can be collected.

5.1.16 Remove plastic slide jars with acetone from –20°C. Place the air dried brain smears slides into one jar and SG smear into a separate jar. Fix tissue smears in acetone at –20°C for 30 minutes. Acetone can be reused only with known negative test samples.

5.1.17 After 30 minutes, remove slide jar from –20°C to cabinet. Open and remove slides to a slide rack and allow drying.

5.1.18 Remove a fixed lyssavirus positive and negative control brain smear from –20°C and also air dry.

5.1.19 Thaw an ampoule of conjugate and add conjugate diluent to 2ml. Connect a 0.2µm filter in to a 3 ml syringe and tip conjugate into the syringe.

5.1.20 Place slides in a slide tray. Add six drops of conjugate to each slide and spread over the tissue smear with an inverted pipette yellow tip if needed. Work with the negative control smear first, then the SG, followed by the test brain smears, then the positive control. Change the tip also in this progression.

5.1.17 Incubate chamber slides at 35 °C in a humidified,5% CO_2 for 30 minutes.

5.1.18 Remove slides from the incubator and tip stain into Pyroneg solution.

5.1.19 Slides are washed by a progression through two jars of PBS ABC.

5.1.20 Start by washing the negative control slide followed by the SG, Brain and finally the positive control smears in the rinse jar. Transfer the test smears to the soak jar and the positive control to the rinse jar. Complete the progression so that all slides are rinsed and allowed to dry.

5.1.21 Arrange a coverslip for each slide on a Kimwipe and add 1 drop of mounting media (see 9.6) to each cover slip. Lower the dried slide on to the coverslip and gently press out excess mounting fluid on to tissue. Wipe the slides and place in a slide tray..

5.2.1 **5.2Virus Isolation in Neuro 2a cell**s Prepare a worksheet for Lyssavirus isolation from Sample Manager.

5.2.2 Virus isolation is carried out by inoculating a suspension of Neuro 2a cells that are transferred to a culture flask after 1 hour incubation.

5.2.3 Obtain a confluent and healthy flask of Neuro 2a cells from the Cell Culture laboratory.

5.2.4 Trypsinise to remove the cells and prepare a 12ml suspension in HMEM + 1% FCS.

5.2.5 Add 2 ml of suspension to each of two 7ml plastic bottles, one labelled control and the other with the sample accession number.

5.2.6 Add 200µl of tissue homogenate from 5.1.15 to one of the bottles. Place both bottles at 35°C for 1 hour with occasional mixing.

5.2.7 Add 8 ml of HMEM + 10% FCS to each of two 25cm^2 flasks. Label “Neuro 2a” “P# ”, Control or appropriate accession number, the tissue sample and date.

5.2.8 After the 1 hour incubation, use a 5 ml syringe equipped with an 18g drawing up needle to resuspend and transfer first the control non-inoculated cells then the inoculated cells to the labelled flasks.

5.2.9 Check the cell cultures after 24 hours for evidence of bacterial contamination. If this occurs, repeat from 5.2.2, using homogenate that has been centrifuged at 2500 rpm for 10 minutes then the supernatant fluid passed through a 0.45µm filter.

5.2.10 Incubate the culture for 4 to 7 days.

5.2.11 At the end of this period examine cells for any cytopathic effects. The Neuro 2a line can detach and form clusters of viable cells in suspension.

5.2.12 In the class 2 cabinet, tap the control flask to remove a good proportion of the cell monolayer. Tip the 8ml of culture medium and cells into a labelled centrifuge tube, leaving 2ml in the flask. Repeat this process for the inoculated flask and centrifuge both tubes at 2000 rpm for 5 minutes in sealed centrifuge pots.

5.2.13 Open the centrifuge pots in the cabinet and remove the tubes. Decant the supernatant medium from the control cells back into the original flask. Similarly decant medium from the inoculated flask.

5.2.14 Add 200µl of PBS A to each tube. Separately resuspend the cell pellet with a glass pipette and transfer about 50µl to a clean, labelled glass slide. Use an inverted yellow tip to spread the cell suspension over the lower half of the slide. Allow to air dry in the cabinet. This process can be accelerated by placing the slide on a metal block removed from a 37°C heater block.

5.2.15 The air-dried cells can be fixed and stained as described for brain tissue smears (see 5.1.16). A positive control must be included. This can be prior fixed lyssavirus positive brain smears or cell culture material.

5.2.16 When slides have been mounted with coverslips, read

5.3 Virus Isolation in suckling mice (Alternative to Neuro 2A isolation)

5.3.1 Suckling mice should be used selectively as an alternative to cell culture for recovery of virus in cases in which isolation has, or might prove to be difficult. In particular, for critically important samples in which low levels of virus are likely to be present due to the nature of the sample, the stage of the disease or characteristics of the infecting virus. All procedures must be in accordance with currently approved Animal Experimentation Ethics Committee protocols.

5.3.2 For each test sample order 1 Swiss White mouse with a newborn litter of six pups at less than 5 days old. These can be ordered through the Small Animal Facility (5783). Complete an AEC2 form and forward to the AEC Secretary.

5.3.3 Mice are inoculated intra-cranially with CSF or clarified 10% tissue suspension (prepared as described 5.1.8).

5.3.4 Work in a class 2 biosafety cabinet. Use a 90mm Petri dish as an inoculation area. Petri dishes can also be used as rests for the syringe and needle between mouse inoculations.

5.3.5 Load a 1.0 ml syringe equipped with a 28g needle with 0.2ml of inoculum. Restrain the mice with padded forceps. Insert the needle slightly to the side of hemisphere cleft and to a depth of about 1mm. Inoculate each mouse with 0.02ml of inoculum.

5.3.6 Observe mice daily at least 28 days. Euthanise any mice which develop signs of illness (ruffled fur, tremors, lack of coordination, paralysis.

5.3.7 Remove brain tissue and examine for antigen by DFA according to the procedure described in 5.1.

5.3.8 Carry out virus isolation in Neuro 2A cells as described in 5.2

5.4 Clean up

Procedures outlined in AAHL Microbiological Safety And Security Standard Operational Procedures should be followed.

In brief:

- Tip all PBS wash buffer into a Biocan with Pyroneg solution.
- ALL SCALPEL BLADES MUST BE DISCARDED INTO A SHARPS CONTAINER.
- Carcasses, tissue samples, paper, petri dishes, paper etc for disposal are placed in an a small autoclave bag, and the bag wiped with Aidal prior to removal from the cabinet. This bag is then placed into a larger bag, sealed in a tote box and promptly autoclaved.
- Disposables including pipette tips, plastic tubes can be placed in the Biocan as well.
- Use wash bottle to add Aidal to PBS jars to decontaminate before soaking in Pyroneg solution.
- When discarding acetone, in the cabinet, tip acetone into a pipette canister containing Aidal, disinfect the outside of the canister with Aidal and leave at room temperature for 24 hours before flushing down the sink with copious amounts of water.
- Instruments are discarded into a bread box containing water and Pyroneg. This is disinfected with Aidal before removal from the cabinet, transferred through to the animal services autoclave with the lid in place and the lid slightly opened immediately prior to sterilisation.
- Non-autoclavable items are disinfected with Aidal before removal from the cabinet. Then soaked Pyroneg solution overnight rinsed and air dried and reused.
- Wipe down cabinet with Aidal.

6. RESULTS.

6.1 Reading

6.1.1 Examine all slides under 20x lens. Entire smears or monolayers should be scanned for specific staining.

6.1.2 Slides are read for staining intensity and antigen distribution as follows:

Staining intensity is graded from 4+ to 1+.
4+ intensity - a glaring, apple green brilliance
3+ intensity - slightly diminished staining intensity
+2, +1 - considerably diminished

Antigen distribution.
4+ - a massive infiltration of large and small inclusions of varying shape in almost every area of the impression.
3+ - inclusions almost every microscopic field
2+ - inclusions 10% to 50% of the microscopic fields and most fields contain only a few inclusions.
+1 - inclusions in <10% of the microscope fields and only a few inclusions are found per field (usually only one or two inclusions per field).

6.1.3 All category 3 submissions must be read separately by two staff.

6.1.4 Record results on the Sample Manager Worksheet.

6.1.5 Note any abnormal staining effects.

6.2 Acceptance of Assay

6.2.1 The positive control should have 4+ intensity and 4+ to 3+ distribution.

6.2.2 Negative control slide should not show any specific staining (note a negative control smear is not normally tested with brain smear tests).

6.2.3 All tests should demonstrate a positive and negative reaction. PCR and Isolation can be used to confirm results. Seek advice from the lab manager and Duty Vet for unusual test results.

6.2.2 If any these conditions have not been met, report problem for acceptance to the laboratory supervisor who will decide if all or part of test must be repeated.

6.3 Interpretation of results

6.3.1 **Brain Smears.** In an accepted assay, interpretations are:

6.3.1.1 Not detected when test brain smear slides from all 4 regions clearly show no fluorescence. Report as “Not detected”.

6.3.1.2 Not detected (incomplete testing) when the four brain regions have not been examined. Report as “Not detected” with qualifying remark.

6.3.1.3 Not detected, atypical fluorescence, Atypical fluorescence should not be read as positive. Most commonly this may be seen as an edge speckled fluorescence effect related to drying, atypical fluorescence appearing in the tract of a blood vessel, atypical fluorescence associated with bacterial contamination. Report only if confident of this interpretation as “Not detected”.

6.3.1.4 Inconclusive, atypical fluorescence. This interpretation is applied if there is uncertainty in interpretation of fluorescence. Report as “Inconclusive” with qualifying remark.

6.3.1.5 Inconclusive, unusual staining. This interpretation is applied if there is discrepancy between stained slides or unusually low levels of stained antigen. Should be retested. Report as “Inconclusive” with qualifying remark.

6.3.1.6 Positive test material will show typical specific speckled or inclusion body fluorescence usually in all slides. Report as "Positive for Lyssavirus antigen". Virus isolation and PCR should be carried out.

6.3.2 **Neuro 2a cell smears.** In an accepted assay, interpretations are

6.3.2.1 Not detected when stained smears clearly show no fluorescence. Report as "Lyssavirus not isolated".

6.3.2.2 Not detected (incomplete testing) when culture fails due to contamination or other causes. Address problem and repeat testing, do not report.

6.3.2.3 Not detected, atypical fluorescence, Atypical fluorescence should not be read as positive. Report only if confident of this interpretation as "Not detected".

6.3.2.4 Inconclusive, atypical fluorescence or unusual staining. Repeat testing, do not report.

6.3.2.5 Positive smears will show typical specific speckled or inclusion body fluorescence. Report as "Positive for Lyssavirus isolation".

6.4 Reporting and Record Storage

6.4.1 Results are recorded on the Lyssavirus FAT worksheet and entered into the Sample Manager**.**

6.4.2 The interim report should be printed, examined for errors, initialled and dated before the test is authorised.

6.4.3 Results entered into Sample Manager must be authorised by the laboratory supervisor or authorised delegate.

6.4.4 Interim Sample Manager reports are filed together with the SAN and test worksheet in the appropriate sequence folder located in an approved laboratory or in the Records Archiving Compactus.

7. SPECIMEN RETENTION

7.1 Retain all original tissues and 10% suspensions at –80°C. Note storage and disposal on Sample Manager.

7.2 Lyssavirus positive material should be entered into the Microstores databsase and stored appropriately as original tissue, 10% homogenate and virus isolate.

7.3 Samples should be disposed of in accordance with requirements of Section 5 of the AAHL Microbiological Security Manual.

8. QUALITY ASSURANCE

8.1 Progressive Monitoring

On-going assay performance is monitored using spreadsheets (Lyssa FAT and Lyssa Isolation) located in the folder O:\DIAG\iso9000\Progressive Test QC Records \Virologyxls. Sheets must be updated after completion of each test with test material and control results. The sheet must be completed before authorising of results.

8.2 Neuro 2a cell monolayers should be subjected to 6 monthly check testing for sensitivity by titration of rabies virus with known titre. Records of this testing should be kept in " O:\DIAG\iso9000\Progressive Test QC Records \Virology .

8.3 External Proficiency Testing

External Proficiency Testing for Rabies/Lyssavirus antigen detection is currently undertaken as requested.

9 APPENDICES

9.1 Neuro-2a cell Growth Medium

HMEM medium	400ml
NEAA	4.0ml
Glutamine	4.0ml
Pen/strep	1.0ml
Fungazone	2.0ml
Foetal Calf Serum(FCS)	10%

Obtain components from tissue culture stocks

Alternatively Invitrogen supplies these

	Catalogue #
HMEM	11575-032
Glutamine	
Anti mycotic/microbial	15240062

Prepare using sterile technique.
Label bottle with date and additions
Store at +4°C for up to 1 month..
Add FCS to volume of media required just prior to use

9.2 Conjugate Diluent (PBS/BSA with 0.005% Evans blue)

PBS A	90ml
PBS + 10% BSA	10ml
0.5% Evans Blue in DD H_20	1ml

Store at + 4 °C.

PBSA and PBS+10%BSA obtained from stores
Evasns Blue: Sigma

Alternative Mounting Medium:

VECTASHIELD : Abacus ALS product can be used, mounting medium with DAPI cat#VEH1200
Specifically formulated for FITC labelled conjugates

9.4 0.5% Evans Blue in DD H_20

Weigh 0.20g of Evans Blue Powder
Add to 40mL of sterile Tissue Culture water.
Mix well filter through a 0.45µm filter
Store at 4°C.

9.5 Lyssavirus FAT positive & negative control slides

Lyssavirus positive control slides are prepared from stored lyssavirus positive brain material located in Microstores cabinet 23 in the Rabies Laboratory (Cabinet 23). Brain smear slides can be prepared in small batches (up to fifteen) fixed in acetone and stored at -20°C in the Rabies Laboratory in a slide box. Record on the box lid the microstores number of the preparation used, the date of preparation and initials of the person responsible, Store slides at quality of stained antigen in these positive control slides can decline after 3 months, so slides should not be used if stored longer than this period.

Negative control slides are prepared in the same manner. Use a current known negative brain to make the smears.

Record the batch numbers and testing in O:\DIAG\iso9000\Progressive Test QC Records \Virology

9.6 Fujirebio (Centocor) Anti Rabies Monoclonal FITC Conjugate (1:5 Dilution)

9.6.1 The Fujirebio (Centocor) Anti-Rabies Monoclonal FITC Conjugate (Fujirubio Diagnostics, Inc. CENTOCOR FITC-Anti-Rabies Monoclonal Globulin Catalog #800-090. A mixture of two IgG2a monoclonal antibodies is obtained from:

Fujirebio Diagnostics
201 Great Valley Parkway
Malvern, PA 19355
USA.

9.6.2 A copy of the import permit can be obtained from Records.

9.6.3 The import permit, together with the completed purchase requisition, is taken to Purchasing from which the order will be placed. Generally, more than one kit is ordered.

9.6.4 The Anti-Rabies Monoclonal FITC Conjugate is received in freeze-dried form. Reconstitute this to 5ml in sterile distilled water according to kit insert instructions.

9.6.5 Previously untested batch lots should be titrated for potency (see 9.11)

9.6.6 Dilute the 5ml of reconstituted conjugate to 25ml in Conjugate Diluent.

9.6.7 Dispense the 1:5 diluted conjugate in 0.5ml volumes into 1.8ml tubes. Label these CENTOCOR anti rabies FITC conjugate. Diluted 1:5. Batch Number. Date.

9.6.8 Store this at –80°C in a box marked Conjugate.

9.7 Titration of Fujirebio (Centacor) conjugate.

9.7.1 The conjugate is titrated to establish that the working dilution when used with CVS rabies virus infected BHK cells. This confirms the activity of the conjugate relative to previous batches and provides a point of reference should it be necessary to establish the usefulness of conjugate that is beyond the use by date.

9.7.2 Titrations can be carried out in conjunction with RFFIT serology (see protocol 13-4-61). Seed one 8-well slide with BHK cells infected with approximately 50 $FFID_{50}$ of CVS rabies virus.

9.7.3 After 22 hours, discard medium. Do not remove chamber housing. Rinse cells with PBA ABC and fix 20 minutes with (400ul per well) 10% formaldehyde solution in PBS ABC containing 0.1% NP40. Rinse cells four times with PBS ABC, tap on tissue to drain.

9.7.4 Thaw 0.5mL volume of conjugate for testing and make up to 2.0mL with conjugate diluent. Prepare 2 fold dilutions from neat to 1/128. Add 150µl per well to the fixed cells.

9.7.5 Incubate at 37°C for 30 minutes. Remove housing, rinse twice in PBS ABC, mount and read for fluorescence.

9.7.6 Score fluorescence for intensity from 4+ to negative. Fluorescence should be of sufficient intensity for use in RFFIT serology to a dilution of 1/4 to 1/8 from neat (taken as the 2.0mL volume of diluted stock). Any concerns regarding potency of the conjugate should be referred to the laboratory supervisor.

9.7.7 Records of testing should be kept in the reagent test folders in laboratory 3L3160.

Worksheet Form Rabies/Lyssavirus Diagnosis For use with AAHLQA/13-4-93

SAN		**DATE**		**Sample Identification**	
SAMPLES				**CATEGORY**	
Sample Condition					

FAT	Positive Control Slide	Negative Control Slide	Conjugate	Conjugate Diluent
BATCH				

Tissues	**Intensity**	**Distribution**	**Intensity**	**Distribution**
Cerebral cortex (CC)				
Hippocampus (H)				
Cerebellum (C)				
Brain stem (BS)				
Salivary gland (SG)				
Parotid gland				

Positive control				
Negative control				
Operator				

Histology:

Date Fixed | **Date sent/collected** | **Formalin Batch**

Samples Fixed:_____

☐ **Isolation:Generate a Sample manager Worksheet –complete worksheet**

PCR

Samples Prepared		**MagMax batch**		**Date sent**	

Test Authorisation
Comments:_____

Signed:_____ **Date:**_____

Worksheet Form Rabies/Lyssavirus Isolation For use with AAHLQA/13-4-93

SAN:_____
DATE:_____ SUPERVISOR:_____
ROOM:_____ CATEGORY:_____
Cell Line:_____ Date/Passage:_____
Media/ Supplemented:_____
Supplements:_____
Inoculum Details:_____
_____
_____

Record of CPE
Pass Number:_____ Date

Sample Identification							
OPERATOR							

Record of FAT
DATE:_____ OPEARTOR:_____

FAT	Positive Control Slide	Negative Control Slide	Conjugate	Conjugate Diluent
BATCH				

Sample Identification	**Intensity**	**Distribution**	**Intensity**	**Distribution**

Positive control				
Negative control				
Operator				

Test Authorisation

Comments:_____

Signed:_____ **Date:**_____

END

附件三

RABIES RAPID FLUORESCENT FOCUS INHIBITION TEST (RFFIT) FOR THE MEASUREMENT OF SERUM NEUTRALISING ANTIBODY TO RABIES VIRUS (SEROTYPE 1 LYSSAVIRUS)

AUSTRALIAN ANIMAL HEALTH LABORATORY

DIAGNOSIS, SURVEILLANCE AND RESPONSE

Rabies Rapid Fluorescent Focus Inhibition Test (RFFIT)

REVISION PAGE

Versions

**Main Author is defined as the principle writer / contributor to the document and who takes responsibility for its technical content.*

Version	***Main** *(Responisble)* **Author**	**Authorised by**	**Authorisation Date** (dd/mm/yy)
O:\DIAG\DXPROT\RABRFFIT	P Selleck	A.J. Forman	May 1995
QA-13-4-61	R. Lunt	P. Daniels	2 April 1998
Version 3	Ross Lunt	Peter Daniels	28 May 2001
Version 4	Ross Lunt	Peter Daniels	6 August 2002
Current Version	Ross Lunt	Mike Johnson	6 September 2005
Current Version	Paul Selleck	Paul Selleck	11 April 2008

Document Details : Creation / Modifications (Details of changes)

**Modification Author is defined as a writer / contributor who makes changes to the document but does not take on the Main Author responsibility.*

Date	***Modification Author/s**	**Modification Details**	**Authorisation**
8-Jun-00	R. Lunt	Pool update	P. Daniels
5 April-01	R. Lunt	Document revision	P. Daniels
28-May-01	R. Lunt	Use of OIE standard in place of WHO standard, update to sample preparation, 6.2.6 addition, 9.9 addition.	P. Daniels
6-Aug-02	R. Lunt	Update of pool reference number, text correction to worksheet 9.2, deletion of CUSUM and replacement with more general graphical analysis as QCEL is no longer functioning;6.3.1 text correction;5.1.9 text correction	P. Daniels
22-Aug-05	R. Lunt	4.1.2 Acceptance/Rejection Criteria, Use of RFFIT progress.xls and Test QC Records 3L3114.xls for test monitoring, see 8. Quality Assurance. Replacement of PBS/Glycerol with 9.4 Buffered Tris Glycerol Mounting Medium pH 9.0. Range of other minor editing changes.	M. Johnson
11-April-08	Paul Selleck	Reviewed – no modifications identified	Paul Selleck

1 INTRODUCTION

1.1 Disease

Rabies is an infectious zoonotic disease characterised by encephalitis that may be associated with paralysis, behavioural changes and other neurological manifestations prior to an ultimately fatal outcome.

1.2 Assay

The rapid fluorescent focus inhibition test (RFFIT) is an *in vitro* assay for neutralising antibody to rabies virus. The assay, which is set up in 8 well chamber slides, is read following immunofluorescent staining of cells as an indicator of viral growth and takes 24 hours to complete.
The assay is used for:

- Investigations of acute rabies infection by testing serum or CSF
- Monitoring post vaccination serum antibody response in humans to check for adequate response or current status
- As a basis for vaccination certification in dogs and cats

While the RFFIT may be used to detect cross reacting antibody to rabies related lyssaviruses such as Australian Bat Lyssavirus, antigenic differences between the viruses can affect assay sensitivity.

Lyssaviruses are classified for work at biosafety level 3z.
Initial dilution of serum received for routine investigation of post vaccination immune response may be carried out in laboratory 114. All other procedures are generally carried out in the Rabies Laboratory (Isolation room 7) by appropriately vaccinated staff. (Note: Under conditions of restricted entry, it is possible to work with rabies virus in laboratory 3L3114, however, for practical purposes it is currently more convenient to undertake this work in the Rabies Laboratory.

All potentially infectious work is to be carried out in a biosafety cabinet and contaminated materials autoclaved in generally in the Animal Services the room before removal from the South Suite..

1.3 Results

Results are expressed as an international unit equivalence level relative to the OIE standard serum.

1.4 References

Smith, JS Yager, PA and Baer, M (1973) A rapid reproducible test for determining rabies neutralizing antibody. Bull. Wld. Hlth. Org., 48, 535-541.

2 EQUIPMENT

Lab-Tek 8 Well Chamber Slides, Permanox, cat # 177445.

Water bath (GA01033 in lab 3L3114) set to 56 °C

Coplin jars

Burn bin

Pipettes – currently verified for use

Incubators

- GA00423 Forma CO_2 or NA00410 Forma CO_2 3L3 114
- QC0346 Nuaire CO_2 Incubator (for test incubation), 3L3 128

Freezers/Refigerators

- GAF0273 Forma –80°C (for reagent storage), 3L3 128 - Iso 7
- QC0064 Westinghouse –20°C Freezer (for acetone fixation step), 3L3 128
- QC0063 Westinghouse Refrigerator (for reagent storage), 3L3 128
- NA00632 Westinghouse Freezer (for reagent storage), 3L3-114
- GA00336 Phillips Refrigerator (for medium storage), 3L3-114
- 3L3 136 Cool Room (for medium storage))

3. REAGENTS

3.1 Chemicals

3.1.1 Pyroneg detergent – Store item 2786 (for use in bread box.)
3.1.2 Aidal Plus (21g/L glutaraldehyde) for general decontamination. Store Item 2783.
3.1.3 Acetone (Store Item) in Coplin jar at – 20 °C.
3.1.4 Acetone 80% in PBS ABC at room temperature
3.1.5 Glycerol, AnalR Store Item 2571.
3.1.6 Sigma Trizma® Pre-set crystals pH 9.0 cat# T1444-10PAK

3.2 Biologicals

Microstores numbers refer to reagents stored in labelled boxes in the -80 °C freezer in the Rabies room (3L3128).

3.2.1 Control sera
Normal Human Serum - 8706-18-0010 and GAF0273 Forma –80°C.
Normal Cat serum (lab 3L3114 –20°C) see 9.7
Weak Positive Human Serum - 8706-18-0011
OIE reference serum 3L3 128, -80°C (diluted to 0.5 IU/mL) see 9.6

3.2.2 Virus (see 9.8)
Rabies CVS 11 Virus - GAF0273 Forma –80°C as 50µl per tube working stock. See O:\Diag\Virol\Test Monitoring\3L3114\RFFIT progress.xls for Pool Reference Number and dilution for use.

3.2.3 Culture medium
Basal Medium Eagle eg. GIBCO cat # 21010-046
BME + 5% TPB (see 9.1)
BHK cell growth medium (see 9.2)

3.2.4 Cells
BHK21 BTV Vaccine Strain Cells: suspension at 0.75 X 10^6 per ml.

3.2.5 Store items
1% w/v DEAE Dextran (10,000 ug/ml) in DD H_2O
PBS A
PBS ABC
PBSA + 10% BSA
Sterile TC water

3.2.6 For cell staining
0.5% Evans Blue in DD H_20 (see 9.9)
Centocor Anti-Rabies Monoclonal FITC Conjugate (see 9.10).
Conjugate diluent (see 9.3)
Buffered Glycerol Mounting Medium pH 8.5 (see 9.4)
Coverslips
Glycerol as lens immersion fluid

4. PREPARATION

4.1 Preparation of Samples

4.1.1 **Receipt.** Samples received by laboratory 114 staff may include human, cat, dog, bat and occasionally other animal sera. Cerebro-spinal fluid (CSF) may also be received in the investigation of acute human rabies. On delivery to the laboratory, samples are matched with the corresponding Job number from the Specimen Advice Notice (SAN)or other paperwork by reference to sample number/bar code stickers supplied in Sample Assessions and accompanying sample container(s). **The sample paperwork is examined to identify the nature and required testing for the submitted sample. Note: all paperwork accompanying samples may be viewed from Sample Manager using "View Graphic File" in Explorer for that Job.**

Sealed tins containing samples <u>for disease investigation</u> should be opened in a biological safety cabinet. The secondary containers (plastic bags, tins, etc) of serum samples for RFFIT may be opened with care on the laboratory bench. Examine the condition of the sample and record on the SAN/paperwork any sample leakage, poor quality or condition that may indicate a discrepancy between the received sample and the material identified in the accompanying paperwork. Report any discrepancies to the laboratory supervisor.

4.1.2 **Acceptance/Rejection Criteria.** Samples should not be tested without recourse to the Laboratory Supervisor if there is cause to believe that the sample quality or identification is deficient or compromised, for example, serum grossly contaminated by haemolysis. Sample acceptance will be based on complete confidence that the submitted material is correctly identified, is serum or CSF and remains fit for testing having accounted for transport and storage conditions. Whole bloods should not be submitted, however these may be tested after separation of serum, providing there is not excess haemolysis. Any concerns regarding the quality of the sample, including the receipt of whole blood must be noted on the sample paperwork and as a qualifying remark added to the Analysis Comment section of the Sample Manager Report.

4.1.3 **Numbering and storage.** The identification on each sample should be compared with the paperwork. Identifying numbers on the paperwork that correspond with those on the sample should be marked with highlighter pen on the paperwork. Other unique identifying numbers etc may be handwritten on to the front of the SAN/paperwork. Label each sample with the correct sample number/bar code sticker for that Job. If sample numbers have not been assigned by Sample Accessions to each sample in the Job, the sample number is assigned and written according to the order of listing on the original paperwork to indicate correspondence of samples with numbering. Complete the sample handling record on the back of the SAN/paperwork. Samples arriving as serum, and should be stored at +4^{o}C to await testing.

4.1.4 To separate clotted bloods by centrifugation, spin vials a t2000k (approx) for about 15 minutes. If the sample is not in a separator tube, transfer serum to a new tube labelled the same as the original sample container. In this case, retain original tube at least until issue of the test report.

4.1.5 All serum samples should be heat inactivated at 56°C for 30 minutes, prior to testing. **Note: indicate that a sample has been heat inactivated by drawing a single red line across the cap**

4.2 Preparation of Staff

4.2.1 Staff working with rabies, rabies related viruses and other lyssaviruses must be familiar with the relevant sections of the Microbiological Security and Safety Manual, in particular, "Standard Operating Practices: Biosecurity Level 3(Z) Rabies" (Section 5.18 Special Conditions for Rabies, Rabies-like viruses and Australian Bat Lyssavirus).

4.2.2 Staff working with live virus must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

4.2.3 Staff who undertake the determination of antibody in human serum are recommended to have been vaccinated against hepatitis B virus.

5. PERFORMANCE OF THE TEST

5.1 Virus Neutralisation Stage

5.1.1 Commence VNT Test Cover Sheet (see forms folder, O:\General\iso9000\Forms\Vector Borne Diseases) and list sera (normally four sera per slide) on Sample Record And Results Sheet. One slide will be required for titration of international standard, and one slide for virus back titration.

5.1.2 Serum is inactivated in Room 3L3114 in the South Suite by heating samples at 56°C for 30 minutes in water bath. Containers of serum removed from package in the rabies room (see 4.1.3) may be externally decontaminated according to AAHL Microbiological Safety And Security Standard Operational Procedures, then brought to lab 3L3114 for heat inactivation.

5.1.3 Do not heat inactivate CSF before testing.

5.1.4 Identify the required chamber slides by numbering with pencil according to the Sample Record And Results Sheet.

5.1.5 Each test serum is normally screened at dilutions of 1:20 and 1:200. For endpoint titration a two-fold dilution series may be used (eg. from 1:20 to 1:2560). The 0.5 IU/ml OIE international unit control serum is tested at dilutions from 1:8 to 1:64. Serum dilutions are calculated on the final serum concentration after the addition of virus and may be prepared in the chamber slides or, if large numbers of sera are required to be tested, in microtitre plates and transferred to the chamber slides.

5.1.6 To screen sera at dilutions of 1:20 and 1:200 add 100µl of BME with 5% TPB (see 9.1) to each of two wells. Add 11µl of serum to the first well to give a 1:10 dilution. Mix thoroughly before transferring 11µl from the first well into the second well. Mix and discard 11µl with the tip.

5.1.7 The dilution series for the international standard and the virus addition and back titration are carried out in the rabies lab. However medium can be added to the chamber slides in preparation while in lab 3l3114.

5.1.8 To prepare for the OIE international standard dilution series, add 300µl of BME with 5% TPB into the top left hand well and 100µl into the six bottom wells of a chamber slide.

5.1.9 To prepare the chamber slide for the virus back titration add 100µl of BME with 5% TPB to the first six wells of a chamber slide, and 200µl to the final 2 wells for the cell control (see Sample Record And Results Sheet).

5.1.10 AT THIS STAGE WORK IS TRANSFERRED TO THE RABIES LABORATORY. Take the slides and sufficient BME + 5% TPB to dilute virus and do the back titration (1ml per slide + ~ 2ml).

5.1.11 In the rabies lab, remove one ampoule of International Standard and one ampoule of a 50µl volume of CVS rabies virus from –80° C freezer. Allow reagents to thaw in cabinet.

5.1.12 All work is in the class 2 cabinet unless otherwise stated.

5.1.13 Complete the international standard dilution series by adding 100µl of 0.5 IU/ml OIE international standard serum to the 300µl of medium in the first well to give a 1:4 dilution. Distribute 100µl to the top right hand well, and 100µl to each of the two wells immediately below. Continue the two fold dilutions on each side of the slide to give duplicates of the four dilutions. The final dilutions, following the addition of 100µl of virus, will be 1:8, 1:16, 1:32, 1:64.

5.1.14 Rabies CVS 11 virus (0112-03-1701) is diluted in BME with 5% TPB so as to contain 50 50% Fluorescing Focus Doses (i.e. 50FFD_{50}/0.1ml). Check the Progressive Monitoring Sheet O:\Diag\Virol\Test Monitoring\3L3114\RFFIT progress.xls for dilution for use. Add 100µl of diluted virus to all wells containing serum and to 2 wells of the back titration slide. Use multi-stepper pipette with 2 tips. Take care not to splash virus from wells.

5.1.15 Make 2, 10-fold dilutions of the working dilution of virus (eg 25µL working dilution + 225µL BME/5% TPB, for 5 FFD_{50}/0.1mL, then 25µL from this dilution + 225µL BME/5% TPB, for 0.5 FFD_{50}/0.1mL) and add 100µl of each dilution to 2 wells of the back titration slide.

5.1.16 If infection with a rabies related virus is suspected the serum may be tested against these viruses by substituting the appropriate virus for rabies virus in this method. The virus pools and their working dilutions are Mokola (9206-17-1600) 1:32, Lagos Bat (9206-17-1610) 1:20, Duvenhage (9206-17-1620) 1:1000 and Duvenhage RA (9206-17-1630) 1:1000. **Note: these viruses must only be worked with in the rabies laboratory.**

5.1.17 Incubate chamber slides at 35 °C in 5% CO_2 for 90 (± 15) minutes.

5.1.18 During this incubation period, there is opportunity to return to lab 3L3114 to prepare the BHK cell suspension.

5.1.19 Using a flask of healthy BHK cells which were passed two days earlier, prepare a suspension of BHK (vaccine) cells containing 0.5 $X10^6$ cells/ml in BHK growth medium (see 9.2), allowing 2mls for each chamber slide. Hold the cell suspension at +4°C until required.

5.1.20 Return to the rabies lab with cell suspension. After the virus/serum incubation treat cell suspension by adding for each ml of cells, 1µl of 1% DEAE dextran (final concentration of 10µg/ml). Mix thoroughly prior to adding 200µl of treated cells to each well (use multi-stepper pipette with 2 tips). Take care not to splash virus from wells.

5.1.21 Incubate at 35°C in 5 % CO_2 for 22 to a maximum of 24 hours.

5.2 Cell Fixation Stage

5.2.1 In rabies laboratory, working in the class 2 cabinet:

5.2.2 Remove covers from chamber slides and discard.

5.2.3 Gently tip medium from chamber slide cultures into bread box with Pyroneg solution.

5.2.4 Pipette 200 to 400µl of PBS ABC into each well.

5.2.5 Gently tip PBS from chamber slides into bread box with Pyroneg solution.

5.2.6 Pipette 200 to 400µl of 80 % acetone into each well. And leave slides for 2 minutes.

5.2.7 Tip 80 % acetone into a pipette discard with about 200 – 300ml of Aidal, cover with lid

5.2.8 Remove plastic chamber housing with forceps and discard this into bread box with Pyroneg solution or Burn Bin. Usually the gasket will peel off with the housing. If this does not occur, remove this with forceps and discard.

5.2.9 Place slides into slide rack..

5.2.10 Transfer slide rack to acetone at – 20 °C by removing Coplin jar of acetone from – 20 °C to cabinet, placing rack into jar, and returning jar to – 20 °C.

5.2.11 Fix cells at – 20 °C for at least 30 minutes.

5.2.12 Remove Coplin jar with slides to cabinet and remove slide rack. Return jar to – 20 °C and allow slides to air dry in cabinet.

5.3 Staining fixed cells.

5.3.1 In the rabies lab, remove a 0.5ml aliquot of conjugate from – 80°C and allow to thaw.

5.3.2 Dilute the conjugate 1:20 by making up to 10ml in Conjugate Diluent (see 9.3) to give a final conjugate dilution of 1:100. Filter through a 0.45µm filter before use.

5.3.3 Place slides in a slide tray. Add conjugate over each square of fixed cells, then spread over staining area with an up-turned yellow tip. Add additional stain if necessary, but be careful not to over fill slide, as stain will drain from the edge. Approximately 1ml of stain is used per slide.

5.3.4 Incubate slide tray at 35 C in 5% CO_2 for 30 minutes.

5.3.5 Tip the conjugate from slides into the bread box. Then rinse slides by 5 dunks in PBS. As the slides tend to float from a slide rack, this is more conveniently done by rinsing slides individually.

5.3.6 Air dry slides, add 2 drops of mounting fluid (see 9.4) to slide and cover with cover slip. Gently press out excess fluid on to tissue.

5.3.7 Add 2 drops of glycerol immersion fluid on to the cover slip before reading.

5.4 Clean up

5.4.1 Procedures outlined in AAHL Microbiological Safety And Security Standard Operational Procedures should be followed. In brief:

- Tip all PBS wash buffer into bread box with Pyroneg.
- Use wash bottle to add Aidal to PBS Coplin jar and leave 10 – 15 minutes in cabinet.
- When changing acetone from – 20°C Coplin jar, in the cabinet, tip acetone into a pipette canister containing Aidal, disinfect the outside of the canister with Aidal and leave at room temperature for 24 hours before flushing down the sink with copious amounts of water. Decontaminate Coplin jar as above.
- Instruments, pipette tips and other disposables are discarded into the bread box containing water and Pyroneg. This is decontaminated with Aidal before removal of the closed box from the cabinet. The box is transferred through to the animal services autoclave with the lid in place but unclamped immediately prior to sterilisation.
- Non-autoclavable items are disinfected with Aidal before removal from the cabinet.
- Wipe down cabinet with Aidal and turn on UV light. Always ensure cover is in place when UV lamp is on.

6. RESULTS.

6.1 Reading

6.1.1 Turn on UV microscope 10 minutes prior to reading slides to allow lamp to warm up.

6.1.2 Examine all slides under 20x glycerol immersion lens by epifluoresence.

6.1.3 Observe 20 low-power microscopic fields in each chamber well and count the number of fields which contain fluorescing cells.

6.1.4 Record results on a RFFIT records and results sheet.

6.1.5 Note any wells showing poor cell coverage eg to toxic effects of serum.

6.2 Acceptance of Assay

6.2.1 Read the control slides first.

6.2.2 The cell controls should have no fields with fluorescing cells.

6.2.3 The 50 FFD_{50}/0.1ml wells should have 18 - 20 positive fields, the 5 FFD_{50}/0.1ml wells should have 10 - 20 positive fields and the 0.5 FFD_{50}/0.1ml should have less than 10 positive fields.
(The back titration estimation of the FFD_{50} should show the test as containing between 30 to 90 FFD_{50}).

6.2.4 The neutralisation end-point titre of the International Standard serum is estimated by counting the number of fluorescing foci at each dilution and then using these figures in the Reed and Meunch formula to calculate a 50% end-point, being the predicted dilution of this standard which will result in 10 fluorescing fields.
For the OIE standard, this 50 % end point dilution should be within 6.5 international units of the running mean maintained for that standard in the RFFIT sheet of O:\DIAG\iso9000\Progressive Test QC Records 3L3114.xls.

6.2.5 If any these conditions have not been met, report problem for acceptance to the laboratory supervisor who will decide if all or part of test must be repeated.

6.2.6 Consecutive failure of the test to pass control standards, or indications that results will be unduly delayed by any other cause must be promptly reported to the Technical Manager.

6.3 Interpretation of results and use of spread sheet

6.3.1 The test serum results are expressed in International Units per ml by the ratio of test serum with the International Standard Serum times 0.5 to convert the result to IU/ml, i.e.

$$Test \quad Serum\,(IU/ml = \left[\frac{test\ serum\ 50\%\ titre}{Int.std\ serum\ 50\%\ titre}\right] X\ 0.5$$

6.3.2 A spread sheet has been set up to facilitate calculation of results: O:\DIAG\VIROL\rffit\RFFIT.XLS.

6.3.3 To use the sheet, first load Excel and locate the file on the protocol drive. Then use the sheet to calculate the titre of the 0.5IU/ml standard serum by entering results from the IU slide by entering the well counts into C8 to D11. The test serum initial dilution and dilution scale are normally 20 and 10 respectively. Then enter the fluorescent focus counts into G6 and G7. The titre is given in F10.

6.3.4 Record control information as follows in O:\DIAG\VIROL\Test Monitoring\3L3114\RFFIT progress.xls: Test Date, Operator, Virus input, IS titre and mean counts at each dilution of the IS serum. Run Macro 1 to copy and save data to the O:\DIAG\iso9000\Progressive Test QC Records 3L3114.xls. sheet.

6.3.5 Calculate results for individual sera by entering the counts at both dilution levels. If serum dilution levels other than 1:20 and 1:200 have been used, the serum dilution boxes on the sheet should also be altered.

6.3.6 Levels of neutralisation of greater than 0.5 IU/ml are considered significant.

6.3.7 Animals held in quarantine should have been vaccinated prior to importation into Australia, and should also have previously tested antibody positive. Testing in Australia is for confirmation or otherwise of this antibody status.
Samples for which testing indicates antibody levels of less than 0.5 IU/ml should be re-assessed to ensure the validity of the initial result.

6.4 Reporting

6.4.1 Results are recorded on the "RFFIT Record and Results Sheet" and entered into the Sample Manager LIMS in accordance with the Operating Manual instructions for that package.

6.4.2 The draft report should be printed, examined for errors, initialled and dated before the test is authorised.

6.4.3 ***Results entered into Sample Manager must be authorised by the laboratory supervisor or authorised delegate.***

6.4.4 Sample Manager draft reports are filed with the SAN/paperwork in the appropriate sequence folder, located in laboratory 3L3 114 or in the Records Archiving Compactus..

6.4.5 Test Cover Sheet and RFFIT Record and Results Sheet are filed in the RFFIT folder in the lab 114 office, or in the Records Archiving Compactus.

7. SPECIMEN RETENTION

7.1 Disease Investigation

7.1.1 Retain all sera at –20 °C and review after 6 months.

7.2 Survey Sera

7.2.1 Retain all sera at +4 °C or –20 °C. Survey sera of interest to more than one laboratory should not be discarded without general agreement form supervisors.

7.3 Animal certification

7.3.1 Retain all sera at –20 °C and review after 2 months.

7.4 Disposal and Record of Samples.

7.4.1 Samples should be disposed of in accordance with Section 5 of the AAHL Microbiological Security Manual.

7.4.2 Records will be maintained of sample disposal by marking the RFFIT test sheet which records testing of the samples for disposal.

8. QUALITY ASSURANCE

8.1 Progressive Monitoring

On-going assay performance is monitored using a running sheet located in the folder "O:\Diag\Virol\Test Monitoring\3L3114\RFFIT progress.xls. This sheet must be updated after completion of each test with standard serum titre and virus input details. The sheet must be completed before authorising of results.

8.2 Control Charting.

Graphical methods are used to assess trends. A spreadsheet of the test performance is maintained in the RFFIT graph sheet of O:\DIAG\iso9000\Progressive Test QC Records 3L3114.xls

8.3 External Proficiency Testing

AAHL participates in external proficiency testing of the RFFIT through the UK Pet Travel Scheme. Ross Lunt coordinates participation in the program. Procedures for external proficiency testing are in Quality Manual section 6-2-2.

9. APPENDICES

9.1 BME with 5% TPB

BME medium	400ml
TPB	20ml
Hepes	4.0ml
Glutamine	4.0ml
Pen/strep	1.0ml
Fungazone	2.0ml

Prepare using sterile technique.
Label bottle with date and additions
Store at +4°C.

9.2 BHK Cell Growth Medium

BME with 5% TPB (see 9.1)
Heat inactivated foetal calf serum to 10%

9.3 Conjugate Diluent (PBS/BSA with 0.005% Evans blue)

PBS A	90ml
PBS + 10% BSA	10ml
0.5% Evans Blue in DD H_20 (see 9.9)	1ml

Store at + 4 °C.

9.4 Buffered Tris Glycerol Mounting Medium pH 9.0

a) 10X Stock Tris Saline (0.5M Tris, 1.5M NaCl) pH 9.0

Sigma Trizma® Pre-set crystals pH 9.0 cat# T1444, 1 pack	Contents of 1 pack
NaCl	17.56g

Dissolve and make up to 200 mL with sterile Tissue Culture water
Store at room temperature for up to 1 year.

b) Working Stock Mountant (0.05 M Tris-buffered saline pH 9.0 with 20% glycerol

	Parts	For 50mL
Sterile Tissue Culture Water	7 parts	35mL
10X Stock Tris Saline	1 part	5mL
Glycerol (AnalaR 10118.4K, AAHL 002571)	2 parts	10mL

Store at room temperature for up to 2 months.

Glycerol should be replaced at yearly intervals because the pH changes slowly with time.

9.5 WHO International Standard Serum

The use of the WHO standard as the operating standard in the RFFIT at AAHL has been discontinued and replaced with the OIE Standard (from May 2001). However the WHO standard remains a valid and recognised standard reference. Stocks are held at AAHL and additional standard can be obtained as indicated below.
The International Standard for rabies immunoglobulin (2nd international standard preparation) was obtained from:

NIBSC
(National institute for Biological standards and Control)
PO Box 1193,
Blanche Lane,
South Mimms, Potters Bar,
Herts, EN6 3QH, UK.

Email: standards@nibsc.ac.uk; web site: http://www.nibsc.ac.uk/

Product Code: RAI.

9.6 OIE International Standard

The OIE Standard serum is available from:

Dr Florence Cliquet,
AFSSA Nancy
Domaine de Pixerecourt
B.P. 9,
F-54220 Malzeville
France

Fax 33 3 8329 8959

Reconstitute standard sera in sterile ddH_2O. Dilute to the required IU level (2.0 IU/mL for the WHO standard and 0.5 IU/mL for the OIE standard) in PBS A + 1%BSA. Dispense 130µl of diluted standard in labelled tubes and store at –80 in GAF0273.

9.7 Normal Cat Serum

Normal cat serum pool ex SPF cat from tissue culture. Serum harvested non aseptically on 19/02/02 for use as a negative control for RFFIT. 4mL vols. (Two pools available; see also 0202-19-1710)

9.8 Viruses

9.8.1 Passage history of viruses

a) CVS rabies virus

870430 0003	original
29/5/89	mouse
9006291000	BHK1
20/11/90	BHK2
14/12/90	BHK3
9101211430	BHK4
0112031701	BHK5

9.8.2 CVS Rabies

The CVS rabies virus was obtained in 1986 from the Commonwealth Serum Laboratories (Melbourne, Australia) and has been determined to be antigenically indistinguishable from CVS-31 (Dr J. Smith, personal communication). The designation of this virus as CVS-11 in the previous protocol does not appear to have any recorded basis. The virus was passaged once in mice and five times in BHK cells at AAHL.

Stocks of the virus 9101281500 are held at – 80 °C in the rabies lab in ¼ oz containers. This material is dispensed into Sarstedt tubes in 50µl volumes which forms the working virus stock. These are held in a box at – 80 °C labelled RFFIT antigen.

9.8.3 Other viruses

Mokola (9206-17-1600), Lagos bat (9206-17-1610), Duvanhage (9206-17-1620) and Duvenhage RA (9206-17-1630) viruses, were obtained from Dr J. Smith (CDC Atlanta) and passaged 3 times in BHK cells.

9.8.4 Titration of virus for use in RFFIT

The RFFIT requires a virus input of between 25 and 100 FFD50/0.1ml, with a target of 50 FFD50/0.1ml.

To establish virus titre

- perform serial10 fold virus dilutions
- add 0.1ml in duplicate to wells of chamber slides
- add 0.1ml of BME + 5% TPB
- add 0.2ml of cells as described in 5.1.20
- fix, stain and read as described in 5.2
- the dilution for 1 FFD50/0.1ml is that which results in 10 positive fields in twenty counted. Obtain a mean positive count at the virus dilutions above and below the target (10) and calculate the exact titre by the method of Reed and Muench:

where C1 = mean FF counts at the virus dilution immediately below the target (i.e. < 10 FF)
D1 = $\log_{10}$ virus dilution for C1, eg -6

C2 = mean FF counts at the virus dilution immediately above the target (i.e. > 10 FF) eg. -5
D2 = $\log_{10}$ virus dilution for C2
P1= Proportion below target = 100*(C1/20)
P2 = Proportion above target = 100*(C2/20)

$$Virus\,Titre\ (\log_{10} FFD/0.1ml) = \left[\left(\frac{P2-50}{P2-P1}\right) X\left[D2-D1\right]\right] - D2$$

9.9 0.5% Evans Blue in DD PBS A

Evans Blue 0589-60
Obtained from Koch-Light Laboratories Ltd. England.
0.1g is added to 20 ml PBS A.
Mix and allow overnight to dissolve.
Filter through 0.45µm filter.
Store at 4°C for up to 3 years.

9.10 Centocor Anti-Rabies Monoclonal FITC Conjugate (1:5 Dilution)

9.10.1 The Centocor Anti-Rabies Monoclonal FITC Conjugate is obtained from:

CENTOCOR INC.,
244 Great Valley Parkway
Malvern, PA 19355
USA.

9.10.2 A copy of the import permit can be obtained from Records.

9.10.3 The import permit, together with the completed purchase requisition are taken to Purchasing from which the order will be placed. Generally, more than one kit is ordered.

9.10.4 The Centocor Anti-Rabies Monoclonal FITC Conjugate is received in freeze-dried form. Reconstitute this to 5ml in sterile distilled water according to kit insert instructions.

9.10.5 Previously untested batch lots should be titrated for potency (see 9.11)
9.10.6 Dilute the 5ml of reconstituted conjugate to 25ml in Conjugate Diluent.

9.10.7 Dispense the 1:5 diluted conjugate in 0.5ml volumes into 1.8ml tubes. Label these CENTOCOR anti rabies FITC conjugate. Diluted 1:5. Batch Number. Date.

9.10.8 Store this at –80°C in the rabies room in a box marked Conjugate.

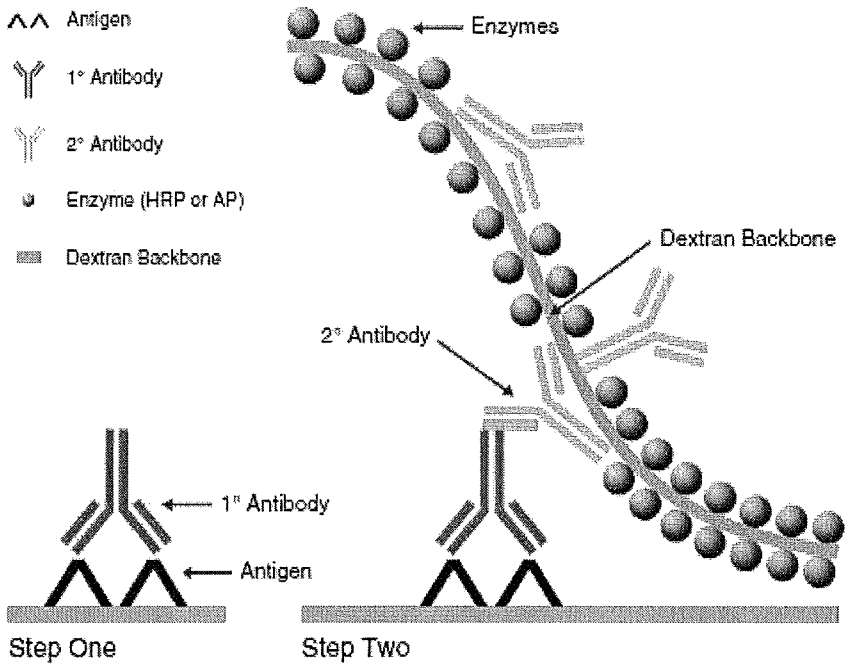
END

Immunohistochemistry.

Purpose
Visually identify the presence of antigen within a tissue. IHC is of benefit when you wish to identify the type of cell that the antigen is associated with or when only fixed tissue is received.

Basic principles.
Immunohistochemistry detects the presence of antigen by binding labelled antibodies to the antigen in question and then detecting the presence of the label. The labels can be detected through fluorescence, radioactivity or through the use of chromogens. We use chromogens in our routine work. Layers of antibodies can be utilised to improve the specificity of the test. For example rather than just detecting whether the primary antibody has bound to antigen, a labelled secondary antibody to the primary antibody can be added and this can be detected.

1. Primary antibody against the antigen to be detected is applied to the section using a predetermined dilution. The antibody will bind to the antigen. This is illustrated in step one of the picture below. Rabbits and mice are most often the host species for these antibodies.
2. The section is washed well to remove unbound antibody.
3. A labelled secondary antibody is applied to the section and will bind to the primary antibody if present. The secondary antibody should be conjugated with a label. The label that we use is horse radish peroxidise (HRP). The secondary antibody is bound to a dextran backbone that has multiple HRP sites. This improves the sensitivity of the test. This can be seen in step two of the picture below. The secondary antibody must also be against the host of the primary antibody. For example, if we are detecting Australian Bat Lyssavirus and the antibody was produced in a mouse, we must use an anti-mouse secondary antibody. Ideally neither of the host species for the antibodies used should be the same as the species of the animal that we are testing as this can produce background staining.
4. The section is again washed to remove unbound secondary antibody.
5. The chromogen is added to the section. We use AEC which in the presence of the HRP will react to form a red colour which can then be visualised microscopically.



Two-Step Polymer Method (EnVision™).

Picture taken from; DAKO. Education Guide: Immunochemical Staining Methods. 5th Edition

The system that we use at AAHL is Dako Envision FLEX.

Treatments prior to performing immunohistochemistry.

Dewaxing.
Sections used for immunohistochemistry are cut from paraffin blocks. Before immunohistochemistry is performed, the sections must be taken through a series of X3B (a xylene derivative) and ethanols to water in order to remove the wax.

Antigen retrieval – Demasking of antigen sites.
While formalin is the fixative of choice for the preservation of tissue morphology, the method in which it stabilises the tissue also hinders immunohistochemistry. Formalin works by forming cross-links between proteins within the tissue. These cross-links that are formed are often referred to as formaldehyde bonds. Formaldehyde bonds can mask the antigen sites and prevent the antibody from reaching them. In order to successfully perform immunohistochemistry on these tissues by allowing the antibody to reach the antigen, these cross-links need to be removed. This process, known as antigen retrieval, can be performed through variety of methods;

- Microwave antigen retrieval
- Pressure cooker antigen retrieval
- Heat induced antigen retrieval e.g. waterbath.
- Proteolytic enzyme digestion e.g. proteinase K.

Each method has advantages and disadvantages. The two methods that we commonly use at AAHL are proteolytic enzyme digestion through the use of proteinase K and heat induced retrieval using the PT link.
Proteinase K- used at a concentration of 5µg/mL, proteinase K is thought to somehow break down the formaldehyde bond masking the antigen allowing the antibody to reach the antigen.
Dako PT link- is a unit which heats a commercial buffered retrieval solution for a set period of time. The unit has the ability to be configured to different temperatures, times and has a timer function. Our PT link is set to heat to 67°C prior to placing the slides in the bath and then will heat to 97°C for 30 minutes (just below boiling is believed to be an ideal temperature for antigen retrieval) before cooling down. The retrieval solution used in the PT link also has the capability of dewaxing the slides.

Blocking of endogenous peroxidises.
Peroxidases can be found naturally in normal and neoplastic tissues e.g. erythrocytes and leucocytes. These peroxidises can react with the chromogen to produce colour which may be confused with detection of the antigen. In order to prevent this from occurring the activity of these peroxidises may be blocked. This can be done through the application of hydrogen peroxide for 5 minutes prior to the addition of the primary antibody.

References.

- Bancroft J. and Gamble M. Theory and Practice of Histological Techniques. 5th Edition. Churchill Livingstone. 2002.
- Dako. *Education Guide: Demasking of Antigens.* 2nd Edition. http://www.dako.com/au/00092_demasking_antigens_row.pdf
- Dako. Education Guide: Immunohistochemical Staining Methods. http://www.dako.com/au/08002_ihc_staining_methods_5ed.pdf

Reagents Used.

Blocking of endogenous peroxidises: 3% H_2O_2

Antigen retrieval:	Proteinase K Supplied by MERCK Used at 5µg/mL
	Dako EnVision FLEX Target Retrieval Solution High pH Supplied by Dako Used in Dako PT link
Wash Buffer:	Dako EnVision FLEX Wash Buffer. Supplied by Dako.
	Or
	TRIS Buffered Saline pH 7.6 In-house solution. See recipe on following page.
Primary Antibody:	HAM MAb. 5DF12-3D6-3. S35PFHM-11. 98.9.16 Produced in-house.
Antibody diluent:	Dako EnVision FLEX Antibody Diluent Supplied by Dako
	Or
	Skim milk diluted in TRIS buffered Saline (0.01g/mL)
Secondary Antibody:	Dako EnVision FLEX/HRP (dual link) Suppled by Dako.
AEC:	Dako AEC+ High Sensitivity Substrate Chromogen Ready to use. Supplied by Dako.
Mounting media:	Dako Faramount Aqueous Mounting Medium. Ready to use. Supplied by Dako.
Haematoxylin:	Lillie-Mayer Haematoxylin Supplied by Australian Biostain PTY LTD.
	Or
	Lillie-Mayer Haematoxylin (Mod) In-house solution. See recipe on following page.
Scott's Tap water:	In-house solution. See recipe on following page.

Tris-buffered saline

10X Tris-buffered Saline Stock Solution
Ingredients:

Trizma Base (Sigma, T-1503)	13.9g
Trizma HCl (Sigma, T-3253)	60.6g
Sodium Chloride	87.66g
Tween 20	5.0mL
1N Hydrochloric Acid	20mL
Distilled water	1.0L

Tris-buffered Saline Working Solution (In-house)

10X Tris-buffered Saline Stock Solution	1.0L
Distilled water	9.0L

Once the working solution has been made, ensure that the pH is 7.6.

Mayer's Haematoxylin (Lillies modification)

Reagents

Haematoxylin (CI no. 75290)	5.0gm
Ammonium Alum	50.0gm
Glycerol	300.0mL
Water	700.0mL
Sodium iodate (chemical ripening agent)*	0.2-0.4gm
Acetic acid	20.0ml

Procedure
Dissolve haematoxylin in half the water with gentle heat.
Dissolve ammonium alum (or potassium alum) in remaining water with heat if necessary.
Cool both solutions to 25°C or room temperature, in running tap water.
In a 2 litre conical flask slowly add the ammonium alum to the haematoxylin while stirring. The stain should be a rich violet-red colour.
Add the sodium iodate.
Add the glycerol and acetic acid.

Note 1: The stain is ready to use immediately. The stated life of the stain may be months to years.
Note 2: Lillie recommends with iodate ripening try 40mg for each gram of haematoxylin used and test the batch for staining, allowing graded intervals of 2, 5, 10, 20, 60 min. If satisfactory staining is attained only with longest interval, add another 10mg $NaIO_3$/gm haematoxylin and test again. In this way over oxidation can be avoided.

Scotts Tap Water

Reagents

$NaHCO_3$	7.0gm
$MgSo_4$	40.0gm
Distilled water	2.0 Litres

Rabies Serology at AAHL – Assessment of the Fluorescent Antibody Virus Neutralisation (FAVN)

Antonio Di Rubbo
The Diagnostic Services Response Group (DSR)
The Australian Animal Health Laboratory (AAHL)

Presentation Overview

Principles of Serum Neutralisation Tests

Determination of Titres and International Units per mL Using the FAVN Test

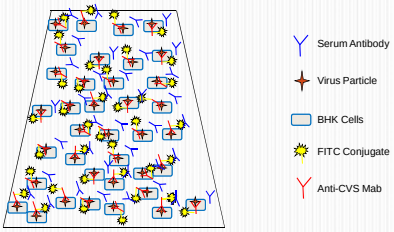
Determination of Titres and International Units per mL Using the RFFT Test

The FAVN and the RFFT Give Equivalent Results

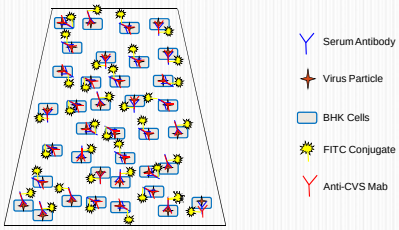
FAVN Versus RFFT: Determining the Choice

Conclusions

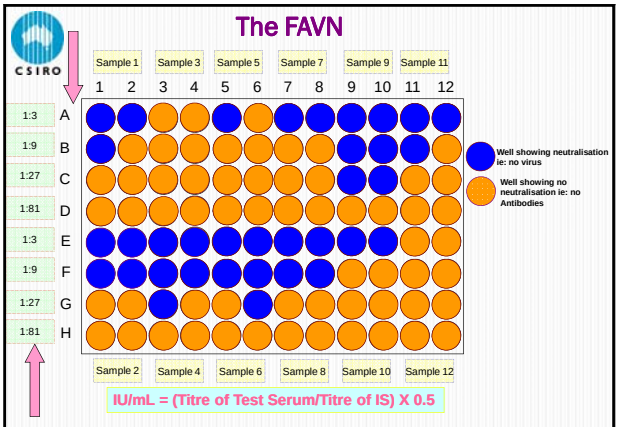
Principles of VNTs



Principles of VNTs



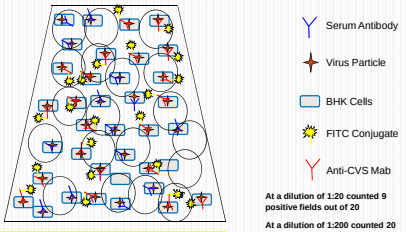
The FAVN



IU/mL = (Titre of Test Serum/Titre of IS) X 0.5

The RFFIT

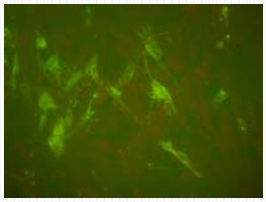
IU/mL = (Titre of Test Serum/Titre of IS) X 0.5



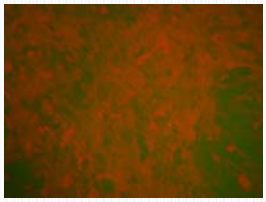
The Titre of a Serum is Given by the Predicted Dilution that Will Result in 10 Fluorescing Fields

Typical Antibody Negative and Positive Samples

Fluorescing Virus Particles: No Antibodies Present in Serum



No Fluorescing Virus Particles: Neutralising Antibodies Present

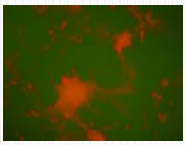


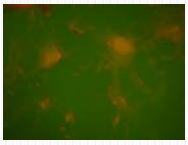
Advantages of the FAVN Versus RFFIT

Costs

Reduced Cell Toxicity Caused by Sera

Examples of Serum Toxicity to the Cells





Advantages of the FAVN Versus RFFIT

Costs

Reduced Cell Toxicity Caused by Sera

Rapidity and Ease of Execution

High Reproducibility

A Comparison of Two Serological Methods for Detecting the Immune Response after Rabies Vaccination in Dogs and Cats being Exported to Rabies-free Areas

Deborah J. Briggs,* Jean S. Smith,† Francis ⌊ Mueller, James Schwenke,‡ Robin D. Davis,* Chandra R. Gordon,* Kristen Schweitzer,* Lillian A. Orciari,† Pamela A. Yager* and Charles E. Rupprecht†

animal sera. Inconsistent results had been obtained for a panel of animal sera tested by the RFFIT in different serology laboratories while more reproducible results were later obtained for a survey of laboratories using the FAVN.[5] No direct comparison of the reproducibility of results obtained by a single laboratory performing both methods was conducted and the source of the inconsistency was not identified. With the development of the FAVN and the recommendation that this test be used for all tests of animals transported internationally, a direct comparative analysis of the two tests is needed. Our study was designed to evaluate the FAVN and RFFIT and to determine if the new methodology has a selective advantage over the RFFIT for titration of rabies antibody in vaccinated dogs and cats.

Briggs *et al,* 1998.

Results Between RFFIT and FAVN are Comparable

	RFFIT				FAVN			
Serum #	Run 1	Run 2	Run 3	Run 4	Run 1	Run 2	Run 3	Run 4
1	1.5	1.32	1.41		1.10	1.60	1.60	1.60
2	<0.5				1.60	0.53	0.53	0.53
3	1.61	1.5			1.60	1.60	1.60	1.60
4	0.86	1.5			1.10	0.77	0.53	*
5	1.51	1.5			1.11	2.30	1.60	1.60
6	<0.5	<0.5			0.18	0.37	0.53	0.31
7	1.61	1.5			1.11	1.60	1.60	1.60
8	1.83	1.61	1.5		1.60	1.60	1.60	1.60
9	<0.5	<0.5	0.5	0.69	0.26	0.53	0.53	0.53
10	1.5	1.5			1.60	1.10	1.60	1.60
11	1.61	1.5	1.43	1.61	0.63			
12	0.5	1.32	0.88		0.36			
13	1.31	1.5	1.17		1.08			
14	0.51	1.41	1.17		1.88	1.08		
15	0.5	1.5	1.65		1.88			
16	0.5	1.22	1.01		1.08			
17	1.2	1.5	1.55		1.08	1.08		
18	1.41	1.32	1.55		1.08			
19	1.51	1.7	1.65		1.08			
20	1.61	1.5	1.65		1.88			

Titres are expressed in IU/mL

Advantages of the RFFIT

A 24 Hour Test Means Results are Available Earlier

Less Serum Needed for the Test

Less Reagents Used for the Wash (PBS ABC)

Conclusions

Both the RFFIT and FAVN are Effective Tools for Rabies Serology

The FAVN was Preferred Method

- Cheaper to Run
- Less Tedious
- Less Toxicity
- More Reproducible

Acknowledgments

Darren Schafer Running Most of the RFFIT

Ross Lunt: Laboratory Supervisor

DERL: K. Newberry; A. Bagnara; J. Cooke; C. Duch; S. Juzva; M. Hosking; L. McNabb

FLUORESCENT ANTIBODY VIRUS NEUTRALISATION TEST (FAVN)

RABIES VIRUS (SEROTYPE 1 LYSSAVIRUS)

AUSTRALIAN ANIMAL HEALTH LABORATORY

DIAGNOSIS, SURVEILLANCE AND RESPONSE PROJECT

Rabies FAVN

NATA Accreditation Class of Test 20.25.09

1. INTRODUCTION

1.1 DISEASE

Rabies is an infectious zoonotic disease characterised by encephalitis that may be associated with paralysis, behavioural changes and other neurological manifestations prior to an ultimately fatal outcome.

1.2 ASSAY

The Fluorescent Antibody Virus Neutralisation Test (FAVN) is an *in vitro* assay for neutralising antibody to rabies virus. The assay is set up in 96-well microtitre plates, and is read 48 hours after set-up following immunofluorescent staining of cells for virus growth.

Origin of the method
The method for this protocol is derived from the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2010) Chapter 2.1.13 part 2.a) "Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (a prescribed test for international trade)"

Allowed Deviations from Standard
a) Test and control serum dilutions
Required by standard: Test sera and control sera are assessed in quadruplicate from 1/3 using a three-fold dilution scale.
Change in this protocol: Test sera are assessed in duplicate at dilution levels from 1/3 using a three-fold dilution scale. OIE standard is tested in quadruplicate from 1/3 using a three-fold dilution scale.
Justification: The OIE Standard method uses replicates to improve the estimate of the titre. As the precision of measurement is critical at the threshold level and as the large majority of test sera are well beyond the threshold (greater than 2 IU/mL), the degree of precision is unnecessary for most measurements. However all sera with an initial titre assessment of less than 1.2 will be retested, in quadruplicate to confirm the initial estimate.
b) Fixative
Required by standard: Fixation in 80% acetone.
Change in this protocol: Fixation using formalin-NP-40.
Justification: Formalin-NP40 produces equivalent quality of staining, but is preferable for qualities of virus inactivation and ease of disposal.

Purpose of assay
The assay is used for:
- Provision of supporting laboratory evidence for vaccination immunity required in the certification process for the international movement of dogs and cats
- Investigations of suspect acute rabies infection in humans and other animals by testing serum or Cerebrospinal fluid (CSF)
- Verifying or monitoring for adequate post vaccination immune serum antibody levels in humans
- The FAVN can be used to detect antibody to lyssaviruses other than serotype 1 (rabies virus). In general, these tests should use virus homologous to the serotype/genotype under investigation. Antibody against Australian Bat Lyssavirus can cross-react in the rabies FAVN, however antigenic differences between the viruses may affect assay sensitivity of detection and interpretation of results.

Validation and Verification
This is a standard method for which full validation is not required.
The assay is verified by:
- Use of an external reference standard and conversion of test serum titres to a ratio of this standard

- A verification file comparing results with an alternative method (Rabies RFFIT)
- Use and monitoring of control sera
- Participation in external proficiency testing

Restrictions
Lyssaviruses are classified for work at physical containment (PC) level 3.
There are training and vaccination requirements.
There are restrictions on the location of work.

1.3 RESULTS

Results are expressed as an international unit equivalence level relative to the Office International des Epizooties (OIE) standard serum or equivalent reference serum.

1.4 REFERENCES

Smith, JS Yager, PA and Baer, M (1973) A rapid reproducible test for determining rabies neutralizing antibody. Bull. Wld. Hlth. Org., 48, 535-541.

OIE Manual of Diagnostic Tests Chapter 2.01.13_RABIES.pdf

2. EQUIPMENT

All calibrated equipment and temperature controlled devices used in the setup and performance of the assay on the must be listed in the O:\GENERAL\iso9000\Scientific\EQUIPMENT_LIST.XLS spreadsheet. Use of the equipment must be in compliance with Suite Co-ordinator and Microbiological Security requirements.
The room or rooms in which the equipment is housed as designated on the equipment list must be identified.

2.1 Calibrated and Other Listed Equipment

2.1.1 Single channel micropipettes - calibrated for 25 and 50 µl volumes
2.1.2 Multi-channel micropipettes - calibrated for 25 and 50 µl volumes
2.1.3 Biosafety cabinet, class II
2.1.4 Incubator 37°C ± 2°C
2.1.5 Water bath set to 56 °C
2.1.6 Refrigerator +4°C ± 2°C storage, fridge or cool room
2.1.7 Bench top centrifuge
2.1.8 Freezer at -80°C ±10°C
2.1.9 Cool Room +4ºC (medium storage, test sample storage)
2.1.10 Cold Room –20°C for sample storage
2.1.11 Consumables and other items
2.1.12 Any suitable, round-bottom, 96 well microtitre plate

2.2 Consumables and other items

2.2.1 Plate covers
2.2.2 Tips, as required for above pipettes
2.2.3 Tip discard e.g. Burnbin
2.2.4 Reservoirs, any brand, sterile
2.2.5 50ml centrifuge tubes, e.g. Falcon brand
2.2.6 Disposable plasticware, 6ml sterile bottle
2.2.7 Glassware, sterile: pipettes, range of sizes, bottles
2.2.8 Autoclave bags
2.2.9 Pipetting aid

3. REAGENTS

3.1 Chemicals

3.1.1 Pyroneg detergent – Store item 2786 (for use in bread box.)
3.1.2 Aidal Plus (21g/L glutaraldehyde) for general decontamination. Store Item 2783.
3.1.3 40% w/v Formaldehyde Merk Cat. 4.10012.2500
3.1.4 Nonidet P40 BDH Prod 56009

3.2 Biologicals

3.2.1 Control sera
Normal Human Serum - 8706-18-0010 .
Normal Cat serum (DERL –20°C) see 9.7
Weak Positive Human Serum - 8706-18-0011
OIE reference serum, -80°C (diluted to 0.5 IU/mL) see 9.6

3.2.2 Virus (see 9.8)
Rabies CVS 11 Virus - GAF0273 Forma –80°C as 75µl per tube working stock. See "O:\DIAG\iso9000\Progressive Test QC Records DERL.xls" for Pool Reference Number and dilution for use.

3.2.3 Culture medium
Basal Medium Eagle eg. GIBCO cat # 21010-046
BME + 5% TPB (see 9.1)
BHK cell growth medium (see 9.2)

3.2.4 Cells
BHK21 BTV Vaccine Strain Cells: suspension at 0.4 X 10^6 per ml. Use a flask of healthy BHK (vaccine) cells which were passed two days earlier or cells revived from Liquid Nitrogen.

3.2.5 Store items
PBS A
PBS ABC
PBSA + 10% BSA
Sterile TC water

3.2.6 For cell staining
0.5% Evans Blue in DD H_20 (see 9.9)
Centocor Anti-Rabies Monoclonal FITC Conjugate (see 9.10).
Conjugate diluent (see 9.3)

4. PREPARATION

4.1 Preparation of Samples

4.1.1 **Receipt.** Samples received by accession staff may include human, cat, dog, bat and occasionally other animal sera. Cerebrospinal fluid may also be received in the investigation of acute human rabies. On delivery to the laboratory, samples are matched with the corresponding Job number from the Specimen Advice Notice (SAN) or other paperwork by reference to sample number/bar code stickers supplied in Sample Accessions and accompanying sample container(s). **The sample paperwork is examined to identify the nature and required testing for the submitted sample. Note: all paperwork accompanying samples may be viewed from Sample Manager using "View Graphic File" in Explorer for that Job.**

Sealed tins containing samples <u>for disease investigation</u> should be opened in a biological safety cabinet. The secondary containers (plastic bags, tins, etc) of serum samples for FAVN may be opened with care on the laboratory bench. Examine the condition of the sample and record on the SAN/paperwork any sample leakage, poor quality or condition that may indicate a discrepancy between the received sample and the material identified in the accompanying paperwork. Report any discrepancies to the laboratory supervisor.

Containers of serum removed from package in the rabies room (see 4.1.3) may be externally decontaminated according to AAHL Microbiological Safety and Security Standard Operational Procedures then brought to DERL for heat inactivation.

4.1.2 **Acceptance/Rejection Criteria.** Samples should not be tested without recourse to the Laboratory Supervisor if there is cause to believe that the sample quality or identification is deficient or compromised, for example, serum grossly contaminated by haemolysis. Sample acceptance will be based on complete confidence that the submitted material is correctly identified, is serum or CSF and remains fit for testing having accounted for transport and storage conditions. Whole bloods should not be submitted, however these may be tested after separation of serum, providing there is not excess haemolysis. Any concerns regarding the quality of the sample, including the receipt of whole blood must be noted on the sample paperwork and as a qualifying remark added to the Analysis Comment section of the Sample Manager Report.

4.1.3 **Numbering and storage.** The identification on each sample should be compared with the paperwork. Identifying numbers on the paperwork that correspond with those on the sample should be marked with highlighter pen on the paperwork. Other unique identifying numbers etc may be handwritten on to the front of the SAN/paperwork. Label each sample with the correct sample number/bar code sticker for that Job. If sample numbers have not been assigned by Sample Accessions to each sample in the Job, the sample number is assigned and written according to the order of listing on the original paperwork to indicate correspondence of samples with numbering. Samples arriving as serum should be stored at +4^{o}C prior to testing.

4.1.4 To separate clotted bloods by centrifugation, spin vials at 2000k (approx) for about 15 minutes. If the sample is not in a separator tube, transfer serum to a new tube labelled the same as the original sample container. In this case, retain original tube at least until issue of the test report.

4.1.5 All serum samples should be heat inactivated at 56°C for 30 minutes, prior to testing. **Note: indicate that a sample has been heat inactivated by drawing a single red line across the cap.**

4.2 Preparation of Staff

4.2.1 Staff working with rabies, rabies related viruses and other lyssaviruses must be familiar with the relevant sections of the Microbiological Security and Safety Manual, in particular, “Standard Operating Practices: Biosecurity Level 3(Z) Rabies” (Section 5.18 Special Conditions for Rabies, Rabies-like viruses and Australian Bat Lyssavirus).

4.2.2 Staff working with live virus must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

4.2.3 Staff operators who undertake the determination of antibody in human serum are recommended to have been vaccinated against hepatitis B virus.

5. PERFORMANCE OF THE TEST

Note: a summary version of this protocol can be found at the end of this document (9.13 Summary of FAVN Method).

5.1 Virus Neutralisation Stage

5.1.1 Open Sample Manager and print the worksheet listing of samples to be tested. Check that this corresponds to the available samples.

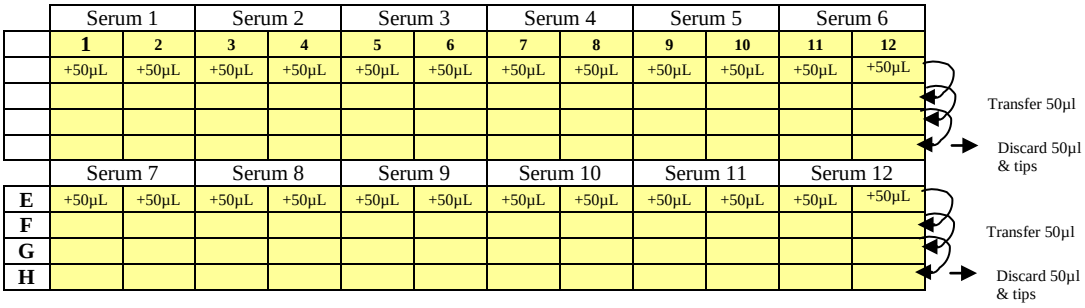
5.1.2 Serum is inactivated in DERL by heating samples at 56°C for 30 minutes in water bath. Do not heat inactivate CSF.

5.1.3 Commence VNT Test Cover Sheet (see forms folder O:\AAHL ISO Quality Assurance (QA) Systems\ISO 17025 QA Doc Control\DERL and list sera (normally twelve sera per plate) on Sample Record and Results Sheet. The titration of the international standard and virus back titration requires an additional plate.

5.1.4 Label plates with a sequence number identified on the worksheet and the date.

5.1.5 Preparing test sera dilutions (in duplicate threefold at 1/3, 1/9, 1/27 and 1/81):

- Add 100µl of BME with 5% TPB (see 9.1) to a block of 8 wells for each serum.
- Add 50µl of serum to the first two wells of each dilution series to give duplicate wells of a 1/3 serum dilution
- Prepare serial dilutions by transferring 50µl from the 1/3 through the well series. The schematic for serum dilution is shown below



5.1.6 The dilution series for the international standard and the virus addition and back titration are carried out in the rabies laboratory. However medium can be added to the microtitre plate in preparation while in DERL.

5.1.7 Preparing International Standard (IS) dilutions (in quadruplicate-threefold dilutions at 1/3, 1/9, 1/27, 1/81, 1/243, 1/729, 1/2187and 1/6561):

- Add 100µl of BME with 5% TPB into 4 columns of a microtitre plate
- Add 50µl of IS to the first 4 wells, mix and transfer 50µl to the 4 wells below. Continue the titration down to the end of the plate. Discard the last 50µl volumes together with the tips.

Note: Use of a secondary standard to replace the IS is acceptable, provided it has been sufficiently calibrated against the IS and provided there are periodic checks of this calibration.

5.1.8 To prepare the plate for the virus back titration. Add 300µl of BME with 5% TPB to 7 wells of a plate (from B1 down to H1), missing the first well.

5.1.9 AT THIS STAGE WORK IS TRANSFERRED TO THE RABIES LABORATORY. Take the plates and sufficient BME + 5% TPB to dilute virus and do the back titration (5.5ml per plate).

5.1.10 Remove one ampoule of International Standard and one ampoule of CVS rabies virus from –80° C freezer. Allow reagents to thaw in the cabinet.

5.1.11 All work is in the class 2 cabinet unless otherwise stated.

5.1.12 Complete the international standard dilution series as indicated in 5.1.7.

5.1.13 Rabies CVS 11 virus is prepared in BME with 5% TPB so to contain 100 Tissue Culture Infective Doses in 50µL ($TCID_{50}$). Check the Progressive Monitoring Sheet "O:\DIAG\iso9000\Progressive Test QC Records DERL.xls" for dilution for use.

5.1.14 Preparing Back Titration (BT): Titrate in a 10-fold dilution by adding 33.3µl of diluted working stock virus (i.e. $TCID_{50}$) to the first well (B1) of the plate prepared for the BT described in 5.1.8. Pipette up and down and discard the tip. With a clean tip, transfer 33.3µL from B1 to C1. Pipette up and down again, discard the tip and continue the titration down to F1. Discard the tip together with the last 33.3µL. Row H will be the cell control. Transfer 50µL from column 1 to columns 1 to 6. Add 50µL of the working stock virus to the first 6 wells of the plate (i.e. A1 to A6). Add 100µL of BME with 5% TPB to all wells from A1 to H6.

5.1.15 Addition of virus: Add 50µL the working stock virus to all serum containing wells and incubate the plates at 35 °C in 5% CO_2 for 1h.

5.1.16 Prepare a suspension of BHK cells containing 4 x 10^5 cells/mL in growth medium (see 9.2), allowing 5.5mL for each plate. Hold the cell suspension at +4°C until required.

5.1.17 Add 50µl of cells to each well (use the electronic multi-stepper pipette). Take care not to splash virus from wells.

5.1.18 Incubate at 35°C in 5 % CO_2 for 48 hours.

5.2 Cell Fixation Stage

5.2.1 In rabies laboratory, working in the class 2 cabinet: Tip plates content into a bread box containing pyroneg.

CONTAMINATION RISK: Minimise risk of contamination of work area by:
- **operator training the technique**
- **use of double gloves**
- **awareness of decontamination procedures**

5.2.2 Using the electronic multichannelled pipette wash the plates twice with 200µL and 400µL PBS ABC for the first and the second time respectively. During each washing step remove the PBS solution from the plates by tipping it into the bread box as described in step 5.2.1.

5.2.3 Add 400µL of formalin fixing solution to all wells and leave in the cabinet for 1h. Following this period, work may be continued in DERL, but plates must be enclosed in plastic bags and transported in appropriate sealed containers.

5.2.4 Discard the content in **an empty bread box** and wash the plates twice as before. (**Note:** This bread box can be used until the end of the test to discard wash solutions through the various steps and be discarded into the sink at the end of the day).

5.2.5 Tip plates on a paper towel unfolded next to the bread box to dry the plates. Dilute fujiribo anti-rabies monoclonal FITC conjugated antibody 1/20 in the conjugate diluent and add 50µL of conjugate solution to all wells.

5.2.6 Incubate plates at 35°C for 30min 5 % CO_2 incubator. Wash plates like before and add 50µL of PBS to all wells.

5.2.7 Take plates to the microscope room and with the inverted UV microscope score the plates beginning with the Back Titration followed by the International Standards. Score wells as being positive for the presence of fluorescent foci or otherwise score them as negative.

5.3 Clean up

5.3.1 Procedures outlined in AAHL Microbiological Safety and Security Standard Operational Procedures should be followed. In brief:

- Instruments, pipette tips and other disposables are discarded into the bread box containing water and Pyroneg. The outside of the closed box is decontaminated with Aidal before removal from the cabinet. The box is autoclaved with the lid in place but unclamped immediately prior to sterilisation.
- Non-autoclavable items are disinfected with Aidal before removal from the cabinet.
- Wipe down cabinet thoroughly with Aidal.

6. RESULTS

6.1 Reading

6.1.1 Note any wells showing poor cell coverage eg to toxic effects of serum under the light microscope. This step may also be performed before beginning the fixing stage.

6.1.2 Turn on UV microscope 10 minutes prior to reading plates to allow lamp to warm up.

6.1.3 Beginning with the Back Titration and International Standard wells and following with all other wells, examine all wells scrutinising the entire area of the wells for the presence of fluorescent foci. For test sera, begin reading wells at the highest dilution.

6.1.4 Score the presence of any fluorescent focus as a positive well for the presence of virus.

6.1.5 Record results on a FAVN records and results sheet.

6.1.6 Use FAVN.xls located in AAHL ISO Quality Assurance (QA) Systems\ISO 17025 QA Doc Control\DERL to calculate results for virus back titration, control and test sera.

6.2 Acceptance of Assay

6.2.1 The cell controls should have no fields with fluorescing cells.

6.2.2 The back titration has a target input of 2.0 (log_{10}) and should be within the range 1.5 to 2.5.

6.2.3 The naïve serum should show no neutralisation of virus in any wells

6.2.4 The IU Standard serum and other positive control sera should have a titre within 2 SD of the progressive mean

6.2.5 If any these conditions have not been met, report problem for acceptance to the laboratory supervisor who will decide if all or part of test must be repeated.

6.2.6 Consecutive failure of the test to pass control standards, or indications that results will be unduly delayed by any other cause must be promptly reported to the Technical Manager.

6.3 Interpretation of results and use of spread sheet

6.3.1 The Back Titration denotes the amount of virus input into the test, which must be between $\log_{10}$ 1.5 and $\log_{10}$ 2.5. The test sera results are expressed in International Units per ml by the ratio of test serum with the International Standard Serum times 0.5 to convert the result to IU/mL. This achieved using the Spearman-Kärber formula: i.e.

$$Test \quad Serum\ IU/ml = \left[\frac{test\ serum\ 50\%\ titre}{Int.std\ \ serum\ 50\%\ titre} \right] X\, 0.5$$

6.3.2 A spread sheet has been set up to facilitate calculation of virus input and test sera results in the controlled folder O:\AAHL ISO Quality Assurance (QA) Systems\ISO 17025 QA Doc Control\DERL

6.3.3 In general, levels of neutralisation of greater than 0.5 IU/ml are considered significant.

6.3.4 Samples for which testing indicates antibody levels of less than 1.0 IU/ml to ensure the required level of precision at critical levels proximate to the threshold (see Assay).

6.4 Reporting

6.4.1 Results are recorded on the "FAVN Record and Results Sheet" and entered into the Sample Manager LIMS in accordance with the Operating Manual instructions for that package.

6.4.2 The draft report should be printed, examined for errors, initialled and dated before the test is authorised.

6.4.3 ***Results entered into Sample Manager must be authorised by the laboratory supervisor or authorised delegate.***

6.4.4 Sample Manager draft reports are filed with the SAN/paperwork in the appropriate sequence folder, located in DERL or in the Records Archiving Compactus..

6.4.5 Test Cover Sheet and FAVN Record and Results Sheets are filed in the FAVN folder in DERL, or in the Records Archiving Compactus.

7. SPECIMEN RETENTION

7.1 Records of sample tracking and retention are maintained using the Sample Manager – sample location function

7.2 Samples are discarded in accordance with minimum retention periods and Section 5 of the AAHL Microbiological Security and Safety Manual.

7.3 Retention Periods and Storage

Category	**Holding period**	**Location / Temp**
Importation / Routine	Hold for a minimum of 30 days	DERL -20°C
Disease investigation	Positive – Retain for reference purpose, or discard after approval from laboratory supervisor	DERL or Level 2, -20°C
	Negative - Hold for a minimum of 30 days	DERL -20°C
Animal surveillance /	Hold indefinitely. Discard only	DERL or Level 2, -20°C

NAQS	after approval from laboratory supervisor	

Note: A three-month system of boxed storage is maintained in DERL -20°C for transient holding of routine-test sera. At the end of this period sample discard occurs and is recorded in Sample Manager.

8. QUALITY ASSURANCE

8.1 Internal Controls

Internal controls (cells, virus, positive and negative sera) are included in each assay run. Results are compared with expected values to determine assay acceptance.

8.2 Progressive Monitoring

On-going assay performance is monitored using a running sheet located in the folder "O:\DIAG\iso9000\Progressive Test QC Records DERL.xls". This sheet must be updated after completion of each test with standard serum titre and virus input details. The sheet must be completed before authorising of results

8.3 External Proficiency testing

AAHL participates in external proficiency testing of the FAVN. Testing is conducted through the European Union Reference Laboratory ANSES, Nancy Laboratory for Rabies and Wildlife Technopôle Agricole et Vétérinaire BP 40 009 54220 Malzéville Cedex FRANCE

8.4 Retest checks

Retests are conducted on all samples with IU/mL values of less than 1.0.

9. APPENDICES

9.1 BME with 5% TPB

BME medium	500mL
TPB	25mL
Hepes	5.00mL
Glutamine	5.00mL
Pen/strep	1.25ml
Fungazone	2.50mL

Prepare using sterile technique.
Label bottle with date and additions
Store at +4°C.

9.2 BHK Cell Growth Medium

BME with 5% TPB (see 9.1)
Heat inactivated foetal calf serum to 10%

9.3 Conjugate Diluent (PBS/BSA with 0.005% Evans blue)

PBS A	90ml
PBS + 10% BSA	10ml
0.5% Evans Blue in DD H_20 (see 9.9)	1ml

Store at + 4 °C.

9.4 WHO International Standard Serum

The use of the WHO standard as the operating standard in the RFFIT at AAHL was discontinued and replaced with the OIE Standard (from May 2001), which is now used in the FAVN. However the WHO standard remains a valid and recognised standard reference. Stocks are held at AAHL and additional standard can be obtained as indicated below.
The International Standard for rabies immunoglobulin (2nd international standard preparation) was obtained from:

NIBSC (National institute for Biological standards and Control)
PO Box 1193, Blanche Lane, South Mimms, Potters Bar,
Herts, EN6 3QH, UK. Email: standards@nibsc.ac.uk;
Web site: http://www.nibsc.ac.uk/
Product Code: RAI.

9.5 OIE International Standard

The OIE Standard serum is available from:
Marine WASNIEWSKI, Scientific Officer
Head of Immunology-Virology Division / Community Reference Institute
ANSES Site de Nancy
LERRPAS , Technopôle Agricole et Vétérinaire
BP 40 009 54220 Malzéville Cedex
FRANCE
Phone : +33.3.83.29.89.50
Fax : +33.3.83.29.89.58
email : m.wasniewski@nancy.afssa.fr

Reconstitute standard sera in sterile ddH_2O. Dilute to the required IU level (2.0 IU/mL for the WHO standard and 0.5 IU/mL for the OIE standard) in PBS A + 1%BSA. Dispense 160µl of diluted standard in labelled tubes and store at –80.

9.6 Fixing Solution

10% formaldehyde solution in PBSA containing 0.1% NP40

PBSA	450 ml
40% w/v Formaldehyde	50ml
Nonidet P40	0.5ml

9.7 Normal Cat Serum

Normal cat serum pool ex SPF cat from tissue culture. Serum harvested non aseptically on 19/02/02 for use as a negative control for RFFIT. 4mL vols. (Two pools available; see also 0202-19-1710).

9.8 Viruses

Passage history of Rabies stock viruses

9.8.1 CVS rabies virus

The CVS rabies virus was obtained in 1986 from the Commonwealth Serum Laboratories (Melbourne, Australia) and has been determined to be antigenically indistinguishable from CVS-31 (Dr J. Smith, personal communication). The designation of this virus as CVS-11 in the previous protocol does not appear to have any recorded basis. The virus was passaged once in mice and five times in BHK cells at AAHL.

870430 0003 original
29/5/89 mouse

9006291000	BHK1
20/11/90	BHK2
14/12/90	BHK3
9101211430	BHK4
0112031701	BHK5

Stocks of the virus 9101281500 are held at – 80 °C in the rabies lab in ¼ oz containers. This material is dispensed into Sarstedt tubes in 50µl volumes which forms the working virus stock. These are held in a box at – 80 °C labelled RFFIT antigen.

9.8.2 Other viruses

Mokola (9206-17-1600), Lagos bat (9206-17-1610), Duvanhage (9206-17-1620) and Duvenhage RA (9206-17-1630) viruses, were obtained from Dr J. Smith (CDC Atlanta) and passaged 3 times in BHK cells.

9.9 0.5% Evans Blue in DD PBS A

Evans Blue 0589-60
Obtained from Koch-Light Laboratories Ltd. England.
0.1g is added to 20 ml PBS A.
Mix and allow overnight to dissolve.
Filter through 0.45µm filter.
Store at 4°C for up to 3 years.

9.10 Centocor Anti-Rabies Monoclonal FITC Conjugate (1/5 Dilution)

9.10.1 The Centocor Anti-Rabies Monoclonal FITC Conjugate is obtained from:

CENTOCOR INC.,
244 Great Valley Parkway
Malvern, PA 19355
USA.

9.10.2 A copy of the import permit can be obtained from Records.

9.10.3 The import permit, together with the completed purchase requisition are taken to Purchasing from which the order will be placed. Generally, more than one kit is ordered.

9.10.4 The Centocor Anti-Rabies Monoclonal FITC Conjugate is received in freeze-dried form. Reconstitute this to 5ml in sterile distilled water according to kit insert instructions.

9.10.5 Previously untested batch lots should be titrated for potency (see 9.11)

9.10.6 Dilute the 5ml of reconstituted conjugate to 25ml in Conjugate Diluent.

9.10.7 Dispense the 1/5 diluted conjugate in 0.5ml volumes into 1.8ml tubes. Label these CENTOCOR anti rabies FITC conjugate. Diluted 1/5. Batch Number. Date.

9.10.8 Store this at –80°C in the rabies room in a box marked Conjugate.

9.11 Summary of FAVN Method

Dilution of test sera and negative control in duplicate is performed directly in the microtitre plate: (*please follow directions on table 1 representing the microtitre test plate*)

1. When testing 12 sera: add 100µL of BME supplemented with no FCS in all wells.
2. Add 50µL of each serum to 2 wells of row A and row E.
3. Using a multichanelled pipette, mix up and down and transfer 50µL from row A to row B.
4. Mix up and down and continue the titration down to row D.
5. Discard tips and the last 50µL.

Table 1: Microtitre Plate Showing Dilution of Sera. All Wells Contain 100µL of Media at Start

	Serum 1		Serum 2		Serum 3		Serum 4		Serum 5		Serum 6	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL
B												
C												
D												
	Serum 7		Serum 8		Serum 9		Serum 10		Serum 11		Serum 12	
E	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL	+50µL
F												
G												
H												

Transfer 50µl

Discard 50µl & tips

Transfer 50µl

Discard 50µl & tips

Dilution of International Unit Standard (IUS) is performed in quadruplicate, beginning at 1/3 dilution, three folds down the plate (*please follow directions on table 2 representing the microtitre IS plateand Back Titration*)

1. Add 100µL of media to all wells of four columns. Generally the last four columns of a microtitre plate are selected.
2. Add 50µL of International Standard to the first four wells of the column.
3. Using a multichannelled pipette, mix up and down and transfer 50µL down to the next row.
4. Continue the titration down the plate, discard the last 50µL and tips.

Table 2 Microtitre Plate Showing assigned wells for the International Unit Standard

	Back titration						In house control		International unit standard			
	1	2	3	4	5	6	7	8	**9**	**10**	**11**	**12**
A									+50µL	+50µL	+50µL	+50µL
B												
C												
D												
E												
F												
G												
H												

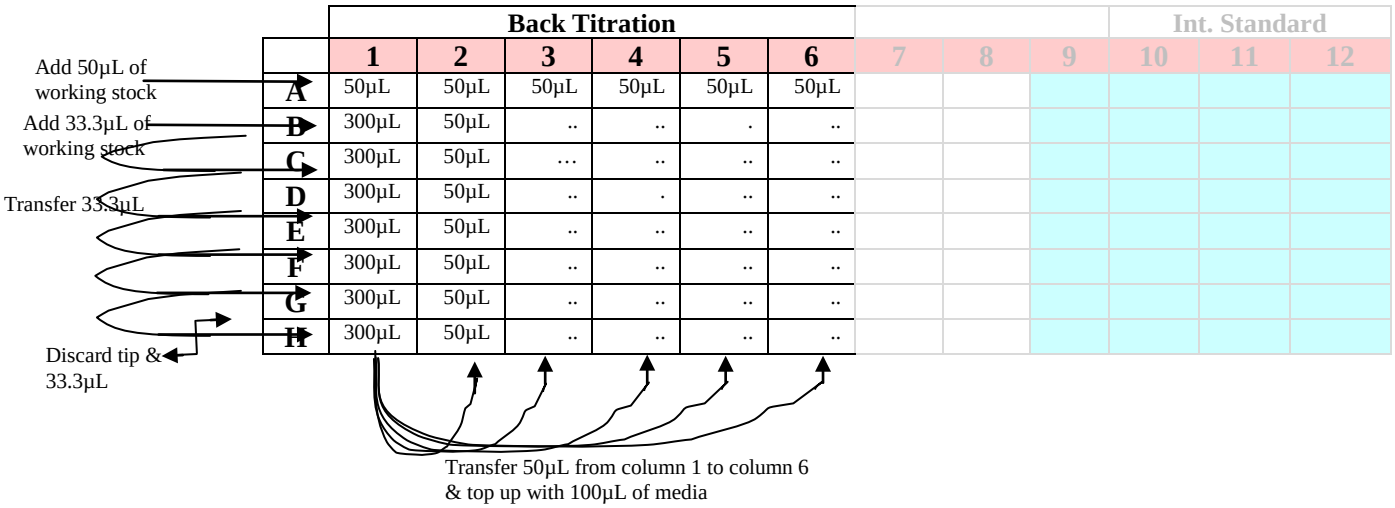
Transfer 50 µl

Discard 25µl & tips

The Back Titration (BT) is performed directly in a microtitre plate. (*please follow directions on table 2 representing the microtitre IS plateand Back Titration*)

1. Add 300µL of media in the first column (B1-H1) of the microtitre plate.
2. Add 50µL of working stock to A1 down to A6.

3. Add 33.3µL of working stock virus in the first well (B1), pipette up and down and discard the tip.
4. Transfer 33.3µL from B1 into C1, pipette up and down and discard the tip.
5. Continue the serial dilution down to G1 pipette up and down and discard the 33.3µL together with the tip.
6. Using a multichennelled pipette, transfer 50µL from the first column to all following columns up to column 6 so that each well contains 50µL.
7. Add 100µL of media to all wells.



Complete Neutralisation stage

1. Add 50µL of working stock virus to all wells except the BT half plate (ie, to all serum containing wells).
2. Incubate at 37°C with 5% CO_2.
3. Add 50µL of BHK cell suspension prepared at 4.0 X 10^5/mL **(**or 2.0 X 10^6per plate).
4. Incubate for 48h.

Fixing and Detection Stage

Note: This step requires extra care given the large volumes and titres of virus to be handled. Use double glove and be fast & resolute when tipping plates. Minimise the number of times to move in & out the cabinet.

1. Tip plates content into a bread box containing pyroneg.
2. Using the electronic multichannelled pipette wash the plates twice with 200µL and 400µL of PBS ABC for the first and the second time respectively.
3. Add 400µL of fixing solution to all wells and leave in the cabinet for 1h.
4. Discard the content in **an empty bread box** and wash the plates twice as before.
5. Tip plates on a paper towel. Dilute fujiribo anti-rabies monoclonal FITC conjugated antibody 1:20 in the conjugate diluent and add 50µL of conjugate to all wells.
6. Incubate at 37°C for 30min.
7. Wash the plates with PBS ABC like before and add 50µL of PBS in all wells.
8. Take plates to the microscope room and with the inverted UV microscope score the plates beginning with the Back Titration followed by the International Standards. Score wells as being positive for the presence of fluorescent foci or otherwise score them as negative.

REVISION PAGE

Versions

**Main Author is defined as the principle writer / contributor to the document and who takes responsibility for its technical content.*

Version	***Main** *(Responsible)* **Author**	**Authorised by**	**Authorisation Date** (dd/mm/yy)
TRIM v.1	Antonio Di Rubbo/Ross Lunt	Ross Lunt	26 October 2010

Revision History (Details of and reason for changes)

**Modification Author is defined as a writer / contributor who makes changes to the document but does not take on the Main Author responsibility.*

Author/s	**Date**	**Revision Details** *(Note: CARs, etc. relating to changes must be cited):*
Antonio Di Rubbo / Ross Lunt	26/10/10	New Procedure

Synopsis of electronic components within this document

Explain all object linkages / formats imbedded, and any linkages to web sites that have been imbedded within this document. This information is important for the current and future management (i.e. readability and preservation) of this electronic document.

Page	**Details of electronic object imbedding or linking**
4	http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.13_RABIES.pdf
5	O:\GENERAL\iso9000\Scientific\EQUIPMENT LIST.XLS
7, 16, 17	O:\AAHL ISO Quality Assurance (QA) Systems\ISO 17025 QA Doc Control\DERL

Molecular Diagnosis at CSIRO Livestock Industries, Australian Animal Health Laboratory

Nucleic Acid Detection for Disease Diagnosis and Emergency Disease Investigation

QA 13-80-6

LYSSAVIRUS CONVENTIONAL PCR (INCLUDING RABIES VIRUS)

REVISION PAGE

Versions

**Main Author is defined as the principle writer / contributor to the document and who takes responsibility for its technical content.*

Version	Main *(Responsible)* Author	Authorised by	Authorisation Date (dd/mm/yy)
7 Jun 2005	Hans Heine	Hans Heine	7 June 2005
13 Nov 2008	Jianning Wang	Ian Pritchard	8 Dec 2008
20 Jul 2010	Som Walker	Jianning Wang	18 Oct 2010
1 Nov 2010	Som walker	Jianning Wang	2 Nov 2010

Revision History (Details of and reason for changes)

**Modification Author is defined as a writer / contributor who makes changes to the document but does not take on the Main Author responsibility.*

Author/s	Date	Revision Details (Note: CARs, etc. relating to changes must be cited)
Hans Heine	7 Jun 2005	Subdivision of previous long document 13-4-80 version 27 August 2004. Content of this new document is identical to Part 2, Sections 12.2 of the old long document.
Jianning Wang	13 Nov 2008	Removed all links. Removed Appendix 1 with Links. Checked all primers by blast search.
Som Walker	20 Jul 2010	Change the format of the manual. Update Table of Content, Quick Reference sheet. Change the PCR work-sheet and add Appendix 5: Comparison of cDNA synthesis with primary PCR and One-Step RT-PCR. The appendix 5 is located in O:\GENERAL\Molecular Diagnosis - PCR\Test Validation\Lyssavirus PCR
Som walker	1 Nov 2010	Correct an error in the conventional worksheet..

Synopsis of electronic components within this document

Explain all object linkages / formats imbedded, and any linkages to web sites that have been imbedded within this document. This information is important for the current and future management (i.e. readability and preservation) of this electronic document.

Page	Details of electronic object imbedding or linking
10	Blast search for identification of gene sequences Internet: http://www.ncbi.nlm.nih.gov/BLAST/Nil
12	Blast sequence analysis via the Internet http://www.ncbi.nlm.nih.gov/BLAST/ with specific links for each virus
12	jpg image of positive gel

TABLE OF CONTENTS:

1. INTRODUCTION

1.1 AETIOLOGY

The genus Lyssavirus belongs to the family Rhabdoviridae having a genome consisting of negative sense ssRNA. There are seven genotypes of lyssavirus including rabies and rabies-related genotypes as listed below. See NCBI Lyssavirus (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=11286)

Rabies virus (RV)

Genotype 1: Rabies virus (worldwide, not Australia);

Rabies related viruses (RRV)

Genotype 2: Lagos bat virus (Africa);
Genotype 3: Mokola virus (Africa);
Genotype 4: Duvenhage virus (South Africa);
Genotype 5: European bat virus ELB1;
Genotype 6: European bat virus ELB2;
Genotype 7: Australian bat lyssavirus ABLV (pteropid & insectivorous bat)

Rabies virus isolates (genotype 1) consist of at least 11 (1995) distinct co-circulating phylogenetic lineages of rabies virus isolates, correlating with geographical origin or species of isolation as follows: Europe Middle East; Africa 1a; Africa 1b; Vaccine 2 (CVS; AVO1); Latin America 1; Vaccine 1 (PV; SAD); Africa 3; Arctic; Latin America 2; Africa 2; Asia.

1.2 DISEASE

Rabies and rabies-like viruses cause fatal diseases of all warm-blood animals, including human. Classical rabies has only been diagnosed on 2 occasions in recent years in Australia. On both occasions, infection was contracted overseas.

1.3 ASSAY

This protocol describes a fully nested RT-PCR for the detection of all lyssaviruses including rabies virus (RV), rabies related virus (RRV) strains and Australian bat lyssaviruses (ABLV). The assay is a modification of a heminested generic lyssavirus PCR described by Heaton et al. 1997 for the PCR amplification of the rabies virus genotype 1 and rabies related genotypes 2-6 (Lagos bat virus, Mokola virus, Duvenhage virus, and European bat virus 1 and 2). RNA is extracted from suitable virus-inactivated diagnostic samples (mainly brain or CSF) followed by RT-PCR amplification using redundant primer sets homologous to conserved regions of the lyssavirus N gene. The identity of amplified PCR fragments and the corresponding genotype are determined by DNA sequencing of amplified fragments extracted from agarose gel followed by DNA alignment with known genotypes and Blast search of GenBank database.

This protocol also contains the original heminested PCR described by Heaton et al. including correction of an error in one of the published Heaton primers (JW12). Limited evaluations of the tests as well as comparison to ABLV TaqMan assays are described in validation of generic lyssavirus PCR (available at O:\GENERAL\Molecular Diagnosis - PCR\Test Validation\Lyssavirus PCR). The tests were evaluated for the amplification of Australian bat lyssaviruses (pteropid and insectivorous ABLV) as lyssavirus genotype 7 was unknown when the original test was published.

1.4 RESULTS

Results are obtained at completion of the PCR by observing presence or absence of correct size of amplified PCR fragments in an agarose gel electrophoresis. Amplified PCR fragments of the correct expected size are eluted from agarose gel and the DNA sequences determined. Sequence identity and genotype are determined by Blast search of the GenBank database.

1.5 REFERENCES

Bourhy H., Kissi B., Tordo N. (1993). Molecular diversity of the *Lyssavirus* genus. Virology 194, 70-81.

Badrane H., Bahloul C, Perrin P, Tordo N (2001). Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J Virol. 75:3268-3276.

Gould A.R., Hyatt A.D., Lunt, R., Kattenbelt J.A., Hengstberger S., Blacksell S.D. (1998). Characterisation of a novel lyssavirus isolated from Pteropid bats in Australia. Virus Research 54, 165-187.

Heaton P.R., Johnstone P., McElhinney L.M., Cowley R., O'Sullivan E., Whitby J.E. (1997). Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J.Clin.Microbiol. 35, 2762-2766.

Kissi B., Tordo N., Bouhy H. (1995). Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 209, 526-537.

Mannen et al. (1991). Conserved nucleotide sequence of rabies virus cDNA encoding the nucleoprotein. Virus Genes 5, 69-73.

McColl K.A., Gould A.R., Selleck P.W., Hooper P.T., Westbury H.A., Smith J.S. (1993). Polymerase chain reaction and other laboratory techniques in the diagnosis of long incubation rabies in Australia. Australian Veterinary Journal 70, 84-89.

Sacramento D., Bourhy H., Tordo N. (1997). PCR technique as an alternative for diagnosis and molecular epidemiology of rabies virus. Molecular and Cellular Probes 5, 229-240.

Tordo N., Poch O., Ermine A., Keith G. (1986). Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. Nucleic Acids Research 14, 2671-2683.

Whitby J.E., Heaton P.R., Whitby H.E., O'Sullivan E., Johnstone P. (1997). Rapid detection of rabies and rabies-related viruses by RT-PCR and enzyme-linked immunosorbent assay. J.Virol.Meth. 69, 63-72.

Foord AJ., et al. (2006). Molecular diagnosis of lyssaviruses and sequence comparison of Australian bat lyssavirus samples. Australian Veterinary Journal 84, 225-230.

2. EQUIPMENT

2.1 PCR EQUIPMENT LIST

Please refer to the Master Equipment List.

2.2 PCR CONSUMABLES LIST

Please refer to the PCR Consumables List.

3. REAGENTS

3.1 CHEMICALS

Chemicals used for molecular diagnosis are listed in the Reagents and Consumables list. Refer to MSDS for safety instructions as part of the approved chemical list.

3.2 BIOLOGICALS

Biological reagents for molecular diagnosis are purchased from the Approved List of Suppliers.

Positive control samples for rabies virus PCR consist of ABLV infected tissue homogenate when rabies virus is tested. Do not use inactivated rabies virus tissue culture homogenate of CVS strain to avoid potential cross-contamination with sample. Use ABLV positive control for rabies suspect diagnostic samples to be able to differentiate sample and control by DNA sequencing.

Positive controls are either Pteropid ABLV (Mouse brain homogenate positive for this virus by FAT, PCR and DNA sequencing SAN 96-0648) for testing samples from Pteropid bats, or Insectivorous ABLV (Mouse brain homogenate positive for this virus by FAT, PCR and DNA sequencing (SAN 96-1256) for testing samples from Insectivorous bats.

4. PREPARATION

4.1 PREPARATION OF SAMPLES

In accordance with the AAHL Microsecurity Manual, samples for the diagnosis of lyssaviruses (rabies, ABLV) are processed in an isolation room in the south suite. All details on the sample identification and the accompanying SAN documentation are checked for consistency and recorded on the SAN sheet.

Samples are stored at -80°C, or processed immediately to extract RNA in accordance with the Standard Operating Procedures. Preferred samples include fresh or frozen central nervous tissue (brain, spinal cord) and salivary glands, or other samples as deemed appropriate by the molecular diagnosis technical supervisor. Inactivated samples are transported (in an appropriate transport container) to the PCR suite for processing.

4.2 STAFF TRAINING AND APPROVALS

1. The operator must have been approved by the Technical Manager as having appropriate level of training and experience. Refer to Staff Records.
2. Staff working in the Diagnostic PCR Suite must be familiar with the relevant sections of the Microbiological Security and Safety Manual, in particular, “Standard Operating Practices: Biosecurity Level 3(Z)”.
3. Staff must have undertaken appropriate OHS & E training, be familiar with the relevant MSDSs, and have current Microbiological Spills training.

5. ASSAY PROCEDURE

5.1 SAMPLE PREPARATION

In the south suite isolation room, 100 µl aliquot of the sample (10% homogenate) is added to the appropriate extraction buffer. Inactivated sample in sealed container is transported to PCR suite.

5.2 NUCLEIC ACID EXTRACTION

Please refer to 13-4-80-2 for Extraction Methods

5.3 Optional cDNA Synthesis

cDNA synthesis (TaqMan Reverse Transcription Reagents including MultiScribe reverse transcriptase; Applied Biosystems, Cat# N808-0234) using random hexamer; refer to 13-4-80-2 for cDNA synthesis.

5.4 Conventional PCR

The following specific reagents are required to prepare primer mixes as described in the appendix:

Primer RV D007 (=modified JW12; Heaton et al., 1997)
5' ATGTAACACCYCTACAATG 3'

Primer RV D008 (= JW6 (DPL); Heaton et al., 1997)
5' CAATTCGCACACATTTTGTG 3'

Primer RV D009 (= JW6 (E); Heaton et al., 1997)
5' CAGTTGGCACACATCTTGTG 3'

Primer RV D010 (= JW6 (M); Heaton et al., 1997)
5' CAGTTAGCGCACATCTTATG 3'

Primer RV D011 (= JW10 (DLE2); Heaton et al., 1997)
5' GTCATCAAAGTGTGRTGCTC 3'

Primer RV D012 (= JW10 (ME1); Heaton et al., 1997)
5' GTCATCAATGTGTGRTGTTC 3'

Primer RV D013 (= JW10 (P); Heaton et al., 1997)
5' GTCATTAGAGTATGGTGTTC 3'

Primer RV D017 (new fwd primer for fully nested PCR)
5' AGATCAATATGAGTAYAARTAYCC 3'

Redundant nucleotide code: R=A,G Y=C,T

Primary RT-PCR

LyssaMIX 1 (RV D007, RV D008, RV D009, RV D010)

Use 3 µl of LyssaMIX 1 per 25 µl PCR reaction

The amplification parameters are:

1 cycle 48°C 30 min, 95°C 2 min;
5 cycles 95°C 90 sec, 45°C 90 sec, 50°C 20 sec, 72°C 60 sec;
40 cycles 95°C 30 sec, 45°C 60 sec, 50°C 20 sec, 72°C 60 sec;
1 cycle 95°C 30 sec, 45°C 90 sec, 50°C 20 sec, 68°C 10 min.

Secondary PCR (fully-nested*)

LyssaMIX 2 (RV D017, RV D011, RV D012, RV D013)

Use 3 µl of LyssaMIX 2 per 25 µl PCR reaction

The amplification parameters are:

1 cycle 95°C 15 min;
5 cycles 95°C 90 sec, 45°C 90 sec, 50°C 20 sec, 72°C 60 sec;
25 cycles 95°C 30 sec, 45°C 60 sec, 50°C 20 sec, 72°C 60 sec;
1 cycle 95°C 30 sec, 45°C 90 sec, 50°C 20 sec, 72°C 7 min.

***The fully nested secondary PCR has been validated on classical rabies and ABLV.**

Alternative Secondary PCR
(heminested; as published Heaton et al., 1997)

LyssaMIX 3 (RV D007, RV D011, RV D012, RV D013)
Use 3 µl of LyssaMIX 3 per 25 µl PCR reaction

For samples suspect of genotypes other than classical rabies or ABLV, the heminested secondary PCR should be included.

The amplification parameters (same as secondary PCR above) are:

1 cycle 95°C 15 min;
5 cycles 95°C 90 sec, 45°C 90 sec, 50°C 20 sec, 72°C 60 sec;
25 cycles 95°C 30 sec, 45°C 60 sec, 50°C 20 sec, 72°C 60 sec;
1 cycle 95°C 30 sec, 45°C 90 sec, 50°C 20 sec, 72°C 7 min.

5.5 Agarose Gel Electrophoresis

Please refer to 13-4-80-2 for gel electrophoresis procedure.

5.6 Agarose Gel Extraction of PCR Products

Please refer to 13-4-80-2 for gel extraction procedure.

5.7 DNA Sequencing

Primary PCR product

A. forward primer

RV D007	all genotypes

B. reverse primers

RV D008	insectivorous ABLV, rabies, lagos bat and duvenhage
RV D009	EBLV 1 and 2
RV D010	pteropid ABLV and Mokola

Secondary PCR product

A. forward primer

RV D017	all genotypes

B. reverse primers

RV D012	pteropid and insectivorous ABLV
RV D011, D012 & D013	rabies and unknown lyssavirus genotypes.

5.8 Blast Search for Identification of Gene Sequences

Internet: http://www.ncbi.nlm.nih.gov/BLAST/

5.9 DNA Sequence Alignment

See Appendix for GenBank accession number for one strain of each genotype.

6. RESULTS

6.1 READING

At the completion of the PCR, aliquots of all samples are run on an agarose gel, and photographed. Specific PCR fragments of the correct size are identified and the DNA sequence of the lyssavirus specific fragments are characterized by a Blast search of the GenBank database.

6.2 INTERPRETATION AND RECORDING

Agarose gel analysis is interpreted in the following way:

- negative control — no amplification of specific product
- positive control — amplification of specific products of the correct size
- test samples — amplification of PCR product of the correct size as shown in the table below

PCR	Fragment size
Primary PCR	606 bp
Secondary PCR (Fully nested)	498 bp
Alternative secondary PCR (heminested)	582 bp

A test is valid when

- The negative control sample must have no evidence of specific amplified products for both primary and nested PCR, and
- The positive control samples yield specific fragments for the primary and/or the nested PCR.

Amplified PCR fragments of the correct size from controls and samples are then eluted from the gel, and sent for sequencing.

Sequence identity and genotype are determined by a Blast search of the GenBank database.

6.3 Reporting

If the assay data are acceptable and there is no evidence of contamination, then data can be entered in Sample Manager.

A copy of the report is also stored in the folder in the south suite reading room with the SAN and sample sheets. Any unusual or borderline results must be discussed with the technical manager before being reported.

7. Specimen Retention

7.1 Negative Samples

Negative samples are retained for 1 month after the final testing has been completed. They are stored at -80°C.

7.2 Positive Samples

Positive samples are retained for 6 months after the final testing has been completed. They are stored at -80°C.

QUICK REFERENCE SHEET LYSSAVIRUS AND RABIES VIRUS PCR

General information	
Taxonomy	Family Rhabdoviridae; Genus Lyssavirus (contains 7 genotypes, including rabies and ABLV)
Genome	(-) ssRNA
Host species	Mammals (human, dog, bat, cat, others)
Suitable samples	Inactivated 10 % homogenates of nervous tissue, brain, salivary glands, CSF
AAHL info/"expert"	John Bingham, Ross Lunt and Hans Heine
Assay	Nested PCRs to detect all genotypes of lyssavirus including rabies and ABLV. Primer sequences are based on the lyssavirus N gene.
References	Heaton et al. (1997) J Clin Microbiol 35: 2762
GenBank accession #	Rabies virus strain PV (X03673); InABLV (AF081020) and PtABLV (AF006497)
CAUTION!!!	**Level 3Z agent**
Positive control	Mouse brain homogenate; SAN# 96-1256 for InABLV or SAN# 96-0648 for PtABLV
Sample preparation	Add 100 µl of inactivated sample to 262 µl MagMAX (MM) buffer
RNA extraction	Use 180 µl of sample in MM buffer for MagMAX extraction
Primary RT-PCR	
PCR reagent	SuperScript III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen Cat No. 12574-026)
PCR primers	Use 3 µl of LyssaMIX 1 (RV D007+ RV D008, RV D009, RV D010) in 25 µl PCR reaction
PCR cycles	1 cycle: 48°C 30 min, 95°C 2 min 5 cycles: 95°C 90 s, 45°C 90 s, 50°C 20 s, 72°C 60 s 40 cycles: 95°C 30 s, 45°C 60 s, 50°C 20 s, 72°C 60 s 1 cycle: 95°C 30 s, 45°C 90 s, 50°C 20 s, 68°C 5 min
PCR product	606 bp
Sequencing primers	Fwd = RV D007, Rev = RV D008 + RV D009 + RV D010
Sequence analysis	http://www.ncbi.nlm.nih.gov/BLAST/
Secondary PCR (fully nested)	
Secondary PCR	HotStar Taq Master Mix Kit (QIAGEN Cat No. 203445) Use 2 µl of primary PCR product as template in 25 µl PCR reaction
PCR primers	Use 3 µl of LyssaMIX 2 (RV D017+ RV D011, RV D012, RV D013) in 25 µl PCR reaction
PCR cycles	1 cycle: 95°C 15 min 5 cycles: 95°C 90 s, 45°C, 90 s, 50°C, 20 s, 72°C 60 s 25 cycles: 95°C 30 s, 45°C, 60 s, 50°C, 20 s, 72°C 60 s 1 cycle: 95°C 30 s, 45°C, 90 s, 50°C, 20 s, 72°C 10 min
Nested PCR product	498 bp
Sequencing primers	Fwd = RV D017, Rev = RV D011 + RV D012 + RV D013
OPTIONAL: Heminested PCR	
Heminested PCR	HotStar Taq Master Mix Kit (QIAGEN Cat No. 203445) Use 2 µl of primary PCR product as template in 25 µl PCR reaction
PCR primers	Use 3 µl of LyssaMIX 3 (RV D007+ RV D011, RV D012, RV D013) in 25 µl PCR reaction
PCR cycles	same as secondary PCR
PCR product	582 bp
Sequencing primers	Fwd = RV D007, Rev = RV D011 + RV D012 + RV D013

WORKSHEET FOR PRIMARY RT-PCR AND NESTED PCR FOR RABIES AND LYSSAVIRUS

SANs: .....

MASTERMIX OPERATOR: ..... **DATE:** .....

A. PRIMARY RT-PCR

REACTION COMPONENTS	VOLUME PER REACTION (µL)	VOLUME FOR ..... RXS (µL)
Nuclease-Free Water	6.5	
2X Reaction MIX (Invitrogen Cat No. 12574-018, 026) Lot #	12.5	
Primer name: **LyssaMIX 1** MicroStores No.	3.0	
SuperScript III RT/Platinum *Taq* Mix	1.0	
Total Volume	**23**	
Template RNA	2	

One-Step RT-PCR Program

1 cycle	48°C 30 min, 95°C 2 min
5 cycles	95°C 90 s, 45°C 90 s, 50°C 20 s, 72°C 60 s
40 cycles	95°C 30 s, 45°C 60 s, 50°C 20 s, 72°C 60 s
1 cycle	95°C 30 s, 45°C 90 s, 50°C 20 s, 68°C 5 min

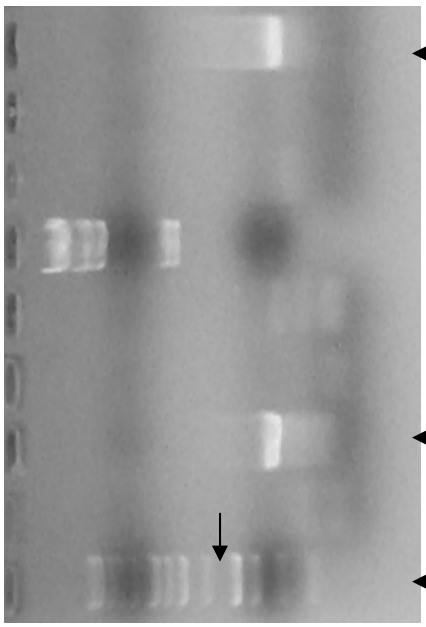
B. NESTED PCR

REACTION COMPONENT	VOLUME PER REACTION (µL)	VOLUME FOR ..... RXS (µL)
Nuclease-Free Water	7.5	
HotStar *Taq* Master Mix (QIAGEN Cat No. 203445) Lot #	12.5	
Primer name: **LyssaMIX 2** MicroStores No.	3.0	
Total Volume	**23**	
Primary PCR Product	2	

Nested PCR Program

1 cycle	95°C 15 min
5 cycles	95°C 90 s, 45°C, 90 s, 50°C, 20 s, 72°C 60 s
25 cycles	95°C 30 s, 45°C, 60 s, 50°C, 20 s, 72°C 60 s
1 cycle	95°C 30 s, 45°C, 90 s, 50°C, 20 s, 72°C 10 min

APPENDIX 1: GEL IMAGES OF POSITIVE TEST (EXAMPLE SAN 01-1692)



APPENDIX 2: DNA SEQUENCES FOR ALIGNMENT

GenBank accession# and link to GenBank for representative strain of each genotype:

Genotype 1 Rabies virus; strain PV, Pasteur vaccine strain rabies (= X03673)
Genotype 2 Lagos bat virus (= U22842)
Genotype 3 Mokola virus (= S59448)
Genotype 4 Duvenhage virus (= U22848)
Genotype 5 European bat virus 1
POL. EBV1-8615Pol (= U22844)
FRA. EBV1-8918Fra (= U22845)
Genotype 6 European bat virus 2
FIN. ELB2-9007Fin (= U22846),
HOL. ELB2-9018Hol [U22847] (= U22847)
Genotype 7 Australian bat lyssavirus (ABLV)
Insectivorous (= AF081020)
pteropid bat (= AF006497)

Blast sequence analysis via the internet
http://www.ncbi.nlm.nih.gov/BLAST/

APPENDIX 3: PRIMER MIXES FOR LYSSAVIRUS PCR

Primer mixes are prepared from single primer stocks (18 µM).

LyssaMIX 1: primary PCR;

LyssaMIX 2: fully-nested PCR;

LyssaMIX 3: hemi-nested PCR (backup only).

Use 3.0 µl of primer mixes per 25 µl PCR reaction.

PRIMER MIXES FOR LYSSAVIRUS CONVENTIONAL PCR

Primer (18 µM)	Redundancy of primers	Volume per 3 µl of primer mixes (µl)	Volume per 300 µl of primer mixes (µl)
LyssaMIX 1 Primary PCR			
RV D007	2	1.0	100
RV D008	1	0.5	50
RV D009	1	0.5	50
RV D010	1	0.5	50
Water		0.5	50
LyssaMIX 2 Fully-nested PCR			
RV D017	8	1.5	150
RV D011	2	0.5	50
RV D012	2	0.5	50
RV D013	1	0.5	50
Water		0	0
LyssaMIX 3 Hemi-nested PCR			
RV D007	2	1.0	100
RV D011	2	0.5	50
RV D012	2	0.5	50
RV D013	1	0.5	50
Water		0.5	50

APPENDIX 4: OTHER LYSSAVIRUS PCR PRIMERS IN THE DIAGNOSTIC PCR SUITE

(Update HH 10/04/02)

Name	Sequence 5' to 3'	Gene/position	Virus genus specificity	Remarks
RV D001	CAATGGATGCCGACAA	NP68	Rabies	Modified NP68; McColl et al 1993
RV D002	CTCTATCCTGTCTGCAATG	NP581	Rabies	NP581; McColl et al 1993
RV D003	GTAGGATGCTATATGGG	NP1013	Rabies	NP1013; McColl et al 1993
RV D004	GTCACTCGAATATGTCTTGTTTAG	NP1426	Rabies	Modified NP1426; McColl et al 1993
RV D005	GGAAAGATGGTTCCTCAG	GP3310	Rabies	Modified NP3310; McColl et al 1993
RV D006	GTGTAGAGACTCTTCATATCTG	GP3713	Rabies	NP3713; McColl et al 1993
RV D014	GGAATGAATGCTGCAAAGCTG	NP175	Pteropid ABLV	Original LYSF-FF TaqMan (QH G.Smith); sequencing primer
RV D015	GAACGCCGCGAAGTTGG	NP180	Insectivorous ABLV	Original LYSF-YB TaqMan (QH G.Smith); sequencing primer
RV D016*	GGCAGAYCCCCTCAAATAACTC	NP256	Pteropid & insectivorous ABLV	Original LYSR TaqMan (QH G.Smith); sequencing primers
RV D018	ATGTAACACCYCTACAATGGA	NP(-16)	Generic lyssavirus	2 nt longer than RV D007
RV D019*	GGGTAYTTRTACTCATAYTGATC	NP92	Generic lyssavirus	Reverse complement of RV D017

*R=AG Y=CT

PCR PRIMER COMBINATIONS

Primers	Fragment length (bp)	Specificity	Remarks
RV D001 + RV D002	513	Rabies NP	
RV D003 + RV D004	413	Rabies NP	
RV D005 + RV D006	403	Rabies GP	
V D018 + RV D019	108	Generic lyssavirus NP	

Molecular Detection of Lyssaviruses

Adam Foord CSIRO, AAHL

Outline of Presentation

- Introduction to Lyssaviruses and situation in Australia
- Conventional PCR for detection of all lyssaviruses
- Taqman real-time PCR assay for detection of Australian Bat Lyysavirus
- Comparison of Convention and Taqman assays

Lyssaviruses introduction

… situation Before 1996

6 established Genotypes

Genotype 1 Rabies virus

Genotype 2 Lagos bat virus
Genotype 3 Mokola Virus
Genotype 4 Duvenhage Virus

Genotype 5 European bat lyssavirus 1
Genotype 6 European bat lyssavirus 2

Genotype 1 Classical Rabies virus

- Best known of the Lyssavirues
- Has a near world wide distribution with Australia and New Zealand notable exceptions.
- A minor outbreak of Rabies occurred in Tasmania in 1897 (Dogs/Child)
- Two confirmed cases of rabies in humans were reported in Australia both contracted outside our borders

Australian Bat Lyssavirus

..After 1996

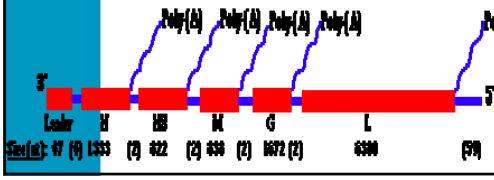
- In 1996 a novel lyssavirus was discovered in a flying fox in Ballina N.S.W
- Isolated as a result of investigations into the reservoir for Hendra virus (Graham Fraser et al)
- ABLV has now been isolated in all 4 major species of Pteropid bats (Flying foxes) *and* the Yellow bellied sheath tail. (Insectivorous bat)
- Two strains of ABLV exist -Insectivorous ABLV
  - Pteropid ABLV

ABLV

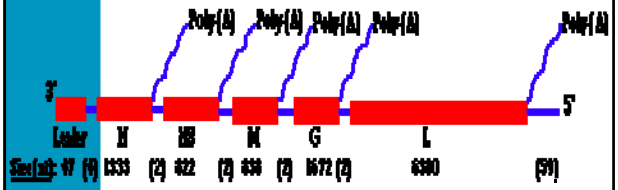
- ABLV has now been isolated in Bats from many regions of Australia, Along Eastern seaboard, S.A, N.T and most recently Kimberly region of W.A
- ABLV is genetically simular but distinct to rabies Virus and forms the seventh Genotype of Lyssavirus
- Caused the death of two Humans 1996 & 1998 with the disease course and clinical manifestations simular to Classical rabies virus

Genus *Lyssavirus*

- Family Rhabdoviridea
- SS RNA viruses
- Negative sense
- All genotypes approx 12 kb in length



Genomic organisation



Fully-Nested RT-PCR for detection of all genotypes of Lyssavirus

- Developed and validated a Nested PCR for detection of all genotypes of Lyssavirus
- Modification of a published Hemi-nested RT-PCR to form a Fully-nested format.

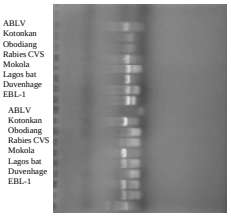
Hemi-nested Format

Template

Fully-Nested format

Template

Specificity of Nested RT-PCR



Nested PCR

Primary PCR

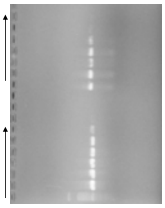
Assay Specific for all tested Genotypes for Lyssavirus

Technical sensitivity comparison

Using Rabies CVS as a template (RT-PCR with total of 75 rounds amplification)

10-fold dilutions of Viral RNA

10-fold dilutions of Viral RNA



Hemi-nested RT-PCR

Fully-Nested RT-PCR

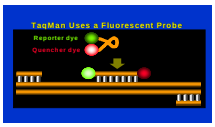
- Fully-nested format at least or slightly more sensitive than Hemi-nested

Taqman assay for detection of ABLV

Two Taqman assays designed

- One specific for Insectivorous ABLV
- One specific for Pteropid ABLV

As per all TaqMan assays
Consist of Primer pairs with Probe designed to bind
To sequence B/T these primers.



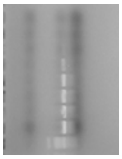
Technical sensitivity and comparison to Conventional nested RT-PCR

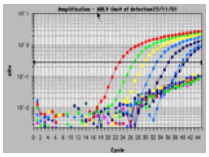
- We compared the relative sensitivities for the TaqMan assay Vs Conventional Nested RT-PCR

Nested RT-PCR

Pteropid ABLV Taqman assay

Template dilution





•Taqman assay is approx. 10-fold more sensitive

CSIRO Livestock Industries

Multiplexing and Internal controls

• Our instrument ABI 7700 has the ability to muliplex reactions
(records emissions from reporter dyes emitting different wavelengths post excitation)

• We use this to incorporate a TaqMan assay for the detection of 18 S rRNA.

• Control for the
- •Extraction of nucleic acid
- •Integrity of RNA
- •Absence of major quantities of PCR inhibitors

• Circumvents the need to process positive controls (nucleic acid extraction ETC) At the same time as samples (Cross-contamination risk reduction)

CSIRO Livestock Industries

Some General Taqman assay observations

In ~100 + assays for ABLV (2.5 years, From March 2001)

•Time: approx 4 hours from sample acquisition to result.

•Contamination issues: None observed!

•Standard reaction conditions enables the simultaneous Diagnosis for different agents on the same sample e.g for Bat samples; ABLV, Hendra and Nipah.

CSIRO Livestock Industries

Summary

•Developed Fully-nested RT-PCR for detection of all Lyssavirues

TaqMan assays for detection of ABLV are

•Rapid ~4 hours compared to ~20 hours for nested RT-PCR + sequencing

•Sensitive -approx 10 genome equivalents/ul (at least as sensitive as nested RT-PCR)

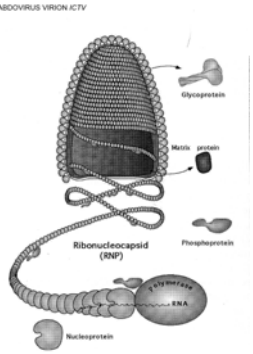
•Specific

•Robust

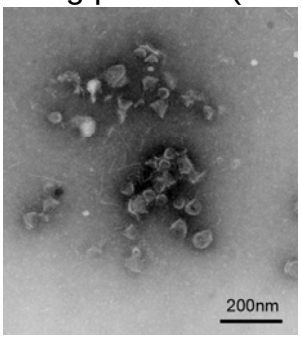
CSIRO Livestock Industries

Rhabdoviridae

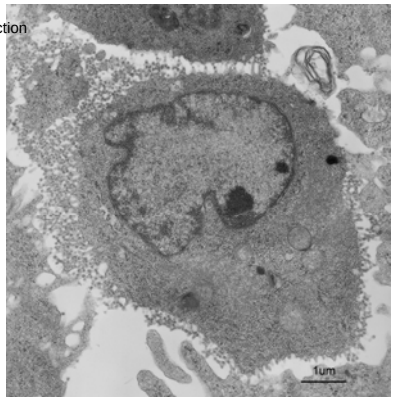
- Bullet or cone shaped
- Helical
- Enveloped
- 70 – 190 nm
- eg. Lyssa - Rabies, YHV (Roni)



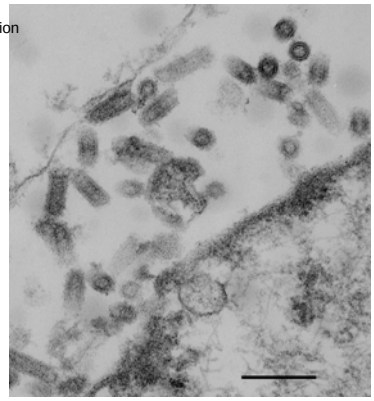
Rhabdoviridae – defective interfering particles (NCEM)



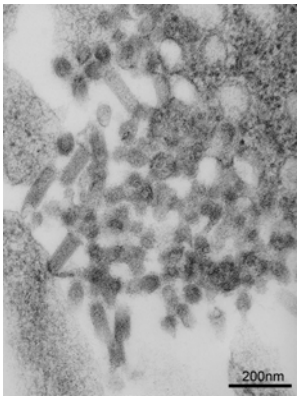
Thin section



Thin section

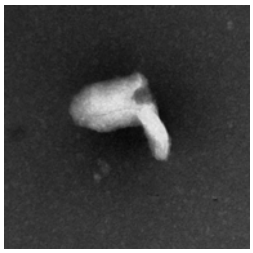


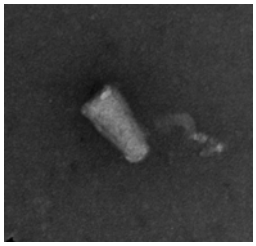
Thin section

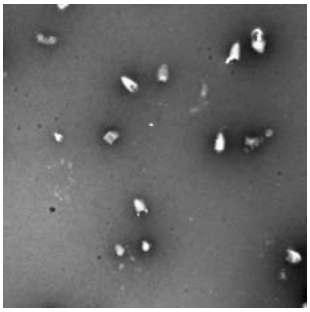


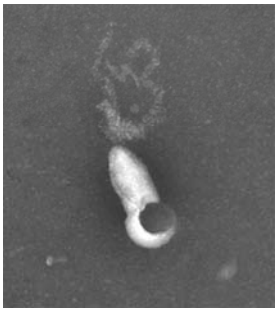
Bovine ephemeral fever virus (BEFV)

- Genus Ephemovirus
- Negative contrast electron microscopy









Rabies and Lyssaviruses

John Bingham
CSIRO - Australian Animal Health Laboratory

In this lecture I will:

1. Give an overview of lyssaviruses, particularly bat lyssaviruses
2. Review the diagnostic tests for lyssaviruses
3. Review the pathogenesis and clinical signs
4. Review the treatment for rabies

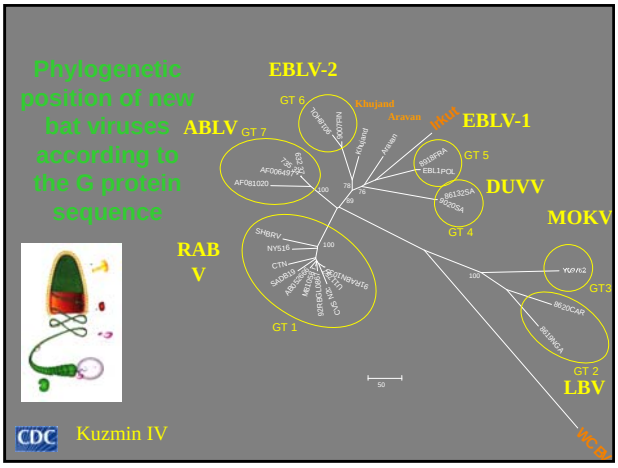
The genotypes of Lyssaviruses

	Name	Principal host	Geographical distribution
1	Rabies	Carnivora, bats	Worldwide (with exceptions)
2	Lagos bat	Fruit bats	Africa
3	Mokola	Not known	Africa
4	Duvenhage	Microchiroptera	Southern Africa
5	EBL 1	*Eptesicus serotinus*	Europe
6	EBL 2	*Myotis* spp.	Europe
7	ABL	Mega- and microchiroptera	Australia

EBL = European bat lyssavirus
ABL = Australian bat lyssavirus

Unclassified Bat Lyssaviruses

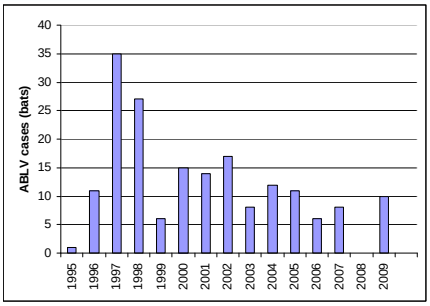
- Aravan Virus - Kyrgzstan 1991: Lesser Mouse Eared bat (*Myotis blythi*)
- Khujand - Tajikistan 2001: Whiskered bat (*Myotis mystacinus)*
- Irkut - East Siberia 2002: Greater tube-nosed bat (*Murina leucogaster*)
- Western Caucasian bat virus – Krasnodar 2002: Schreiber's bat (*Miniopterus schreibersii*)



Australian bat lyssavirus (ABLV)

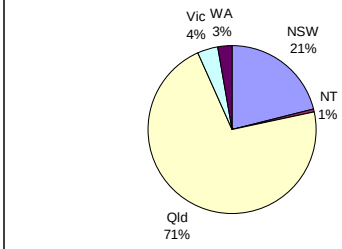
Grey headed flying fox (*Pteropus poliocephalus*)

ABLV - History



Source of data: ABLV Bat Stats (Dec 2009)

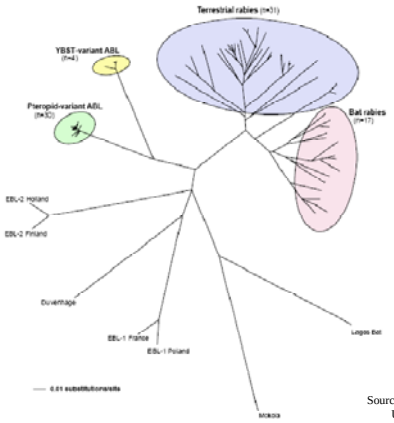
ABLV Distribution (1995 – 2009)



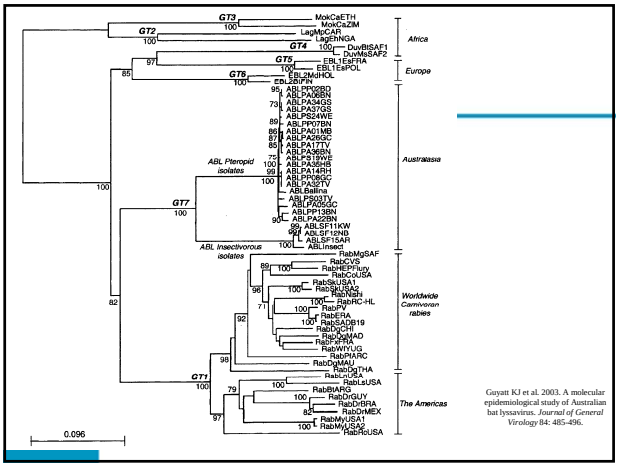
ABLV: Host range

- Maintenance hosts:
  - *Pteropus* spp (Flying foxes)
  - 4 isolates from Yellow-bellied sheathtail bat (maintenance host ?)
- Two humans cases
- Experimental infection of mice
- Other mammals?

Lyssaviruses: phylogenetic tree



Source: JL Barret 2004, PhD thesis, University of Queensland



Guyatt KJ et al. 2003. A molecular epidemiological study of Australian bat lyssavirus. *Journal of General Virology* 84: 485-496.

ABLV control

- Control of ABLV is based on:
  - Avoidance of bats: Public awareness
  - Destruction and testing of suspect bat cases
  - Vaccination of in-contact humans
  - Destruction or monitoring of in-contact pets
- Bat are protected species in Australia and are not culled for the purpose of controlling lyssavirus

Lyssavirus: Laboratory diagnosis at AAHL

- I will discuss:
  - The different diagnostic tests done at AAHL for lyssavirus (including rabies)
  - Their relative values and limitations
  - The samples needed for a reliable diagnosis

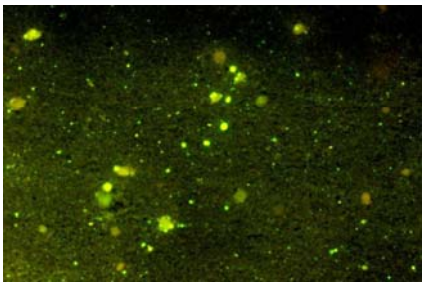
Diagnostic tests at AAHL

- Fluorescent antibody test (FAT)
- PCR (real-time and conventional)
- Immunohistochemistry / histopathology
- Virus culture
- Serology

FAT

- Method:
  - Smear of brain material on slide
  - Fix with acetone
  - Stain with fluorescein-labeled conjugate
  - Examine under ultraviolet microscope
- Sensitivity: >99%
- Specificity: >99%
- Turn-around time: Hours
- Limitations:
  - Less sensitive with formalin fixed tissue
  - Requires an ultraviolet microscope

FAT



Fluorescent antibody test - rabies

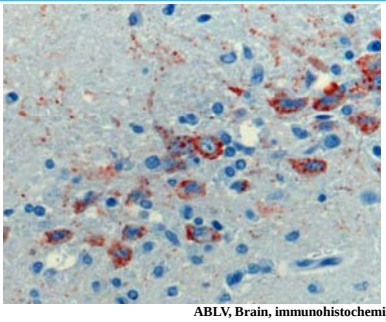
PCR

- Different types of PCR:
  - Nested RT-PCR: broad reactivity
  - TaqMan (real time): type-specific
- Sample: brain suspension
- High sensitivity and specificity
- Rapid turn-around times
  - Same day for TaqMan
  - 2 days for nested RT-PCR
- Limitations:
  - Reduced sensitivity in formalin-fixed samples

Immunohistochemistry (IHC)

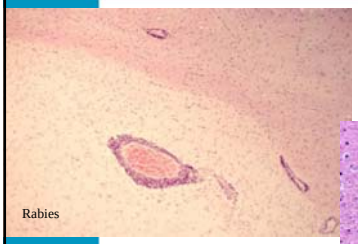
- Method:
  - Formalin-fixed paraffin-embedded sections used
  - Antigen retrieval by enzyme digestion
  - Indirect immunoperoxidase test
  - Primary antibody: broad-reacting monoclonal antibody against rabies nucleoprotein
- High sensitivity and specificity
- Turn-around time: days
- Histopathology examination included

Immunohistochemistry (IHC)

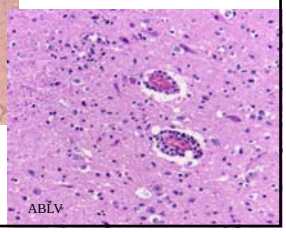


ABLV, Brain, immunohistochemistry

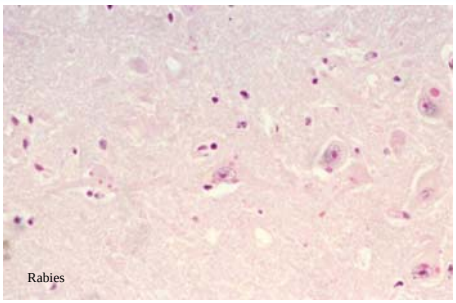
Histopathology



Histopathology
Haematoxylin-eosin stain



Histopathology



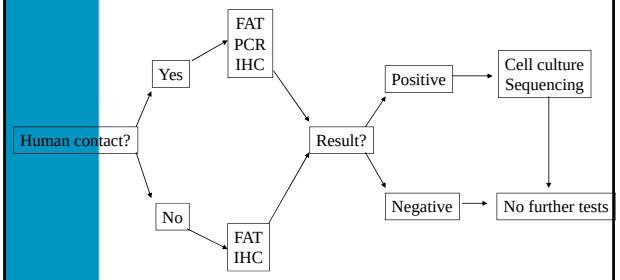
Virus culture

- Cell cultures (mouse neuroblastoma)
- Method:
  - Tissue suspension inoculated onto monolayers
  - Cells incubated for 5 days
  - Cells fixed in acetone and virus detected by FAT
- Turn-around time: 1 week
- Sensitivity high
- Limitations:
  - Sample condition may lower sensitivity
  - Does not work with fixed tissue

Serology

- Used to determine vaccinal response
- Of limited diagnostic value for clinical disease
  - Can be used in human cases maintained on life-support
  - Need to distinguish with vaccination responses: much higher titre in clinical disease and titre present in CSF
- Antibody detection tests
  - Rapid fluorescent focus inhibition test (RFFIT) (virus neutralisation assay)
  - Fluorescent antibody virus neutralisation (FAVN) test
  - ELISA (not routinely used at AAHL)

Test flow chart for AAHL ABLV diagnosis



Reporting

- Interim report(s)
  - Same day as receipt of specimen
  - Human contact: FAT and PCR results
  - Without human contact: FAT only
  - Suitable for purposes of medical management
- Final report

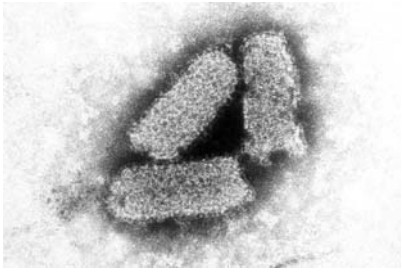
Tissues for diagnosis

- Brain (especially brain stem)
- Other:
  - Spinal cord
  - Ganglia
  - Peripheral nerves
  - Salivary gland

Treatment of tissues for diagnosis

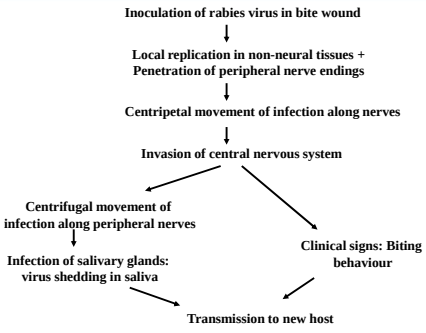
- Divide brain in half:
  - Half fresh chilled
  - Half in neutral buffered formalin

Pathogenesis and clinical signs



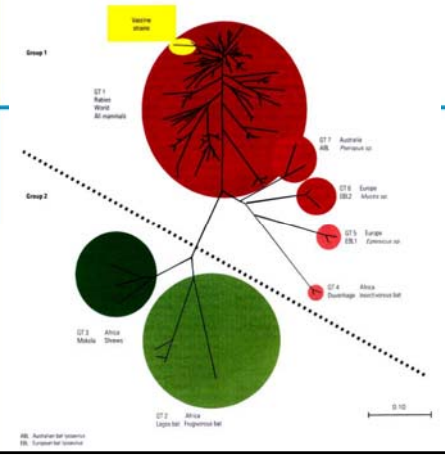
Rabies virions

Pathogenesis of lyssavirus



Treatment of rabies

Louis Pasteur



Principles of treatment for rabies

Treatment is based on:

- Urgent induction of immunity after exposure
- Active (vaccination) and passive (immunoglobulin) immunisation
- Pre-exposure immunisation in high-risk situations
- Palliative care during clinical disease

Survival following bite contact

In the absence of any post-exposure treatment, only 10 – 70 % of humans will develop clinical rabies

Development of disease will depends on :

- Dose of virus
- Proximity of bite to central nervous system

WHO Treatment Categories

Category I	
Touching or feeding animals Licks on intact skin	No treatment
Category II	
Minor abrasions without bleeding Licks on broken skin	Vaccine
Category III	
Transdermal bites or scratches Contamination of mucous membranes	Vaccine + immunoglobulin

Source: WHO Expert Committee on Rabies, Eighth Report 1992

Treatment Guidelines

Do not wait to start treatment !

Wash wound thoroughly with soap and water, disinfectant solution or water alone. Apply ethanol or aqueous iodine

Administer immunoglobulin by infiltration around the bite wound and intramuscularly (Day 0 only)

Administer vaccine intramuscularly in the deltoid area, or other recommended area on Days 0, 3, 7, 14 and 30 (or other recommended schedule)

Source: WHO Expert Committee on Rabies, Eighth Report 1992

Thank you!