終期研究報告 **(Final Research Report)**

研究主題 (Research topic)：

通過數字聚合酶鏈反應開發血液中AR-V7檢測試驗作為去勢抵抗性前列腺癌患者重要的生物標誌物 (The development of AR-V7 detection test in blood by dPCR as an important biomarker for castration-resistant prostate cancer patients)

由香港防癌會資助 (Supported by The Hong Kong Anti-Cancer Society)

研究人員 (Investigators)：

首席研究員 / Principal investigator： CHO Chi Shing William

共同研究員 / Co-investigators： CHAN T Timothy, LAU Yuen Ting Eunice, TAI Y Anna, LAM H Martin, YUEN Y Tony

資助金額 (Grant amount)： HK$114,468.80

研究開始日期 (Research start date)： 2019年4月1日 (1 April 2019)

研究期間 (Research duration)： 2019年4月1日至2021年4月29日 (1 April 2019 to 29 April 2021)

研究簡介 (Introduction)：

抑制雄激素受體 (AR) 信號傳導是前列腺癌的全身治療。在AR導向療法治療的患者中出現了許多分子改變。這些分子改變可能表明治療耐藥性的出現。在西方國家，發現雄激素受體剪接變體7 (AR-V7) 與去勢抵抗性前列腺癌 (CRPC) 對激素治療的耐藥性有關。定量血液中循環的AR-V7核糖核酸 (RNA) 顯然比評估循環腫瘤細胞 (CTCs) 更敏感，因為它的假陰性更少。而且，與腫瘤活檢相反，它允許進行連續測量以說明腫瘤基因型和表型隨時間可能發生的變化。但是，香港這方面的研究數據有限。故這項先導研究旨在評估AR-V7是否可以作為香港CRPC患者對激素治療耐藥的預測指標，並開發一種微創實用且可靠的方法，通過數字聚合酶鏈反應 (dPCR) 直接定量血液中的AR-V7，無需捕獲CTC。在前瞻性研究中繼續檢查該生物標記物將進一步有助於臨床應用。

Suppression of androgen receptor (AR) signaling is a systemic treatment of prostate cancer. A number of molecular alterations arise in patients treated with AR-directed therapies. These molecular alterations may indicate the emergence of treatment resistance. In Western countries, the AR splice variant 7 (AR-V7) is found to be associated with resistance to hormonal therapy in castration-resistant prostate cancer

(CRPC). Quantification of AR-V7 ribonucleic acid (RNA) circulating in blood is apparently more sensitive than assessing circulating tumor cells (CTCs), as it has fewer false negatives. Moreover, in contrast to tumor biopsies, it allows serial measurements to account for possible shifts in tumor genotype and phenotype over time. However, data in Hong Kong population is limited. This pilot study aims to evaluate whether AR-V7 can be act as a predictor of resistance to hormonal therapy in Hong Kong patients with CRPC and to develop a minimally-invasive practical and robust approach for the direct quantification of AR-V7 in blood by digital polymerase chain reaction (dPCR) without the need for CTC capture. Continued examination of this biomarker in prospective studies will further aid clinical utility.

研究目標 **(Objective)**：

在白種人的CRPC患者中，AR-V7與激素治療耐藥性有關。我們假設AR-V7可能也是亞洲CRPC患者的治療選擇生物標誌物。另一方面，我們假設血液中的AR-V7 RNA可以通過dPCR進行定量，其準確性、敏感性和特異性均高於實時定量逆轉錄聚合酶鏈反應(RT-qPCR)。這項研究的目的是開發一種檢測測試，以通過高敏感度液滴dPCR (ddPCR)評估CRPC患者血液中的AR-V7，並研究AR-V7是否可以作為荷爾蒙治療耐藥性的預測指標。

AR-V7 has been found to be associated with resistance to hormonal therapy in Caucasian patients with CRPC. We hypothesize that AR-V7 may also be a treatment selection biomarker for Asian patients with CRPC. On the other hand, we hypothesize that AR-V7 RNA in blood can be quantified by dPCR with a higher accuracy, sensitivity, and specificity than reverse transcription quantitative real-time polymerase chain reaction) (RT-qPCR). This study aims to develop a detection test to assess AR-V7 by highly sensitive droplet dPCR (ddPCR) in the blood of CRPC patients and investigate whether AR-V7 can be act as a predictor of resistance to hormonal therapy.

研究材料和方法 **(Materials and methods)**：

1. 受試者募集 (Subject recruitment)

   招募了29名將在腫瘤科門診接受荷爾蒙治療的CRPC患者，他們的10 mL血液樣本在基線和隨訪期間會被收集用於這項研究。

   29 CRPC patients who would be treated with hormonal therapy in the oncology out-patient clinic were enrolled. Their 10 mL of blood were collected at the

baseline and follow-up visits for this research project.

2. 細胞培養 (Cell culture)

人前列腺癌細胞株22Rv1、VCaP和PC-3購自American Type Culture Collection (Manassas, VA, USA)。LNCaP Red-FLuc購自PerkinElmer (Waltham, MA, USA)。22Rv1和LNCaP Red-FLuc在RPMI-1640培養基中生長，VCaP在DMEM培養基中生長，PC-3在F-12K培養基中生長；所有培養基均在濕潤氣氛、37°C和5% CO_2下補充了10%的胎牛血清和1%的青黴素-鏈黴素 (Invitrogen, Carlsbad, CA, USA)。

Human prostate cancer cell lines 22Rv1, VCaP, and PC-3 were purchased from American Type Culture Collection (Manassas, VA, USA). LNCaP Red-FLuc was obtained from PerkinElmer (Waltham, MA, USA). 22Rv1 and LNCaP Red-FLuc were maintained in RPMI-1640 medium, VCaP was grown in DMEM medium, PC-3 was maintained in F-12K medium; all medium were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at humidified atmosphere, 37°C, and 5% CO_2.

3. 血液處理 (Blood processing)

通過在室溫下將收集在EDTA管中的外周血以120×g離心20分鐘來製備富含血小板的血漿。通過在室溫下以400×g離心10分鐘，從富含血小板的血漿中分離出血小板。將血漿在4°C下以16,000×g進一步離心10分鐘以去除細胞碎片。將血小板存儲在TRIzol (Invitrogen) 中，而將雙離心血漿存儲在TRIzol LS (Invitrogen) 中。立即分離RNA或將樣本保存在-80°C直至提取RNA。

Platelet-rich plasma was prepared by centrifuging peripheral blood collected in EDTA tubes at 120×g for 20 min at room temperature. The platelets were isolated from the platelet-rich plasma by centrifugation at room temperature for 10 min at 400×g. Plasma was further centrifuged at 16,000×g for 10 min at 4°C to remove cellular debris. Platelets were stored in TRIzol (Invitrogen) while double centrifuged plasma was stored in TRIzol LS (Invitrogen). RNA was isolated immediately if available or specimens were stored at -80°C until RNA extraction.

4. RNA提取和反轉錄 (RNA extraction and reverse transcription)

用TRIzol (Invitrogen) 提取細胞系的總RNA。使用QIAamp循環核酸試劑盒 (QIAGEN, Hilden, Germany) 和TRIzol分離血漿RNA。使用SuperScript III逆轉錄酶

(Invitrogen) 逆轉錄洗脫的RNA。

Total RNA from cell line was extracted with TRIzol (Invitrogen). Plasma RNA was isolated using QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) and TRIzol. Eluted RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen).

5. RT-qPCR

通過使用 LightCycler 480 系統 (Roche, Basel, Switzerland) 進行的 QuantiNova 探針 PCR 試劑盒 (QIAGEN) 定量 AR-V7、總 AR 和前列腺特異性抗原 (PSA) 表達。引物和探針的設計如下：
AR-V7 正向：CGGAAATGTTATGAAGCAGGGATGA；
AR-V7 反向：CTGGTCATTTTGAGATGCTTGCAAT；
AR-V7 探針：FAM-CGGAATTTTTCTCCCAGA-MGB；
總 AR 正向：GGAATTCCTGTGCATGAAAGC；
總 AR 反向：CGATCGAGTTCCTTGATGTAGTTC；
總 AR 探針：VIC- CTTCAGCATTATTCCAGTG-MGB；
PSA 正向：CAGTCTGCGGCGGTGTTTT；
PSA 反向：GCAAGATCACGCTTTTGTTCCT；
PSA 探針：FAM-CCCCAGTGGGTCCTCACAGCTGC-TAMRA。
所有實驗均重複三次。

AR-V7, total AR, and prostate specific antigen (PSA) expression were quantified by QuantiNova Probe PCR Kit (QIAGEN) which was performed by using LightCycler 480 system (Roche, Basel, Switzerland). Primers and probes were designed as follows:
AR-V7 forward: CGGAAATGTTATGAAGCAGGGATGA;
AR-V7 reverse: CTGGTCATTTTGAGATGCTTGCAAT;
AR-V7 probe: FAM-CGGAATTTTTCTCCCAGA-MGB;
Total AR forward: GGAATTCCTGTGCATGAAAGC;
Total AR reverse: CGATCGAGTTCCTTGATGTAGTTC;
Total AR probe: VIC- CTTCAGCATTATTCCAGTG-MGB;
PSA forward: CAGTCTGCGGCGGTGTT;
PSA reverse: GCAAGATCACGCTTTTGTTCCT;
PSA probe: FAM-CCCCAGTGGGTCCTCACAGCTGC-TAMRA.
All reactions were performed in triplicate.

6. ddPCR

在 QX200 液滴數字 PCR 系統 (Bio-Rad) 上測量 AR-V7、總 AR 和 PSA 表達水平。ddPCR 反應的體積為 20 µL，其中包含 10 µL 探針 ddPCR Supermix (Bio-Rad)、900 nM 正向和反向引物和 250 nM 探針。通過使用 QX200 液滴發生器 (Bio-Rad) 添加 70 µL 液滴產生油來產生液滴。之後，PCR 反應使用 C1000 Touch Thermal Cycler (Bio-Rad) 在 95°C 進行 10 分鐘；然後進行 40 個 94°C 30 秒和 55°C 1 分鐘的循環。最後 98°C 10 分鐘。在 QX200 液滴讀取器 (Bio-Rad) 上讀取液滴，並使用 QuantaSoft 軟件 1.7.4 (Bio-Rad) 進行分析。

AR-V7, total AR, and PSA expression level were measured on a QX200 droplet digital PCR system (Bio-Rad). ddPCR reactions were prepared in a 20 µL volume containing 10 µL ddPCR Supermix for Probes (Bio-Rad), 900 nM of forward and reverse primers and 250 nM probe. Droplets were generated by adding 70 µL droplet generation oil using a QX200 droplet generator (Bio-Rad). Afterwards, PCR was performed using the C1000 Touch Thermal Cycler (Bio-Rad) at 95°C for 10 min; then followed by 40 cycles of 94°C for 30s and 55°C for 1 min; finally 98°C for 10 min. Droplets were read on QX200 droplet reader (Bio-Rad) and analyzed with QuantaSoft software version 1.7.4 (Bio-Rad).

7. 統計分析 (Statistical analysis)

QuantaSoft軟件 (Bio-Rad, Hercules, CA, USA) 用於評估AR-V7呈陽性的液滴數量，並根據Poisson分佈計算每個液滴的拷貝數，數據顯示為平均值±標準誤差，Prism 7 (GraphPad Software, San Diego, CA, USA) 用於統計分析。

The QuantaSoft software (Bio-Rad, Hercules, CA, USA) will be used to assess the number of droplets positive for AR-V7 and to calculate the copies per droplet from the Poisson distribution. Data are shown as the mean ± standard error. Prism 7 (GraphPad Software, San Diego, CA, USA) was used for calculations.

研究結果 (Results)：

1. 檢測的優化 (Assay optimization)

要優化AR-V7的檢測，使用ddPCR檢測總AR和PSA表達。確定適當的退火溫度。在55°C時，用於ddPCR反應的退火溫度顯示出與基線簇和陽性簇有最佳分離 (圖1)。

To optimize the detection of AR-V7, total AR and PSA expression with ddPCR.

Appropriate annealing temperature was determined. At 55°C, annealing temperature for ddPCR reactions showed the best separation from baseline cluster and positive cluster (Figure 1).

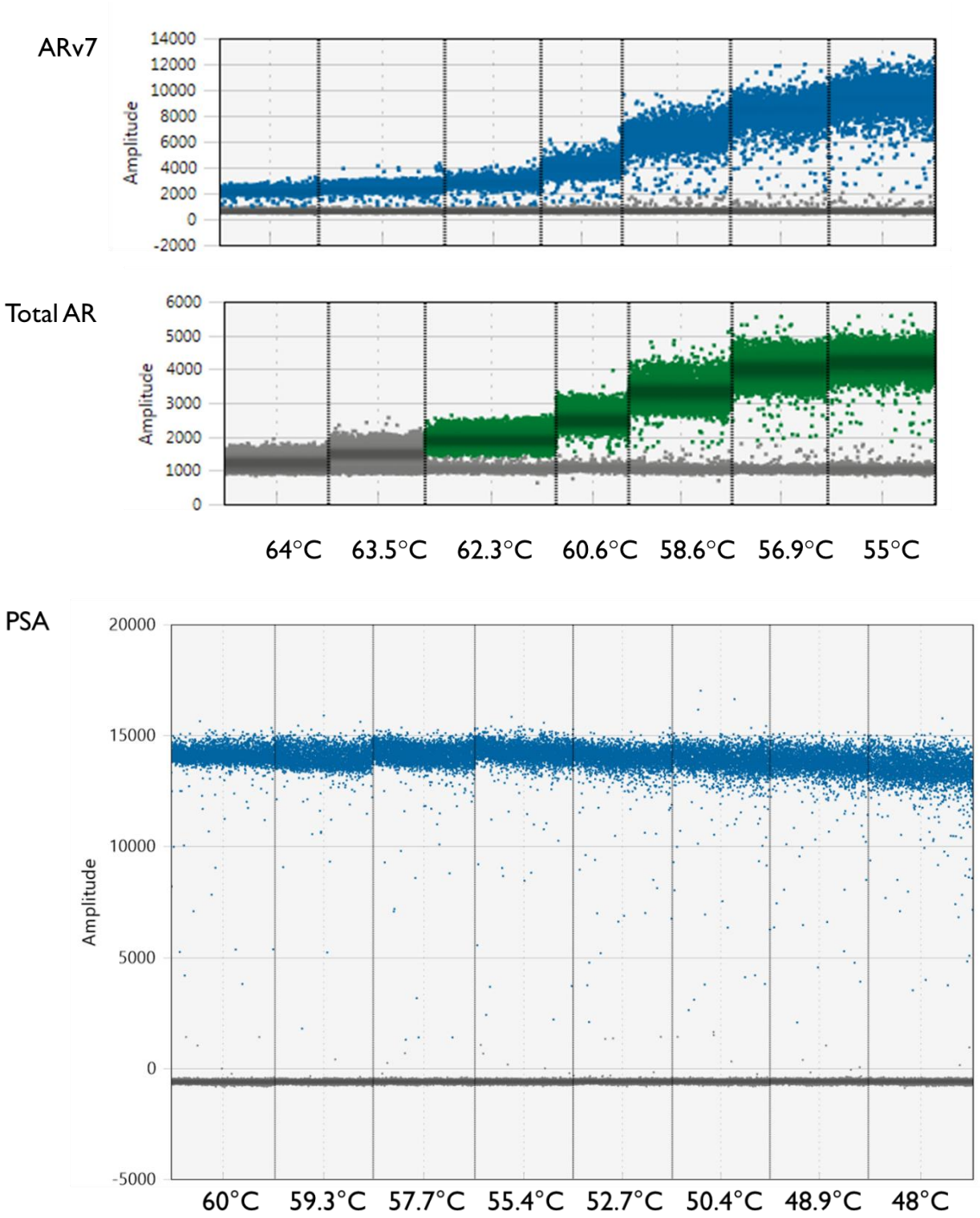


圖 1、退火溫度的優化。(Figure 1. Annealing temperature optimization.)

2. ddPCR的檢測表現 (Performance of ddPCR)

為了評估性能，測量了 2 ng PC-3、LNCaP Red-FLuc、VCaP 和 22Rv1 cDNA。在 AR-V7 陰性細胞株 PC-3 和 LNCaP Red-FLuc 中未觀察到 AR-V7 擴增。在 AR-V7 陽性細胞株 VCaP 和 22Rv1 中觀察到 AR-V7 擴增 (圖 2)。ddPCR 結果顯示了在不同前列腺癌細胞株中測量 AR-V7 和總 AR 表達時的準確性。PC-3 和 LNCaP Red-FLuc 顯示 AR-V7 / 總 AR 的比率為零，VCaP 顯示 AR-V7 / 總 AR 的比率為 0.0103，而 22Rv1 顯示 AR-V7 / 總 AR 的比率為 0.17 (圖 3)。

To evaluate the performance, 2 ng of PC-3, LNCaP Red-FLuc, VCaP, and 22Rv1 cDNA were measured. No AR-V7 amplification was observed in AR-V7 negative cell lines PC-3 and LNCaP Red-FLuc. AR-V7 amplification was observed in AR-V7 positive cell lines VCaP and 22Rv1 (Figure 2). The ddPCR result showed high accuracy when measuring AR-V7 and total AR expressions in different prostate cancer cell lines. PC-3 and LNCaP Red-FLuc showed a zero ratio of AR-V7 / total AR, VCaP showed a 0.0103 ratio of AR-V7 / total AR and 22Rv1 showed a 0.17 ratio of AR-V7 / total AR (Figure 3).

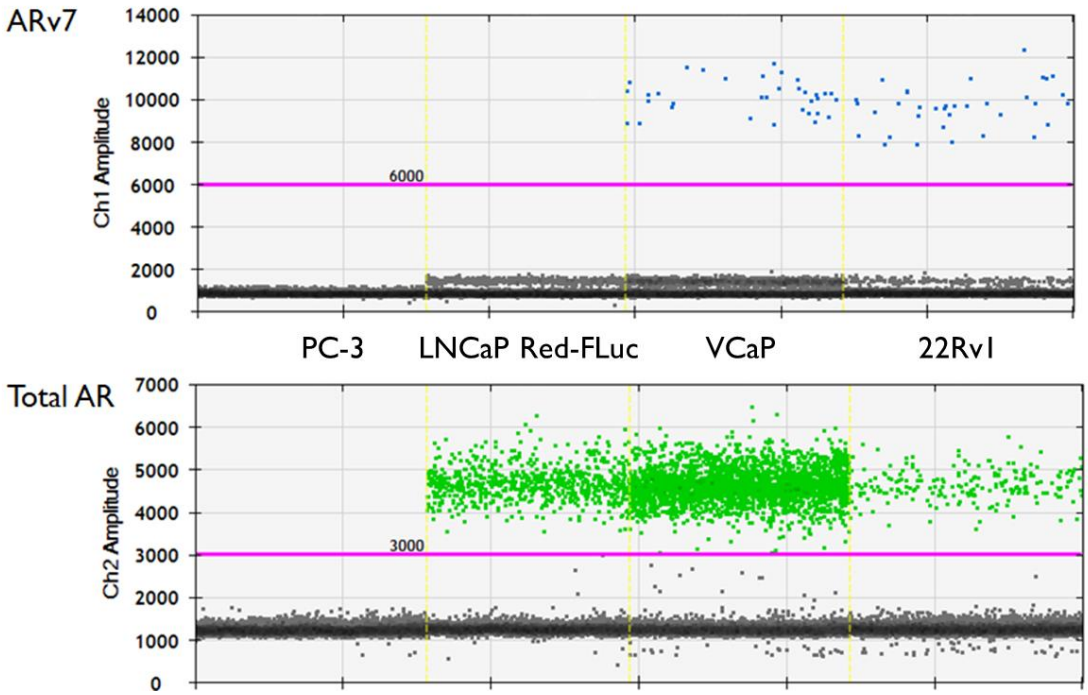


圖 2、通過 ddPCR 檢測 AR-V7 和總 AR。藍點代表 AR-V7，綠點代表總 AR。
(Figure 2. Detection of AR-V7 and total AR by ddPCR. The blue dots represent AR-V7 and the green dots represent total AR.)

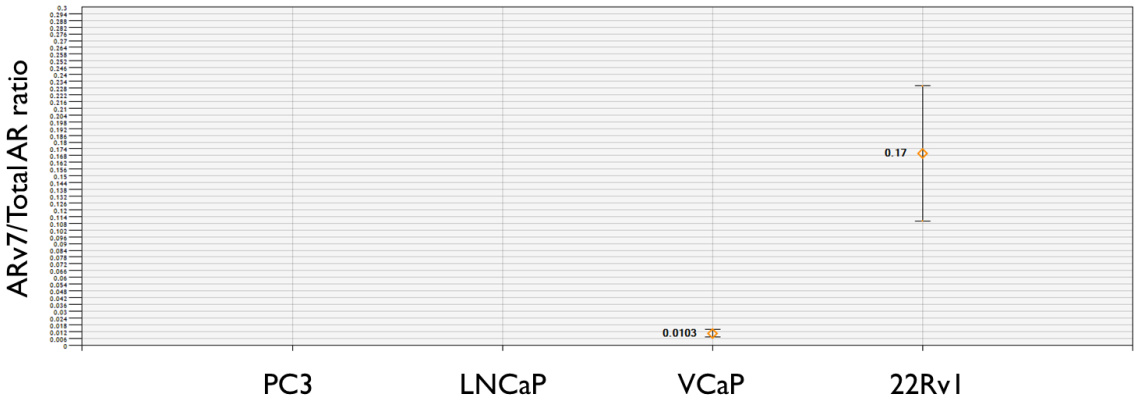
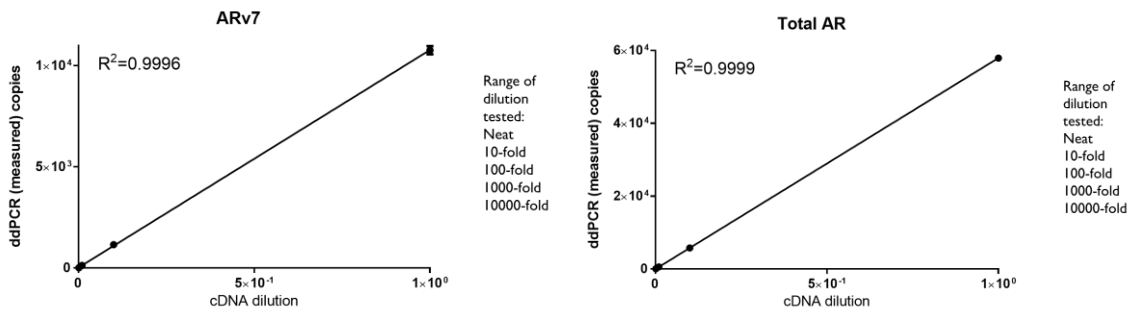


圖 3、前列腺癌細胞株中 AR-V7 / 總 AR 比的測量。
(Figure 3. Measurement of AR-V7 / Total AR ratio in prostate cancer cell lines.)

3. 檢測線性的評估 (Evaluation of assay linearity)

在 22Rv1 和 VCaP cDNA 中評估了檢測的線性。用 22Rv1 cDNA 從純淨至 10,000 倍的連續稀釋來確定 AR-V7 和總 AR 的線性。使用相同稀釋度的 VCaP cDNA 來確定 PSA 線性。在 AR-V7 (R^2 = 0.9996) 、總 AR (R^2 = 0.9999) 和 PSA (R^2 = 0.9978) 中觀察到出色的線性度 (圖 4)。

The linearity of assays was evaluated in 22Rv1 and VCaP cDNA. A range of neat to 10,000-fold dilution of 22Rv1 cDNA was used to determine the linearity for AR-V7 and total AR. Same range of dilution of VCaP cDNA was used to determine the PSA linearity. Good linearity was observed in AR-V7 (R^2 = 0.9996), total AR (R^2 = 0.9999), and PSA (R^2 = 0.9978) (Figure 4).



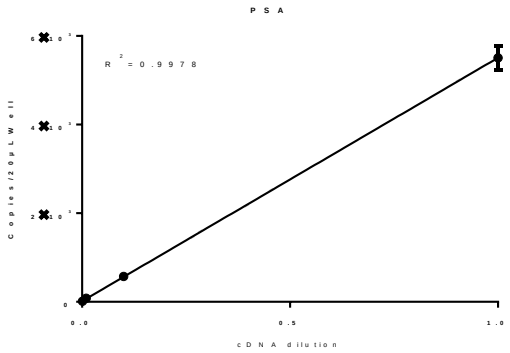


圖 4、線性圖顯示 AR-V7 的實測拷貝數與 cDNA 稀釋度、總 AR 和 PSA 之間的相關性。(Figure 4. Linearity plots showing the correlation between measured copies and cDNA dilution of AR-V7, total AR, and PSA.)

4. ddPCR和RT-qPCR的檢測表現比較
(Comparison of the performace between ddPCR and RT-qPCR)

通過 ddPCR 和 RT-qPCR 檢測了 20 fg 至 20 ng VCaP cDNA 的範圍。在檢測 AR-V7 和總 AR 含量中，ddPCR 可檢測低至 2 pg，而 RT-qPCR 僅檢測到 200 pg (圖 5)。

A range of 20 fg to 20 ng VCaP cDNA were measured by ddPCR and RT-qPCR. ddPCR was able to detect down to 2 pg while RT-qPCR was only able to detect 200 pg for AR-V7 and total AR (Figure 5).

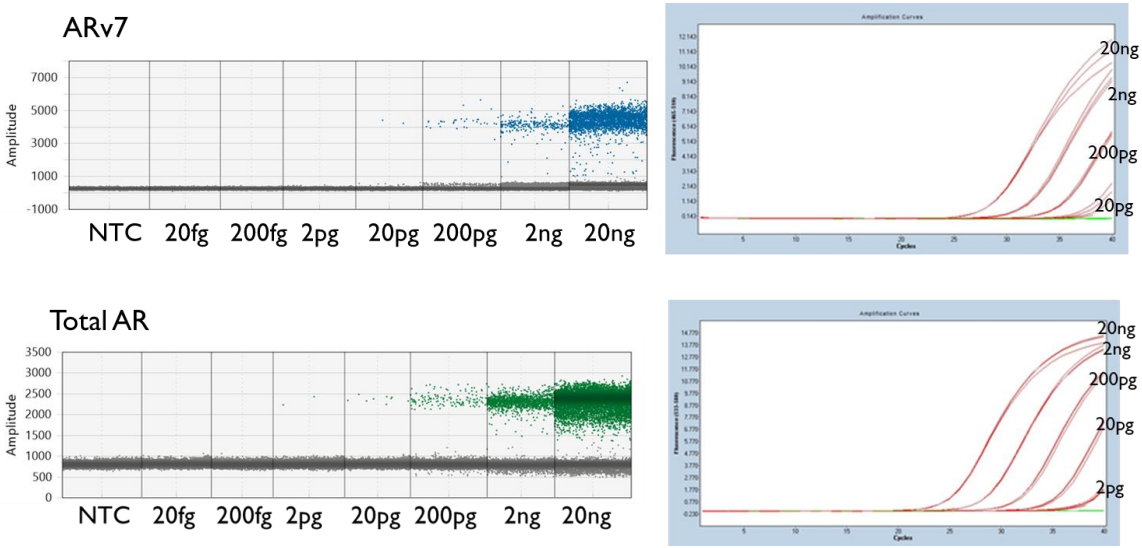


圖 5、ddPCR 和 RT-qPCR 的比較顯示了 ddPCR 的檢測靈敏度優於 RT-qPCR。(Figure 5. Comparsion of ddPCR and RT-qPCR showing the analytical sensitivity of ddPCR outperform RT-qPCR.)

5. 刺入血漿和DMEM培養基 (Plasma and DMEM medium with spike-in)

在提取前將 100 ng 22Rv1 和 VCaP cDNA 以及 2 μg 22Rv1 和 VCaP RNA 刺入到血漿中，僅在帶有 22Rv1 和 VCaP cDNA 刺入物的血漿中觀察到擴增 (圖 6)，這表明 RNA 被血漿中的 RNase 降解。為研究血漿中 RNase 的存在，在提取前將 2 μg 22Rv1 和 VCaP RNA 分別刺入血漿和 DMEM 培養基中。無論使用 TRIzol LS 還是 QIAamp 循環核酸試劑盒，僅在刺入了 22Rv1 和 VCaP RNA 的 DMEM 培養基中觀察到擴增，在刺入了 22Rv1 和 VCaP RNA 的血漿中未觀察到擴增 (圖 7)，這表明血漿中的 RNase 降解了刺入的 RNA。

100 ng of 22Rv1 and VCaP cDNA, as well as 2 μg of 22Rv1 and VCaP RNA were spiked into plasma before extraction. Amplification was only observed in plasma spike-in with 22Rv1 and VCaP cDNA (Figure 6). This implied that RNA was degraded by RNases in plasma. To investigate the presence of RNases in plasma, 2 μg of 22Rv1 and VCaP RNA were spiked into plasma and DMEM medium respectively before extraction. Amplification was only observed in DMEM medium with 22Rv1 and VCaP RNA spike-in, no matter TRIzol LS or QIAamp Circulating Nucleic Acid Kit was used. No amplification was observed in plasma with 22Rv1 and VCaP RNA spike-in (Figure 7). This revealed that RNase in plasma degraded spike-in RNA.

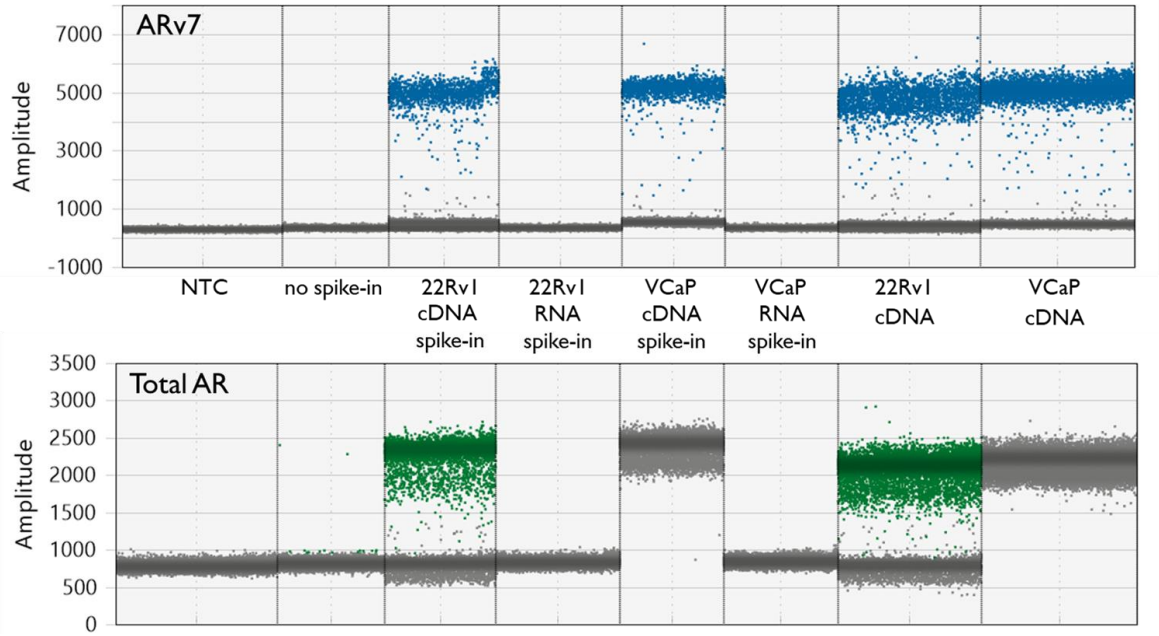


圖 6、ddPCR 圖顯示 22Rv1 和 VCaP cDNA 刺入在血漿中擴增，而 22Rv1 和 VCaP RNA 刺入在血漿中未擴增。(Figure 6. ddPCR plots showing amplification in plasma with 22Rv1 and VCaP cDNA spike-in and no amplification in plasma with 22Rv1 and VCaP RNA spike-in.)

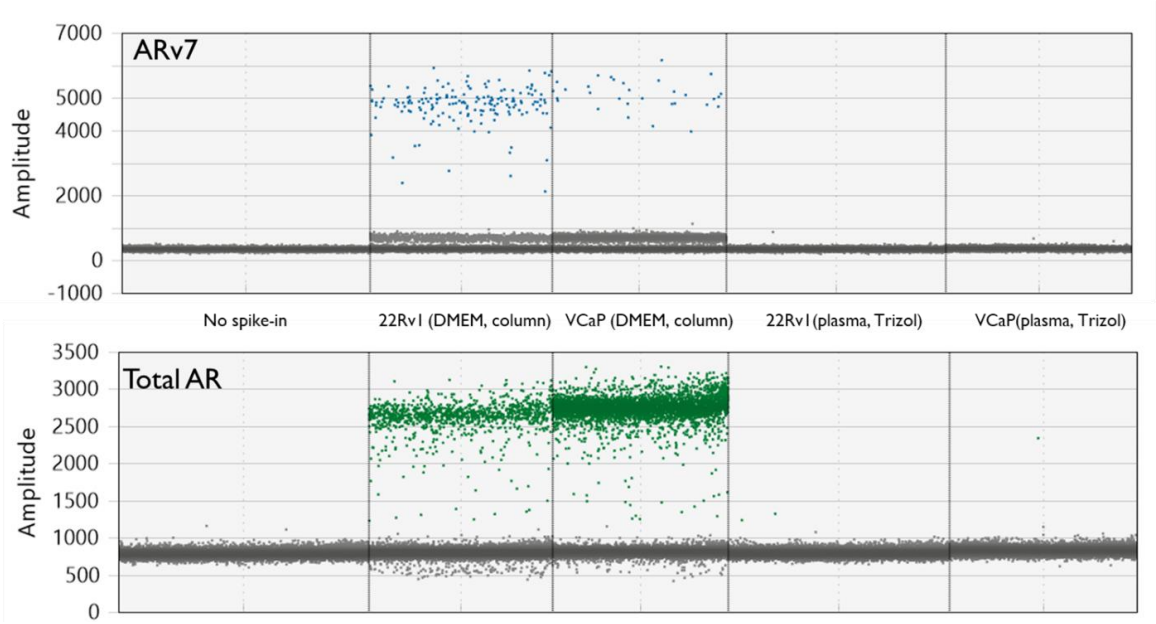


圖 7、ddPCR 圖顯示了使用 QIAGEN 循環核酸試劑盒和 TRIzol LS 時，在 DMEM 中具有 NCI-H2228 RNA 刺入的擴增，在血漿中則沒有 NCI-H2228 RNA 刺入的擴增。(Figure 7. ddPCR plots showing amplification in DMEM with NCI-H2228 RNA spike-in and no amplification in plasma with NCI-H2228 RNA spike-in when using QIAGEN Circulating Nucleic Acid Kit and TRIzol LS.)

6. 比較RT-qPCR和ddPCR在29例CRPC患者的血液樣本中AR-V7的檢測
(Comparison of RT-qPCR and ddPCR for AR-V7 detection in the blood samples of 29 CRPC patients)

我們分別用 RT-qPCR 和 ddPCR 來檢測 CRPC 患者的血漿樣本，結果顯示 RT-qPCR 無法從這些血漿樣品中檢測到 AR-V7，而 ddPCR 則具有較高的 14%檢測度。我們發覺在血漿樣本中不容易檢測 AR-V7，所以我們嘗試也在血小板樣本中檢測 AR-V7，結果顯示血小板比血漿樣本具有更高的檢測靈敏度，RT-qPCR 能夠從 58% 的血小板樣品中檢測到 AR-V7，而 ddPCR 的檢測率更高達 97%。

We performed RT-qPCR and ddPCR on the plasma samples of CRPC patients. We observed that RT-qPCR was unable to detect AR-V7 from these plasma samples, whereas ddPCR yielded a higher detection rate of 14%. We observed that the detection of AR-V7 in plasma samples was not easy, so we also tried to detect AR-V7 in platelet samples. Our results showed that platelet samples have higher detection sensitivity than plasma samples. RT-qPCR was able to detect AR-V7 from 58% of the platelet samples and ddPCR yielded a much higher detection rate of 97%.

7. AR-V7與患者對荷爾蒙治療耐藥性的相關性
(Correlation of AR-V7 with the patients' resistance to hormonal therapy)

我們將 ddPCR 在患者血小板樣本中檢測到的 AR-V7 與患者對荷爾蒙治療藥物 Abiraterone 或 Enzalutamide 的耐藥性相對照，在接收者操作特徵 (ROC) 曲線分析中，曲線下面積為 0.79 (圖 8)。

We correlated the ddPCR detection for AR-V7 in patients' platelet samples with the patients' resistance to hormonal therapeutic drugs, Abiraterone or Enzalutamide. In receiver operating characteristic (ROC) curve analysis, the area under the curve was 0.79 (Figure 8).

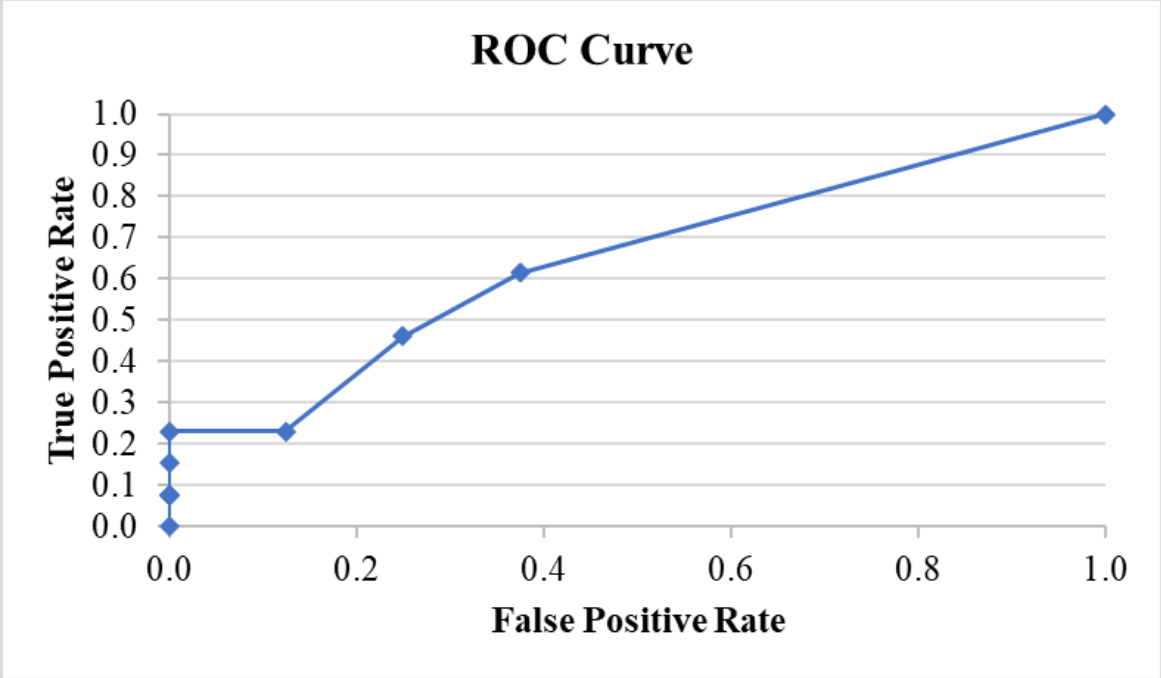


圖 8、ddPCR 在血小板樣本中 AR-V7 的檢測與患者對荷爾蒙治療耐藥性的接收者操作特徵 (ROC) 曲線分析，曲線下面積為 0.79。
(Figure 8. Receiver operating characteristic (ROC) curve analysis of ddPCR detection for AR-V7 in the platelet samples from patients with resistance to hormonal therapy, the area under the curve was 0.79.)

結論 (Conclusion)：

我們發覺在血漿樣本中不容易檢測AR-V7，這或許因為在處理過程中RNase可能會降解RNA。所以我們嘗試在血小板樣本中檢測AR-V7，結果顯示血小板比血漿樣本具有更高的檢測靈敏度。而分別用RT-qPCR和ddPCR來檢測血小板樣本，結果則顯示ddPCR比

RT-qPCR具有更高的檢測度。我們成功開發了一種檢測試驗，可通過高靈敏度ddPCR對CRPC患者的血小板進行AR-V7評估，檢測率高達97%。最重要的是，我們的結果顯示患者血小板樣品中AR-V7的ddPCR檢測結果與患者對荷爾蒙治療耐藥性具一定相關性，提示AR-V7或可作為荷爾蒙治療耐藥性的潛在預測指標。

We observed that the detection of AR-V7 in plasma samples was not easy, this may be due to the possibility of RNA degradation by the RNase during processing. We thus tried to detect AR-V7 in platelet samples, our results showed that platelet samples have higher detection sensitivity than plasma samples. Performing RT-qPCR and ddPCR on platelet samples, higher detection rate was oberved in ddPCR when comparing with RT-qPCR. We successfully developed a detection test to assess AR-V7 by highly sensitive ddPCR in the platelet of CRPC patients, with a high detection rate of 97%. Most importantly, our results observed that there was certain correlation between the ddPCR detection for AR-V7 in patients' platelet samples with the patients' resistance to hormonal therapy, indicating that AR-V7 may act as a potential predictor of resistance to hormonal therapy.