出國報告（出國類別：進修）

英國萊斯特大學
Mayer IgA Nephropathy Laboratories
進修心得報告

服務機關：臺中榮民總醫院腎臟科
姓名職稱：徐佳鈿主治醫師
派赴國家/地區：英國/萊斯特
出國期間：2024 年 6 月 29 日至 2024 年 9 月 29 日
報告日期：2024 年 10 月 4 日

摘　要

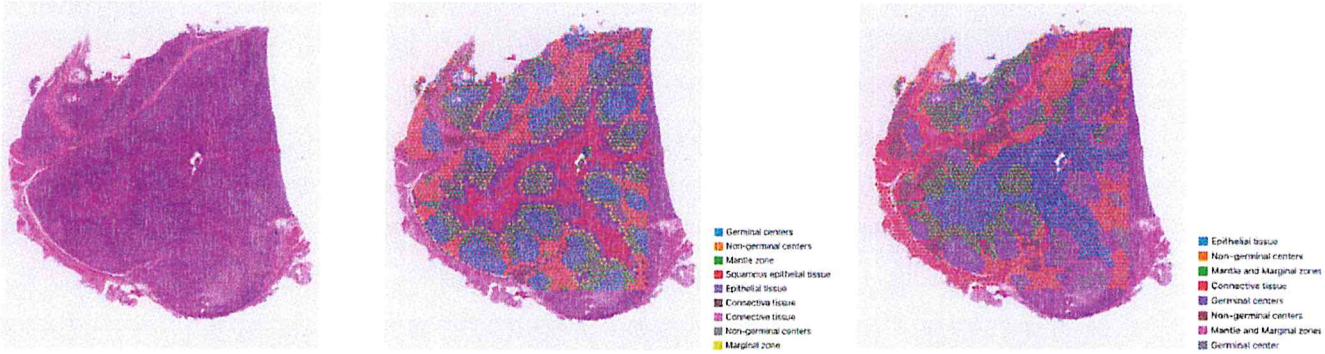
這次進修的目的是學習先進的空間分子學(Spatial Biology)技術，尤其是 GeoMx Digital Spatial Profiling(DSP)，以便應用於腎臟科的精準醫療。特別是 IgA 腎病的研究。此技術結合了表觀遺傳學、轉錄體學、蛋白質體學及代謝體學，能提供檢體內的空間資訊和分子生物資訊。此次進修主要是到英國萊斯特大學的 IgA 腎病實驗室。進修過程從實驗室報到開始，研究者在實驗室內學習了多項基礎實驗設備操作，特別是 pipette 的精準操作，因為 DSP 操作中對試劑加入量的要求極高，且不能有氣泡蓋住檢體。之後還進行了 ELISA 測試，主要測定 Human C5b-9 補體，並參與測試來自全球多個國家的 IgA 腎病臨床試驗檢體。最後進一步學習了 GeoMx DSP 操作，包括樣本準備和 RNA 定量分析，此技術能夠定位腎絲球病變區域內特定細胞的基因表現。此外我也利用空閒時間整理台灣的資料寫了三篇論文投稿。

關鍵字: IgA nephropathy; Digital Spatial Profiler (DSP)

目　次

一、 目的

傳統組織病理學一直是診斷和分期腎臟疾病的重要且成功的工具。腎臟切片的檢查的結果和 IgA nephropathy 的預後習習相關，但是傳統腎臟切片的檢查目前比較是看型態分布及免疫抗體的染色，在切片檢體的全部潛力可能尚未得到充分發揮，目前的技術已經開始往空間分子學 Spatial Biology 演進，利用 epigenetics 表觀遺傳學、transcriptomics 轉錄體學、proteomics 蛋白質體學和 metabolomics 代謝體學等工具，來做進一步分子生物、精準醫療的方向邁進。如下圖左邊是傳統的組織病理學只做型態 morphology 的分辨，中間圖做各種 RNA 的分別，右邊圖則能分辨各種蛋白質在檢體內的分布。這種在檢體上同時得到空間資訊 spatial data 和分子生物資訊的學問就叫 Spatial biology 空間分子學，其中 Digital Spatial Profiling 就是一項目前可以得到這些資訊的技術。



Morphology
H&E-stained image

RNA
Gene expression clustering
(Whole transcriptome)

Protein
Protein clustering
(Immune cell profiling panel)

英國萊斯特大學的 IgA nephropathy 實驗室(Mayer IgA Nephropathy Laboratories)目前已有這方面的技術，所以此次至英國主要是要向 Jonathan Barratt 教授學習這項技術，並參與此技術的臨床研究。

二、 過程

(一) 報到、實驗室介紹

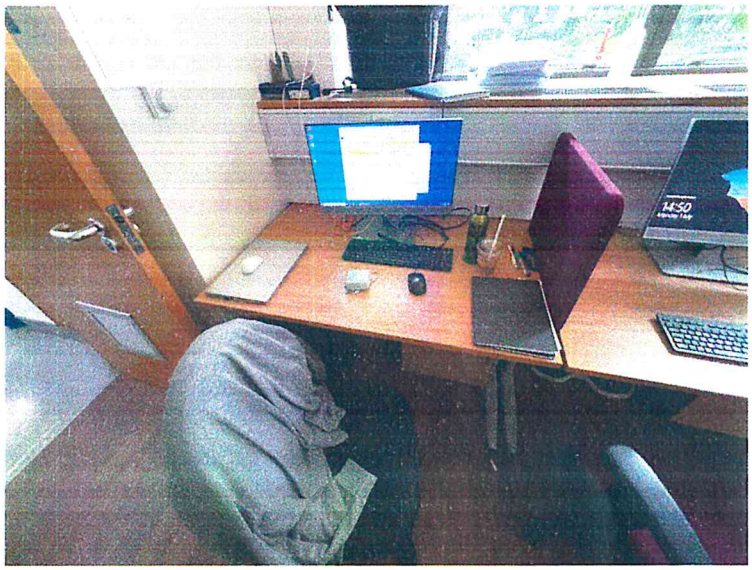
1. 因為有先自假至英國確定住宿，於 113 年 6 月 28 日就先到實驗室報到，和教授見面會談，教授也初步介紹實驗室環境及人員給我認識，並給我識別證。

大門需刷卡才能入內

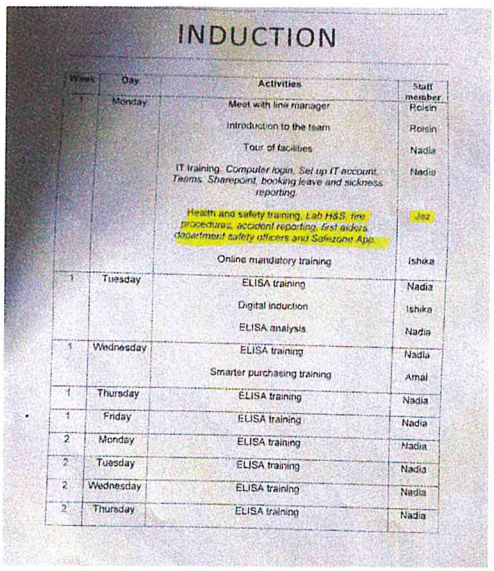
實驗室所在的 Hodgkin Building

2. 因為教授告知週末實驗室沒有開，所以 07/01 上午到實驗室正式上工，因為已經在前一個星期五就先去拜訪過教授及實驗室的經理，所以就可以自己刷卡進入 building 內，之後經理就交代實驗室成員 Amal 帶我到我的位置，有自己的辦公室電腦，登入需要用萊斯特大學給學生或職員的帳號密碼。帳號之前就建好了，但是密碼需要用之前交的資料內的電話打去設定，但是之前在台灣預填時當然是填台灣的手機，無法收到認證的簡訊。所以 Amal 還是決定再我走到主校區的大樓去直接找資訊人員面對面設定密碼。外國人做事情就是按照 check list，一個一個問我之前交的資料，包括台灣的英文地址，郵遞區號等等，英文地址在此之前填了幾次背得出來，但是郵遞區號我只背出四碼 407，所以他又多問了一些個資去比對。接著 Amal 帶我又把大樓中其它和生活習習相關的場所也介紹了一次，從裝開水、泡咖啡、吃午餐的地方，到上下樓層的廁所等等都詳細和我說一次。也介紹 journal club= journal reading 的行程表給我，把當周會遇到的 journal 傳給我讓我先看看，所以第一天上工就先唸了一篇論文。

實驗室(3 樓)



分配到的座位和電腦

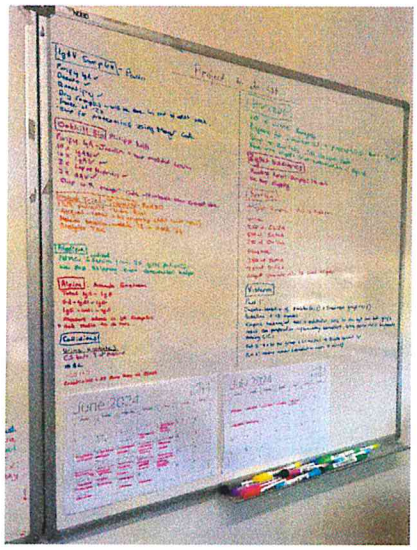


INDUCTION

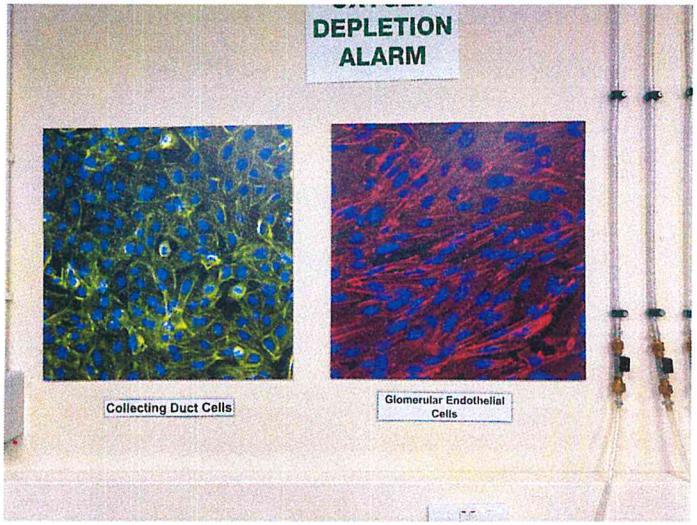
Week	Day	Activities	Staff member
1	Monday	Meet with line manager	Roisin
		Introduction to the team	Roisin
		Tour of facilities	Nadia
		IT training. *Computer login, Set up IT account, Teams, Sharepoint, booking leave and sickness reporting.*	Nadia
		Health and safety training. *Lab H&S, fire procedures, accident reporting, first aiders, department safety officers and Safezone App.*	Jez
		Online mandatory training	Ishika
1	Tuesday	ELISA training	Nadia
		Digital induction	Ishika
		ELISA analysis	Nadia
1	Wednesday	ELISA training	Nadia
		Smarter purchasing training	Amal
1	Thursday	ELISA training	Nadia
1	Friday	ELISA training	Nadia
2	Monday	ELISA training	Nadia
2	Tuesday	ELISA training	Nadia
2	Wednesday	ELISA training	Nadia
2	Thursday	ELISA training	Nadia

表訂第 1、2 周訓練項目及老師

5 樓的 Coffee room(吃午餐裝開水的地方)



白板上是目前<u>正在進行中 IgAN 的 clinical trials</u>，trial 中的檢體很多都會送到實驗室這邊進一步檢驗。



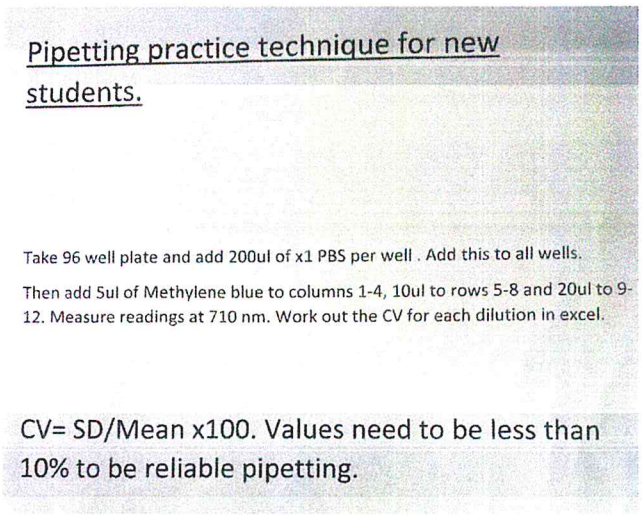
實驗室內也有 cell culture room，有各式各樣的 kidney cell：collecting duct cell、glomerular endothelial cells 等等

3. 和 Jonathan Barratt 教授討論這三個月的學習目標：初步設定第一個月熟悉一般實驗室操作(實驗室成員 Amal 協助)，第二個月 ELISA 檢驗操作(實驗室成員 Nadia 協助)，第三個月 GeoMx DSP 操作 kidney biopsy specimens 看 RNA level(實驗室成員 Max 協助)。

(二) 基礎實驗設備操作之學習

1. 因為 Digital Spatial Profiling 的操作過程會遇到加入試劑的量只有零點幾 μL，所以正確及精準地操作 pipette 就很重要，而且 Digital Spatial Profiling 操作過程中還要不能有氣泡產生蓋住檢體，所以這是需要先訓練 pipette 技術的(尤其在這之前我完全沒有任何實驗室的經驗，大學時期上課離現在已經 20 多年)。除了我之外，所

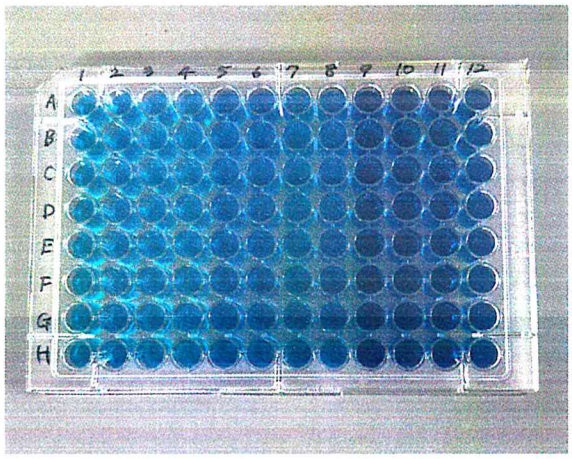
有實驗室新進人員都會先做 pipette 的訓練。



Pipette 器具，pipette tips 和 96 well plate

主要就是將 96 well plate 滴甲基藍，在 200μL 的 PBS 液體中，前 1-4 排加 5μL，中 5-8 排加 10μL，後 9-12 排加 20μL，之後放在 710nm 光譜儀 spectrophotometer 下去看每一相同濃度的差異要小，計算出來的 CV 變異係數 (Coefficient of variation)要小於 10%

實驗室內的光譜儀 spectrophotometer



Pipette 好的樣子

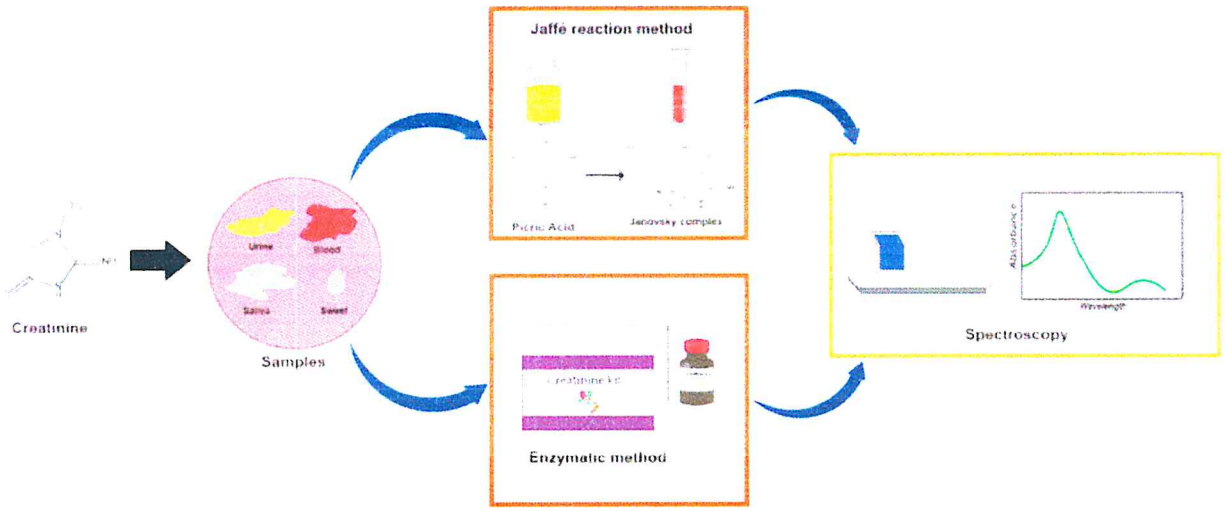
2. 經過一兩天的甲基藍 Pipette 訓練(中間還有實驗室安全規則上課)後，就開始用 improved Jaffe method 來測定 creatinine。因為實驗室安全規則規定不行用手機，也要戴好手套(被資深 Technician Jez 提醒後，不敢明目張膽地邊操作邊照像)，所以有一些機器圖片是後來再去照的，不過後續實驗的確有時會操作揮發性有害物質、易燃物質，所以也不方便照像。Jaffe method 會比 Enzymatic Method 準一些。Jaffe method 是因為 creatinine + Picric acid(經 NaOH 協助催化)後會變成顏色較深的 Janovsky complex，之後一樣利用光譜儀 spectrophotometer 測定剛加入 Picric acid 試

劑時的光度 optical density(OD0)，以及 5 分鐘後的光度 optical density(OD5)就可以算出 creatinine 的濃度。讓我使用的檢體主要是來自各國 IgA nephropathy sparsentan (PROTECT trial)臨床試驗的 urine 檢體，下圖是一天分配給我檢測的量。

920	CA02-03	V11ET_A1	A0138SWGA	USA	12	1	
921	CZ06-02	V9_A1	J012ZYQ7G	Belgium	12	2	
922	CZ06-01	V7_A1	J012VLCMH	Belgium	12	3	
923	CA03-01	V15_A1	A0137LP98	USA	12	4	
924	CA03-04	V13_A1	A013FQJLP	USA	12	5	
925	DE02-01	V11ET_A1	J012VVC8I	Belgium	12	6	
926	DE02-02	V9_A1	J012VVCJ4	Belgium	12	7	
927	CA04-03	V3_A1	A0137LQTS	USA	12	8	
928	CA05-07	V7_A1	A013M1CES	USA	12	9	
929	DE11-07	V13_A1	J012YQTZX	Belgium	12	10	
930	DE11-14	V15_A1	J0130MD7R	Belgium	12	11	
931	CA06-01	V15_A1	A013BVBUJ	USA	12	12	
932	CA06-05	V13_A1	A013FNE9Z	USA	12	13	
933	CA07-02	V3_A1	A0138BEZJ	USA	12	14	
934	CA12-02	V3_A1	A0139BT7K	USA	12	15	
935	ES09-04	V13_A1	J0131GXLT	Belgium	12	16	
936	DE09-01	V15_A1	J012X6AWY	Belgium	12	17	
937	AU09-06	V3_A1	V0003TA0F	Singapore	12	18	
938	DE09-03	V7_A1	J012X6DLP	Belgium	12	19	
939	US02-03	V7_A1	A0136Q07Y	USA	12	20	
940	CA08-03	V9_A1	A0137LR4C	USA	12	21	
941	CA12-02	V11ET_A1	A0139BTAT	USA	12	22	
942	US37-01	V3_A1	A013BVPW0	USA	12	23	
943	US35-10	V7_A1	A013EA07D	USA	12	24	
944	UK09-04	V9_A1	J012ZLFVN	Belgium	12	25	
945	FR02-04	V13_A1	J012YYY5W	Belgium	12	26	
946	UK08-01	V13_A1	J012VS3Z1	Belgium	12	27	
947	US12-01	V15_A1	A0136HULY	USA	12	28	
948	US03-01	V7_A1	A0136GRJ0	USA	12	29	
949	CZ02-04	V7_A1	J012W7N8S	Belgium	12	30	
950	CZ02-06	V9_A1	J012W7NII	Belgium	12	31	
951	SE02-05	V13_A1	J0131HU97	Belgium	12	32	
952	AU01-04	V13_A1	V0003XCAA	Singapore	12	33	
953	CZ05-02	V15_A1	J012VLEWE	Belgium	12	34	
954	IT03-01	V13_A1	J012WL24M	Belgium	12	35	
955	US12-03	V7_A1	A0136HWN2	USA	12	36	
956	AU01-07	V9_A1	V000405VA	Singapore	12	37	
957	CZ06-03	V11ET_A1	J012VLFU7	Belgium	12	38	
958	CA07-01	V13_A1	A0138BEPS	USA	12	39	
959	CA05-07	V15_A1	A013M1D1A	USA	12	40	

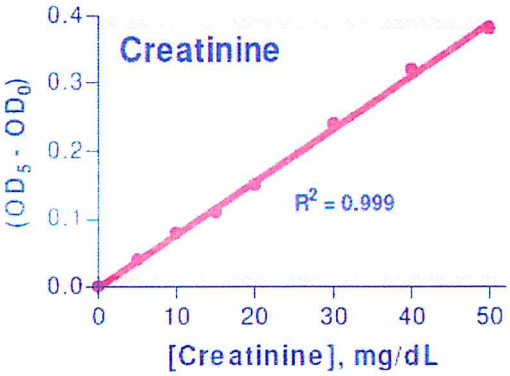
Chia-Lien

使用的試劑是 BioAssay Systems 公司的 QuantiChrom™ Creatinine Assay Kit (DICT-500)，原廠 protocol 如附錄一。



Jaffe method: creatinine + Picric acid(經 NaOH 協助催化)後會變成顏色較深的 Janovsky complex

一組美金 419=13400NTD，可以做 500 次



Standard Curve in 96-well plate

(OD5-OD0)和 creatinine 有很好的 correlation

Number	Barcode	OD0	OD0	VAR	Mean	SD	CV	OD5	OD5	VAR	Mean	SD	CV	Conc (mg/dL)
926	[illegible]	0.182	0.169	-7.1	0.1755	0.009	5.2	0.276	0.254	-8.0	0.265	0.016	5.9	26.24633431
927	[illegible]	0.526	0.457	-13.1	0.4915	0.049	9.9	0.969	0.846	-12.7	0.9075	0.087	9.6	121.9941349
928	[illegible]	0.277	0.278	0.4	0.2775	0.001	0.2	0.486	0.487	0.2	0.4865	0.001	0.1	61.29032256
929	[illegible]	0.296	0.317	7.1	0.3065	0.015	4.8	0.514	0.548	6.6	0.531	0.024	4.6	65.83577713
930	[illegible]	0.302	0.278	-7.9	0.29	0.017	5.9	0.525	0.489	-6.9	0.507	0.025	5.0	63.63636364
931	[illegible]	0.231	0.257	11.2	0.244	0.018	7.5	0.386	0.442	14.5	0.414	0.040	9.6	49.85337243
932	[illegible]	0.321	0.317	-1.2	0.319	0.003	0.9	0.586	0.581	-0.9	0.5835	0.004	0.6	77.5659824
933	[illegible]	0.343	0.348	1.5	0.3455	0.004	1.0	0.619	0.561	-9.4	0.59	0.041	7.0	71.70087977
934	[illegible]	0.182	0.202	11.0	0.192	0.014	7.4	0.285	0.325	14.0	0.305	0.028	9.3	33.13782991
935	[illegible]	0.501	0.484	-2.4	0.4925	0.012	2.4	0.956	0.95	-0.6	0.953	0.004	0.4	135.0439883
936	[illegible]	0.355	0.393	10.7	0.374	0.027	7.2	0.692	0.789	14.0	0.7405	0.069	9.3	107.4780059
937	[illegible]	0.327	0.363	11.0	0.345	0.025	7.4	0.641	0.698	8.9	0.6695	0.040	6.0	95.16129032
938	[illegible]	0.135	0.136	0.7	0.1355	0.001	0.5	0.197	0.205	4.1	0.201	0.006	2.8	19.20821114
939	[illegible]	0.236	0.241	2.1	0.2385	0.004	1.5	0.433	0.433	0.0	0.433	0.000	0.0	57.03812317
940	[illegible]	0.304	0.327	7.6	0.3155	0.016	5.2	0.605	0.659	8.9	0.632	0.038	6.0	92.81524927
941	[illegible]	0.213	0.214	0.5	0.2135	0.001	0.3	0.368	0.372	1.1	0.37	0.003	0.8	45.89442815
942	[illegible]	0.276	0.287	4.0	0.2815	0.008	2.8	0.505	0.524	3.8	0.5145	0.013	2.6	68.32844575
943	[illegible]	0.197	0.21	6.6	0.2035	0.009	4.5	0.325	0.35	7.7	0.3375	0.018	5.2	39.29618768
944	[illegible]	0.278	0.277	-0.4	0.2775	0.001	0.2	0.642	0.63	-2.2	0.636	0.009	1.4	76.20645161

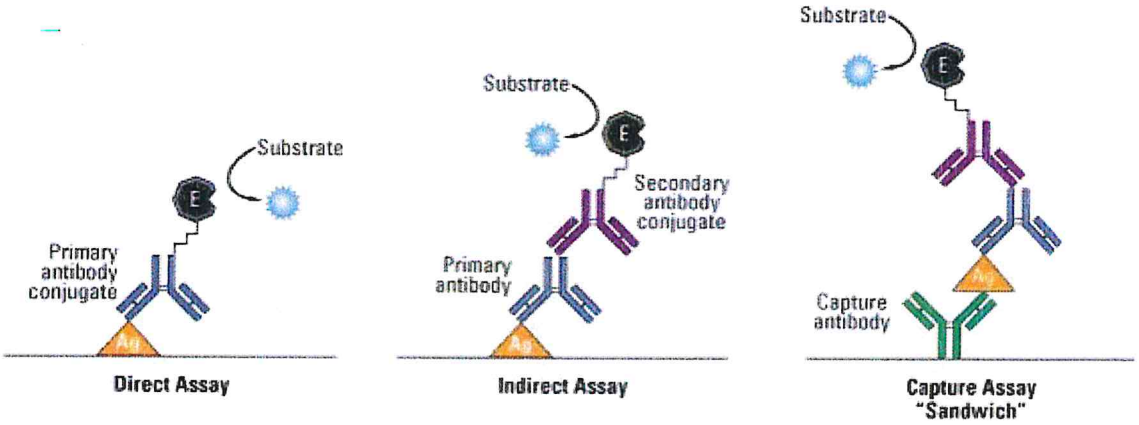
我大約測了 200-300 人，節錄一部分我做的結果，trial 的檢體來自很多國家。

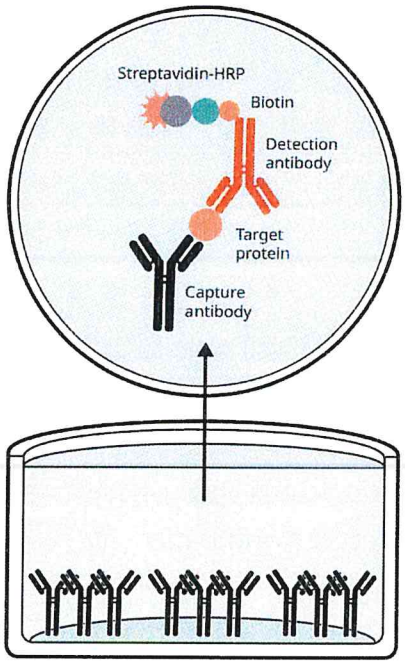
(三) ELISA 檢驗操作

1. 經過幾天的 creatinine 訓練後，就開始 ELISA 的操作，主要是 Human 補體 C5b-9 測定，使用的檢體主要也是來自各國 IgA nephropathy sparsentan (PROTECT trial)臨床試驗的 urine 檢體。教授說會把所有協助幫忙測定的人都在 paper 中致謝，不過其實因為 PROTECT trial phase 3 我們醫院也有參加，所以之前就被列入 PROTECT

Investigators 了。

2. 實驗室 Human C5b-9 ELISA 用的是 BD OptEIA™ Human C5b-9 ELISA Set，Enzyme-linked immunosorbent assay (ELISA)酵素結合免疫吸附分析法，是一種靈敏、特異且廣泛應用於生物學、生化學和醫學研究中的分子生物學分析技術。也是大多數實驗室中常用的一種分子生物檢測技術，它的原理利用抗體與抗原專一性結合的特性，配合呈色劑反應，利用呈色深淺來定量樣品中特定目標蛋白質的含量。ELISA Kit 的優勢在於，只需要少量樣品(100 ul)即可進行檢測、敏感性高並且可以同時檢測多種抗體。而用以標記的酵素被稱為偵測酵素，常見的有辣根過氧化物酶(horseradish peroxidase, HRP)、鹼性磷酸酶(alkaline phosphatase, AP)等。依不同操作方法，ELISA 又可分為直接法 (Direct ELISA)、間接法 (Indirect ELISA)、三明治法 (Sandwich ELISA)、競爭法 (Competitive ELISA) 四種方法。





三明治法 (Sandwich ELISA)

1. 將捕捉抗體 (capture antibody) 固著於塑膠孔盤上，洗去未結合抗體及雜質。
2. 加入檢體，使檢體中抗原與捕捉抗體 (capture antibody) 結合，洗去多餘檢體。
3. 加入另一對抗原專一之一次抗體，使檢體中抗原與此抗體結合，洗去未結合抗體。
4. 加入標記酵素之二次抗體(即 Detection antibody)，使二次抗體與一次抗體結合，洗去未結合二次抗體。
5. 加入酵素受質 substrate 顯色(Streptavidin- HRP-Biotin)，藉由光譜儀 spectrophotometer 測量並計算待測抗原含量。

3. BD OptEIA™ Human C5b-9 ELISA Set 是利用三明治法 (Sandwich ELISA)的方式。三明治法是利用兩種抗體對檢體中的待測抗原進行兩次專一性辨認，因此具有高偵測靈敏度及高專一性。三明治法是將捕捉抗體 (capture antibody) 固定在固體表面上，再加入檢體，捕捉抗體會抓住檢體中具有專一性的抗原，再與一級抗體及酵素標記二級抗體反應。由於這個高度辨識的過程，檢體不需要事先進行純化，

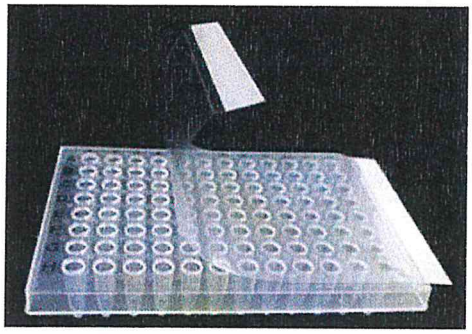
而可直接進行實驗。 然而，需要注意的是，在使用此方法時必須選用特定的一級抗體及二級抗體。為了避免一級抗體跟二級抗體競爭與抗原結合的現象發生，必須確認一級抗體跟二級抗體具有不同的抗原表位 (epitopes, 即抗原上可與抗體結合的位置)，且必須驗證一級抗體跟二級抗體可進行專一性結合，並可同時作用。原廠 Human C5b-9 ELISA protocol 如附錄二，Mayer IgA Nephropathy Laboratories 員工 summarized protocol 如附錄三。一組 set 大約 730 美金，台幣 23346 元。

4. Mayer IgA Nephropathy Laboratories Human C5b-9 ELISA 的步驟：
   (1) 將 Capture Antibody 捕捉抗體按 1:250 稀釋在 Coating Buffer 緩衝液中，將 40μL 的 Capture Antibody 捕捉抗體加入到 9960μL 的 Coating Buffer 緩衝液中，在 96 well plate 的每個 well 都加入 100μL 這個稀釋後(1:250)的溶液。密封 Seal plate (如下一頁圖片)，並在 4°C 下 incubate overnight。請勿稀釋超過實驗所需的捕捉抗體量。
   (2) 放入實驗室內的 96 well plate 自動洗滌機中(如下圖)，機器會吸出 well 中的溶液，用每孔 ≥300μL 的洗滌緩衝液 Wash Buffer 洗滌 3 次。在最後一次洗滌後，將 plate 倒置並用吸水紙吸乾多餘的液體。

實驗室內的 96 well plate 自動洗滌機，各種 ELISA 可以設定各自的 program，機器會自動吸入放在右邊兩個白色筒子內的洗滌緩衝液 Wash Buffer(所以若是測不同的東西，有不同的洗滌緩衝液 Wash Buffer，就要換內容物)

   (3) 在每個 well 加入 ≥200μL 的 Assay Diluent 檢測稀釋液後密封 Seal plate 。於室溫(RT)incubate 一個小時。
   (4) **[在步驟(3) incubate 一個小時快到的 20-25 分鐘前進行]** 用 Assay Diluent 檢測稀釋液來製作 **stock 標準品 standard**, 標準品系列稀釋品和 control 對照品溶液。

將冷凍乾燥的標準品 standard 回溫至室溫後，小心打開 vial 小瓶以避免材料損失。在 vial 中加入 2.1 mL 的 Assay Diluent 檢測稀釋液製作「標準品 standard」，得到 30 ng/mL 的 stock 標準品 standard 溶液。用 Vortex 機器輕輕震盪混合均勻，之後讓混合液作用至少 15 分鐘。(在原 vial 內，所以稱 stock standard)



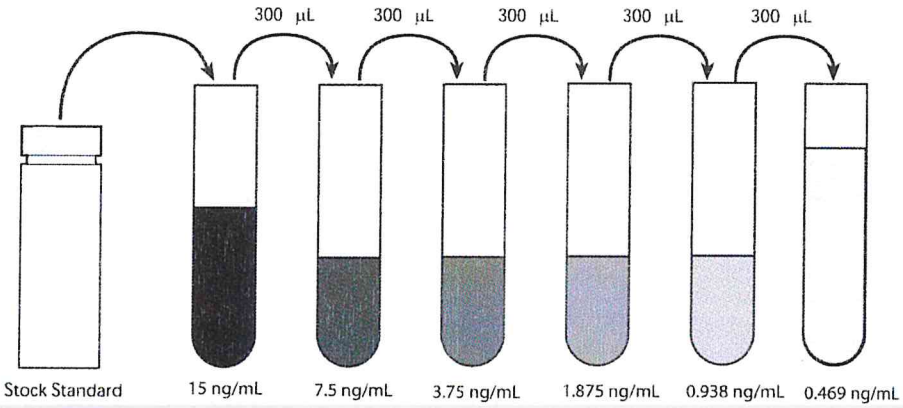
密封
Seal plate

標準品
standard vial

Vortex 機器

Eppendorf tube

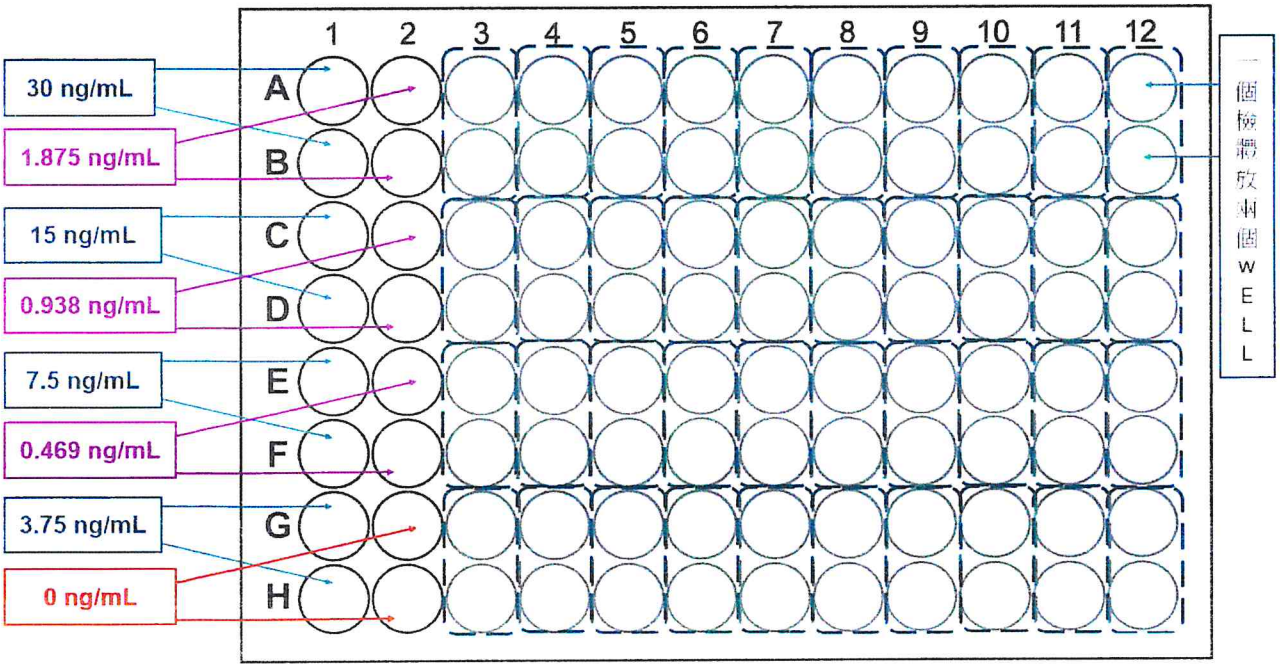
(5) 接著開始製作「**標準品系列稀釋品**」，如下圖，準備 6 個 Eppendorf tube，先在這 6 個 tube 中都分別加入 300μL 的檢測稀釋液 Assay Diluent，接著在剛剛濃度是 30 ng/mL 的 stock standard 中取出 300μL 加入第一個 tube，用 Vortex 機器輕輕震盪混合均勻後，再從第一個 tube 混合液中取取出 300μL 加入第三個 tube 作一系列稀釋到第六個 tube，這樣 stock standard 的濃度是 30 ng/mL，第一個 tube 濃度是 15 ng/mL、第二個 tube 是 7.5 ng/mL、第三個 tube 是 3.75 ng/mL、第四個 tube 是 1.875 ng/mL、第五個 tube 是 0.938 ng/mL 和第六個 tube 是 0.469 ng/mL。



(6) 而 Assay Diluent 檢測稀釋液作為零標準品(0 ng/mL)，即 **control 對照品**溶液。

(7) 步驟(3)incubate 一個小時後接著重覆步驟(2)，將 96 well plate 放入實驗室內的自動洗滌機中洗滌 3 次。一樣在最後一次洗滌後，將 plate 倒置並用吸水紙吸乾多餘的液體。

(8) 如下圖，將 **stock 標準品 standard**(30 ng/mL)、**標準品系列稀釋品**(5 ng/mL、7.5 ng/mL、3.75 ng/mL、1.875 ng/mL、0.938 ng/mL 和 0.469 ng/mL)及 **control 對照品**(0 ng/mL)取 100μL 加至 96 well plate 對應的 well 中。其他剩下的 well 也各加入 100μL 要檢測的 sample(像目前是測 urine)，同一個人的檢體放兩個 wells，之後密封 plate，於室溫下 incubate 二個小時。



(9) [在步驟(8) incubate 二個小時快到的 20-25 分鐘前進行]製作 Working Detector 工作檢測試劑，在 9920μL 的 Assay Diluent 檢測稀釋液中，分別加入 40μL 的 detector antibody 及 40μL 的 Streptavidin-HRP，這樣就作出 1:250 濃度的 detector antibody 及 Streptavidin-HRP 溶液，就個就是 Working Detector 工作檢測試劑。

(10) 步驟(8)incubate 二個小時後接著重覆步驟(2)，將 96 well plate 放入實驗室內的自動洗滌機中洗滌 3 次。一樣在最後一次洗滌後，將 plate 倒置並用吸水紙吸乾多餘的液體。

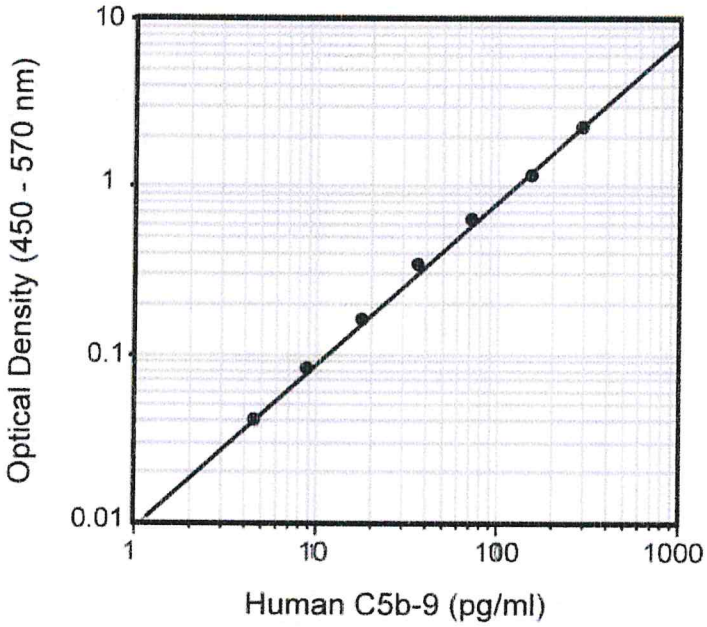
(11) 在 96 well plate 中分別都加入 100μL 步驟(8)做好的 Working Detector 工作檢測試劑，之後密封 plate，於室溫下 incubate 一個小時。

(12) 步驟(11)incubate 一個小時後接著重覆步驟(2)，將 96 well plate 放入實驗室內的自動洗滌機中，但這次要洗滌「七次」，一樣在最後一次洗滌後，將 plate 倒置並用吸水紙吸乾多餘的液體。

(13) 在 96 well plate 中分別都加入 100μL Substrate Solution 受質溶液。將 plate 置於暗處及室溫下 incubate 30 分鐘（這次不用密封膜 without plate sealer）。

(14) 步驟(3)incubate 半個小時後，在 96 well plate 中分別都加入 50μL Stop Solution 終止溶液。在停止反應後的 30 分鐘內，於 450 nm 波長下讀取吸光值(OD)。如果有波長校正功能，從 450 nm 吸光值(OD)中扣除 570 nm 吸光值(OD)。

(15) Human C5b-9 ELISA 的濃度和 optical density (450nm-570nm)有很好的 correlation 可以推算出 Human C5b-9 ELISA 的濃度。

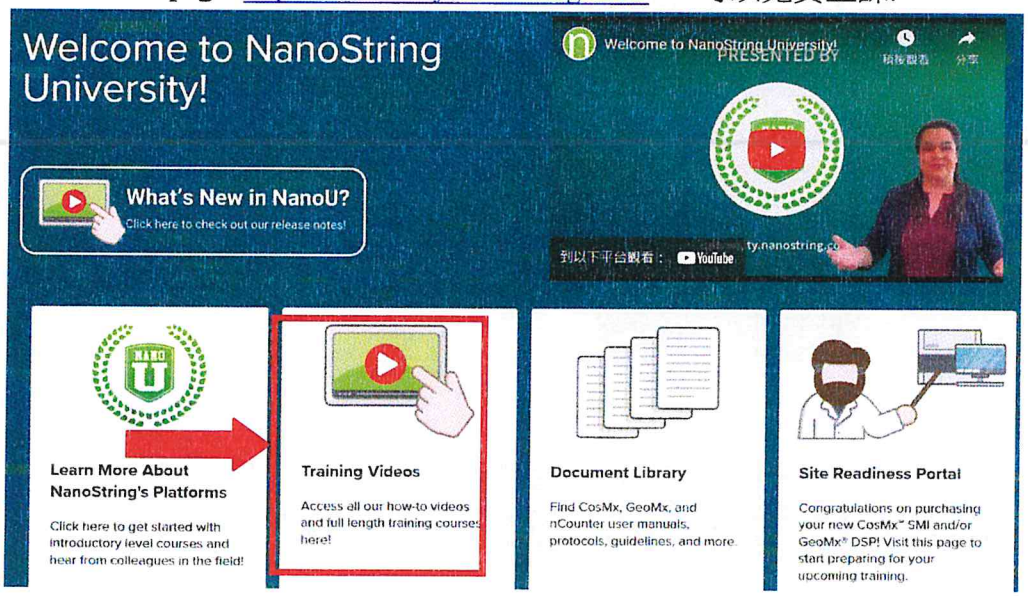


Number	Bar Code	OD1	OD2	VAR	Mean	SD	CV	Interpolation	anti-log	conc (ng/ml)
840	UK07-05	2.593	2.523	-2.7	2.558	0.049	1.9	1.904090488	80.18	80.18
841	AR05-02	0.255	0.237	-7.1	0.246	0.013	5.2	0.742673106	5.53	5.53
842	UK06-06	0.84	0.775	-7.7	0.8075	0.046	5.7	1.363721077	23.11	23.11
843	UK05-03	1.477	1.518	2.8	1.4975	0.029	1.9	1.656063205	45.30	45.30
844	AU08-08	0.467	0.505	8.1	0.486	0.027	5.5	1.112750759	12.96	12.96
845	UK03-01	0.399	0.427	7.0	0.413	0.020	4.8	1.028861209	10.69	10.69
846	US03-03	1.027	1.044	1.7	1.0355	0.012	1.2	1.482636928	30.38	30.38
847	UK01-03	0.2	0.185	-7.5	0.1925	0.011	5.5	0.590860993	3.90	3.90
848	UK01-02	0.632	0.701	10.9	0.6665	0.049	7.3	1.270405937	18.64	18.64

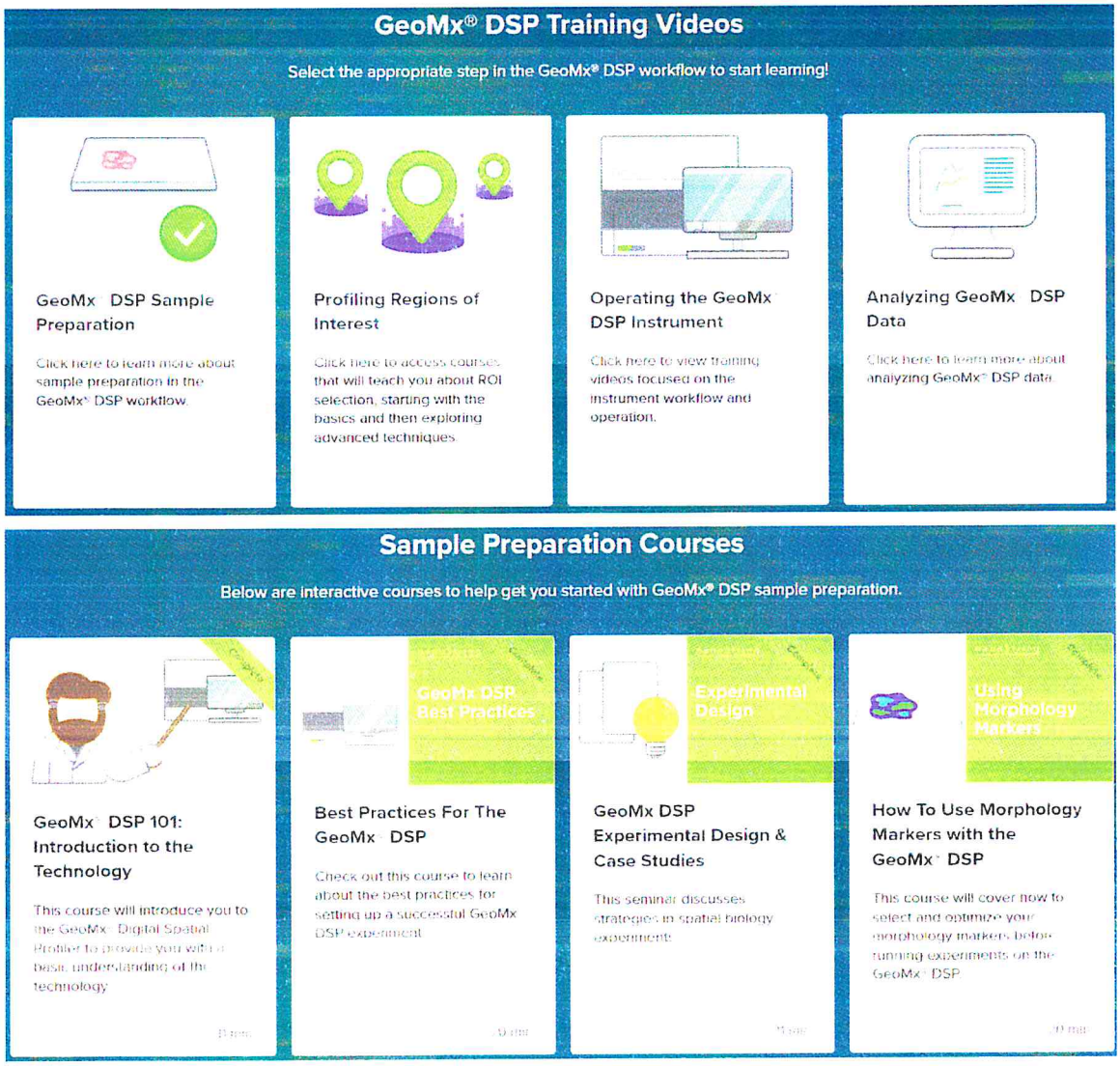
一樣節錄一部分我做的結果

(四) Digital Spatial Profiling 之學習

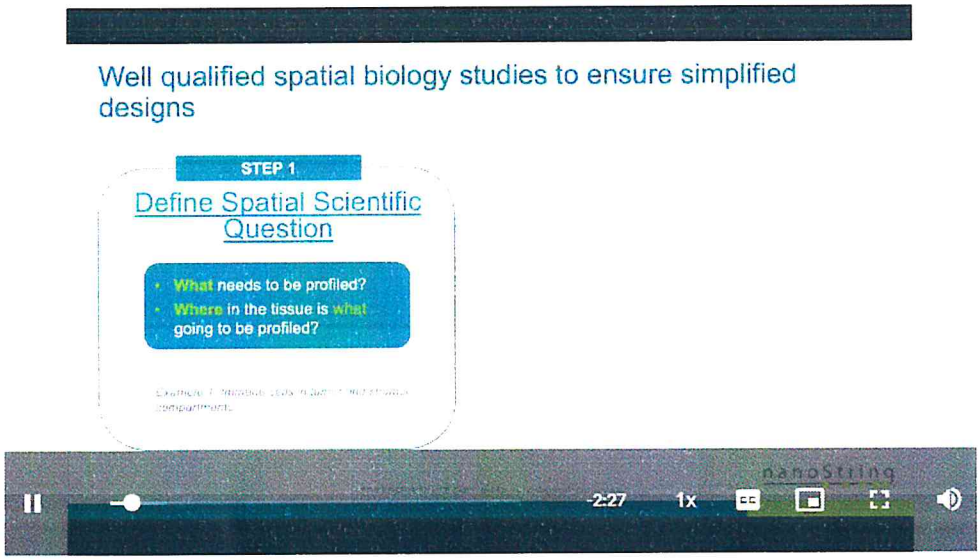
1. 在開始操作 GeoMx® Digital Spatial Profiling 前，實驗室的 Max 博士生(主修小兒腎臟)建議要先了解它的原理，他給我原廠上課網址 GeoMx®DSP manufacturers education page: https://university.nanostring.com/ ，可以免費上課.

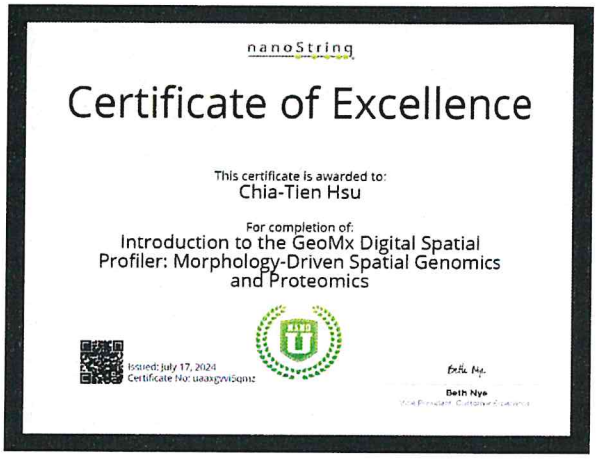


用機構 email 就可以建立一個帳號(像我就是用中榮的 email)，接著就可以上一些 training video，上完課回答一些課程內容的問題後，就會取得 certification.



To expand the video to full screen, click on on the square icon in the bottom right

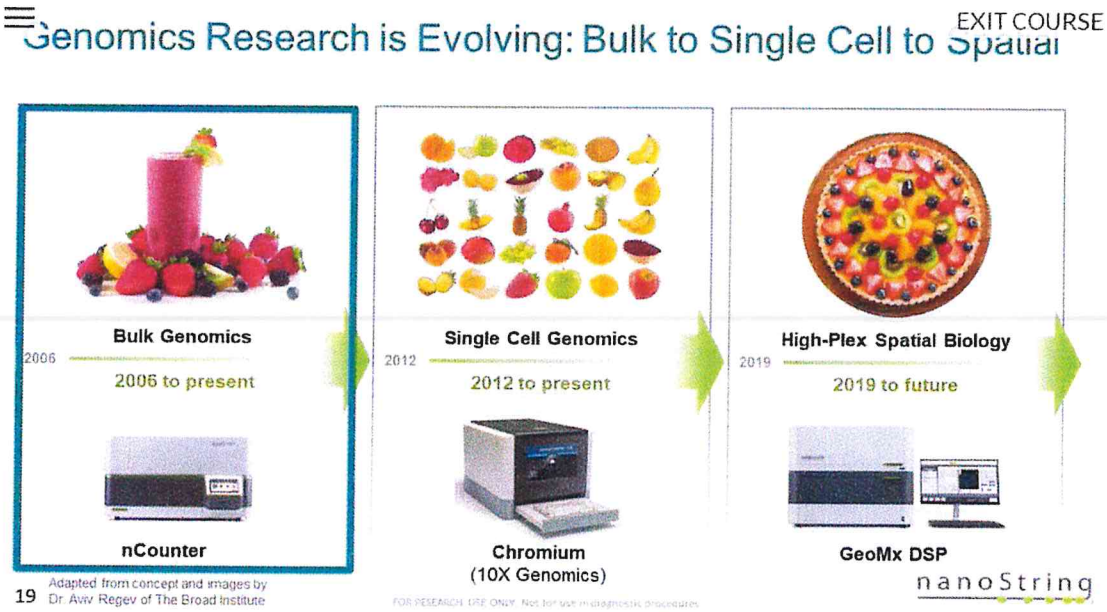




一樣節錄一部分我的 certification

2. 簡單說明一下 GeoMx® Digital Spatial Profiling 是什麼？

(1) 首先介紹目前 genomic research 的進展



- <u>Bulk genomics</u> 把整個 tissue 放入，去看整個 genomics 分佈或變化(混在一起，無法得知單一細胞種類的，也不知道空間上從組織的哪裡來)。
- <u>Single cell genomics</u>，分析單一特定 cell 的 genomics。單細胞定序

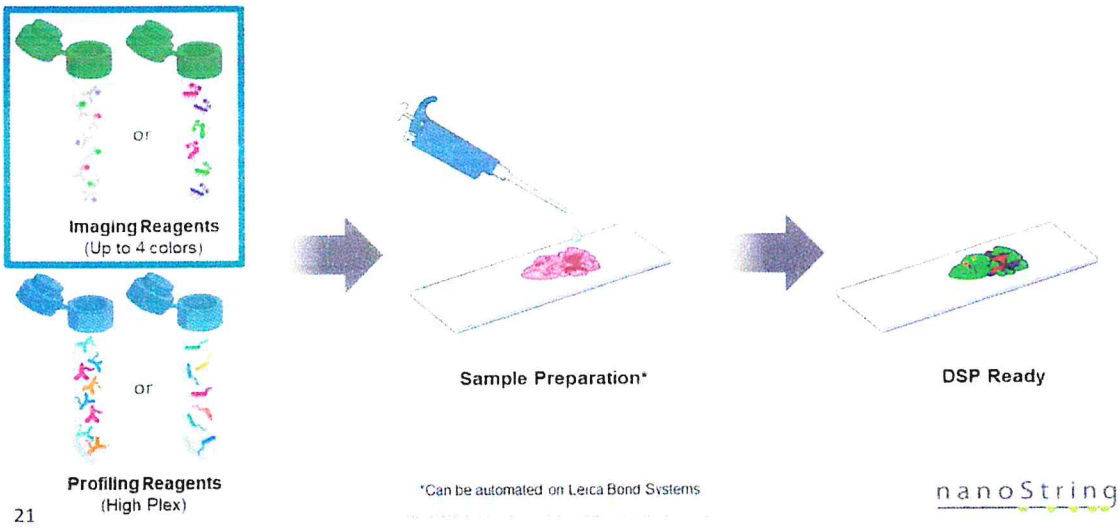
(Single-cell sequencing) 是一門高解析度(high resolution)的測序技術，相較於 bulk sequencing (傳統方法)，沒有把細胞們混在一起，所以我們能從裡面獲取更多資訊，因為即使相似細胞也有不同的表現(異質性 hetergeneous)。*但是 Single-cell sequencing 卻沒有「空間」的資訊，也就是我們不知道我們測定的單一細胞，是在組織中何處來的？是病灶處還是正常處？它們代表的意義不同。*

- High-Plex Spatial biology，可以在 tissue 內圈出有興趣的 ROI(從染色知道是什麼 ROI，例如：都圈 mesangial region)，然後看研究題目去再分要分析 analysis 的數個 AOI(analysis of interest)，分析這些 AOI genomics 分佈或變化，一個 ROI 可以再分成好幾個 AOI。例如：圈好 ROI 是 mesangial region 後，在這個 ROI 內，依細胞不同圈 AOI，像 macrophage 圈一個 AOI，mesangial cell 一個 AOI，其他有核細胞圈一個 AOI(control group?)，然後比較這些 AOI 內不同的 RNA genomic 或你有興趣的蛋白(激素)的定量資料。→*所以同時有空間定位資料，也有定位後定量分析的資料。*
- *一個切片或手術檢體，可能會有病灶區和非病灶區。所以若有空間定位上的資訊，sequencing 分析出來的結果才更能代表「疾病」的情形。Digital Spatial Profiling 有能力直接定位區分出病灶區和非病灶區的特定細胞(只要特定細本來能用特定幾種抗體辨識)，並進一步分析病灶區某一特定細胞的 sequencing。*舉例來說：目前已有很多 Digital Spatial Profiling 的應用，主要還是在腫瘤及免疾類的疾病。如：分析病灶區和非病灶區 T cell sequencing、分析對 immune checkpoint therapy 反應好和反應不好病人組織病灶區 T cell sequencing 找是什麼影響藥物反應等等。目前應用 Digital Spatial Profiling 來分析腎臟疾病的論文還很少，但腎絲球腎炎及腎移植方面(免疾機轉有關的疾病)的基礎研究是目前最適合的方向。

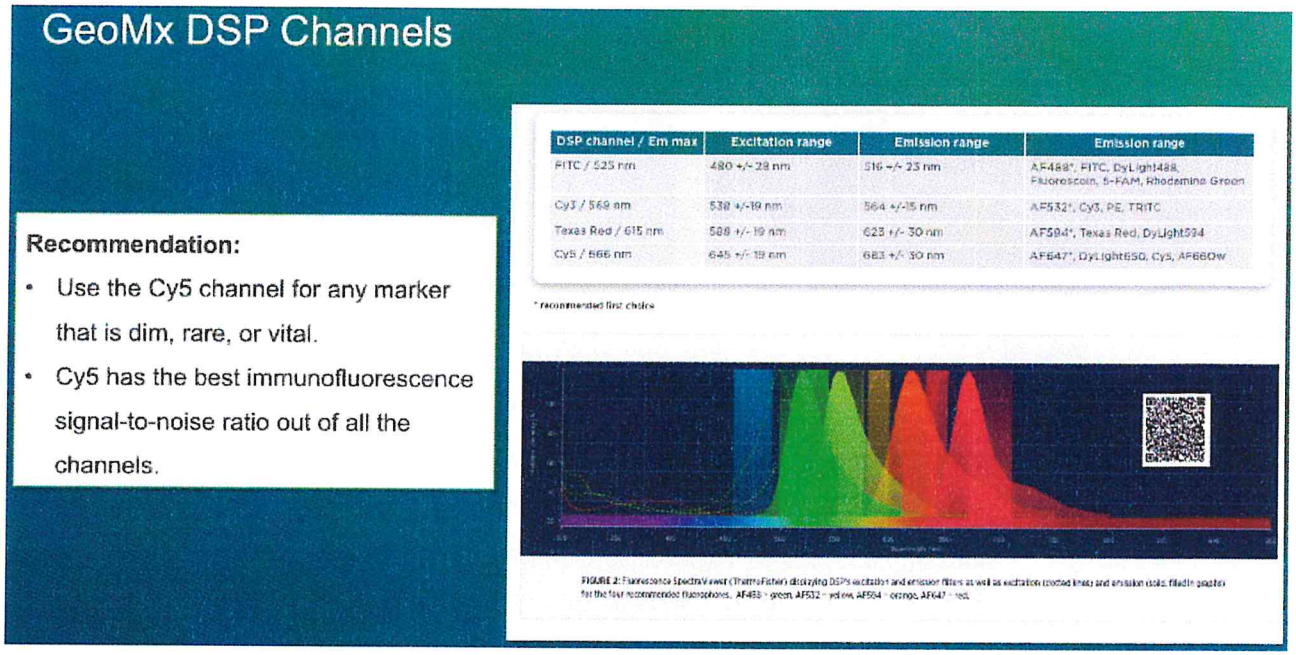
(2) 接著簡單介給一下 GeoMx® Digital Spatial Profiling 原理

- Sample 準備時會用(下圖)

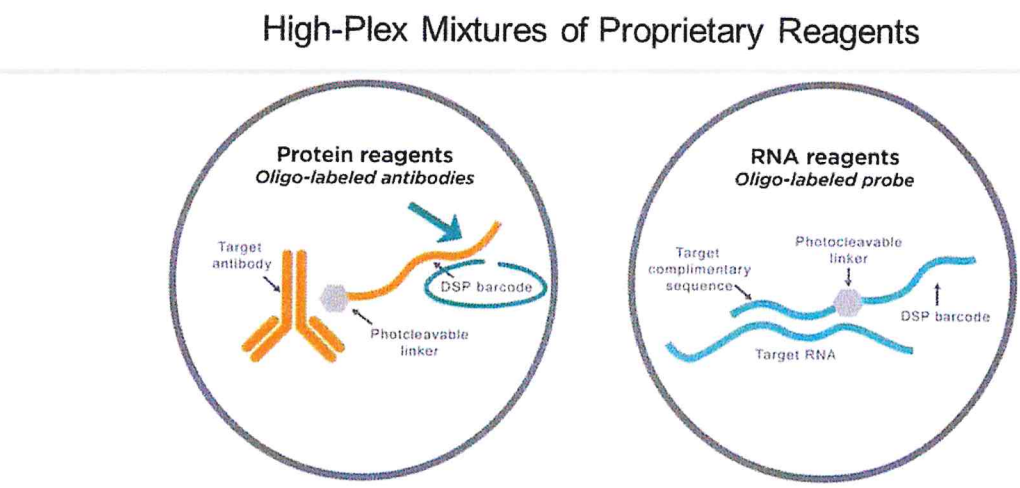
Imaging and Profiling in One Assay Using Standard IHG and ISH Protocols



■ 「第一個」是：imaging reagents(=morphology markers，四種顏色，例如：藍色染 CD58 代表 marcophage, 黃色染 CD10 代表 proximal tubular cell 和 podocyte，綠色染 DNA 代表所有的有核細胞，紅色染 alpha-SMA 代表 mesangial cell)，scan 後就有點像比較多顏色的 IF 染色影像，這樣就可以認出 mesangial region 後續圈 ROI = region of interest，也可以認出各 cell 的分佈。
Morphology markers: 4 channel, Cy5, Cy3, Texas Red, and FITC

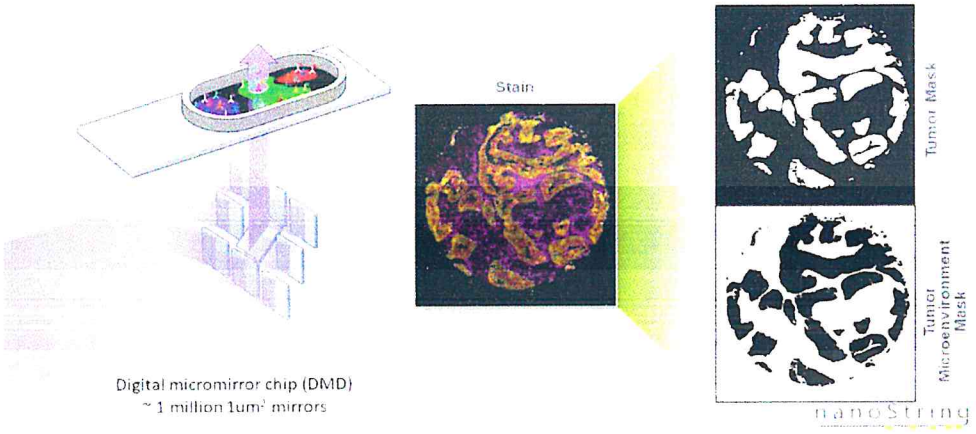


■ 「第二個」是：Profiling Reagents，就是後續圈出 AOI = analysis of interest 看你要分析什麼，分成下圖左邊圈圈中的 protein regents(有些廠商有客製化，有些可以用其他廠商的 antibody or reagent 先 optimize 校正後使用)，或右邊圈圈中 RNA reagents(GeoMx® Human Whole Transcriptome Atlas), 關鍵是下圖的 photocleavable linker 和 DSP barcode，照 UV 紫外光後 DSP barcode 就游離出來，可以收集去定量分析。

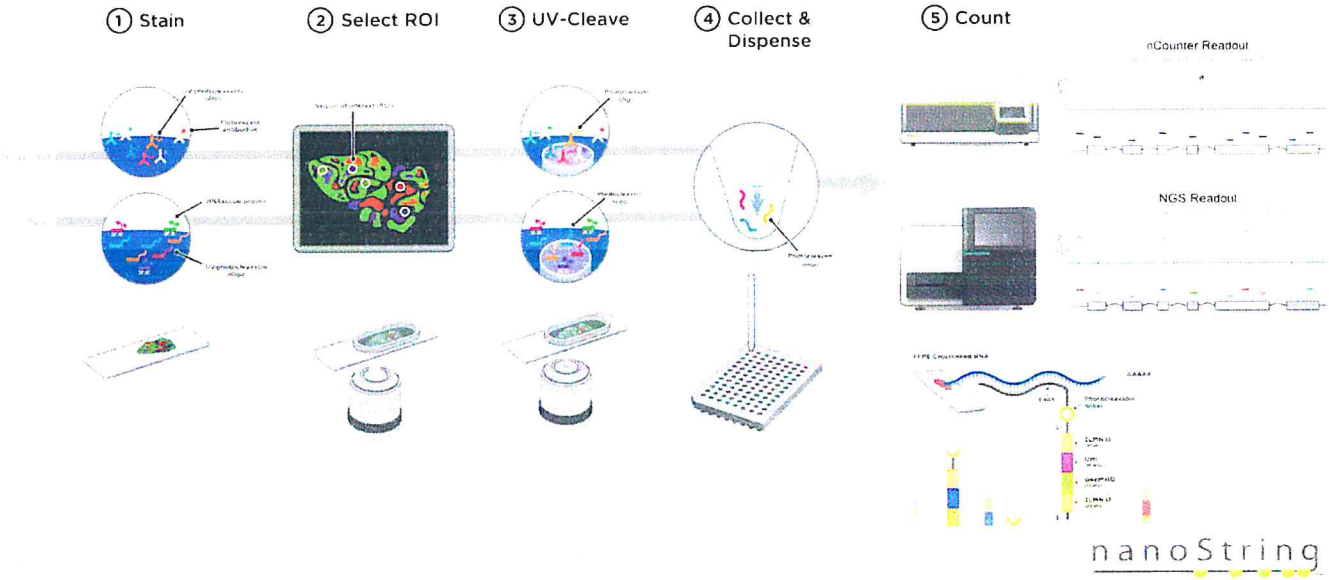


- 為什麼可以這個精準地只把你圈的小小 AOI 範圍照 UV 紫外光，分離出 DSP barcode 去定量分析？關鍵在下圖機器會有一百萬個大約 1um2 大(um 很小)的 digital micromirror chip (DMD)，只有你圈要分析 analysis 的 AOI 的小 mirrors 會變方向，讓 UV 紫化光去照到玻片 slide。

DSP Adaptive Optics Auto-Configure in Your Region of Interest



- 這些分離出來的 DSP barcode(你會知道他代表什麼，例如：AOI 是 marcophage，用 whole genomics RNA reagent，就是定量分析這個時候在 mesangial region 的 marcophage, Human Whole RNA Transcriptome 的分佈及變化)，會在下圖第 4 步被收集，之後在第 5 步被分析 profiling。操作的全部過程要三到四天。

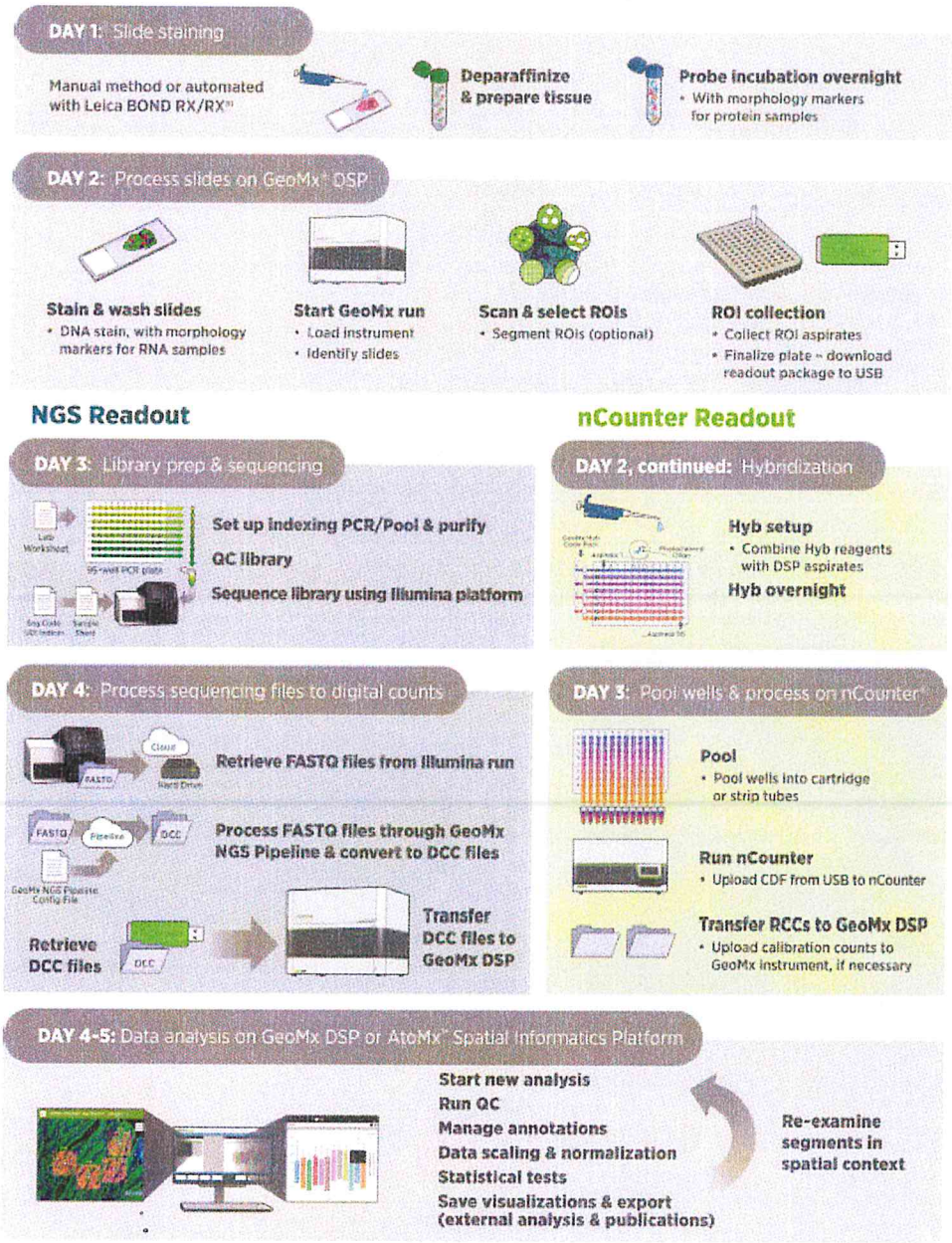


- GeoMx® Digital Spatial Profiling 台灣代理商：冷泉港生物股份有限公司，目前我查台灣只有台大和成大有機器。

3. GeoMx® Digital Spatial Profiling 的原廠說明書，Max 有給我，但是在 https://university.nanostring.com/page/document-library#platform_geomxr-digital-spatial-profiler

也可以下載。另外機器出現問題時，只要接上網路，和原廠人員連絡可以連線遠端維修(在實驗室有遇過一次)。

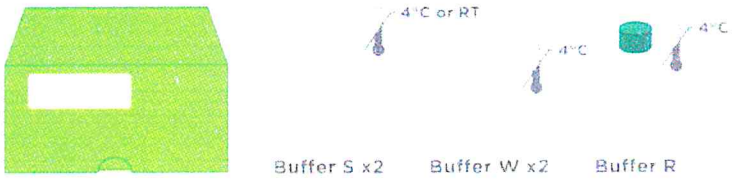
4. Mayer GeoMx® Digital Spatial Profiling 的步驟：



英文版的 Mayer GeoMx® Digital Spatial Profiling Protocol 如附錄四。

- **第一天**(*建議下午一點左右開始操作，這樣 hybridization 和 probe incubation 的時間到第二天早上剛好，不會太長或太短*)

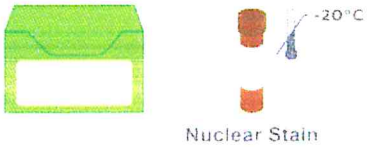
GeoMx RNA Slide Prep Kit



GeoMx Morphology Kit - Human or Mouse RNA compatible (various available)

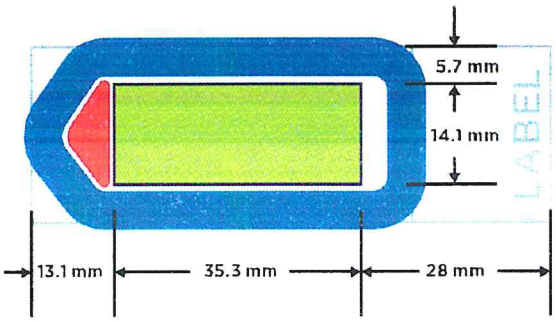
Example morphology kit (Solid Tumor TME)

GeoMx Nuclear Stain Morphology Kit



(1) 在開始準備檢體時 ，確認一下用的玻片大小可以放入 DSP 機器的 gasket for the GeoMx holder 。

實驗室的 GeoMx holder 有四 gaskets，所以一次最多可以分析四個玻片。

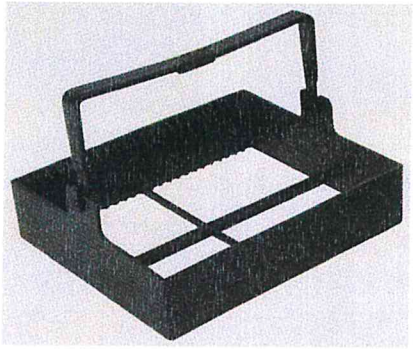


Gasket 可以放一片玻片的大小

若有組織在 gasket 的邊緣可能會造成機器出問題而無法分析。

(2) 將要研究的檢體玻片(最多四片)放在 slide holder 內，之後放入可以設定溫度的烤爐 lab oven 內，設定 60°C 加熱 60-70 分鐘(計時器帶在身上)。注意要記得在 slide holder 下方放一張耐熱的 lab tissue(有點像廚房紙巾的東西)來吸收融化的石蠟 melted paraffin(固定檢體在玻片上的石蠟)，來避免烤爐內後來留下石蠟。

實驗室內的 oven
可以設定溫度時間

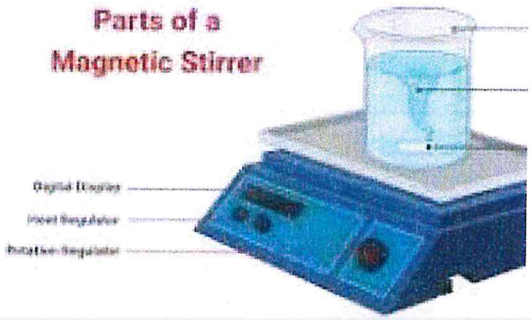


和實驗室一樣的
Slide holder

Lab tissue

(3) 準備福馬林 formalin 停止溶液 stop solution: 把 1.225g 的甘胺酸(Glycine)和 0.75g 的三羥甲基胺基甲烷(Trizma base)加入實驗反應瓶 reagent bottle 中，加入 100ml 的水，之後放入電磁攪拌器 magnetic stirrer bar 磁條，將實驗反應瓶 reagent bottle 整個放在電磁攪拌器 magnetic stirrer 上，用電磁攪拌器混合直到後面要使用福馬林停止溶液 stop solution 的步驟為止。

Reagent bottle



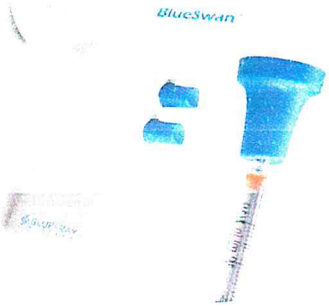
實驗室的電磁攪拌器 magnetic stirrer 及示意圖

(4) 用 70% IMS = 變性酒精(Denatured alcohol) 來消毒後面會使用的「玻片染色罐 coplin jars」，之後用蒸餾水 distilled water 沖洗 rinse 玻片染色罐 coplin jars。


實驗室的玻片染色罐 coplin jars


70% IMS

(5) 用「移液器」取出 20ml of 10X PBS 原廠 10 倍磷酸鹽緩衝生理鹽水(Phosphate buffered saline，簡稱為 PBS) 到另一個實驗反應瓶 reagent bottle，並加入 180ml dH2O 蒸餾水，製作出 200ml of 1X PBS ，即 200ml 的 1 倍磷酸鹽緩衝生理鹽水(1:1 濃度; 1X PBS)。

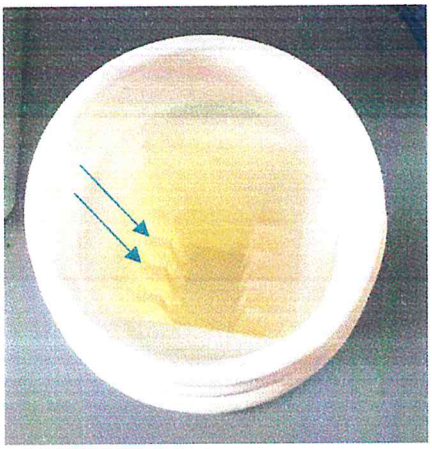


移液器　　原廠 10 倍磷酸鹽緩衝生理鹽水

(6) 把步驟(4)用 70%IMS 消毒好的玻片染色罐 coplin jars ，分別裝入下列溶液並標示好：

a. 2 個玻片染色罐 coplin jars 裝各 50ml 的 100% ethanol 乙醇，並蓋好蓋子 lid。

b. 1 個玻片染色罐 coplin jar 裝 50ml 的 95% ethanol (用 47.5ml 100% EtOH, 加入 2.5ml H_2O 來製作出來) ，並蓋好蓋子 lid。

xylene 二甲苯　　通風櫃 fume hood　　coplin jar 內有分隔玻片的間隔 slide line

c. 3 個玻片染色罐 coplin jars 裝各 50ml 的 xylene 二甲苯，並蓋好蓋子 lid。(注意 Xylene 有揮發性，大量吸入可能使人中毒，所以要在通風櫃 fume hood 操作，所有實際上不用精準裝剛剛好 50ml，如下右圖，只要裝超過玻片染色罐 coplin jar 內的 slide line 以上就好，用倒的不用移液器精準裝，如下右圖 coplin jar 內有分隔玻片的間隔，Xylene 溶液只要倒超過間隔的高度，放入玻片時就一定會全部浸在 Xylene 溶液內)。

d. 1 個玻片染色罐 coplin jar 裝 50ml 的蛋白酶 K(Proteinase K)，記得先用 RNAse ZAP 噴吸量管 pipette 後，才用吸量管 pipette 抽 2.5μL 的原廠 50 μg/μL 蛋白酶 K 加入 50ml 的水，並蓋好蓋子 lid。之後先將這個裝有 50ml 的蛋白酶 K 的 1 個玻片染色罐放入 37°C 的水浴中。

原廠 RNAse ZAP

原廠 Proteinase K

設定 37°C 的水浴

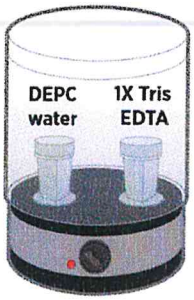
e. 1 個玻片染色罐 coplin jar 裝入步驟(5)製作好的 1 倍磷酸鹽緩衝生理鹽水(1X PBS) 50ml ，並蓋好蓋子 lid。

f. 1 個玻片染色罐 coplin jar 裝入 50ml 的 DEPC 處理水(Diethyl Pyrocarbonate, DEPC-treated Water) **[用 DEPC 處理過且經高壓滅菌後的超純去離子水，會經檢測不含 RNAse、DNAse 和蛋白酶]** ，然後不用蓋子 lid，而用金屬箔 metal foil 輕輕包住洞口。

g. 1 個玻片染色罐 coplin jar 裝入 50ml 的 Tris EDTA ，然後一樣不用蓋子 lid，而用金屬箔 metal foil 輕輕包住洞口。

Tris EDTA

金屬箔 metal foil
輕輕包住洞口

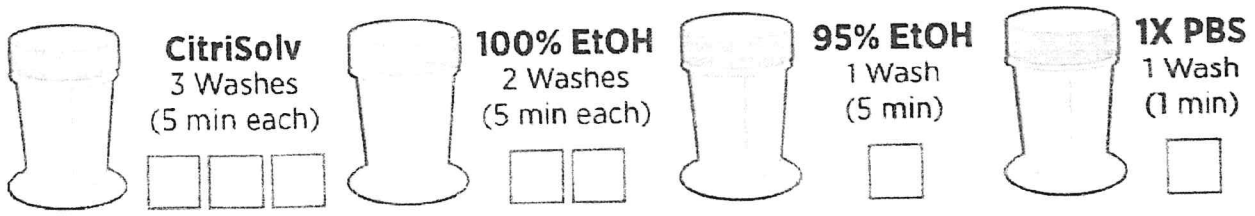


實驗室的蒸鍋 steamer 及示意圖

(7) 將步驟(6)第 f 和 g 步中 Tris EDTA 和 DEPC 處理水的玻片染色罐 coplin jars 放入蒸鍋 steamer 加熱至 100 度。

(8) 將第(2)步驟中，加熱 60-70 分鐘的 slide holder 及玻片從烤爐 lab oven 取出(之後很多都有計時，所以計時器要設定帶在身上，注意時間)。

(9) 將取出的玻片及上述步驟裝好的 3 罐 Xylene、2 罐 100% ethanol 乙醇、1 罐 95% 乙醇和 1 罐 1 倍 PBS 玻片染色罐帶到通風櫃 fume hood 內，用鑷子 tweezers 將玻片一一依照下圖的順序沖洗及置放(CitriSolv = Xylene)。每一個玻片放入時，用用鑷子在罐子內上下沖洗玻片(小心不要把檢體刮掉)至少三次，將所有的玻片都上下沖洗後一起在罐子內置放 5 分鐘。所以 3 罐 Xylene 就至少 15 分鐘，2 罐 100% ethanol 乙醇至少 10 分鐘，1 罐 95%乙醇至少 5 分鐘[**這些動作是在脫蠟 deparaffinization，此種有機溶劑脫蠟法可以將脫蠟後的組織回溶至水相溶液供後續使用**]。這些都完成後放在 1 罐 PBS 玻片染色罐內「至少」靜置 1 分鐘。



(10) 中間等待過程中去注意上一步驟中的蒸鍋 steamer 是否還有水，並用實驗用插入式溫度計 penetration thermometer，穿過金屬箔片插入蒸餾水的玻片染色罐底部的溫度是否已達到 97-100 度。

(11) 確認蒸鍋內 DEPC 處理水的玻片染色罐底部已達 97-100 度後(因為玻片放在 1 倍 PBS 內可以放 1-2 小時，只要至少一分鐘以上就好，所以若溫度未達 97-100 度，可以調整蒸鍋再等 5-10 分鐘再測溫度是否達到，達到後再做下一步即可)，用鑷子將 DEPC 處理水玻片染色罐上方的金屬箔拿掉，再玻片一一放置蒸鍋內 DEPC 處理水玻片染色罐沖洗約 10 秒後，用鑷子將 Tris EDTA 玻片染色罐上方的金屬箔拿掉，將玻片一一移到 Tris EDTA 玻片染色罐內加熱 15 分鐘[**在組織固定時會用福馬林，福馬林會讓 enzymes 去活性並從組織脫落，後續會干擾 RNA target 的測量，而 Tris-EDTA 可以逆轉這一過程，詳見 20012825,** https::wwww.jbc.org/article/S0021-9258(20)49515-8/pdf]。

插入式溫度計 penetration thermometer

(12) 這時可以先準備後面會用的 1 倍 PBS 玻片染色罐，把前面用過的倒掉，裝入新的 1 倍 PBS 溶液在玻片染色罐中。另外也先到水浴機那用實驗用溫度計 thermometer 測量步驟(6) d step 之蛋白酶 k 玻片染色罐底部溫度是否已達 37 度，若還沒有達到就調整機器設定讓溫度達到 37 度。

(13) 第(11)步玻片在 Tris EDTA 玻片染色罐加熱 15 分鐘後，關閉蒸鍋並將玻片一一移到新的 1 倍 PBS 玻片染色罐內靜置 1 分鐘。

(14) 同樣地確認水浴機中的蛋白酶 k 玻片染色罐底部溫度是否已達 37 度後(一樣玻片可以放在 1 倍 PBS 玻片染色罐內 1-2 小時，確認水浴溫度有達到 37 度再進行下一步)，將玻片一一移到水浴機內 37 度的水浴蛋白酶 k 玻片染色罐內靜置 15 分鐘(注意這步是 time dependent，不要超過 15 分鐘)[Proteinase K is a serine protease further facilitates antigen retrieval breaking down the peptide cross links promoted by formalin. It preferentially digests peptide bonds next to the carboxy terminal of aromatic amino acids, hydrophobic amino acids and sulfuric amino acids, so it also has the added benefit of preferentially breaking down ribonucleases, protecting RNA in the tissue]。

(15) 在這 15 分鐘的等待期間先把原廠的 buffer R 從冰箱取出靜置在室溫內，也把 NanoString whole transcriptome atlas probe set 從冰箱取出，但放在碎冰盒上(若是只做 antibody 的 optimization 實驗就不用加 whole transcriptome atlas probe set)。先準備後面會用的 1 倍 PBS 玻片染色罐，把前面用過的倒掉，裝入新的 1 倍 PBS 溶液在玻片染色罐中。

(16) 前一步玻片在蛋白酶 k 玻片染色罐 37 度水浴 15 分鐘後，關閉水浴機並將玻片一一移到新的 1 倍 PBS 玻片染色罐內靜置 5 分鐘。等待的 5 分鐘先去通風櫃 fume hood 將 10% neutral buffered formalin(NBF)倒入約 50ml 到 1 個玻片染色罐內(超過 slide line 玻片放入時可以全部泡入福馬林內就好，不用精準到 50ml，福馬林一樣不用移液器就倒的就好)，然後將步驟(3)製作好的 stop solution 分別裝入 2 個玻片染色罐內，裝入的量也是大約 50ml(一樣超過 slide line 玻片放入時可以全部泡入 stop solution 就好)。

(17) 新的 1 倍 PBS 玻片染色罐內靜置 5 分鐘後，用鑷子 tweezers 將玻片一一依照下圖的順序沖洗及置放。每一個玻片放入時，用用鑷子在罐子內上下沖洗玻片(小心不要把檢體刮掉)至少三次，將所有的玻片都上下沖洗後一起在罐子內置放 5 分鐘。所以 1 罐 10% neutral buffered formalin 就至少 5 分鐘，2 罐 stop solution 就至少 10 分鐘。

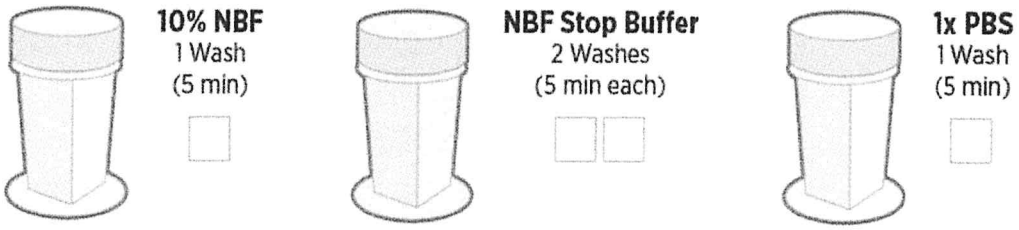
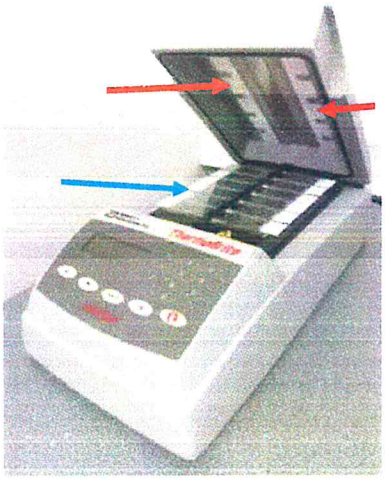


Figure 7: Post-fix wash steps

(18) 上一步驟等待過程可以先準備後面會用的 1 倍 PBS 玻片染色罐，把前面用過的倒掉，裝入新的 1 倍 PBS 溶液在玻片染色罐中。另外開始準備用來 Hybridization probe 雜合核酸探針的 hybridization chamber = incubator 核酸探針雜合機器，先用 DEPC 處理水擦拭一個塑膠托盤 plastic tray，之後打開 hybridization chamber 核酸探針雜合機器取出裡面的泡棉條(foam strips，下右圖紅箭頭有左右各一條白色的泡棉條)，將泡棉條在擦拭好的托盤內浸泡吸收 DEPC 處理水，接著將收 DEPC 處理水的泡棉條放回核酸探針雜合機器內。

實驗室的 hybridization chamber

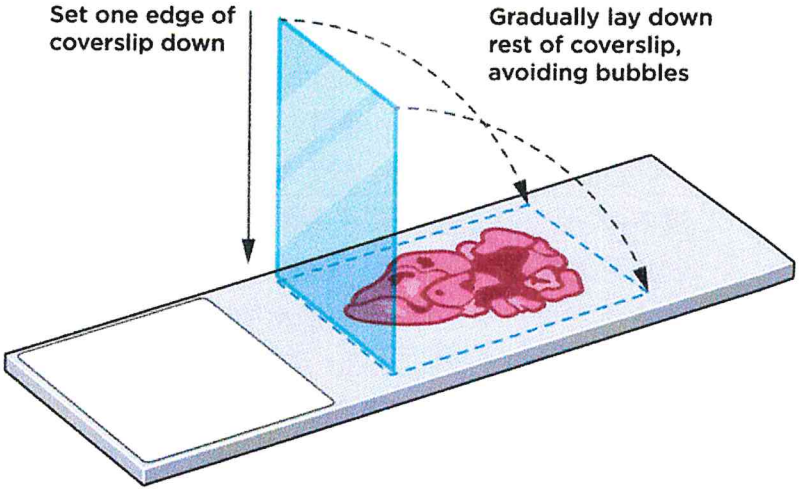


藍箭頭是放玻片的地方

RNAse away

(19) 準備 1.5ml 大小的 Eppendorf tube，每一片玻片要準備 55μL 的 RNA probe mix，所以要加入 44μL 的 buffer R、5.5μL 的 DEPC 處理水，以及 5.5μL 的 NanoString whole transcriptome atlas probe set (or dH2O if practice/optimization)，總共有 55μL(若 2 片玻片就上述數字 x2)，過程注意用量都很微量要好好加入，也儘量避免氣泡產生，另外只有步驟中有和 RNA probe 相關時，使用 pipette 前都要用 RNAse away 噴過。[**其實等一下每片玻片要用 50μL RNA probe mix 而已，準備 55μL 可以避免只有 50μL 全 pipette 時吸入空氣，剩下的 5μL 就是避免氣泡產生的關鍵之一。原廠建議 250μL 每一玻片，不過實際操作發現不用這麼多，所以實驗室修正為 50μL，不然這些試劑也很貴**]。

(20) 在第 17 步玻片在第 2 個 stop solution 玻片染色罐靜置 5 分鐘後，將玻片一一移到新的 1 倍 PBS 玻片染色罐內再靜置 5 分鐘。之後取出第一片玻片，用實驗室細 tissue(=microfiber tissue)吸乾玻片背面的 PBS，玻片正面不是檢體的地方也收，小心擦拭吸乾，不要刮到檢體。在玻片的檢體正上方加入 50μL 的 RNA probe mix，讓檢體在玻片上有被加入的 RNA probe mix 覆蓋住，注意過程一定要避免氣泡產生，若不小心產生就一定要讓檢體正上方沒有氣泡。接著把加好 RNA probe mix 的玻片上蓋上「蓋玻片 cover glass = coverslip」，接著放入 hybridization chamber 核酸探針雜合機器內。第一片玻片完成後才取出第二片玻片擦乾進行同樣加入 50μL 的 RNA probe mix 蓋上蓋玻片後，放入 hybridization chamber 核酸探針雜合機器內，玻片一一完成放入核酸探針雜合機器內後就蓋上機器的蓋子，run program 讓核酸探針雜合到明天早上。



原廠說明書交代如何蓋上「蓋玻片」避免氣泡產生

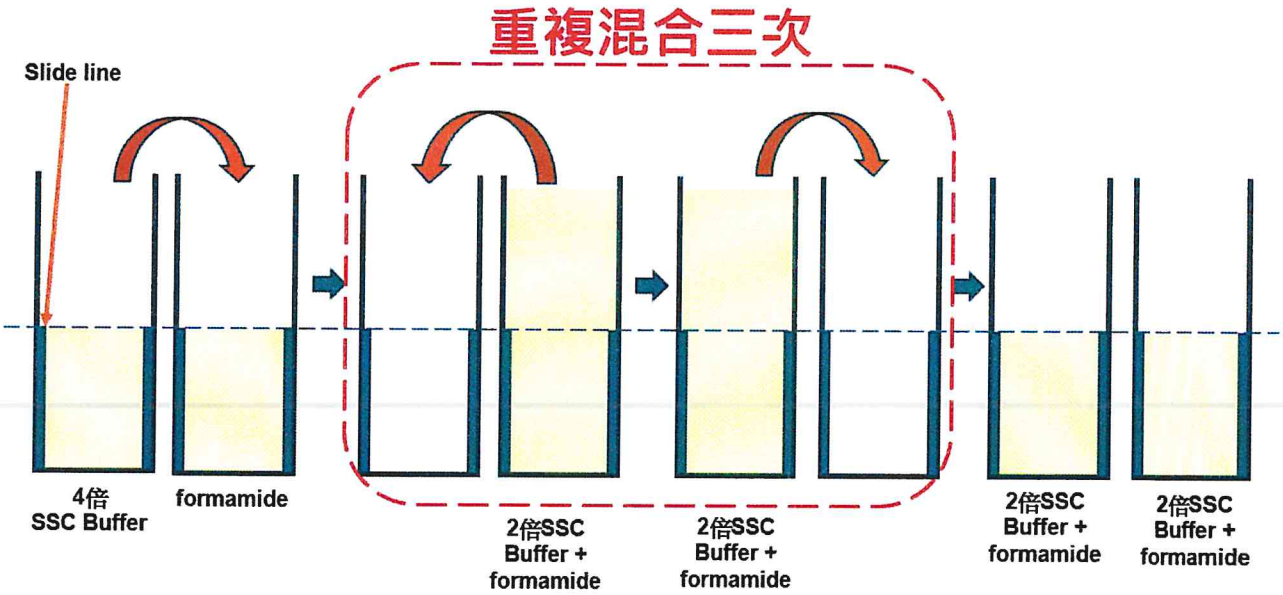
(21) 第一天就最後一步是把明早要用 50ml 的 100%甲醯胺 formamide 先從冷凍櫃移到冰箱退冰。

■ 第二天

(1) 先打開水浴機器，設定在 37 度。並從冰箱取出 Buffer W 室溫靜置。

(2) 製作 4 倍的 SSC Buffer(SSC Buffer is commonly used in nucleic acid hybridization techniques)，用移液器從原廠 20 倍的 SSC Buffer 的取出 35ml 到反應瓶內，之後加入 140ml 的蒸餾水，製作出 175ml 的 4 倍 SSC Buffer 溶液。

(3) 製作 2 罐甲醯胺 formamide 及 2 倍的 SSC Buffer 混合溶液玻片染色罐：



先將 2 個空的玻片染色罐用 RNAse away(dH2O if optimization/practice)清潔乾淨，接下來因為甲醯胺 formamide 有揮發性要在通風櫃操作。如上圖在其中一個空的玻片染色罐加入 4 倍的 SSC Buffer 到 slide line 就好，另外一個則加入甲醯胺 formamide，一樣到 slide line 就好(讓這兩罐量大至上相近)。接著將第一個 4 倍 SSC Buffer 玻片染色罐內的 4 倍 SSC Buffer 全部倒入甲醯胺

formamide 玻片染色罐內混合成 formamide 及 2 倍的 SSC Buffer 混合溶液(因為一個玻片染色罐內大約可以裝 110ml 容液，所以這樣才 8-90 分滿)，接著又將 formamide 及 2 倍的 SSC Buffer 混合溶液全部倒回 4 倍的 SSC Buffer 混合溶液倒出變成空的玻片染色罐內，這樣在 2 個玻片染色罐交替混合至少三次後，才將這個 formamide 及 2 倍的 SSC Buffer 混合溶液平均分配在這兩個玻片染色罐內(大至上就是剛好兩個液面都在 slide line，這樣其實不到 50ml，但足以將玻片都泡在溶液內就好)。最後把這 2 罐甲醯胺 formamide 及 2 倍的 SSC Buffer 混合溶液玻片染色罐(=Stringent Wash 溶液)放入 37 度的水浴機內水浴。

(4) 製作 3 罐 2 倍的 SSC Buffer 玻片染色罐：上一次最多使用 100ml 的 4 倍 SSC Buffer，第(2)步製造的 4 倍 SSC Buffer 最少還有剩下 75ml 以上，不過還是把剩下的 4 倍 SSC Buffer 用移液器測量並移入實驗反應瓶內，之後加入相同容量的蒸餾水來製作 2 倍的 SSC Buffer 並混合(例如：量出 4 倍 SSC Buffer 還有剩下 90ml，就加入 90ml 的蒸餾水，得到 180ml 的 2 倍的 SSC Buffer)，最後將實驗反應瓶內的 2 倍的 SSC Buffer 倒入 3 罐玻片染色罐內，一樣至少超過 slide line 就好。

(5) 都準備好後就停止核酸探針雜合機器，取出所有的玻片放入其中 1 罐 2 倍的 SSC Buffer 玻片染色罐中，放入時用鑷子上下來回幾次小心地移除蓋玻片 cover glass = coverslip(檢體不要被移除)。

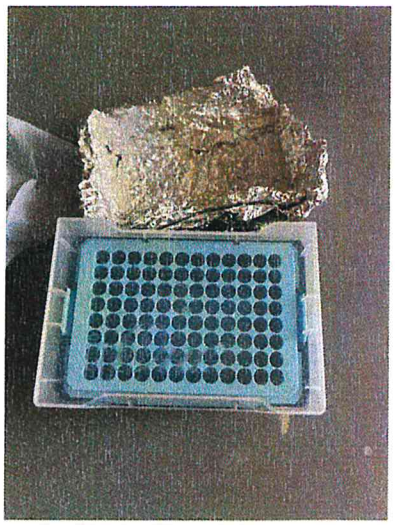
(6) 接著確認水浴中的 2 罐甲醯胺 formamide 及 2 倍的 SSC Buffer 混合溶液玻片染色罐底部是否有達到 37 度，若已達到 37 度就將玻片都移入第 1 罐甲醯胺 formamide 及 2 倍的 SSC Buffer 混合溶液玻片染色罐內，注意放入時每個玻片都上下來回 3 次以上，之後讓玻片維持在 37 度水浴中 25 分鐘。25 分鐘後再移到第 2 罐甲醯胺 formamide 及 2 倍的 SSC Buffer 混合溶液玻片染色罐內，一樣維持在 37 度水浴中 25 分鐘。[(Stringent Wash 這個步驟洗掉沒有雜合好的探針 poorly hybridised，不然它們會造成很強的背景雜訊]。

Figure 10: Stringent washes to remove off-target probes

(7) 在上述等待的第 2 個 25 分鐘內可以用來準備潮濕盒 humidity box，潮濕盒可以用專門的，也可以自製。自製的潮濕盒是用空的 pipette tip 盒子(如下中圖)，在裡面放入 lab tissue 並加入 DECP treated water 後，蓋回下右圖中藍色放 pipette tip 的架子，然後用錫箔紙包住盒子隔絕光線就好。

humidity box/chamber

自製 humidity box
裡面放入 lab tissue 並加入
DECP treated water



上面藍色放 pipette tip 的架子
就是<u>**之後放置玻片的地方**</u>

(8) 水浴中第 2 罐甲醯胺 formamide 及 2 倍的 SSC Buffer 混合溶液玻片染色罐 25 分鐘到時，將玻片移到第一個 2 倍的 SSC Buffer 玻片染色罐內靜置 5 分鐘，接著移到第二個 2 倍的 SSC Buffer 玻片染色罐內靜置 5 分鐘。

(9) 5 分鐘到時將第一個玻片取出，像第一天一樣用實驗室用的細 tissue 擦乾，小心不要擦掉檢體。之後把擦乾的玻片放在剛剛潮濕盒空的藍色架子上，在檢體上方加入 50μL 的 Buffer W 來 blocking，加好後不用蓋上蓋玻片。接著才取出下一片玻片來重複上面的擦乾及加 50μL Buffer W 的步驟，直到全部玻片都完成後才蓋上盒蓋，並用錫箔紙包住潮濕盒隔絕光線靜置 30 分鐘。

(10) 潮濕盒靜置 10 分鐘左右時，可以先將 nuclear stain 及要使用的 morphologic markers(antibodies)拿出來退冰至少 15 分鐘，之後準備 stain solution，nuclear stain 退冰好要用前還要先離心 30 秒(轉速 1000)。準備需要的 1.5ml 大小的 Eppendorf tube(若是 optimization 看不同濃度 morphology antibody stain 的情形時，一種濃度就要一個 Eppendorf tube)，收集所需的 Eppendorf tubes、NanoString nuclear stain，以及所需的 morphology antibodies = **relevant immunofluorescent** antibodies 放在 Eppendorf tube holder 上。

(11) 製作 stain solution，和第一天一樣一個玻片製作 55μL 的 staining solution，其中包括 5.5μL 的 NanoString 原廠 nuclear stain，和 XμL 的 immunofluorescent antibody – exact volume dependent on optimisation experiments，例如：CD138 我們試過 1:10，1:50 和 1:100 三種濃度，最少用 1:50 可以看得清度，1:100 就有點不清楚，所以我們這時算出 CD138 1:50 要加入 1.1μL。剩下不足 55μL 的部分就加入對應的 buffer W 量來補足，例如上面加 5.5μL nuclear stain、1.1μL 的 CD138，buffer W 就要加 48.4μL 來讓最後的 staining solution 變成 55μL。以上是一片玻片的量，有幾片玻片上面的 staining solution 就乘以幾倍，二片就要有 110μL 的 staining solution。

(12) 潮濕盒靜置 30 分鐘時間到後，就將第一片玻片取出，和之前一樣用實驗室

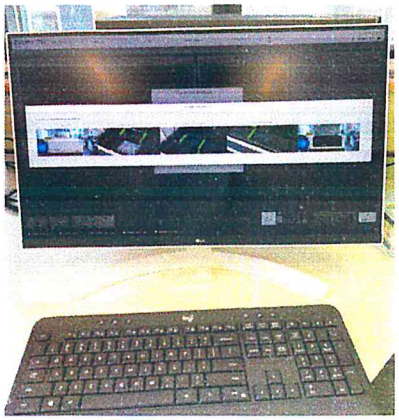
細的 tissue 擦乾放回潮濕盒架子上，之後一樣在檢體上加 50μL 的 staining solution(剩下的 5μL 一樣是用來避免產生氣泡的)，一樣小心不要產生氣泡在檢體上，之後不用蓋上蓋玻片。第一片玻片好了再重複一樣的步驟(擦乾，加 staining solution)完成第二片。在所有的玻片都完成後就蓋上潮濕盒的蓋子及用錫箔紙包住避光靜置 1 小時。

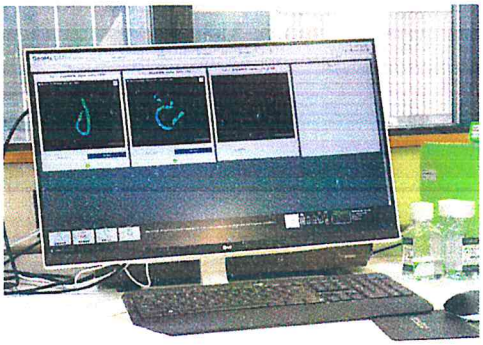
(13) 準備 2 個新的 2 倍的 SSC Buffer 玻片染色罐，上一步靜置 1 小時後就將玻片移入第一個 2 倍的 SSC Buffer 玻片染色罐靜置 5 分鐘，之後再第二個 2 倍的 SSC Buffer 玻片染色罐靜置 5 分鐘。

(14) 之後就可以準備上 DSP 機器了，第二個 2 倍的 SSC Buffer 玻片染色罐靜置 5 分鐘後將第一片玻片取出，用實驗室細的 tissue 擦乾後，玻片的背面用 70%IMS 變性酒精消毒擦拭，之後將擦乾的玻片如下圖放入 DSP 機器的 slide holder 第一個 gasket 中，接著蓋好 gasket，在第一個 gasket 上中空處(紅箭頭處)加入大約 6ml 的 buffer S，將玻片上方用 buffer S 覆蓋。第一片玻片完成後才取下一片重複上面的步驟，最多只能放 4 個玻片。

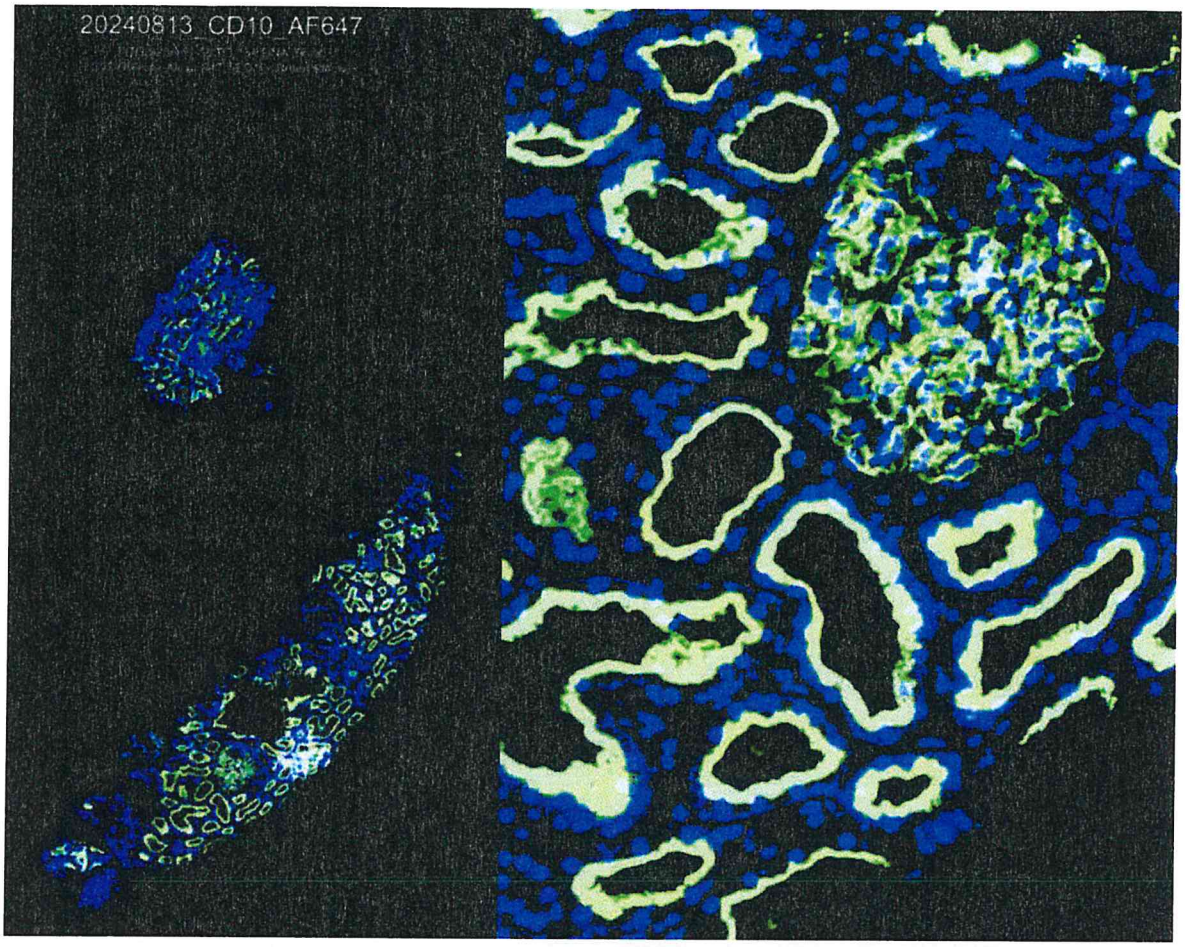
紅箭頭是將 buffer S 加入 gasket 的地方

(15) 將 DSP 機器的 slide holder 放回機器，之後就照 screen 上的指示圈 ROI 等。

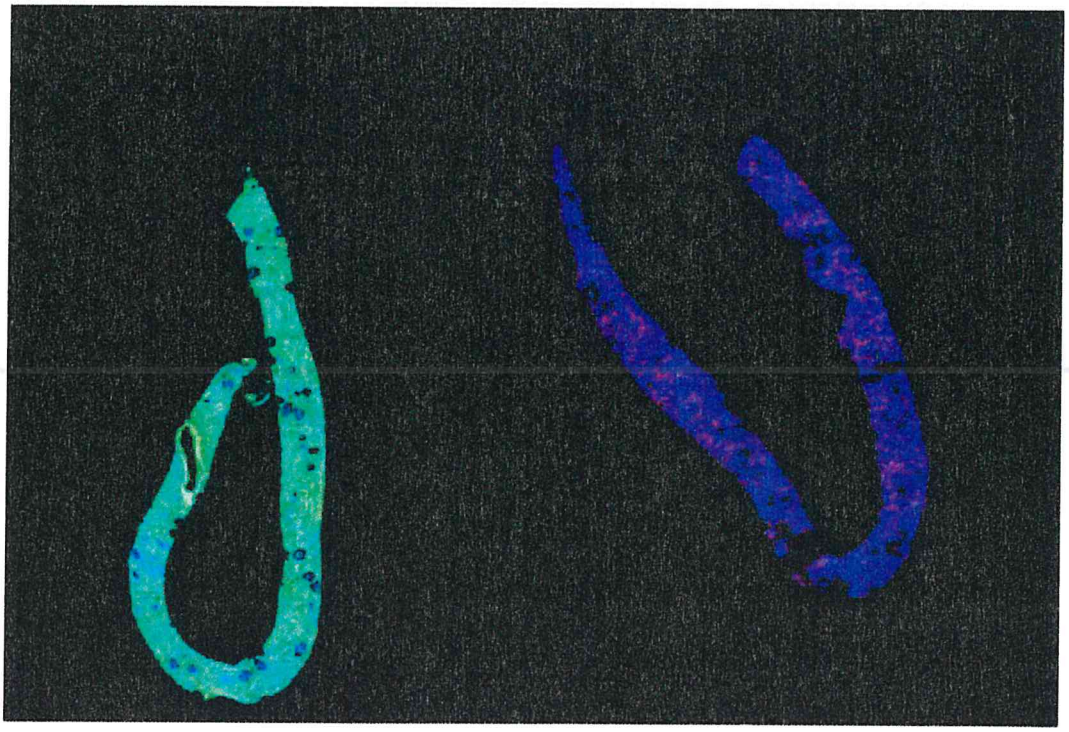




■ 第三天後的操作都在機器上，可以直接看附件即可。



α-SMA CD138



(五) **台灣研究論文撰寫**：在英國期間也用其它空閒時間，將在台灣已有的資料整理、跑統計及寫程式跑機器學習模型，目前已完成三篇論文的撰寫及投稿：

1. 第一篇論文「Clinical Audit-based Corrective Actions to Reduce Insufficient Glomeruli Under Light Microscopy」，已投稿至 *Frontiers in health services*。
2. 第二篇論文「Impact of Patient Education by Nurse Case Managers on Decision Making for Out-of-Pocket Anti-Osteoporotic Pharmaceutical Therapy: a Single-Center Retrospective Study」，已投稿至 *BMC Nursing*。
3. 第三篇論文「Machine Learning Models to Predict Osteoporosis in Patients with Chronic Kidney Disease Stage 3 – 5 and End-Stage Kidney Disease」，已投稿至 *BMC Medical Informatics and Decision Making*。

(六) 陳呈旭主任要我詢問「目前有個 Nefecon 的藥，聽說藥 8000 元台幣，一天四顆，能幫我打聽一下，英國或歐盟賣多少，是否有 indication 限制？」問了 Professor Jonathan Barratt，教授也附上英國 NICE 的建議「Targeted-release budesonide for treating primary IgA nephropathy」文章，並回答我。節錄重點如下：

1. Nefecon is approved in the UK and NICE approved and I am using it in my practice. The current approval is for use in patients with an eGFR >35ml/min and a UPCR >1.5g/g but this is likely to change when the drug has a full approval. The dose is 16mg per day for 9 months, but it is likely that repeated dosing will be required.
2. The list price of targeted-release budesonide is £6,528.54 for a 120 pack of 4 mg modified-release hard capsules. £6,528.54 除以 120 顆，一顆£54.4045 = NTD2295.85，一天要用四顆，所以一天藥價換算台幣 NTD9,183.4。符合 indication 是 NHS 支付 9 個月。

   https://www.nice.org.uk/guidance/ta937

   Nefecon = Targeted-release budesonide，在迴腸才 release 針對腸道 IgA pathogenesis 中的 mucosa-associated lymphoid tissue(MALT)去治療，比傳統的口服 steroid 使用少了很多系統性的副作用。

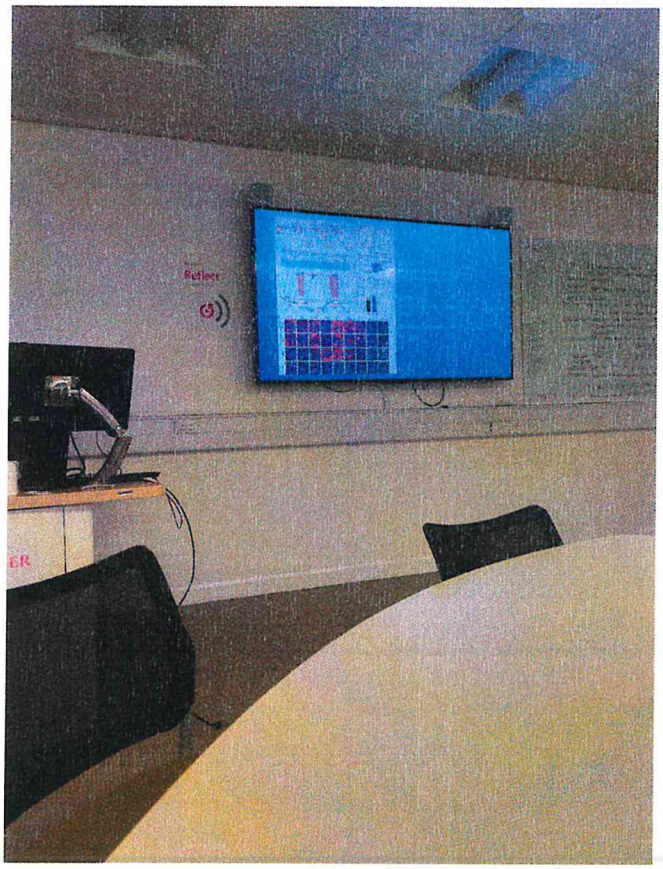
三、 心得

Mayer IgA Nephropathy Laboratories 因為 Professor Jonathan Barratt 是在 IgA nephropathy 的國際知名學者，為多篇 JAMA, NEJM, CJASN 等高分期刊之 corresponding author，也是 IgA nephropathy 許多跨國臨床試驗的總 PI。因此到此學習的同時也有很多來學習的學生及學者來參訪(從 2 星期到 1 年都有)，從高中生、醫學生到研究生，從醫師到醫檢師都有。在我離開前半個月又有新的博士後研究員來實驗室，也是要應用 Digital Spatial Profiling。

實驗室的人員都親切教導，實驗室人員實事求是、態度輕鬆但是做事嚴謹，實驗室內每一處都有標示清楚，讓新進人員及來參訪的人員很快能熟悉器材、用具放在哪？也會熟慣建立 SOP 或 protocol，這樣能快速上手，實驗試劑及耗材使用也不吝嗇讓大家使用，實驗室是由 Jimmy Mayor 捐錢設立，從 2012 年他兒子 David Mayor 知道有 IgA nephropathy 到今年總共捐了超過 400 萬英鎊(大約 1 億 6 千 6 百多萬臺幣)。進實驗室第一天報到，環境介紹取得帳號後，第二天給我實驗室安全手冊閱讀，讓新進及參訪人員每一個都了解實驗室安全規定(如：失

火、有害物質洩漏等等)，也有學校專門的通報 APP。接著當天馬上就用滴管訓練在 96 wells plate 滴出每三排不同的濃度去測，每一濃度區 Coefficient of variation (CV)要控制在 10%(所有新進及來訪人員都會這樣)，之後才開始操作 ELISA 檢驗。第一個月操作 ELISA 檢驗(urine creatinine and C5-C9 及 Sparsantan trial 的血清檢驗)，看檢體來源來各世界各地，是 IgA nephropathy 臨床試驗的檢體。接著用實驗室 health renal biopsy 的檢體來操作 Digital Spatial Profiling 之檢體準備，並嘗試各 custom 抗體(非 GeoMx®原廠其它廠商生產之抗體)之濃度 optimization。

在這三個月中，也參加幾次實驗室團隊的 journal reading，研讀的論文都是基礎研究，輪值報告的人並很少會做投影片，而是先寄給大家後，當天幫大家唸論文重點，之後就開始討論及提問，資深人員也常常指出論文中有哪些需要改進的地方，例如：論文比較 IgA nephropathy 比較嚴重的老鼠某給 gene 表現比疾病比較輕的多，除了這兩組外也要有 health control group 等等，另外針對論文中的文字也是很注重邏輯和有幾分證據說幾分話，常常實驗室資深人員也點出論文中過度推論的地方，所以很注重研究實驗設計及邏輯的正確性。



Journal club 難得一次有看到做投影片報告，這次主題是發表在 Nature 的文章「Design and testing of a humanized porcine donor for xenotransplantation」是之前基因編輯人類腎臟移植的文章。

Team news

Viva success

We are happy to announce that our PhD student, Hareesh Selvaskandan, successfully passed his PhD viva. A viva, an oral examination, is a critical component of earning a PhD degree. During the process, the candidate presents their research and thesis to an examination panel, engaging in a detailed discussion where examiners pose questions about the research methods, findings, and conclusions. It's also an opportunity for the candidate to clarify any ambiguities and defend their research.

HARESH CELEBRATING WITH HIS FAMILY

In his PhD project, Hareesh used Digital Spatial Profiling, which is an exciting new technique for studying which genes are switched on or off in microscopic parts of kidney biopsies, to understand what mechanisms might cause severe forms of IgA nephropathy. We are delighted to share that Dr. Selvaskandan has also been appointed as an Academic Clinical Lecturer at the University of Leicester. He will continue his research with our group alongside his clinical duties as a kidney doctor. Additionally, he has been awarded a Fellowship by the European Renal Association to pursue an MSc in clinical trial design at the University of Oxford. We congratulate him on his outstanding achievements and wish him continued success in all his future endeavours.

New team members

We are excited to welcome new members to our team since the last newsletter.

The Mayer IgA Nephropathy Laboratory

ISHIKA KHAN (RIGHT) AND DR. CHIA-TIEN HSU (LEFT)

Ishika Khan has joined us as a Research Technician. Ishika holds a BSc in Biotechnology and a Master's degree in Applied and Experimental Clinical Immunology. She aims to gain valuable experience in our research laboratory before pursuing a PhD. Ishika has quickly established herself as a hardworking and dedicated team player, and is rapidly learning the unique laboratory techniques we have developed in Leicester.

Dr. Chia-tien Hsu, visiting Researcher:

Here is what Dr. Hsu has to say about his visit:

Hello everyone, I am Dr Chia-tien Hsu from Taiwan, a nephrologist. My primary purpose for coming to the Mayer IgA Nephropathy Laboratories in Leicester University is to learn about the GeoMx Digital Spatial Profiler (DSP) used by Hareesh in his PhD. The GeoMx DSP is an exciting new technique for studying which genes are switched on or off in microscopic parts of kidney biopsies. While research and operations for kidney biopsy samples are still limited, we can use the DSP to understand why IgAN develops and why patients respond to particular treatments. I plan to compare changes in the switching on or off of genes in the kidneys of patients from Taiwan and patients from the UK. While here I also hope to see a little of the UK.

Temporary Staff

We also welcome two new temporary team members to help work on all the studies we have running at the moment: Vlad will return to his undergraduate studies in September, while Alfie plans to continue his education by pursuing a PhD after his role here.

VLAD TORANOV (LEFT) AND ALFIE BARRATT (RIGHT) HAVE JOINED US AS PREVIOUSLY UNDERTAKING WORK EXPERIENCE IN OUR LABORATORY

To unsubscribe click here or email rc121@le.ac.uk

DR ROBIN THOMAS IGA NEPHROPATHY RESEARCH GROUP MANAGER

Team news: 左下是我和另一位也是今年中新上工的 Ishika Khan，她是 Research Technician，生科碩士，主修應用實驗臨床免疫學。之後會在萊斯特大學攻讀博士學位。

四、 建議事項

(一) 邀請 Professor Jonathan Barratt 來本院演講，目前初步和教授約 10 月來台灣至本院演講。

(二) IgA nephropathy 目前有一些補體治療的臨床試驗，本院可以考量是否可以參加。

(三) 另外也可以研究測量 Human C5b-9 ELISA 和疾病嚴重度、預後上的差別，初步有一些 correlation，但需要更多文獻來釐清，我們也可以進行研究。

(四) 擴大和 Mayer IgA Nephropathy Laboratories 合作研究案：後續可以先討論好 Oxford classification, MEST-C 分數 match 台灣和英國的情形，進一步設計個案數多一些的實驗來應用 GeoMx® Digital Spatial Profiling。也可以考量多科合作或台灣跨院合作，目前台大及成大有 GeoMx® Digital Spatial Profiling 機器，主要還是癌症及免疫相關科別的研究比較多。在腎臟科的論文目前還很少，美國、英國、中國、南韓目前有零星腎臟科應用 GeoMx® Digital Spatial Profiling 的論文。

五、 附錄

1. QuantiChromTM Creatinine Assay Kit (DICT-500)原廠 protocol
2. BD OptEIA™ Human C5b-9 ELISA Set 原廠 protocol
3. Human C5b-9 ELISA Set Mayer IgA Nephropathy Laboratories 員工 summarized protocol
4. Mayer GeoMx® Digital Spatial Profiling Protocol

附件1

QuantiChrom™ Creatinine Assay Kit (DICT-500)

Quantitative Colorimetric Creatinine Determination at 510nm

DESCRIPTION

Creatinine is synthesized in the body at a fairly constant rate from creatine, which is produced during muscle contractions from creatine phosphate. In the blood, creatinine is removed by filtration through the glomeruli of the kidney and is secreted into urine. In healthy individuals, creatinine secretion is independent of diet and is fairly constant. The creatinine clearance test has become one of the most sensitive tests for measuring glomerular filtration rate. In kidney disease, creatinine levels in the blood are elevated, whereas the creatinine clearance rate and hence the urine levels are diminished. Creatinine test is most widely used to assess kidney function.

Simple, direct and automation-ready procedures for measuring creatinine concentration in biological samples are becoming popular in Research and Drug Discovery. BioAssay Systems' creatinine assay kit is designed to measure creatinine directly in biological samples without any pretreatment. The improved Jaffe method utilizes picrate that forms a red colored complex with creatinine. The intensity of the color, measured at 510nm, is directly proportional to creatinine concentration in the sample. The optimized formulation substantially reduces interference by substances in the raw sample.

KEY FEATURES

Sensitive and accurate. Use 30 µL samples. Detection limit 0.10 mg/dL (8µM) creatinine in 96-well plate assay.
Simple and high-throughput. The procedure involves addition of a single working reagent and incubation for 5 min. Can be automated as a high-throughput assay for thousands of samples per day.
Improved reagent stability and versatility. The optimized formulation has greatly enhanced reagent and signal stability. Assays can be executed in 96-well plate or cuvet.
Low interference in biological samples. No pretreatments are needed. Assays can be directly performed on raw biological samples.

APPLICATIONS

Direct Assays: urine, serum, plasma and biological preparations.
Drug Discovery/Pharmacology: effects of drugs on creatinine metabolism.

KIT CONTENTS (500 tests in 96-well plates)

Reagent A: 50 mL **Reagent B: 50 mL**
Creatinine Standard: 1 mL 50 mg/dL

Storage conditions. The kit is shipped at room temperature. Store Reagents A and B at 2-8°C and Creatinine Standard at –20°C. Shelf life: 12 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Equilibrate reagents to room temperature prior to use. Please note the difference in standard/sample volume and Working Reagent strength for blood and urine assays. This assay is based on a kinetic Jaffe reaction. To ensure identical incubation time, addition of Working Reagent to standard and samples should be quick and mixing should be brief but thorough. *Use of a multi-channel pipettor is recommended.*

Procedure using 96-well plate:

BLOOD ASSAY (LOW CREATININE LINEAR UP TO 50 mg/dL):

1. Dilute standard to 2 mg/dL by mixing 5 µL 50 mg/dL standard stock and 120 µL distilled water. Transfer 30 µL diluted standard and serum/plasma in duplicate into wells of a clear bottom 96-well plate.
2. Prepare enough Working Reagent by mixing per well reaction at least 100 µL Reagent A and 100 µL Reagent B. Add 200 µL Working Reagent *quickly* to all wells. Tap plate briefly to mix.
3. Read optical density immediately (OD_0) and then at 5 min (OD_5) at 490-530nm (peak absorbance at 510nm).

URINE ASSAY (HIGH CREATININE LINEAR UP TO 300 mg/dL):

1. Transfer 5 µL 50 mg/dL standard and urine in duplicate into wells of a clear bottom 96-well plate.
2. Prepare enough Working Reagent by mixing per well reaction 50 µL Reagent A, 50 µL Reagent B and 100 µL water. Add 200 µL Working Reagent *quickly* to all wells. Tap plate briefly to mix.
3. Read optical density immediately (OD_0) and then at 5 min (OD_5) at 490-530nm (peak absorbance at 510nm).

Procedure using cuvette:

1. Transfer 100 µL 2 mg/dL Standard and serum/plasma samples (*Urine Assay: 15 µL 50 mg/dL Standard and 15 µL urine*) to cuvets.
2. Prepare appropriate Working Reagent as above for the 96-well plate procedures. Add 1000 µL Working Reagent to each cuvet and pipet *briefly* to mix (avoid bubble formation).
3. Read OD immediately (OD_0) and at 5 min (OD_5) at 490-530nm.

CALCULATION

Creatinine concentration of the sample is calculated as

$$= \frac{OD_{SAMPLE\,5} - OD_{SAMPLE\,0}}{OD_{STD\,5} - OD_{STD\,0}} \times [STD] \text{ (mg/dL)}$$

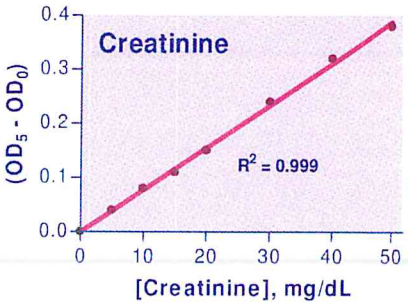
$OD_{SAMPLE5}$, $OD_{SAMPLE0}$, OD_{STD5} and OD_{STD0} are OD_{510nm} values of sample and standard at 5 and 0 min, respectively. [STD] is 2 mg/dL for blood assay and 50 mg/dL for urine assay.
Conversions: 1 mg/dL creatinine equals 88.4 µM, 0.001% or 10 ppm.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipeting devices and accessories (e.g. multi-channel pipettor). Clear bottom 96-well plates (e.g. Corning Costar) and plate reader for the plate procedure. Spectrophotometer and cuvets for measuring OD 510nm for the cuvette procedure.

EXAMPLES

Samples were assayed in duplicate (n = 2) using the 96-well plate protocol. The creatinine concentration (mg/dL) was 0.38 ± 0.01 for rat serum, 0.71 ± 0.02 for rat plasma, 0.79 ± 0.00 for human serum, 0.89 ± 0.04 for human plasma, 1.20 ± 0.04 for goat serum and 136.4 ± 2.9 in a fresh human urine sample.



Standard Curve in 96-well plate

PUBLICATIONS

1. Wang, J.J. et al (2006). Salutary effect of pigment epithelium–derived factor in diabetic nephropathy evidence for antifibrogenic activities. Diabetes 55: 1678-1685.

2. Zhang, S.X. et al (2006). Therapeutic potential of angiostatin in diabetic nephropathy. J Am Soc Nephrol 17: 475–486.

3. Davalos-Misslitz, A.C.M. et al (2007). Generalized multi-organ autoimmunity in CCR7-deficient mice. Eur. J. Immunol. 37: 613–622.

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance from each.

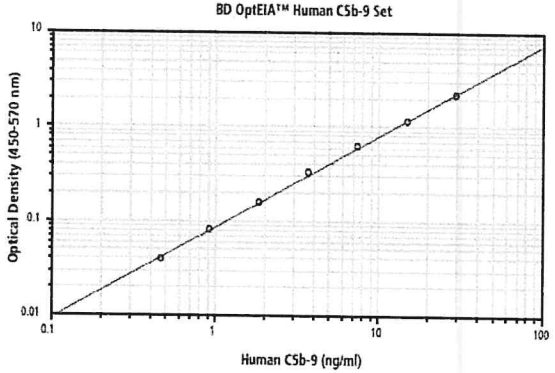
Plot the standard curve on log-log graph paper, with C5b-9 concentration on the x-axis and absorbance on the y-axis. Draw the best fit curve through the standard points.

To determine the C5b-9 concentration of the unknowns, find the unknown's mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the C5b-9 concentration. If samples were diluted, multiply the C5b-9 concentration by the dilution factor.

Computer data reduction may also be employed, utilizing log-log regression analysis.

Typical Standard Curve

This standard curve is for demonstration only. A standard curve must be run with each assay.



Specificity

Cross Reactivity: The following factors were tested in the BD OptEIA™ assay at ≥ 5 µg/mL and no cross-reactivity (value ≥ 470 pg/mL) was identified.

Purified Native Human:

C3, C4, C5, C6, C7, C8 and C9.

558315 Rev.2

Standardization

This immunoassay is calibrated against cobra venom-factor activated human serum.

Assay Optimization

1. BD OptEIA™ Sets allow flexible assay design to fit individual laboratory needs. To design an immunoassay with different sensitivity and dynamic range,the following parameters can be varied: Capture, Detection Antibody titers, Incubation time, Incubation temperature, Assay Diluent formulation, Buffer pH, ionic strength, protein concentration, Type of substrate, Washing technique (i.e., number of wash repetitions and soak times)
2. "Typical Standard Curve" and 5-plate yield were obtained in the BD Biosciences Pharmingen laboratory, using the recommended procedure and manual plate-washing.

Troubleshooting

Possible Source	Corrective Action
Poor Precision	
• Inadequate washing/ aspiration of wells	• Check function of washing system
• Inadequate mixing of reagents	• Ensure adequate mixing
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Incomplete sealing of plate	• Ensure complete seal on plate
Poor Standard Curve	
• Improper standard handling/ dilution	• Ensure correct preparation, storage of standards
• Incomplete washing/ aspiration of wells	• Check function of washing system
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Improper buffer/ diluent used	• Check buffer/ diluent preparation, pH
Low Absorbances	
• Inadequate reagent volumes added to wells	• Check/ calibrate pipettes
• Incorrect incubation times/ temperature	• Ensure sufficient incubation times/ reagents warmed to RT
• Incorrect antibody titration	• Check Capture Ab and Working Detector preparation
• Improper buffer/diluent used	• Check buffer/ diluent preparation, pH
• Overly high wash/aspiration pressure from automated plate-washer	• Utilize manual washing

Limitations of the Procedure

• Samples that generate absorbance values higher than the standard curve should be diluted with Standard Diluent and re-assayed.
• Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.
• BD OptEIA™ Sets are intended for use as an integral unit. Do not mix reagents from different Set batches. Reagents from other manufacturers are not recommended for use in this Set.

BD OptEIA™

Technical Data Sheet

Human C5b-9 ELISA Set

Cat. No. 558315

Materials Provided

The BD OptEIA™ Set for Human C5b-9 contains the components necessary to develop enzyme-linked immunosorbent assays (ELISA) for natural C5b-9 in serum, plasma, and other biological samples. Sufficient materials are provided to yield approximately 5 plates of 96-wells if the recommened storage, materials, buffer preparation, and assay procedure are followed as specified in this package.

Capture Antibody

Purified Anti-Human C5b-9 (250X)

Detection Antibody

Biotin Anti-Human C5b-9 (250X)

Enzyme Reagent

Streptavidin-horseradish peroxidase conjugate (SAv-HRP) (250X)

Standard

Human C5b-9 Standard, lyophilized (5 vials)

United States
877.232.8995

Canada
866.979.9408

Europe
32.2.400.98.95

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

BD
BD Biosciences

BD Biosciences Pharmingen
10975 Torreyana Road
San Diego, CA 92121
Customer/Technical Service
Tel 877.232.8995 (US)
Fax 858.812.8888
www.bdbiosciences.com

別件2

Recommended buffers, solutions

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

The BD OptEIA™ Reagent Set B (Cat. No. 550534) containing Coating Buffer, Assay Diluent, Substrate Reagents A and B, Stop Solution and 20X Wash Buffer Concentrate is recommended.

1. **Coating Buffer-** 0.1 M Sodium Carbonate, pH 9.5
   7.13 g $NaHCO_3$, 1.59 g Na_2CO_3; q.s. to 1.0 L; pH to 9.5 with 10 N NaOH.
   Freshly prepare or use within 7 days of preparation, stored at 2-8°C.
2. **Assay Diluent-** PBS* with 10% FBS†, pH 7.0. The BD Pharmingen™ Assay Diluent (Cat. No. 555213) is recommended.
   *Phosphate-Buffered Saline: 80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0g KCl, q.s. to 10 L; pH to 7.0.
   †Fetal Bovine Serum: Hyclone Cat. No. SH30088 (heat-inactivated) recommended.
   Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
3. **Wash Buffer-** PBS* with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
4. **Substrate Solution-** Tetramethylbenzidine (TMB) and Hydrogen Peroxide. The BD Pharmingen™ TMB Substrate Reagent Set (Cat. No. 555214) is recommended.
5. **Stop Solution** - 1 M H_3PO_4 or 2 N H_2SO_4

Additional Materials Required

1. 96-well Nunc-Immuno® polystyrene Maxisorp ELISA flat bottom plates (ThermoFisher Scientific Cat. No. 442404) are recommended
2. Microplate reader capable of measuring absorbance at 450 nm
3. Precision pipettes
4. Graduated cylinder, one liter
5. Deionized or distilled water
6. Wash bottle or automated washer
7. Log-log graph paper or automated data reduction
8. Tubes to prepare standard dilutions
9. Laboratory timer
10. Plate sealers or parafilm

Storage Information

1. Store unopened reagents at 2-8°C. Do not use reagents after expiration date, or if precipitation or turbidity is evident.
2. Before use, bring all reagents to room temperature (18-25°C). Immediately after use, return to proper storage conditions.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic.

Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

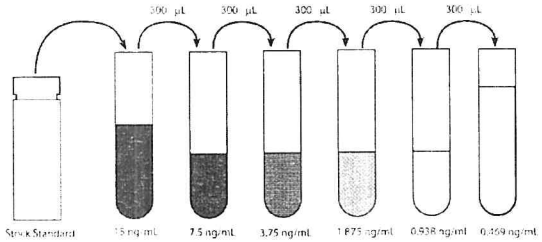
Serum: Use a serum separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin as anticoagulant. Centrifuge for 10 minutes at 1000 x g within 30 minutes of collection. Assay immediately or store samples at ≤-20°C. Avoid repeated freeze-thaw cycles.

Standards Preparation and Handling

1. **Reconstitution:** After warming to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with appropriate amount of assay diluent to yield a 30 ng/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.
2. **Storage/ handling of reconstituted standard:** Use reconstituted standard immediately. Use one vial of standard per 96-well plate.
3. **Standards Preparation for Assay:**
   a. Prepare 30 ng/mL stock standard as described in the Reconstitution section above (step 1). Vortex gently to mix.
   b. Add 300 µL Assay Diluent to 6 tubes. Label as 15 ng/mL, 7.5 ng/mL, 3.75 ng/mL, 1.875 ng/mL, 0.938 ng/mL, and 0.469 ng/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. Assay Diluent serves as the zero standard (0 ng/mL).

Serial dilutions within the plate may also be performed, by pipetting 100 µL of Assay Diluent into each standard well except the highest (30 ng/mL), then adding 100 µL of the 30 ng/mL standard to both that well and the 15 ng/mL well, mixing the well contents by rinsing the pipette tip, and adding 100 µL of the 15 ng/mL standard to the 7.5 ng/mL well. Continue these dilutions to the 0.469 ng/mL standard well, out of which the extra 100 µL should be discarded.



Working Detector Preparation

Note: Working Detector must be prepared within 15 minutes prior to use.

1. Determine volume needed for experiment: 100 µL per well.
2. Determine amount of 250X detector antibody and 250X Streptavidin-HRP to add to working detector mix.
3. Add appropriate volumes of 250X detector antibody and 250X Streptavidin-HRP to Assay Diluent to prepare required volume of Working Detector. Do not dilute more Detection Antibody than is needed for your experiment.
4. Add Working Detector to each well as described in the assay procedure.

Warnings and Precautions

1. Human C5b-9 Standard contains human serum, a potentially biohazardous material. Use universal precautions when handling. Handle as if product were capable of transmitting disease. Materials used in this product have been tested using FDA approved methods and found negative for Human Immunodeficiency Virus (HIV-1 HIV-2), Hepatitis B Surface Antigen (HBSAG) and antibody to Hepatitis C Virus (HCV). However, no known test method can offer complete assurance that specimens of human origin will not transmit infectious disease. When handling or disposing, follow precautions described in CDC and FDA recommendations and OSHA Bloodborne Pathogen recommendations.
2. Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
3. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
4. Capture Antibody contains ≤ 0.09% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
5. Detection Antibody contains BSA, ≤ 0.09% sodium azide and ProClin®-150 as a preservative.
6. Human C5b-9 Standard contains BSA and ProClin®-150 as a preservative.
7. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
8. ***Warning:*** Human C5b-9 lyophilized standard (component 51-9004086) contains 0.03% (w/w) and Detection Antibody Biotin Anti-Human C5b-9 (component 51-9004167) contains 0.002% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).
   Hazard statements
   May cause an allergic skin reaction.
   Precautionary statements
   Wear protective gloves / eye protection.
   Wear protective clothing.
   Avoid breathing mist/vapours/spray.
   If skin irritation or rash occurs: Get medical advice/attention.
   IF ON SKIN: Wash with plenty of water.
   Dispose of contents/container in accordance with local/regional/national/international regulations.

Recommended Assay Procedure

1. Dilute the Capture Antibody 1:250 in Coating Buffer and coat microwells with 100 µL of diluted Capture Antibody per well. Seal plate and incubate overnight at 4°C. Do not dilute more Capture Antibody than is needed for your experiment.
2. Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.
3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room temperature (RT) for 1 hour.
4. Aspirate/wash as in step 2.
5. Prepare standard and sample dilutions in Assay Diluent. See "Standards Preparation and Handling". Be sure to record the reconstituted standard concentraiton for future use.
6. Pipette 100 µL of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hours at RT.
7. Aspirate/wash as in step 2, but with 3 total washes.
8. Add 100 µL of Working Detector to each well. Seal plate and incubate for 1 hour at RT.
9. Aspirate/wash as in step 2, but with 7 total washes.
10. Add 100 µL of Substrate Solution to each well. Incubate plate (without plate sealer) for 30 minutes RT in the dark.
11. Add 50 µL of Stop Solution to each well.
12. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. Add 100 µL diluted Capture Ab to each well. Incubate overnight at 4°C.
2. Aspirate and wash 3 times.
3. Block plates: 200 µL Assay Diluent to each well. Incubate 1 hr RT.
4. Aspirate and wash 3 times.
5. Add 100 µL standard or sample to each well. Incubate 2 hr RT
6. Aspirate and wash 3 times.
7. Add 100 µL diluted Working Detector to each well. Incubate 1 hr RT.
8. Aspirate and wash 7 times.
9. Add 100 µL TMB Substrate Solution to each well. Incubate 30 min RT in dark.
10. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min with λ correction at 570 nm.

附件3

Standards Preparation and Handling

1. Reconstitution: After warming to room temperature, carefully open vial to

avoid loss of material. Reconstitute lyophilized standard with 2.1 mL of assay diluent to yield a 30 ng/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions.

Vortex gently to mix.

2. Storage/ handling of reconstituted standard: Use reconstituted standard

immediately. Use one vial of standard per 96-well plate.

3. Standards Preparation for Assay:

a. Prepare 30 ng/mL stock standard as described in the Reconstitution

section above (step 1). Vortex gently to mix.

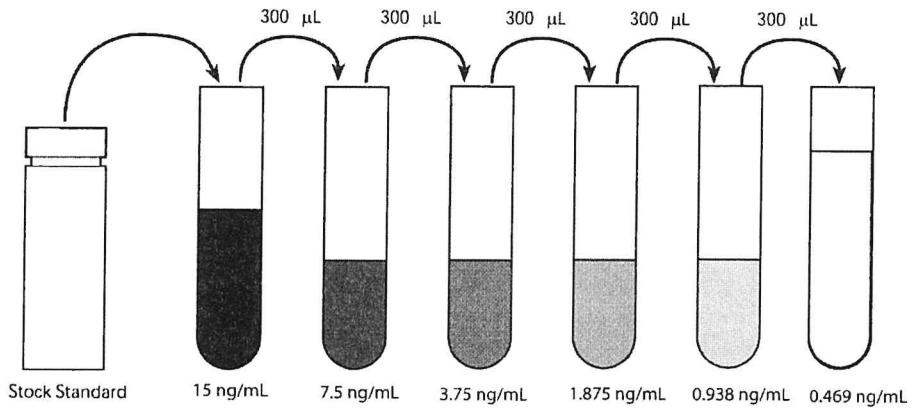
b. Add 300 µL Assay Diluent to 6 tubes. Label as 15 ng/mL, 7.5

ng/mL, 3.75 ng/mL, 1.875 ng/mL, 0.938 ng/mL, and 0.469 ng/mL.

c. Perform serial dilutions by adding 300 µL of each standard to the

next tube and vortexing between each transfer. Assay Diluent serves

as the zero standard (0 ng/mL).



Working Detector Preparation

Note: Working Detector must be prepared within 15 minutes prior to use.

1. Determine volume needed for experiment: 100 mL per well.

2. Determine amount of 250X detector antibody and 250X StreptavidinHRP to add to working detector mix.

3. Add appropriate volumes of 250X detector antibody and 250X

Streptavidin-HRP to Assay Diluent to prepare required volume of Working

Detector. Do not dilute more Detection Antibody than is needed for your

experiment.

Add 40µL of HRP and Detection antibody to 9920µL of reagent diluent.

Recommended Assay Procedure

1. Dilute the Capture Antibody 1:250 in Coating Buffer 40µL into 9960µL of coating buffer and coat microwells

with 100 µL of diluted Capture Antibody per well. Seal plate and

incubate overnight at 4°C . Do not dilute more Capture Antibody than is

needed for your experiment.

2. Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After

last wash, invert plate and blot on absorbent paper to remove any residual

buffer.

3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at room

temperature (RT) for 1 hour.

4. Aspirate/wash as in step 2.

5. Prepare standard and sample dilutions in Assay Diluent. See "Standards

Preparation and Handling". Be sure to record the reconstituted standard

concentration for future use.

6. Pipette 100 µL of each standard, sample, and control into appropriate

wells. Seal plate and incubate for 2 hours at RT.

7. Aspirate/ wash as in step 2, but with 3 total washes.

8. Add 100 µL of Working Detector to each well. Seal plate and incubate

for 1 hour at RT.

9. Aspirate/ wash as in step 2, but with 7 total washes.

10. Add 100 µL of Substrate Solution to each well. Incubate plate (without

plate sealer) for 30 minutes RT in the dark.

11. Add 50 µL of Stop Solution to each well.

12. Read absorbance at 450 nm within 30 minutes of stopping reaction. If

wavelength correction is available, subtract absorbance 570 nm from

absorbance 450 nm.

Calculations

Capture Antibody:

1:250 dilution so...

Full plate needs 10000µL of diluted Ab

10000/250 = 40

So 40µL of capture antibody into 9960µL of coating buffer.

Wash Buffer:

For 2L of 20x wash buffer...

100ml of concentrated wash buffer into 1900ml of dH_2O

Standard:

The vial contains 63ng of standard but 30ng/ml is needed so...

63/30= 2.1

S0 2.1 ml of reagent diluent has to be added to the 63ng of standard powder to achieve 30ng/ml

Working detector:

Both detection Ab and Strep-HRP are 250X so for a full plate...

10000/250=40

S0 40µL of Detection Ab and 40 µL of Strep-HRP will be added to 9920µL of reagent diluent.

Substrate Solution:

For a full plate:

Equal parts reagent A and reagent B so...

5ml of Reagent A and 5ml of reagent B

附件4

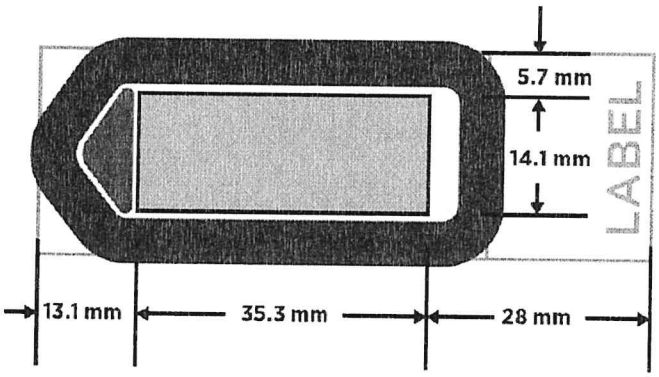
Mayer GeoMx Digital Spatial Profiling Protocol

Day 1

Start: 13:00 Finish: 16:00

1) Ensure tissue fits within the gasket for the GeoMx holder before beginning, see figure 1.

Tissue outside or on the border of the gasket will not be processed and will cause the system to leak.



2) Place slides standing sideways into a slide holder and bake at 60°C for 60 minutes. Place tissue beneath slides to absorb any melted paraffin.

3) Prepare a formalin stop solution:
   - Add 1.225g of glycine and 0.75g trizma base to a 100ml reagent bottle.
   - Add 100ml of water.
   - Place on a magnetic stirrer.

4) Sterilise the required coplin jars with 70% IMS spray. Rinse with distilled water.

5) Create a sufficient quantity of 1X PBS solution:
   - 200ml of 1X PBS required.
   - Combine 20ml of 10X PBS with 180ml dH_2O.

6) Prepare the following washes in the sterilised coplin jars:
   - 2x 100% ethanol (50ml per coplin).
   - 1x 95% ethanol (47.5ml EtOH, 2.5ml H_2O).
   - 3x xylene (50ml per coplin).
   - 1x Proteinase K
     i. Spray pipette with RNAse ZAP.

ii. Add 2.5µL of proteinase K to 50ml of water.

iii. Place in water bath at 37°C.

- 1x PBS (1x concentration, 50ml)
- 1x distilled H_2O (50ml, loosely cover with metal foil rather than a lid)
- 1x Tris EDTA (50ml, loosely cover with metal foil rather than a lid)

7) Prepare the steamer:
   - Fill with water up to the 'lip'. (IMAGE)
   - Place distilled H_2O and Tris EDTA coplins inside.
   - Turn-on steamer (set timer to maximum).

8) Remove slides from the oven and transfer to the fume cupboard.

9) Consecutively place in each xylene wash.
   - 5 minutes per coplin.

10) Consecutively place in each ethanol wash.
   - 5 minutes in each coplin.
   - In descending order of ethanol concentration (100% → 100% → 95%).
   - While completing these washes, ensure there is enough water remaining in the steamer.

When placing slides into washes, dip them up and down a couple of times to ensure that tissue is fully covered.

11) Place in PBS wash for 1 minute.

12) Place slides in steamer washes
   - First check temperature by inserting thermometer through the steamer lid and foil covering the coplin jars.
   - Place slides in distilled H_2O for 10 seconds.
   - Place slides in Tris-EDTA for 15 minutes. Replace foil lid for this duration.

13) Place slides in PBS wash for 5 minutes.

14) Place slides in proteinase-K solution for 15 minutes (in water bath). Complete steps 15 and 16 in the duration.

15) Remove buffer R from fridge.

16) Remove NanoString whole transcriptome atlas from the freezer and place on ice (not necessary if running optimisation experiment).

17) Place in PBS wash for 5 minutes. For the duration, proceed to step 18.

18) Prepare the following washes in the fume cupboard:

- 10% neutral buffered formalin (fill to slide line).
- 2x stop solutions – divide stop solution created in step 3 between two coplin jars.

19) In the fume cupboard, perform the following washes. For the duration of these washes, proceed to step 20 and 21.

- Formalin solution, 5 minutes.
- Stop solution one, 5 minutes.
- Stop solution two, 5 minutes.
- PBS solution, 5 minutes.

20) Prepare the hybridisation chamber:

- Clean a plastic tray with DEPC treated water.
- Turn on the hybridising chamber.
- Remove foam strips from the chamber and saturate with DEPC water on the plastic tray.
- Return strips to the hybridising chamber.
- Run programme 11.

21) Prepare the RNA probe mix in an Eppendorf tube. Approximately 50µL of solution is sufficient to cover each tissue section. Adjust volumes accordingly. For each tissue section:

- 44µL buffer R
- 5.5µL DEPC H_2O

[This makes up 55µL of probe solution – however – only 50µL are required for each tissue section. The extra 5µL reduces the risk of air being drawn into the pipette when applying probe solution to slides.]

- 5.5µL NanoString hWTA (or dH_2O if practice/optimisation).

22) Probe hybridisation:

- Remove slides from PBS wash and dry with microfibre tissue.
- Place in hybridisation chamber.
- Pipette RNA probe mix onto slide. Avoid bubbles whilst pipetting.
- Cover tissue sections with Hybri-slips.

23) Transfer 50ml of formamide out of the freezer and into the fridge to thaw in preparation for day 2.

Day 2

Start: 09:00 Finish: 13:30

1) Turn on waterbath to 37°C.

2) Take buffer W out of the fridge (and formamide if relevant).

3) Make 175ml of 4X SSC solution:
   - Stock solution is 20X
   - ∴ use a 1:4 ratio of SSC to dH_2O
   - ∴ 35ml SSC in 140ml dH_2O

4) Make 2X SSC / Formamide solution:
   - Clean two coplin jars with RNAse away (dH_2O if optimisation/practice).
   - Take to fume cupboard.
   - Fill one coplin with 4X SSC to slide line.
   - Fill the other coplin with formamide to slide line.
   - Transfer the solution in the 4X SSC coplin to the formamide coplin.
   - Transfer the solution in the full coplin into the empty coplin and repeat three times. This will mix the two solutions.
   - Pour the solution from the full coplin into the empty coplin up to the slide line so that the volumes are approximately equal.

5) Place 2X SSC / Formamide coplins into the waterbath.

6) Make 2X SSC solution.
   - Pipette remaining 4X SSC solution into a new reagent bottle. Measure the volume transferred.
   - Add an equal volume of dH_2O.
   - Clean three coplin jars with RNAse away (dH_2O if optimisation/practice).
   - Distribute 2X SSC solution between the three coplin jars.

7) Retrieve slides from the hybridising chamber.
   - End programme on the hybridising chamber.
   - Retrieve slides and place into a 2X SSC coplin to remove the cover slips.
   - Turn off the hybridising chamber.

8) Wash slides in 2X SSC / formamide solution.

- Place slides into a 2X SSC/formamide coplin within the waterbath for 25 minutes.
- Transfer to the other 2X SSC/formamide coplin for a further 25 minutes.

9) Blocking

- Transfer slides from waterbath to a 2X SSC coplin for 2 minutes.
- Prepare the humidity box as demonstrated below
    i. IMAGE
- Transfer slides to a second 2X SSC coplin for a further 2 minutes.
- Dry slide with tissue and place in the humidity box (see below).
    i. IMAGE
- Clean pipette with RNAse away.
- Pipette buffer W onto slide – 50µL per tissue section, avoid bubbles.
- Leave in humidity box for 30 minutes as demonstrated below.
- For this duration proceed to step 10.

10) Prepare stain solution

- Remove nuclear stain from the freezer to thaw (~15 minutes).
- Centrifuge nuclear stain.
- Gather an Eppendorf holder, Eppendorf tube, the nuclear stain and relevant immunofluorescent antibodies.
- Create 55µL of staining solution per tissue section by combining the following:
    i. 5.5µL of nuclear stain.
    ii. *X*µL of immunofluorescent antibody – exact volume dependent on optimisation experiments.
    iii. Make up to 55µL with buffer W.

11) Apply stain to slides

- Remove slides from humidity box.
- Dry slides to remove blocking solution.
- Return slides to humidity box.
- Apply 50µL of stain solution to each tissue section.
- Return slides to humidity box for one hour.

13) Wash slides in 2X SSC

- Two five-minute washes in fresh SSC solution.

14) Load slides into the GeoMx

- Login to the machine *(User: Mayer_IgAN – Pass: Mayer_IgAN_DSP)*
- Start a new run
- Follow the instructions on-screen
- When placing slides into the slide holder ensure:
    i. To dry slides first.
    ii. Clean back of slide with 70% IMS and microfibre tissue
    iii. Place slides into holder
    iv. Slide is covered with 6ml of buffer S
- Follow the instructions on-screen

15) When removing slides, ensure to:

- Pipette buffer out of slide gasket before removing slide from holder.
- Clean slide holder and micromirror array with treated water and microfibre tissue.