Using circulating tumour cells for prostate cancer diagnosis and precision medicine

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Conflict of interest statement

- The studies were partially funded by ANGLE plc

The need for repeated sampling

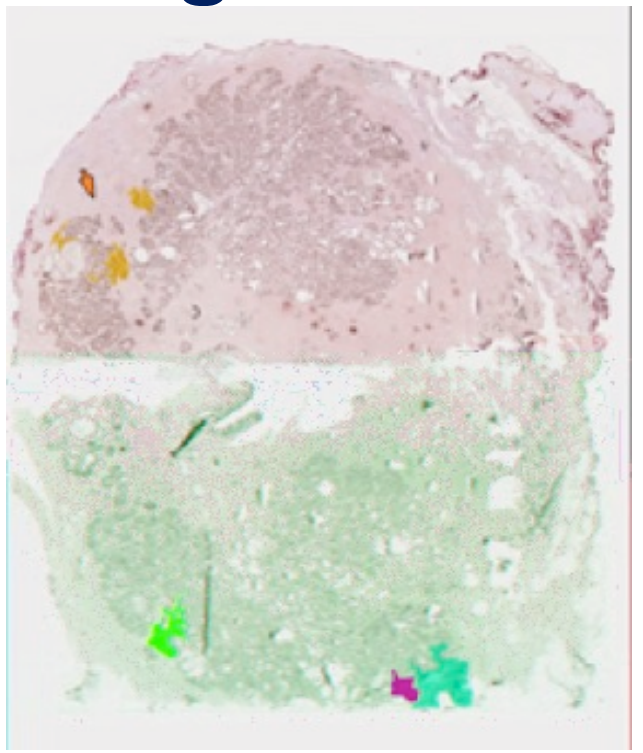
Early diagnosis/detection: periodical screening test

Precision medicine: Right drug to right patient at right time

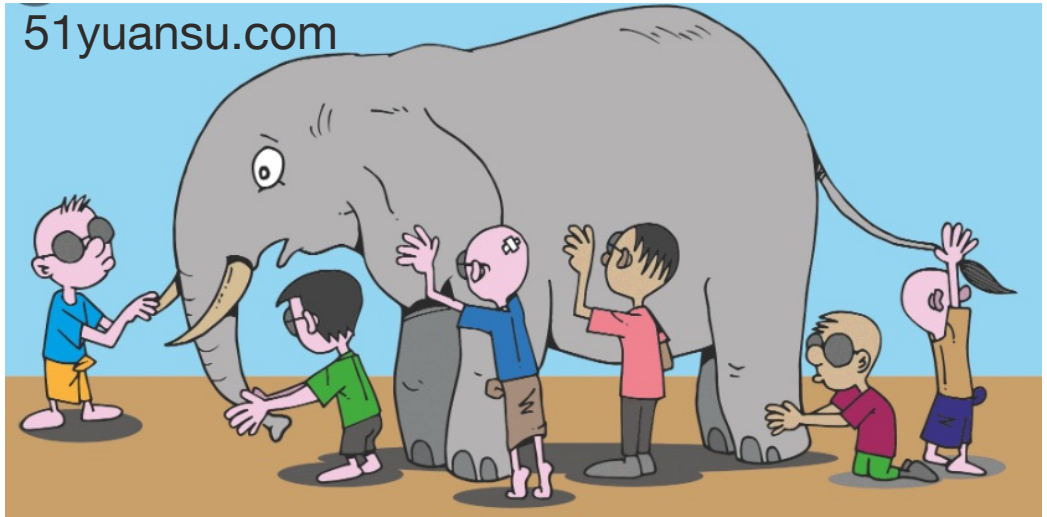
Challenge with traditional tissue based diagnosis

- Very invasive, difficult for frequent sampling- impossible real time monitoring disease status

JNM 2006; 47:287-297

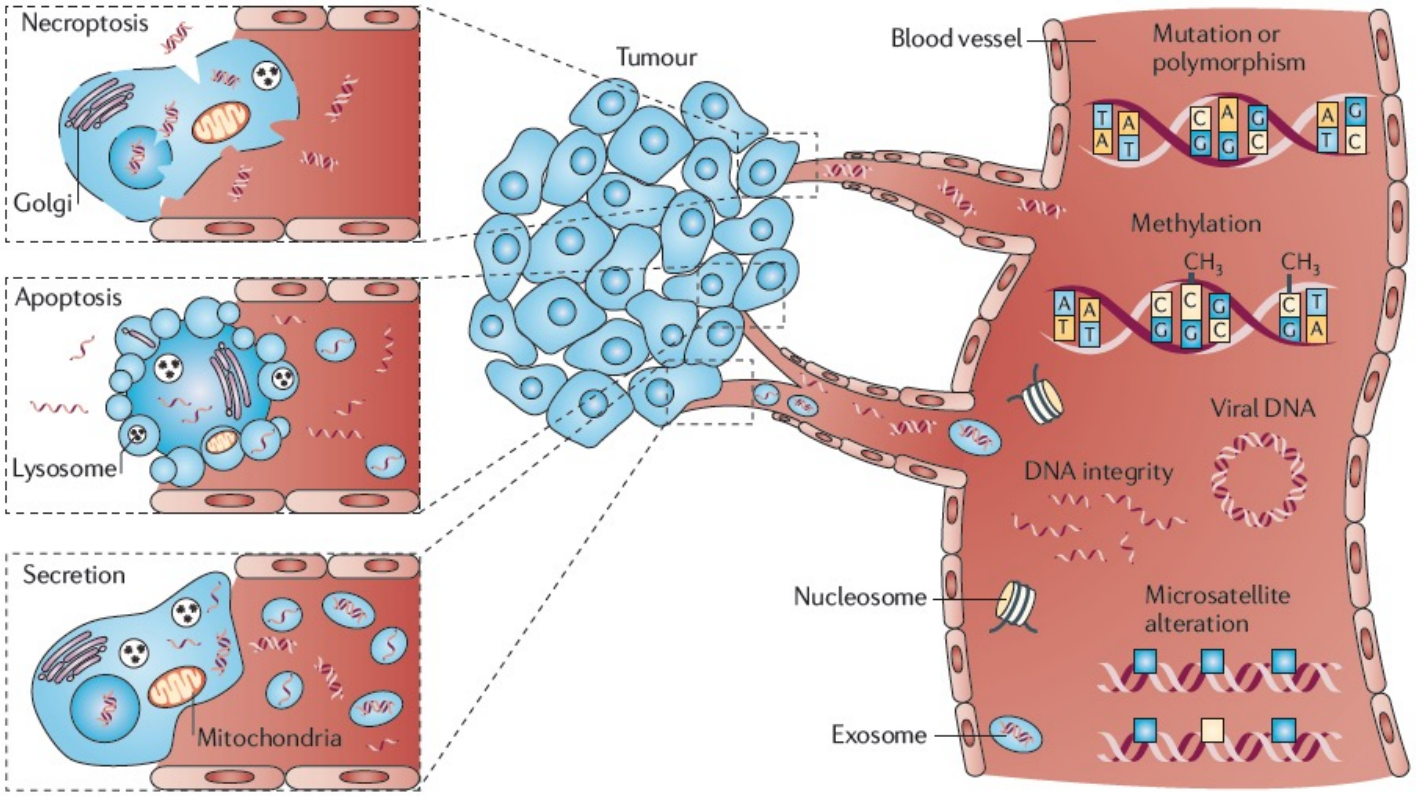


- Intratumour heterogeneity: blind men and an elephant



- The solution: blood text

Circulating biomarkers



(Nature Reviews Cancer 2011; 11:426-437)

Proteins: PSA

Wulfkuhle et al. Proteomic applications for the early detection of cancer. *Nat. Rev. Cancer* **3**, 267; 2003.

Cell-free nucleic acids (ctDNA, ctRNA)

Exosomes (miRNA)

Circulating tumour cell (CTCs)

Other circulating materials

https://www.neoteryx.com/

Why circulating tumour cells are good biomarker

- CTCs: the seeds cancer metastasis, **represent aggressive cancer**
- All genetic/molecular materials from intact cells
- Cell cell difference- Intratumoural heterogeneity
- **Alive cells**, responsible for cancer progression vs death cell released products
- Culturable for *in vitro* drug sensitivity test
- **Detectable at very early stage cancer**

Literature of CTCs biomarker in prostate cancer

- **Advanced cancer prognosis: FDA approval** CellSearch CTC count for advanced colon, breast and **prostate cancer prognosis**
- **Predict/monitor PCa therapy response**

  - CTC change before PSA in treatment response

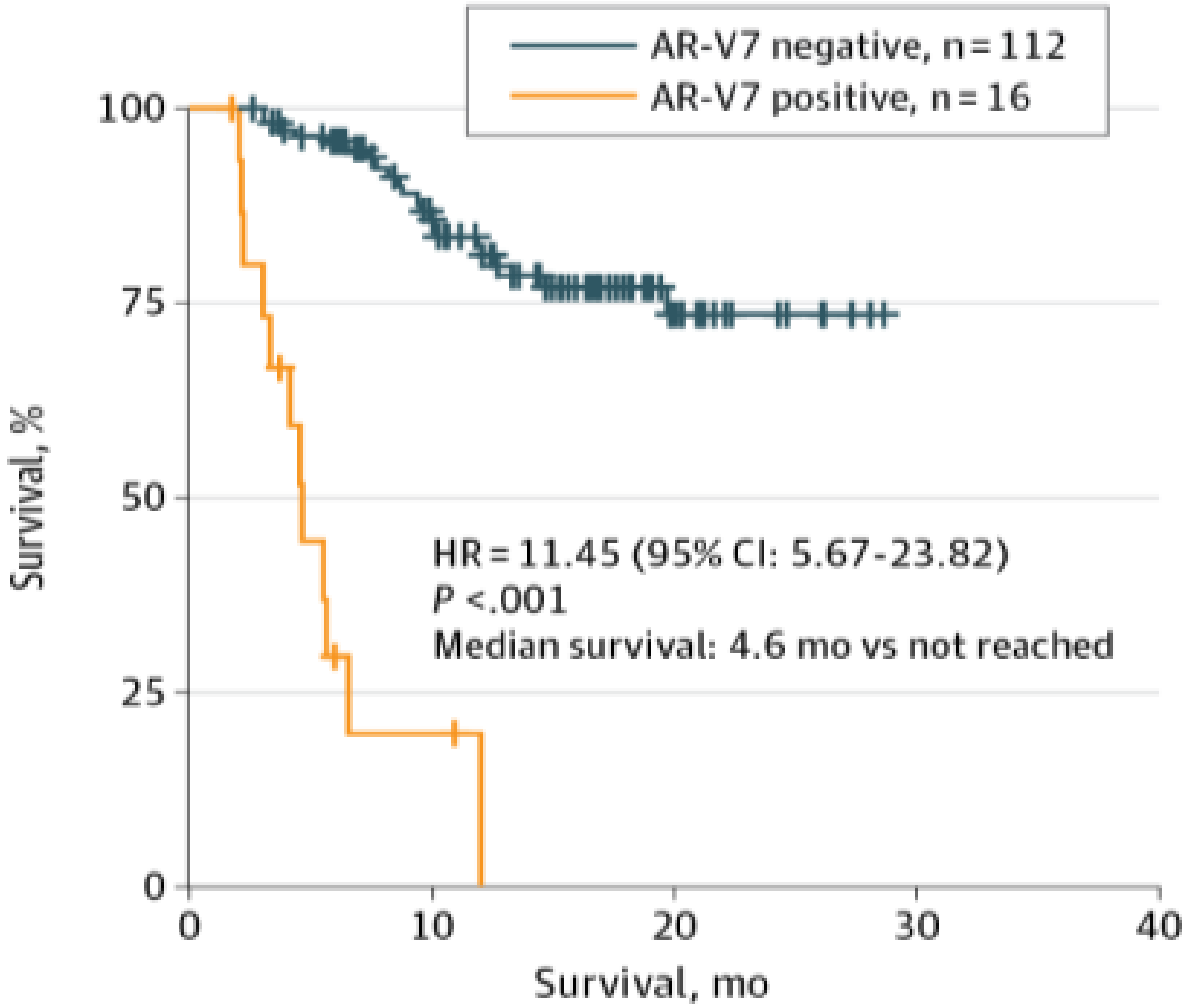
    Shamash J *et al*. Clin Cancer Res. 2012. 8:2352-9.

  - CTC AR-V7 for anti-androgen therapy

    Antonarakis ES *et al*. N Engl J Med. 2014. 371:1028-38.

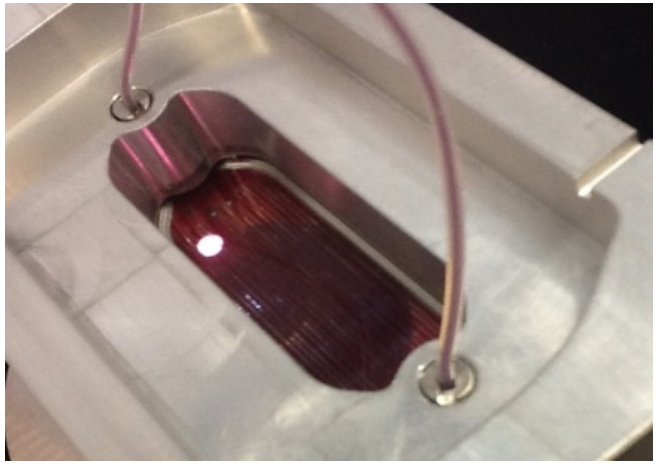
    Scher HI *et al*. JAMA Oncology 2016, 2:1441-9.

    Graf RP *et al.* Eur Urol. 2020. 77:170-177.

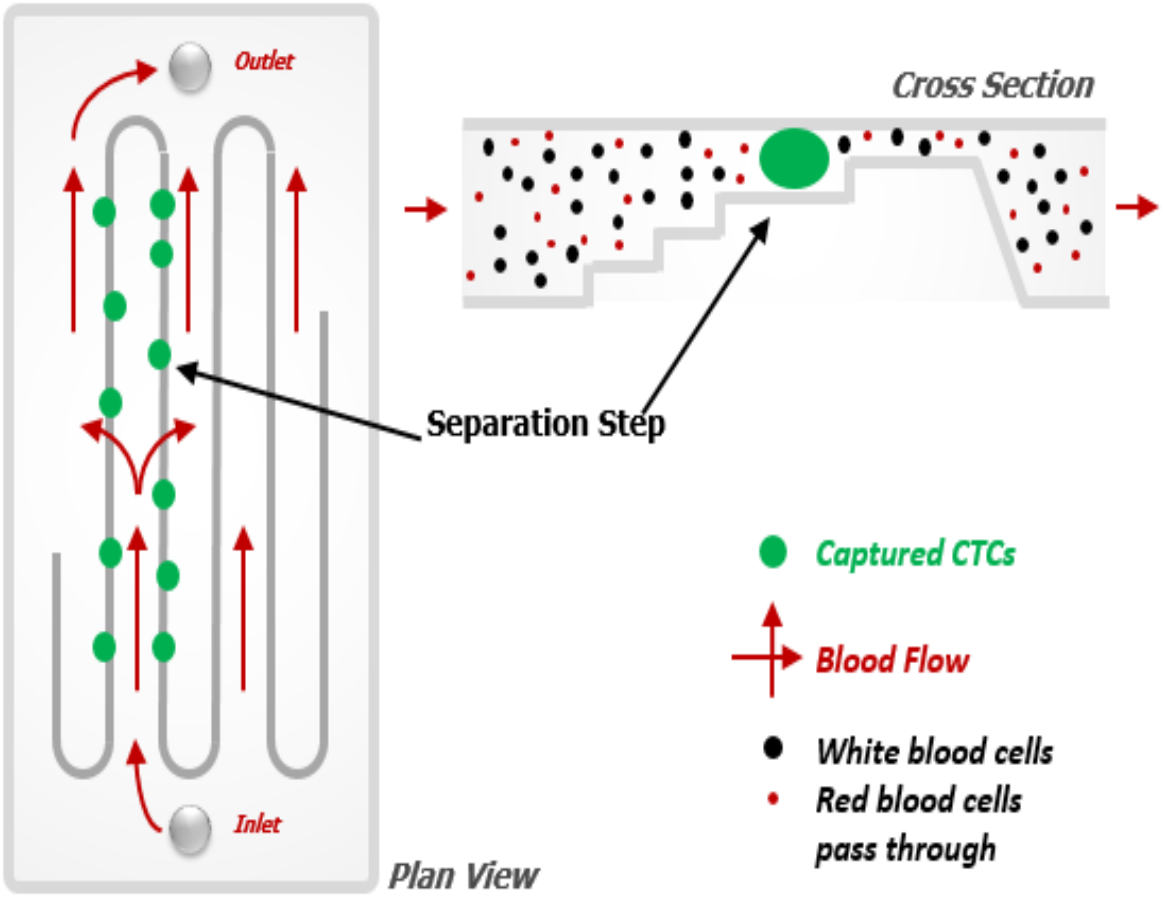


Parsortix- cell size and deformability based CTC isolation

System overview

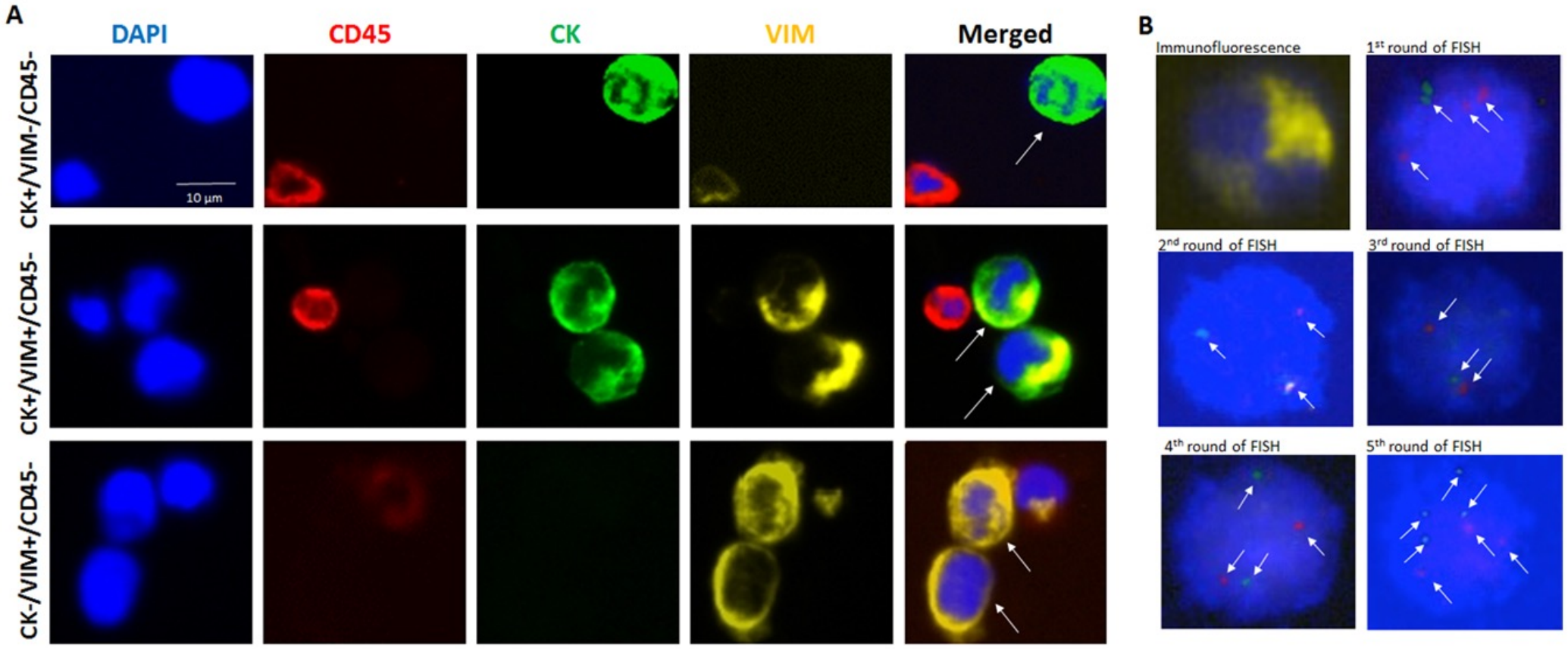


Clamp holding the isolation cassette



Principle for isolation

Three types of CTCs and FISH confirmation

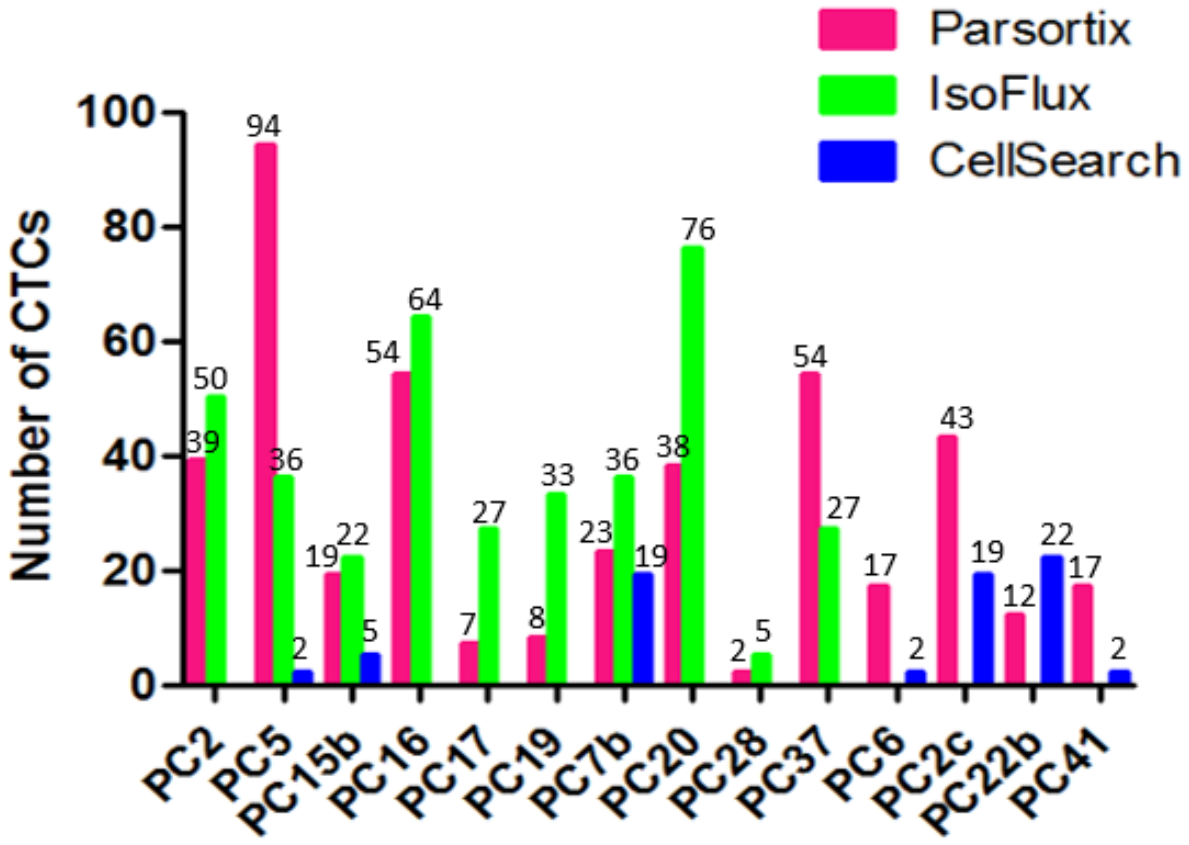


Repeated FISH: similar amount of genomic changes in each subtype

Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

Comparison of Parsortix and EpCaM beads system

Parsortix vs CellSearch (FDA approved) and IsoFlux (additional microfluid design to improve CTC isolation efficiency)



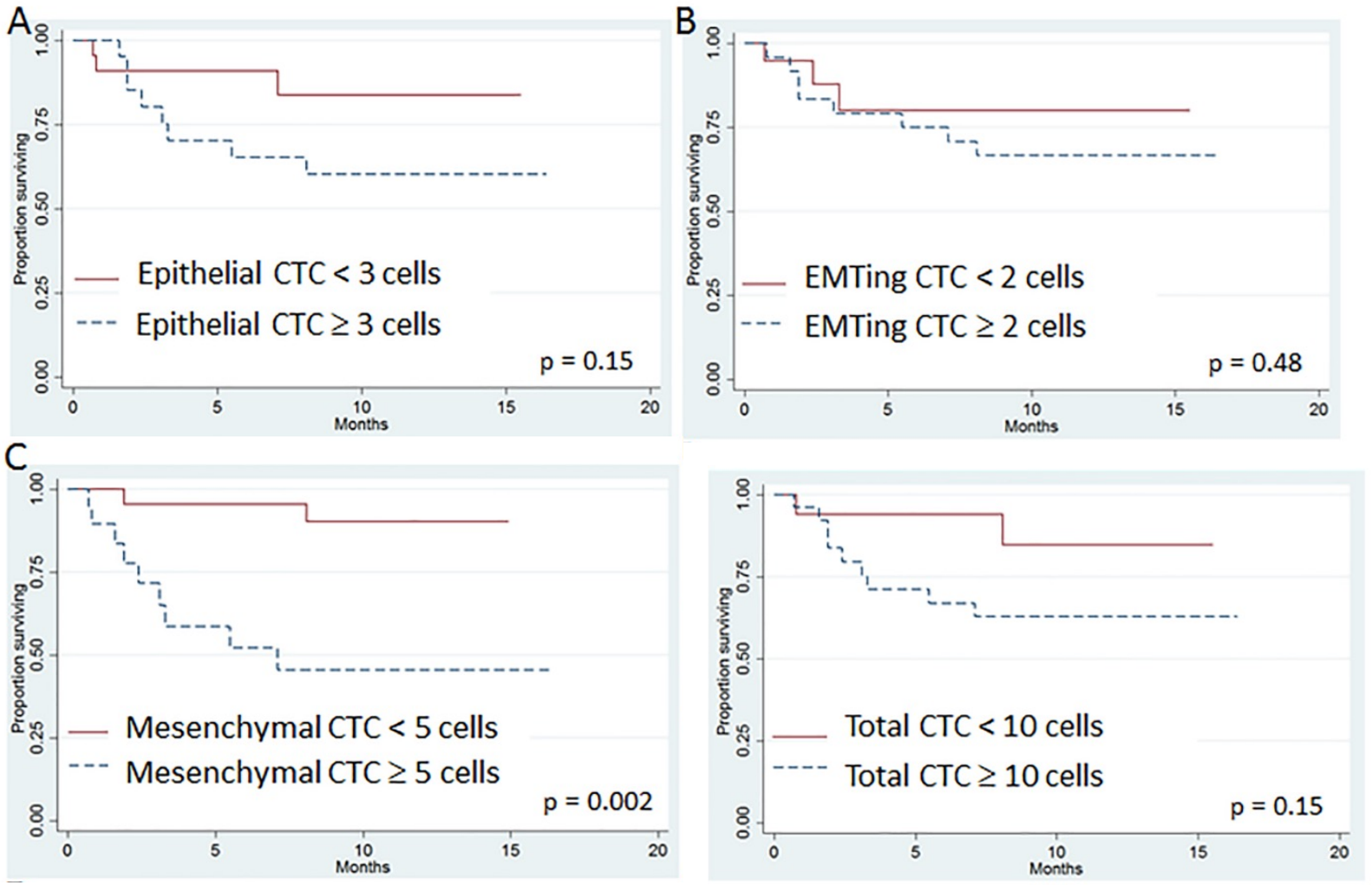
For epithila CTCs

Parsortix (32.1±29.1/patient) Vs CellSearch (10.1±9.3/patient)
p = 0.04

Parsortix (33.8±28.3/patient) Vs IsoFlux (37.6±20.8/patient)
p = 0.33

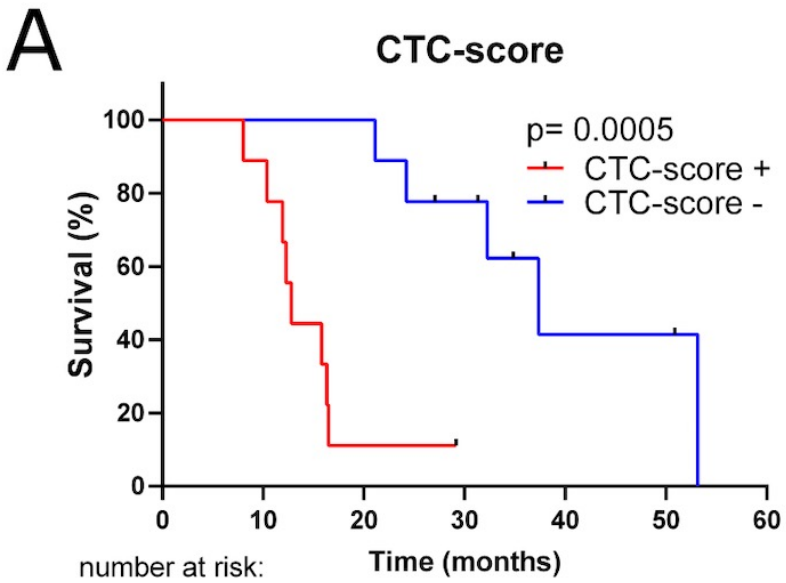
Xu *et al*. PLoS One 2015;10(9):e0138032.

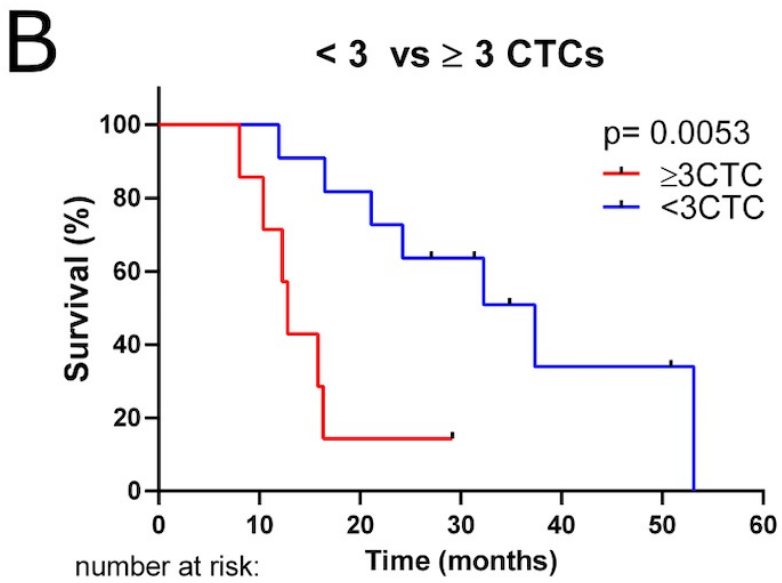
CTC subtypes for prostate cancer patient survival

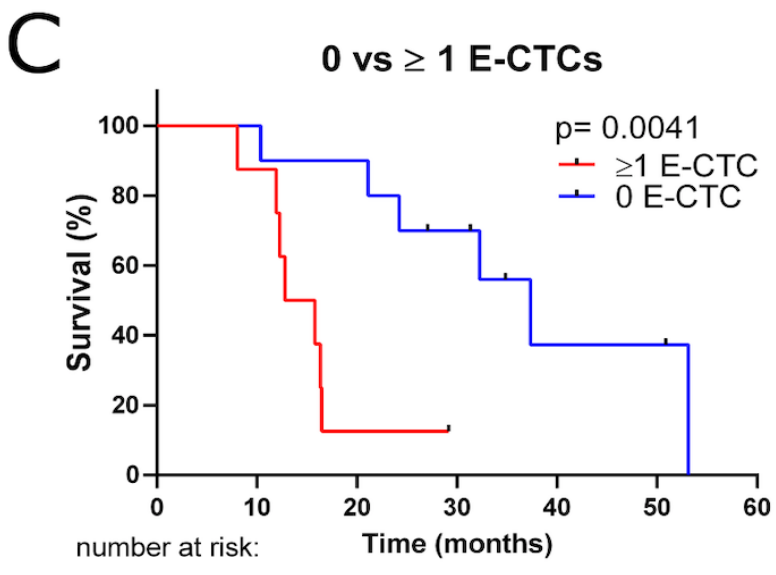


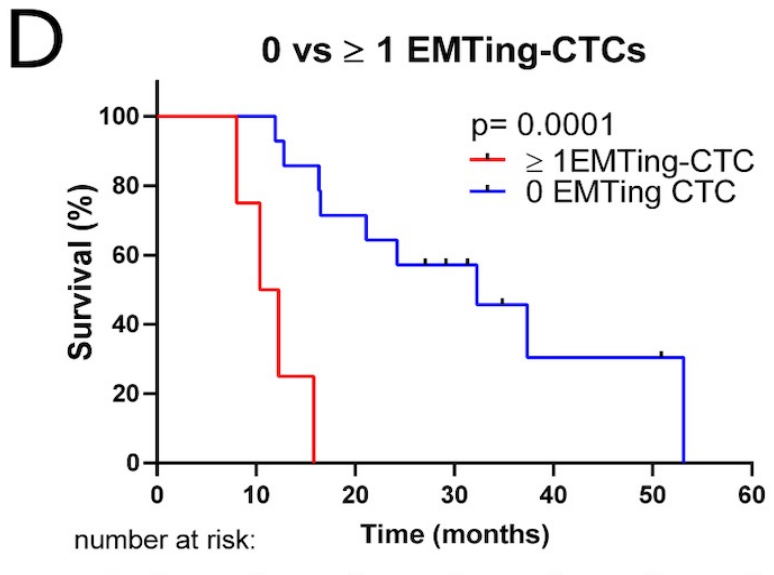
Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

CTCs in predicting docetaxel response- mCRPC



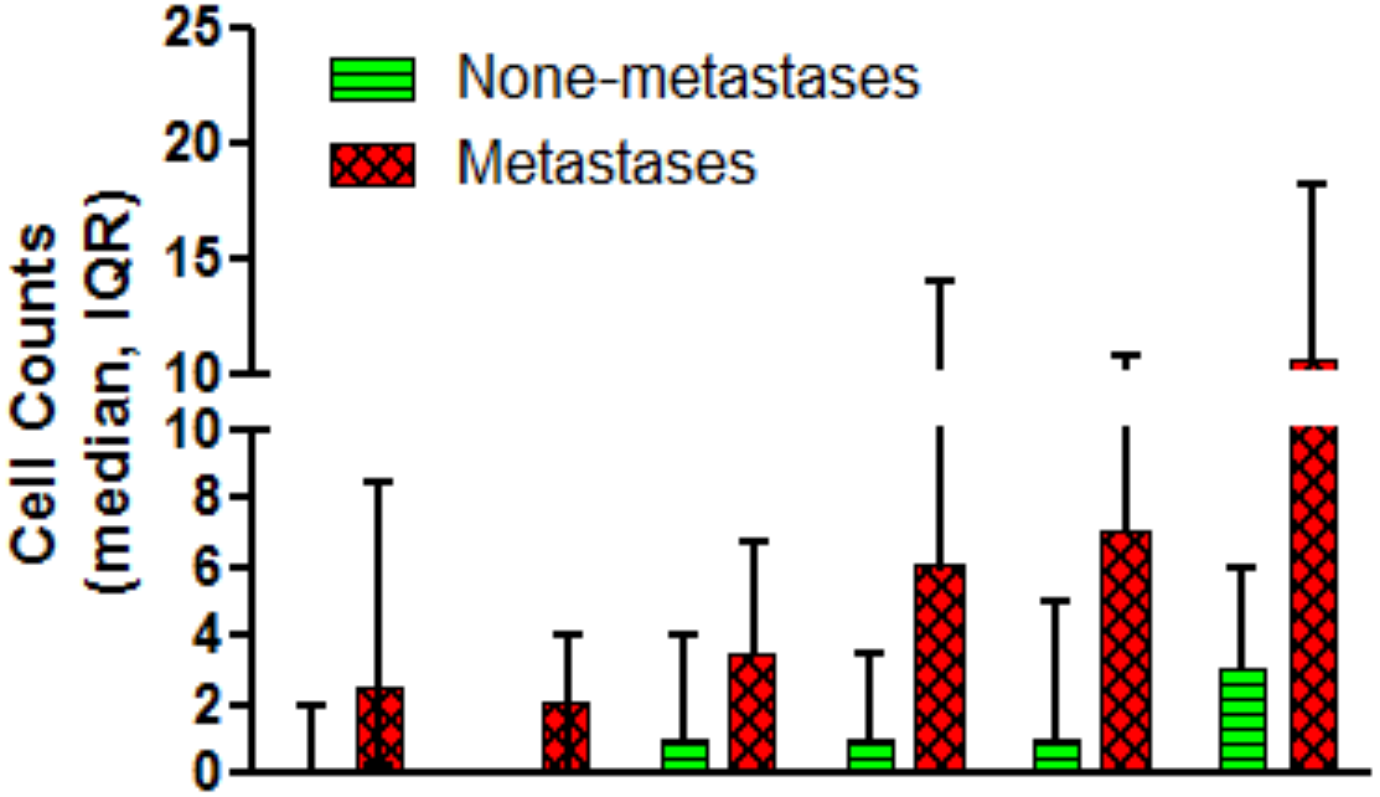






Davis *et al.*
Unpublished data

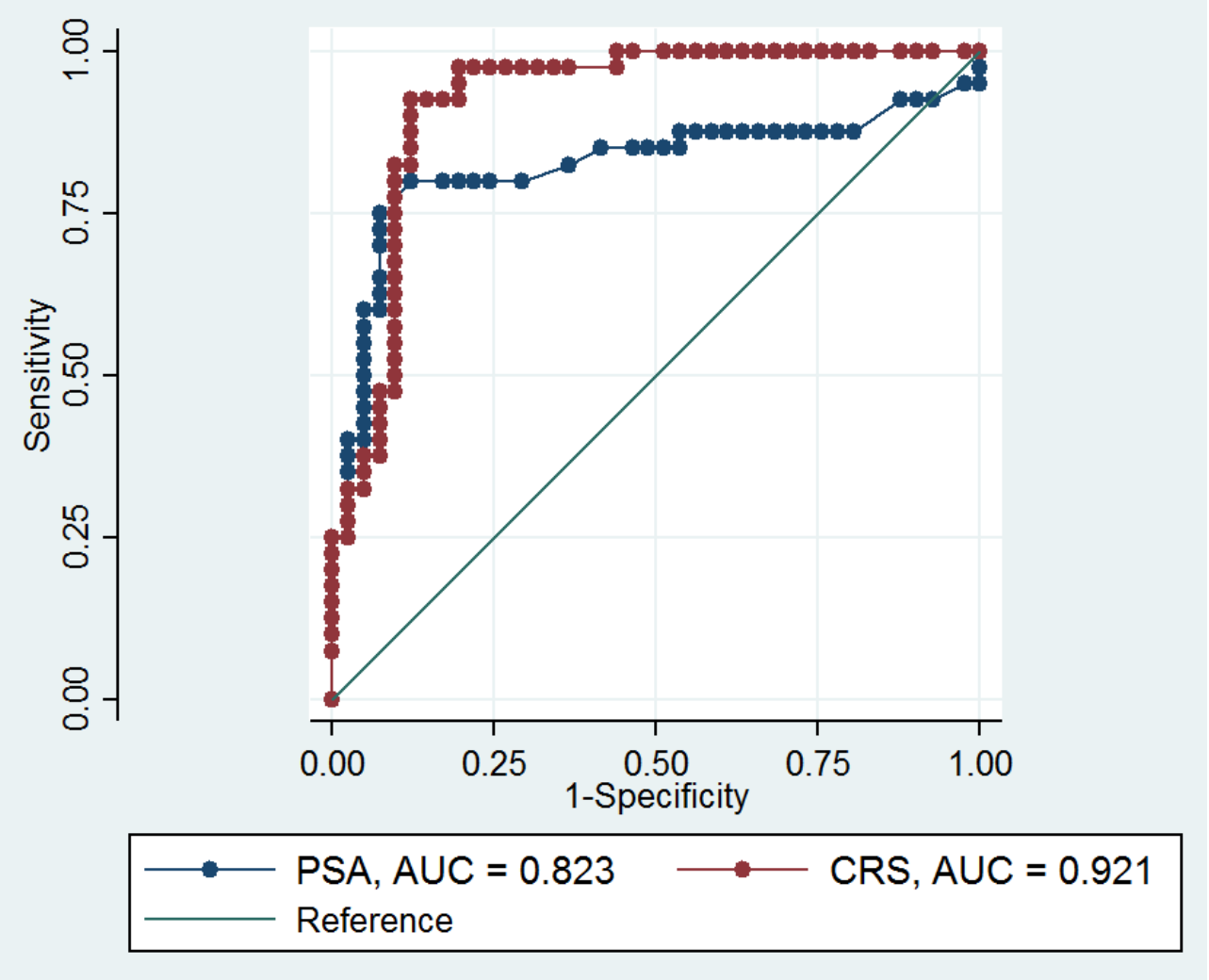
CTC association with cancer metastasis



CTC subtype	CK+/VIM-	CK+/VIM+	CK-/VIM+	All CK+	All VIM+	All
Non-metastases	0(0-2)	0(0-0)	1(0-4)	1(0-3.5)	1(0-5)	3(1-6)
Metastases	2.5(0.25-8.5)	2(0-4)	3.5(1.25-6.75)	6(2-14)	7(3-10.75)	10.5(7-18.25)
p value	0.0017	0.0001	0.0082	0.0001	0.0001	0.0001

Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

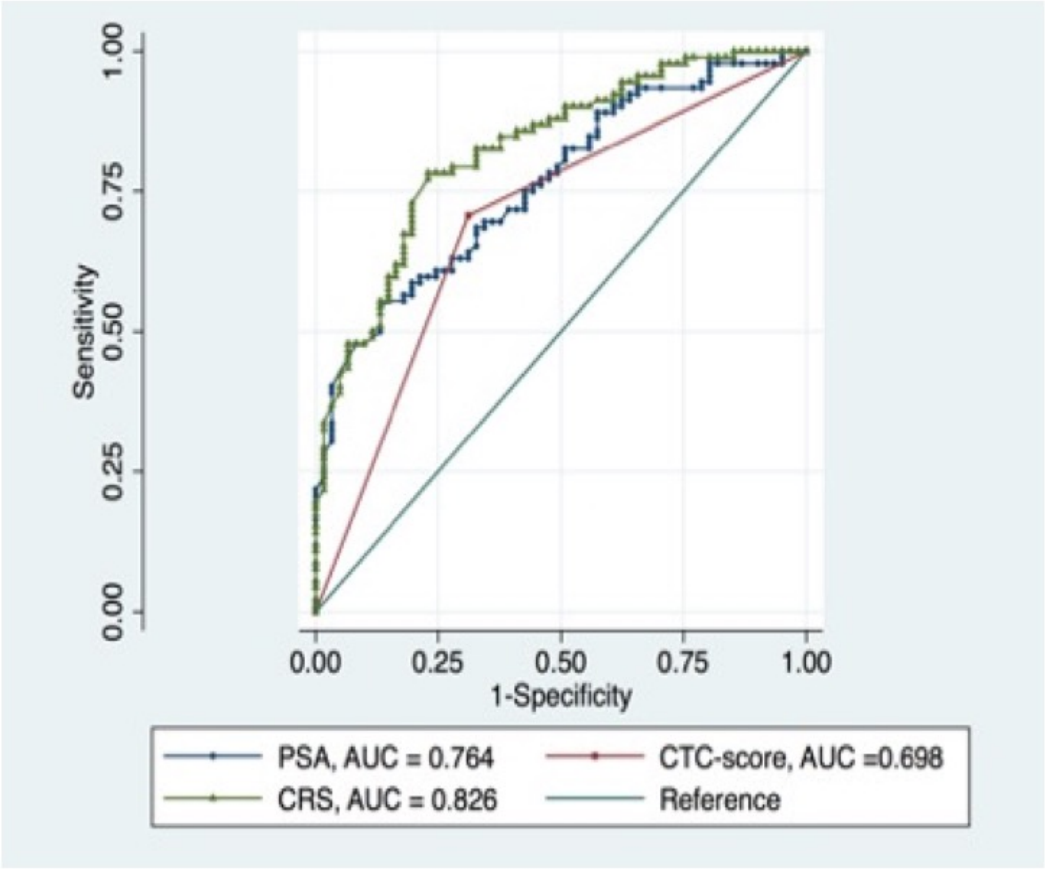
Efficiency to predict metastases- with PSA



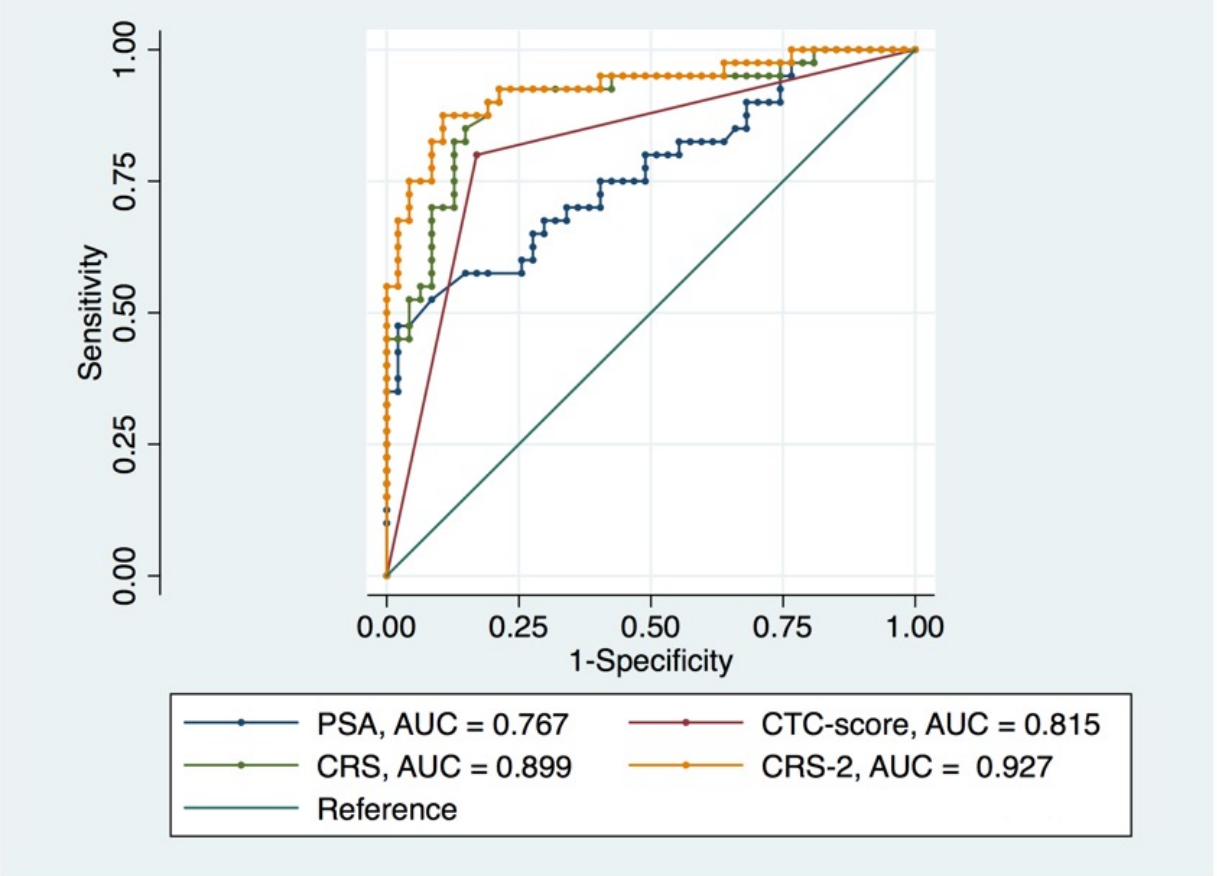
Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

CTCs for diagnosing clinical significant prostate cancer

Prostate cancer diagnosis pathway: PSA-mpMRI-Biopsy



In 155 localised PCa patients



In 98 pre-biopsy patients

Xu *et al. J Urol. 203(1):73-82; 2020*

Circulating tumour cells as biomarkers for treatment stratification of localised prostate cancer

Funder: Prostate Cancer UK, 5 years

Hypothesis:

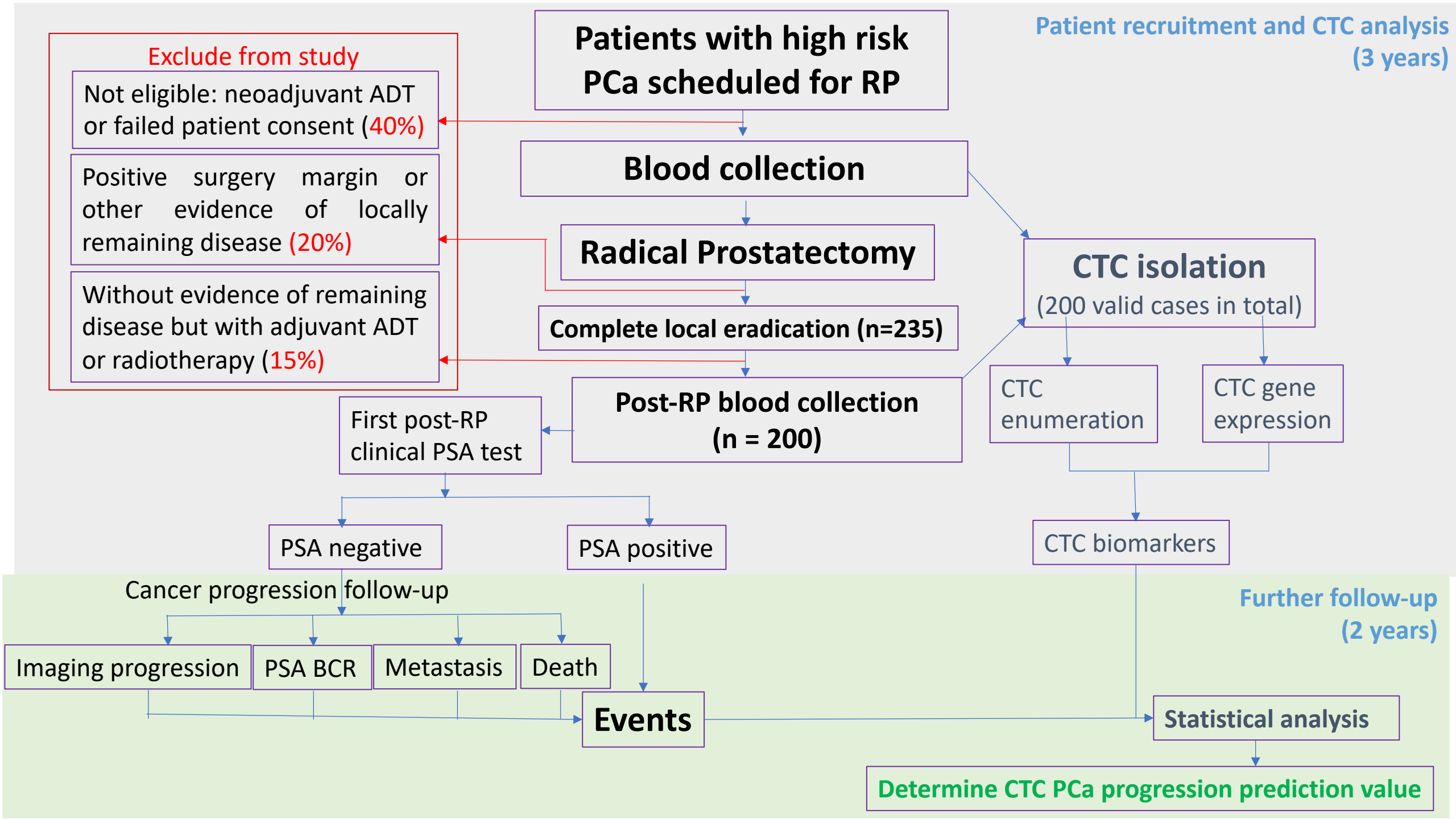
Post radical proctectomy prostate cancer recurrence/metastasis is caused by micro-metastasis

Micro-metastasis can be determined by CTC analysis

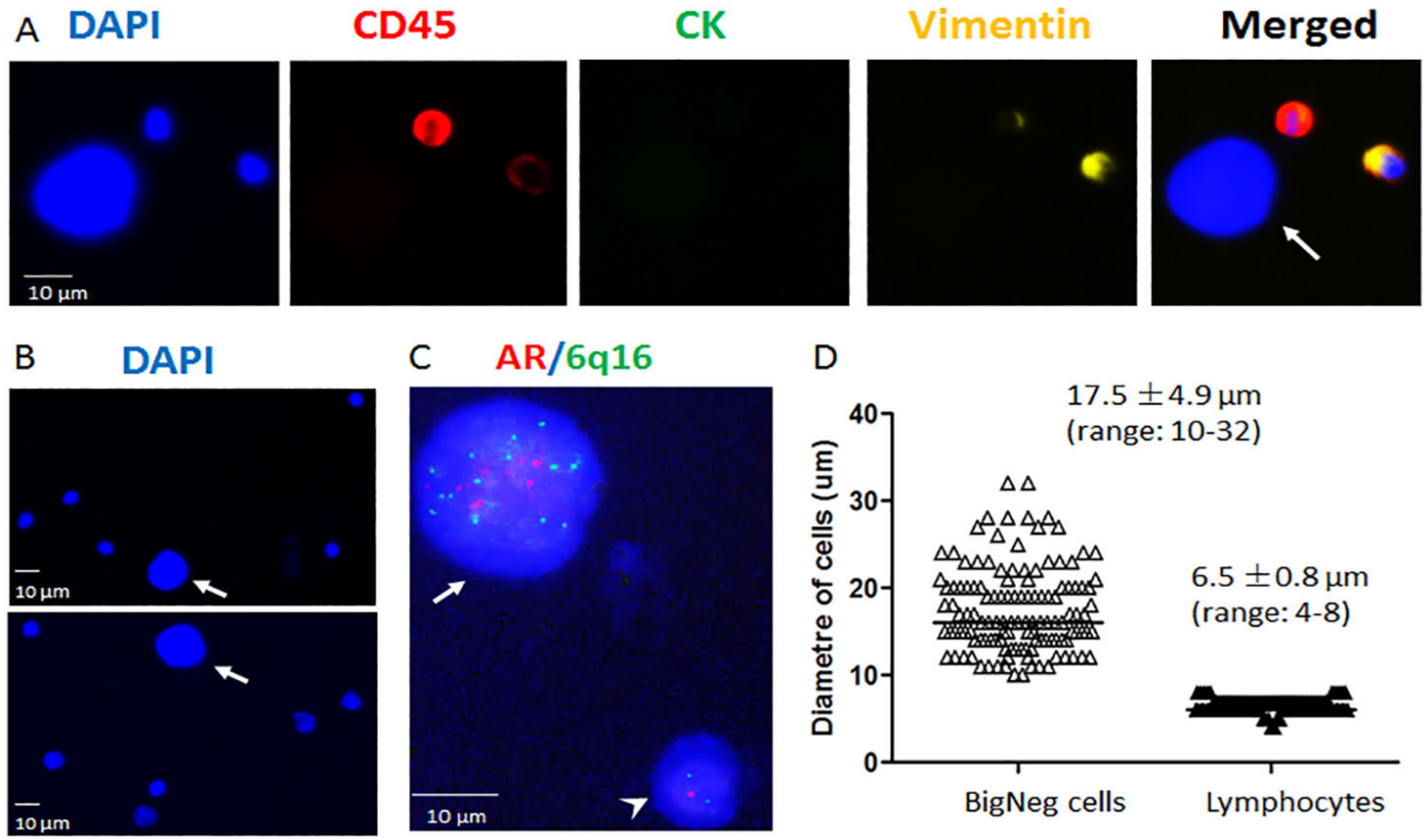
Aims:

CTC test (enumeration and gene expression) in predicting post-RP treatment failure

- time of the development of BCR
- new lesions detected by cancer imaging.

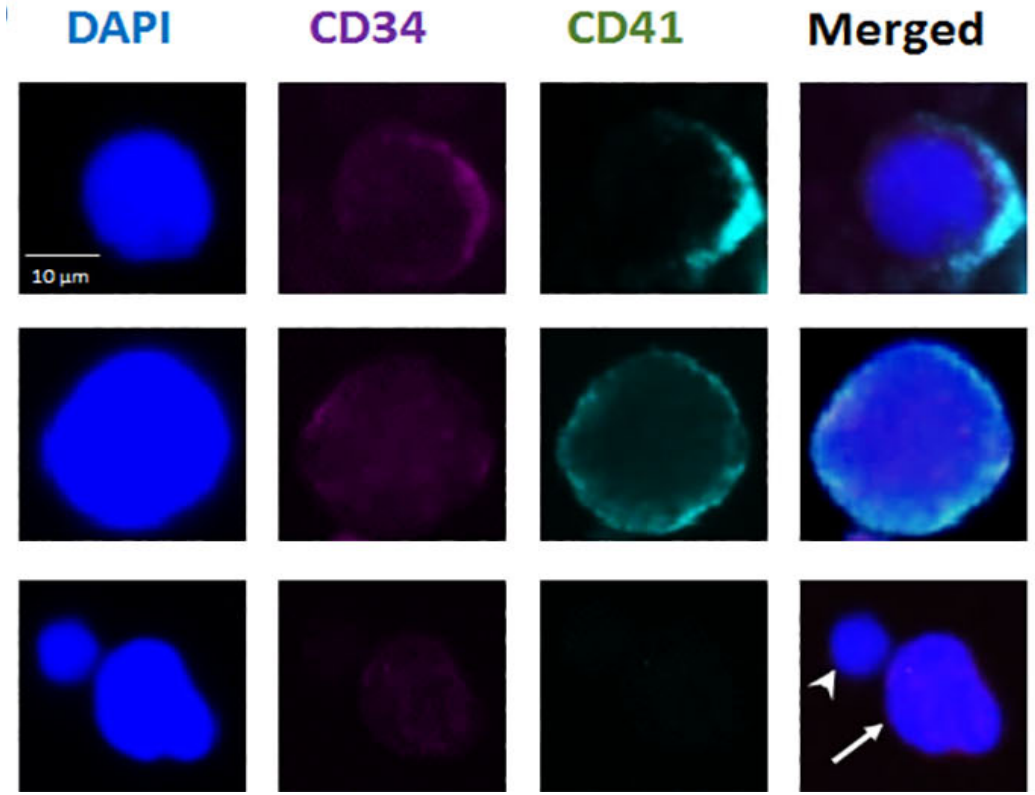

Patient recruitment and CTC analysis (3 years)
Patients with high risk PCa scheduled for RP
Exclude from study
Not eligible: neoadjuvant ADT or failed patient consent (40%)
Positive surgery margin or other evidence of locally remaining disease (20%)
Without evidence of remaining disease but with adjuvant ADT or radiotherapy (15%)
Blood collection
Radical Prostatectomy
Complete local eradication (n=235)
CTC isolation
(200 valid cases in total)
CTC enumeration
CTC gene expression
Post-RP blood collection (n = 200)
First post-RP clinical PSA test
PSA negative
PSA positive
CTC biomarkers
Cancer progression follow-up
Imaging progression
PSA BCR
Metastasis
Death
Events
Further follow-up (2 years)
Statistical analysis
Determine CTC PCa progression prediction value

Surprising things may happen-
Identification of circulating megakaryocytes



Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

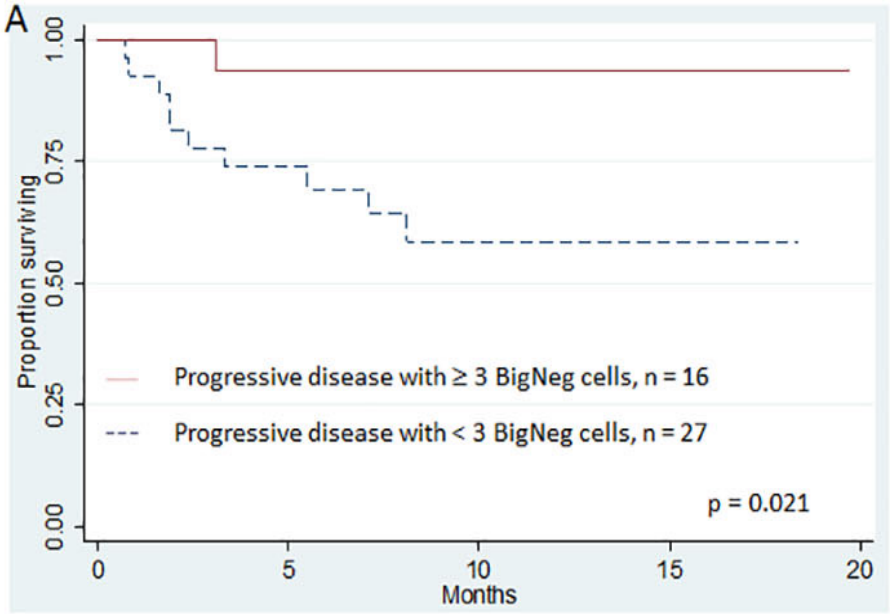
Megakaryocyte nature confirmed by immunofluorescence



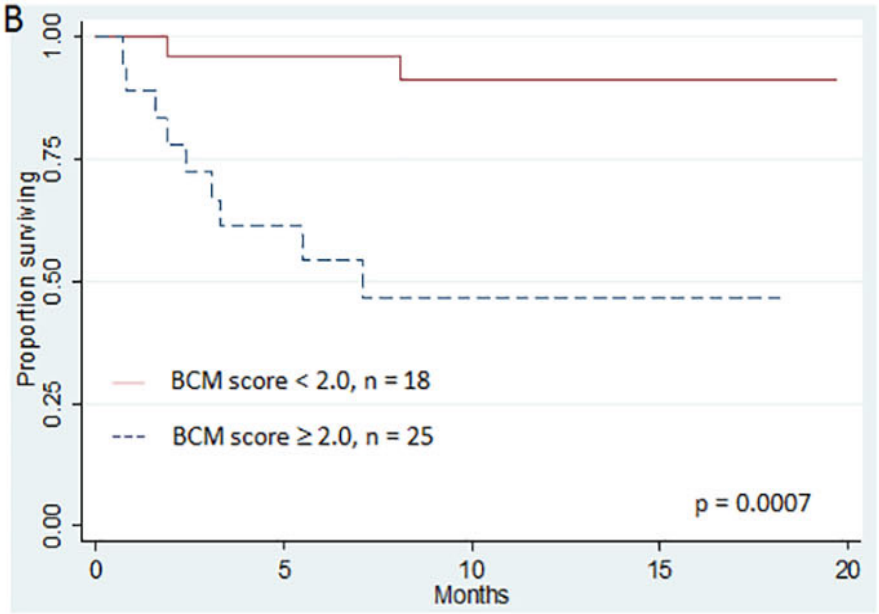
Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

Prognostic value of megakaryocytes in advanced prostate cancer

Megakaryocytes



BCM score (Megakaryocytes and CTC)



BCM (Barts CTC megakaryocyte) score

- Per unit increase: HR 1.282 (95%CI: 1.097-1.499)
- ≥ 2.0: HR 10.170 (95% CI: 2.164-47.789, p = 0.0005)

Xu *et al*. *Clin Cancer Res*. 23(17):5112-22; 2017.

Summary

- CTCs are valuable for prostate cancer treatment prediction/monitoring.
- Analysing subtypes of CTCs are necessary
- The potential for early detection of aggressive cancer
- Looking out for other undiscovered circulating biomarkers

Acknowledgements

My team

- **Dr. Xueying Mao**
- **Dr. Lei Xu**
- **Dr. Tianyu Guo**
- **Dr. Caitlin Davis**
- **Dr. Elzbieta Stankiewicz**
- **Miss Edwina Burke**
- **Dr. Jacek Marzec**
- **Dr. Rachel Lawrance**
- **Dr. Tarek Al-Hammo**uri

Barts Health NHS & UCL Hospitals clinical sites

- Dr. Jonathan Shamash
- Prof. Greg Shaw
- Dr. Alistar Grey
- Dr. Prabhakar Rajan
- Dr. Puiying Chan
- Dr. John Hines
- Prof. Tim Oliver

Barts Cancer Clinical Trail Unit

- Prof. Rhian Gabe
- Mr Tom Duffy
- Ms Bhagyashree Ingle

Orchid Tissue Bank

- Prof. Daniel Berney
- Miss Sakunthala Kudahetti
- Dr. Glenda Scandura
- Mrs Sara Vidal

All healthy donors and patients

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JOURNAL ARTICLE

Development and Analytical Validation of a 6-Plex Reverse Transcription Droplet Digital PCR Assay for the Absolute Quantification of Prostate Cancer Biomarkers in Circulating Tumor Cells of Patients with Metastatic Castration-Resistant Prostate Cancer

Martha Zavridou, Stavroula Smilkou, Victoria Tserpeli, Aggeliki Sfika, Evangelos Bournakis, Areti Strati ✉, Evi Lianidou ✉

Clinical Chemistry, hvac125, https://doi.org/10.1093/clinchem/hvac125

Published: 12 September 2022 **Article history** ▾

Outline

We developed a novel 6-plex RT-ddPCR assay for the simultaneous absolute quantification of ARFL, AR-V7, PSA, and PSMA, HPRT (used as a reference gene), and a synthetic DNA-EC that was included for quality control

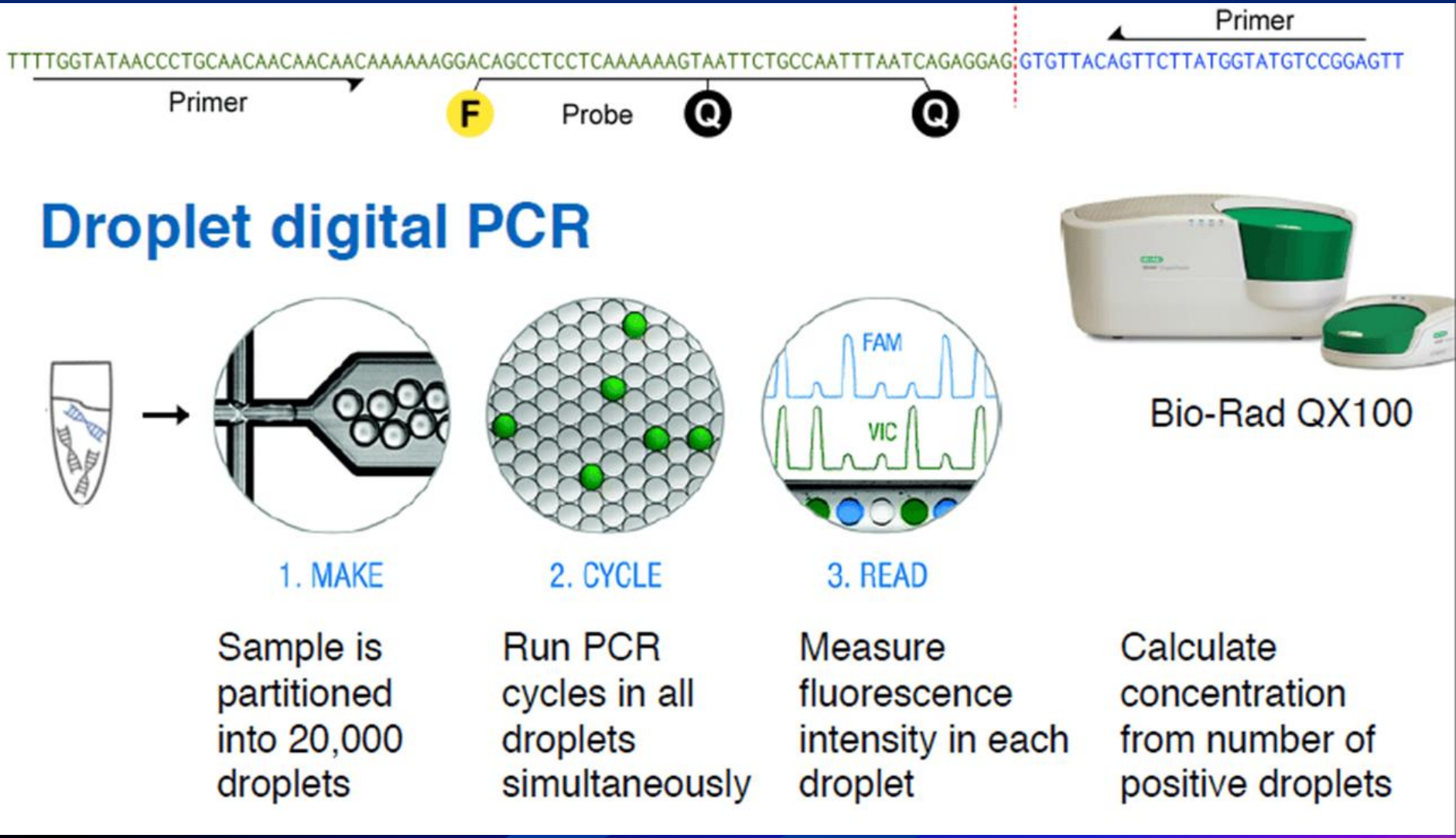
The assay was optimized and analytically validated using DNA synthetic standards for each transcript as positive controls.

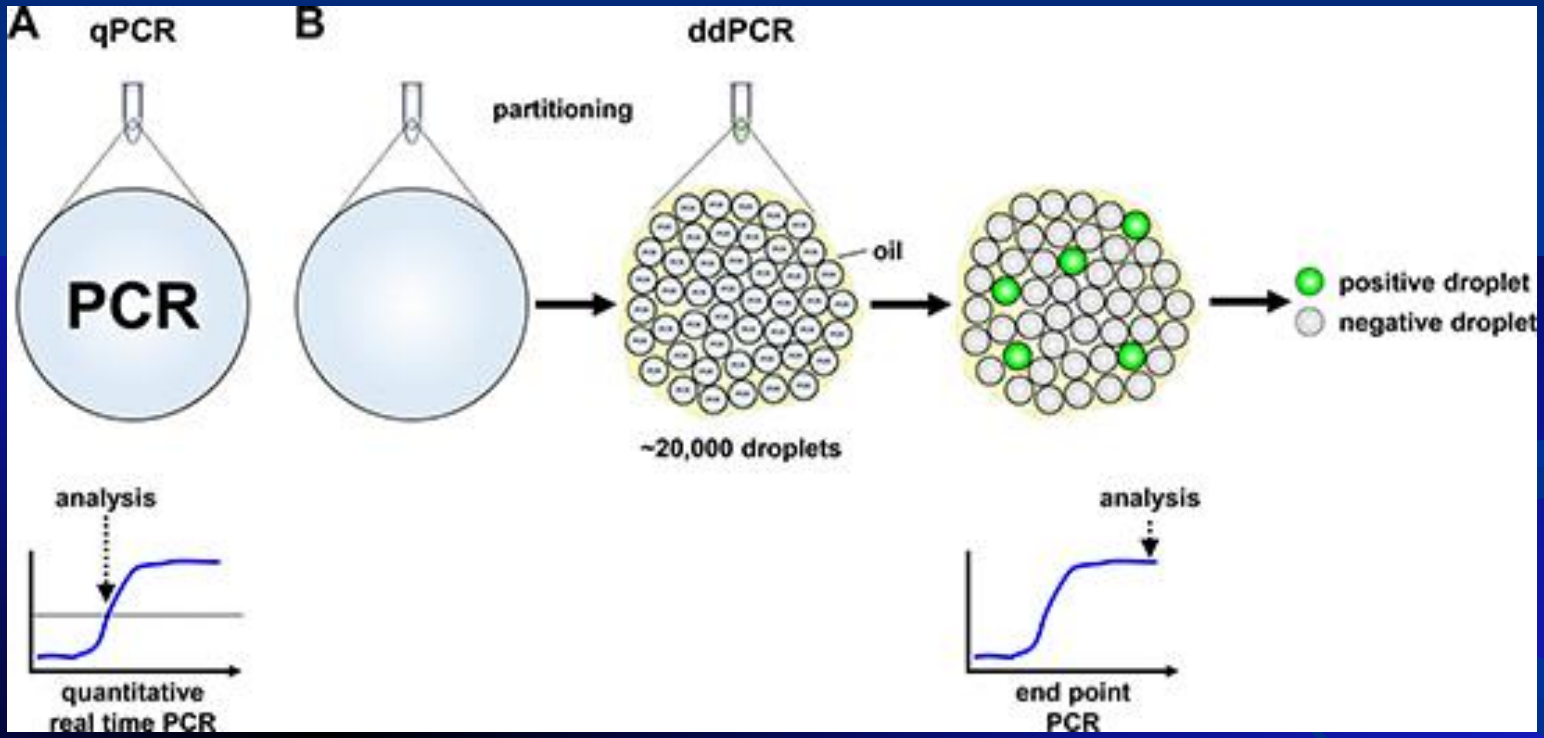
EpCAM-positive CTC fractions isolated from 90 mCRPC patients and 11 healthy male donors were analyzed

Results were directly compared with RT-qPCR for all markers in all samples

ddPCR instrumentation

ddPCR was performed in the QX200 AutoDG Droplet Digital PCR System (Bio-Rad)




A
qPCR
PCR
B
ddPCR
partitioning
oil
~20,000 droplets
positive droplet
negative droplet
analysis
quantitative
real time PCR
analysis
end point
PCR

DNA EXTERNAL CONTROL

DNA-EC was designed de novo and synthesized to evaluate the performance of the developed RT-ddPCR assay

This DNA oligo is a 144 bp double-stranded synthetic DNA that is not present in the human genome

DNA SYNTHETIC OLIGOS

We designed in silico 6 double-stranded synthetic DNA sequences that represent the 6 different transcripts analyzed in this assay, AR-FL, AR-V7, PSA, PSMA, HPRT, and DNA-EC

These synthetic oligos were used as standards of known sequence and concentration for the evaluation of the analytical sensitivity and analytical specificity of the developed multiplex RT-ddPCR assay

Hydrolysis probes

All hydrolysis probes were designed with a 6-carboxyfluorescein (FAM) or hexachlorofluorescein (HEX) fluorophore and ZEN/ Iowa Black FQ quenchers for more efficient quenching

Multiplexing and probe mixing

6-plex RT-ddPCR in a ddPCR instrument that has only 2 emission channels.
we evaluated the specificity of the assay using different ratios of FAM and HEX fluorescent configurations and finally selected the following:

AR-V7 (100% FAM),

PSA (80%FAM/20% HEX),

AR-FL (50% FAM/50% HEX),

PSMA (35% FAM/65% HEX),

DNA-EC (35% FAM/65% HEX),

HPRT (100% HEX).

Based on these ratios, the discrimination of 6 different clusters was achieved

Analytical Validation

- Linear dynamic range
- Limit of detection
- Limit of quantification
- Intra- and interassay precision
- Analytical specificity
- Analytical validation experiments were performed for each marker

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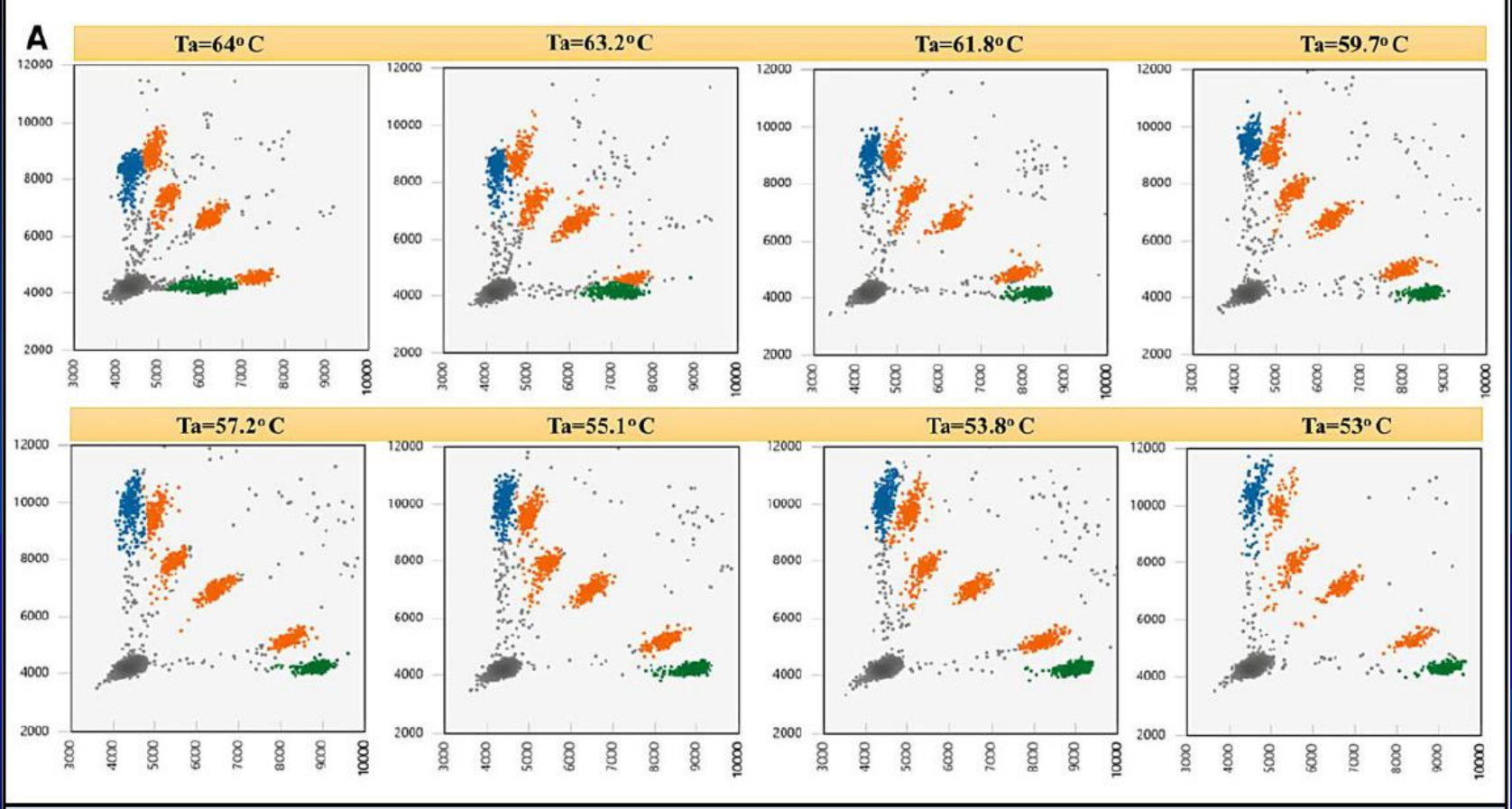

Fig. 1. Thermal gradient of 6-plex RT-ddPCR assay by applying 53°C to 63°C temperature range: (A) Two-dimensional plots derived at 8 different Ta and (B) the corresponding copies/μL. (C), Analytical specificity of the 6-plex RT-ddPCR.

At high Ta, poor separation and low fluorescence amplitude were observed in both channels (Fig. 1, A), while at low Ta, although the separation and fluorescence amplitude were satisfactory, the number of detected transcripts was lower (Fig. 1, B). Based on these results, we selected 55°C as the optimal annealing/extension temperature.

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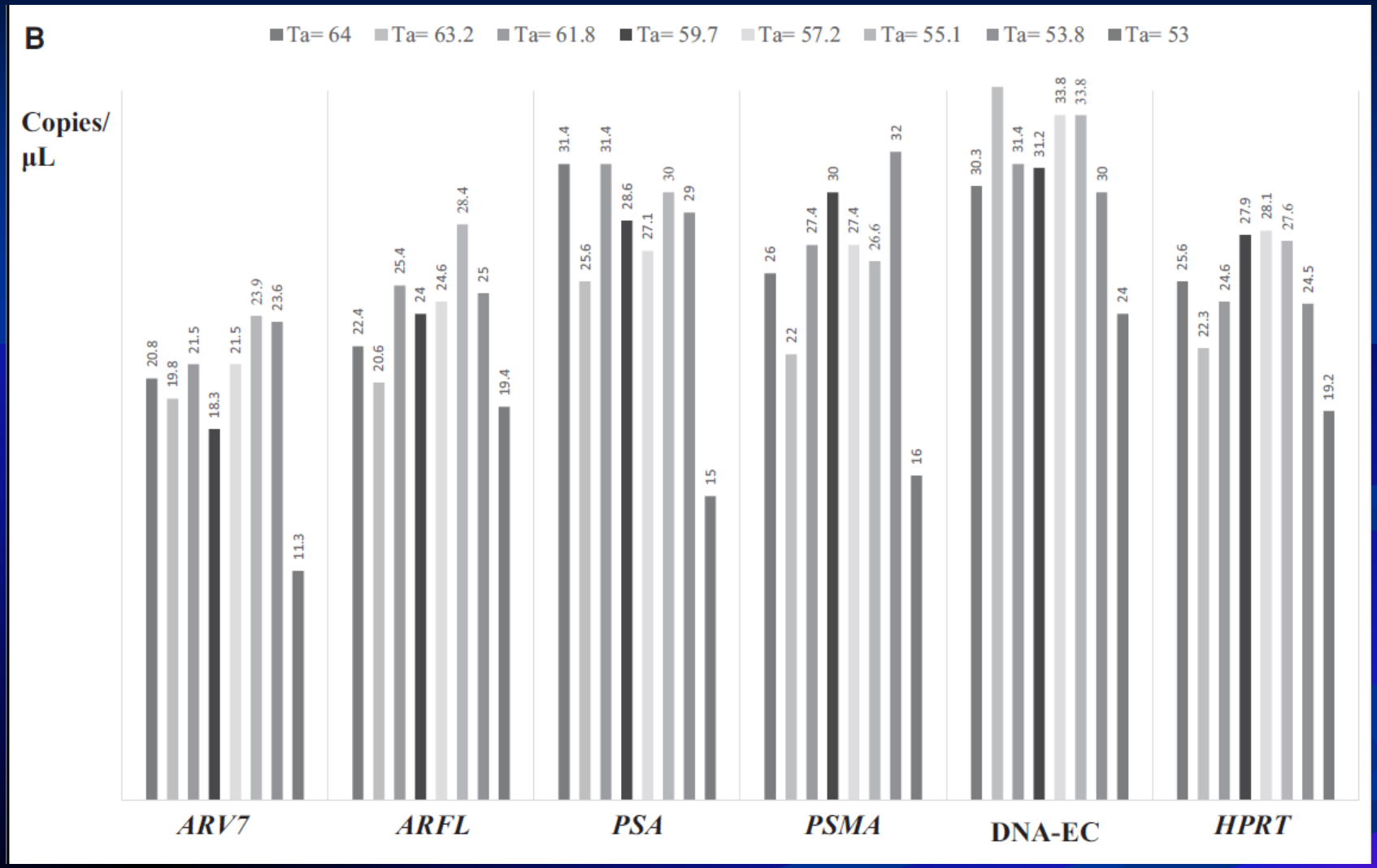

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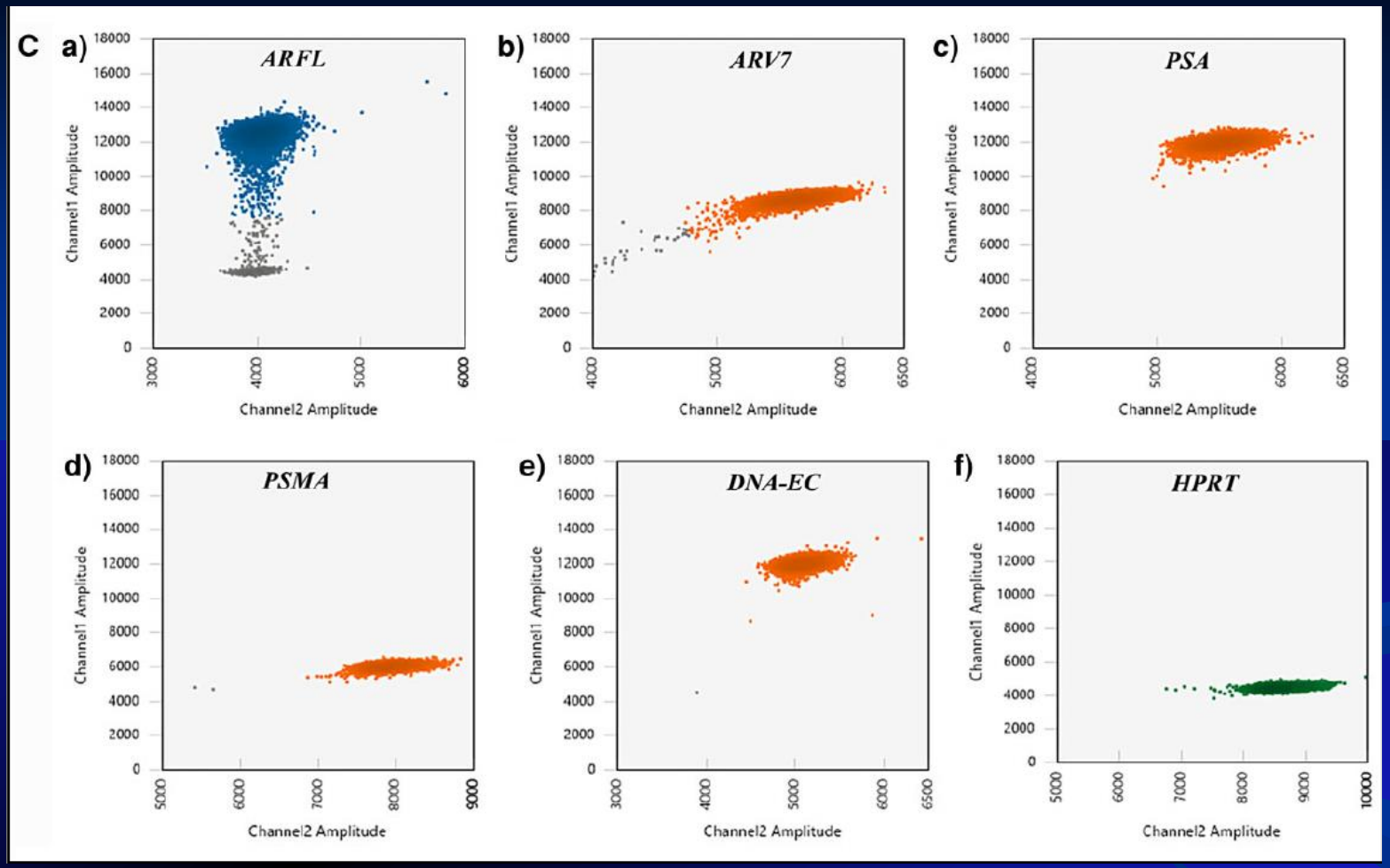
Analytical specificity



We evaluated the analytical specificity of the assay by using synthetic DNA standards corresponding to AR-FL, AR-V7, PSA, PSMA, HPRT, and DNA-EC.

Each pair of primers and probe was found to specifically amplify each gene in the presence of all other primers and probes

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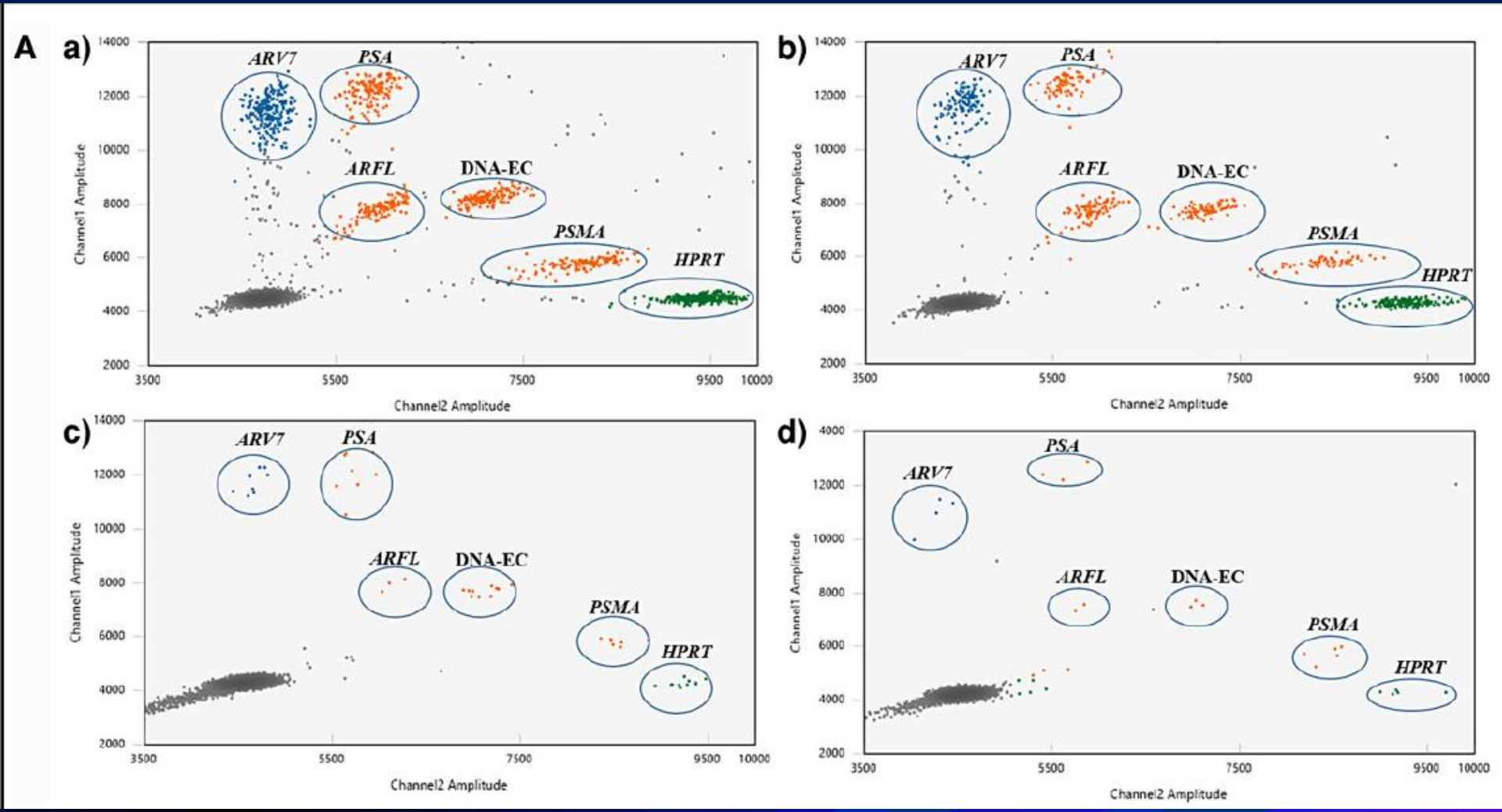
Analytical sensitivity



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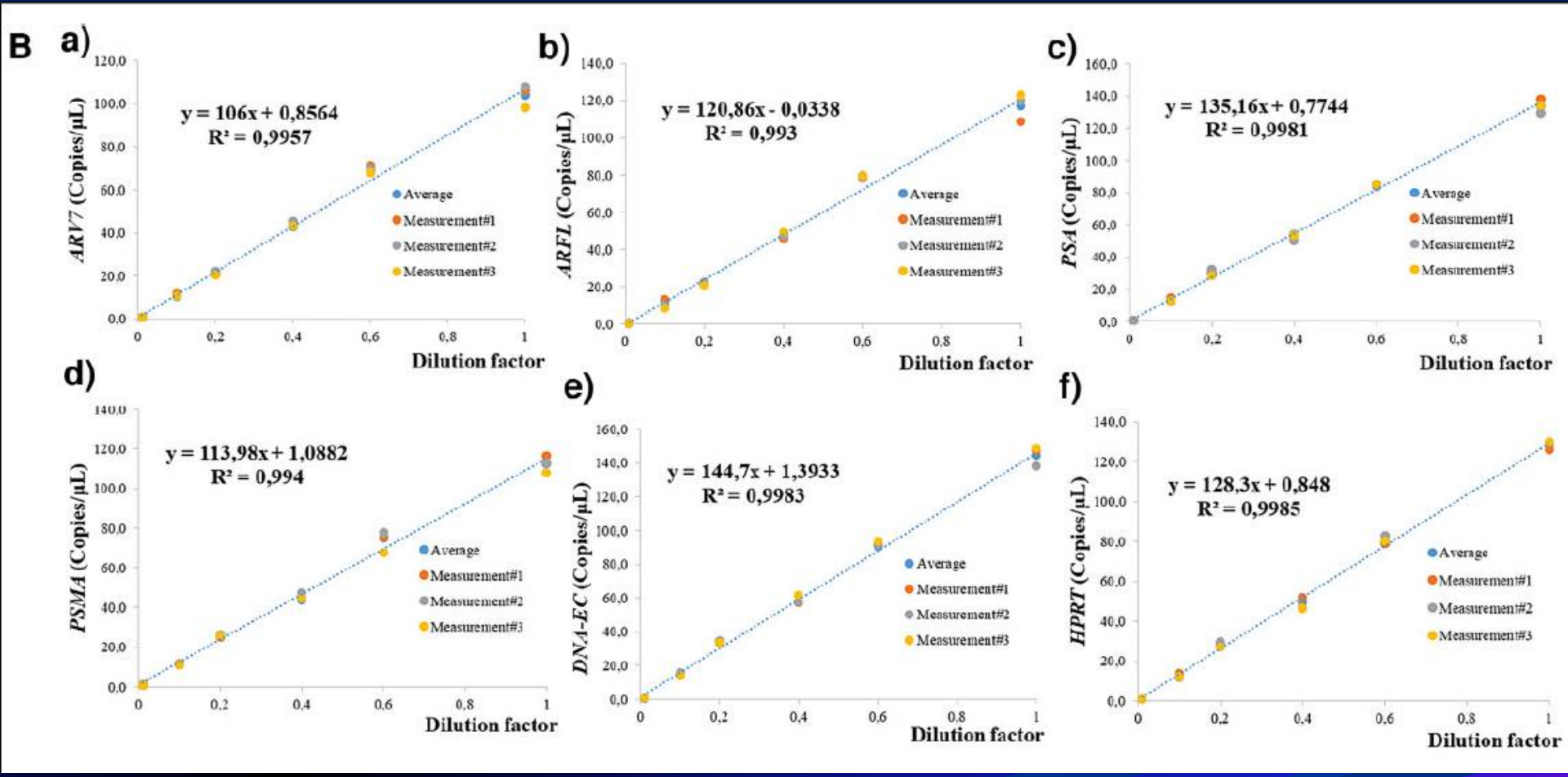
Linear dynamic range (LDR)



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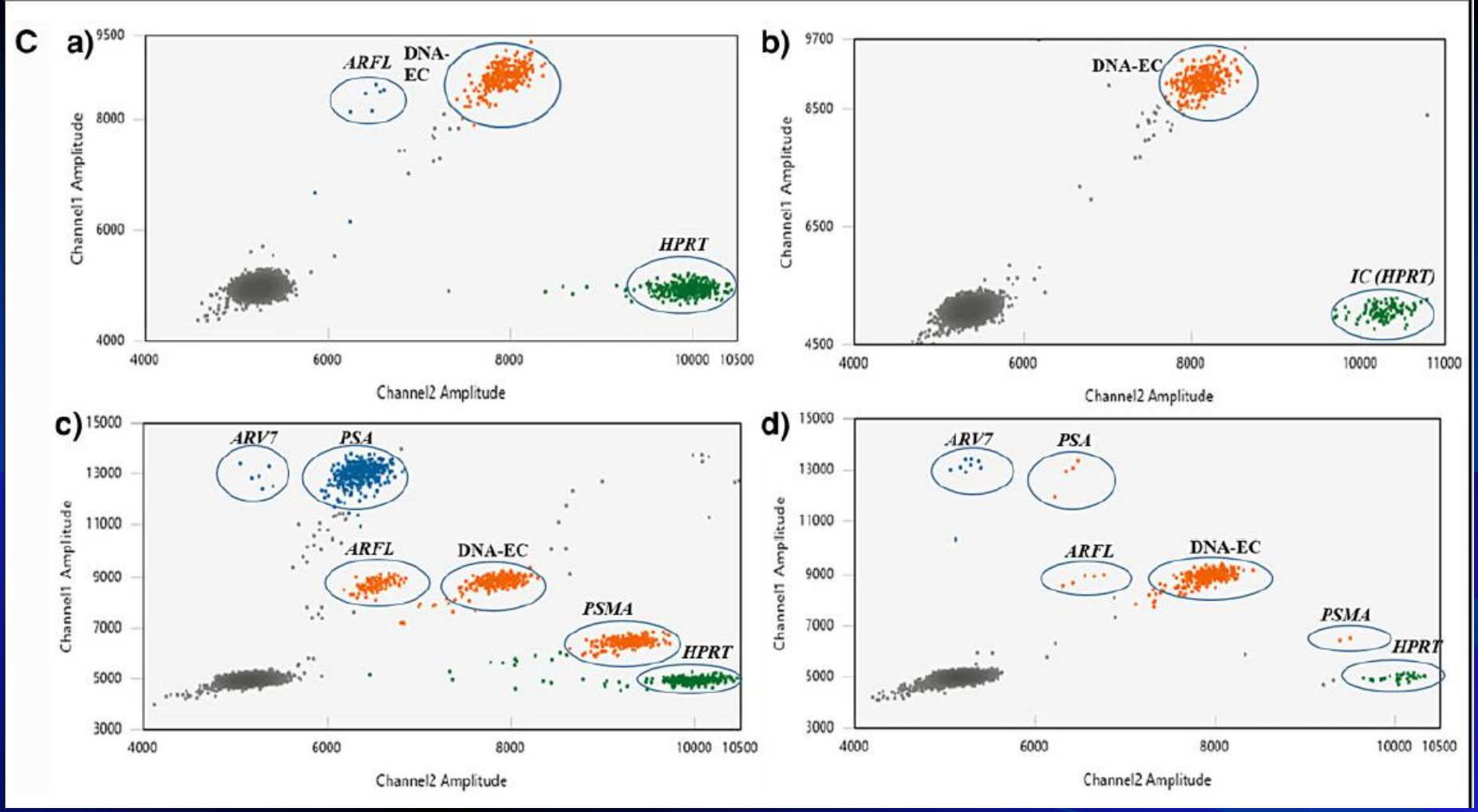

(a) HD sample
(b) a negative sample for all prostate markers
(c) a positive sample of high copy number of AR-FL, PSA, and PSMA and low copy number of AR-V7
(d) a positive sample of low copy number for all prostate markers

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Results

- ***AR-FL*: (71/90; 78.9%)**
- ***AR-V7*: (28/90; 31.1%)**
- ***PSA*: (41/90; 45.6%)**
- ***PSMA*: (38/90; 42.2%)**
- ***HPRT*: (90/90; 100%)**
- ***DNA-EC*: concentration was constant across all samples**

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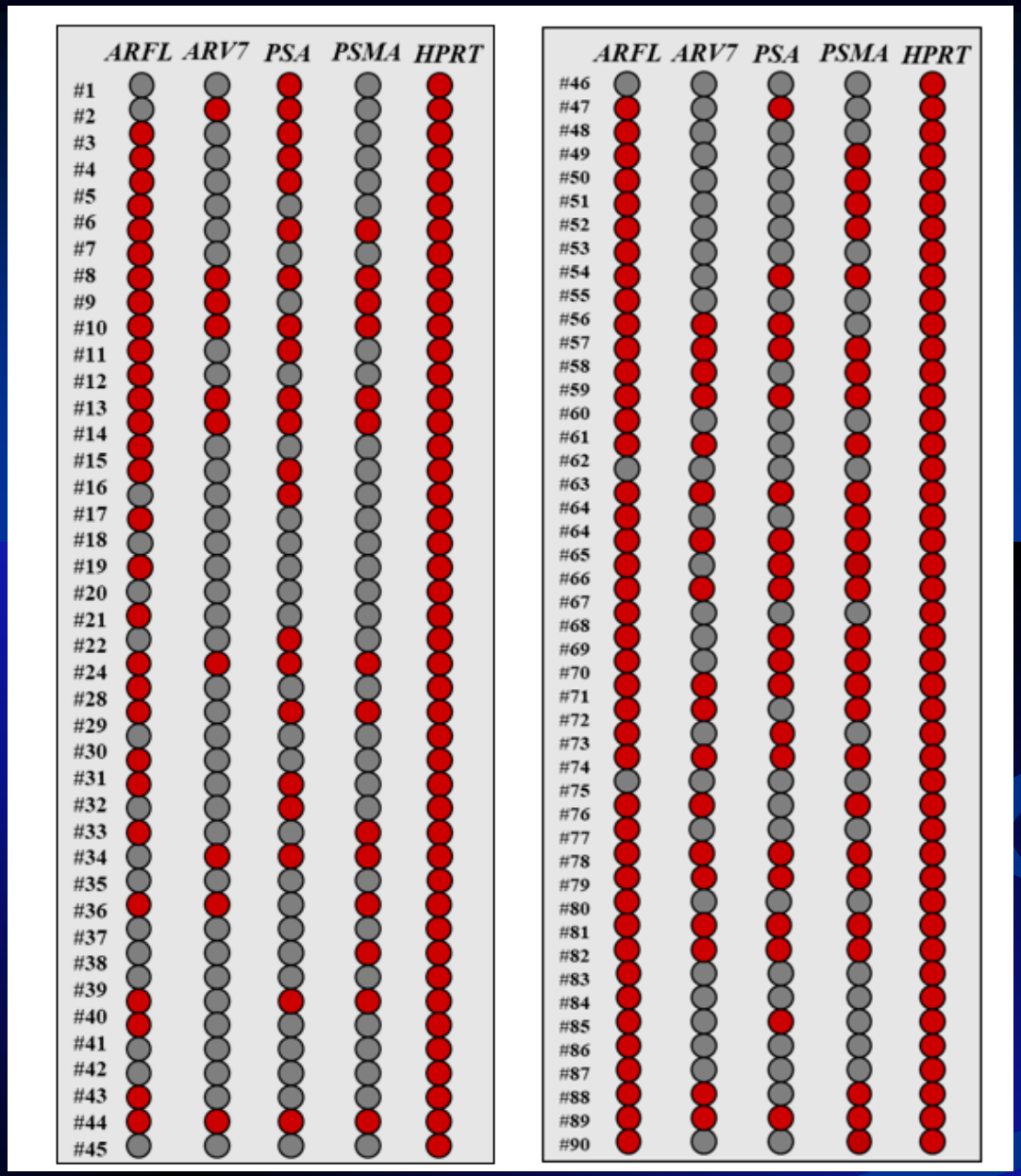

Prostate cancer gene markers in the EpCAM+ CTC fraction from mCRPC patients (n = 90) using 6-plex RT-ddPCR

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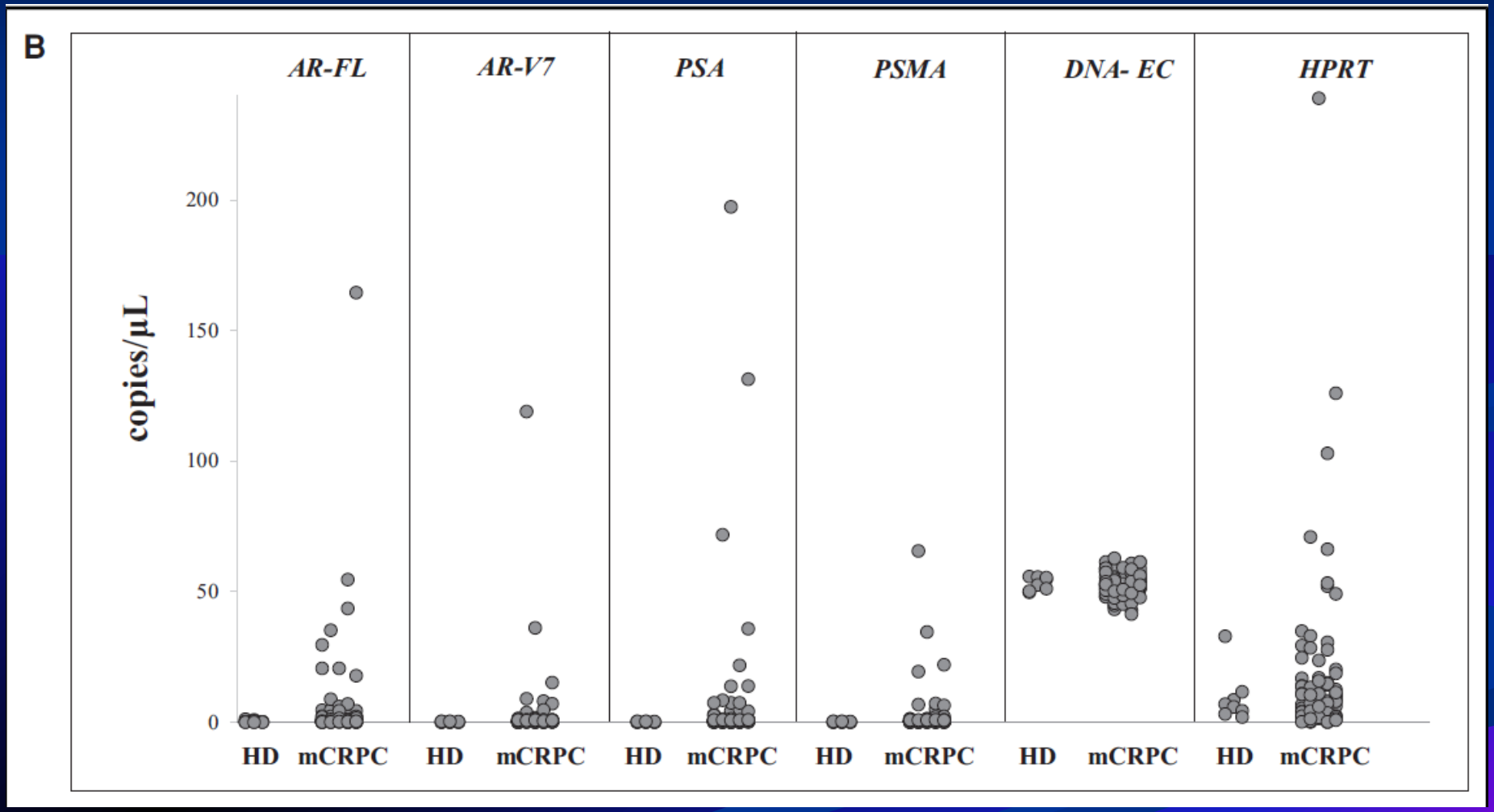
Absolute quantification of each transcript



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Quality control: Levey-Jennings chart for calculated DNA-EC derived from 90 measurements

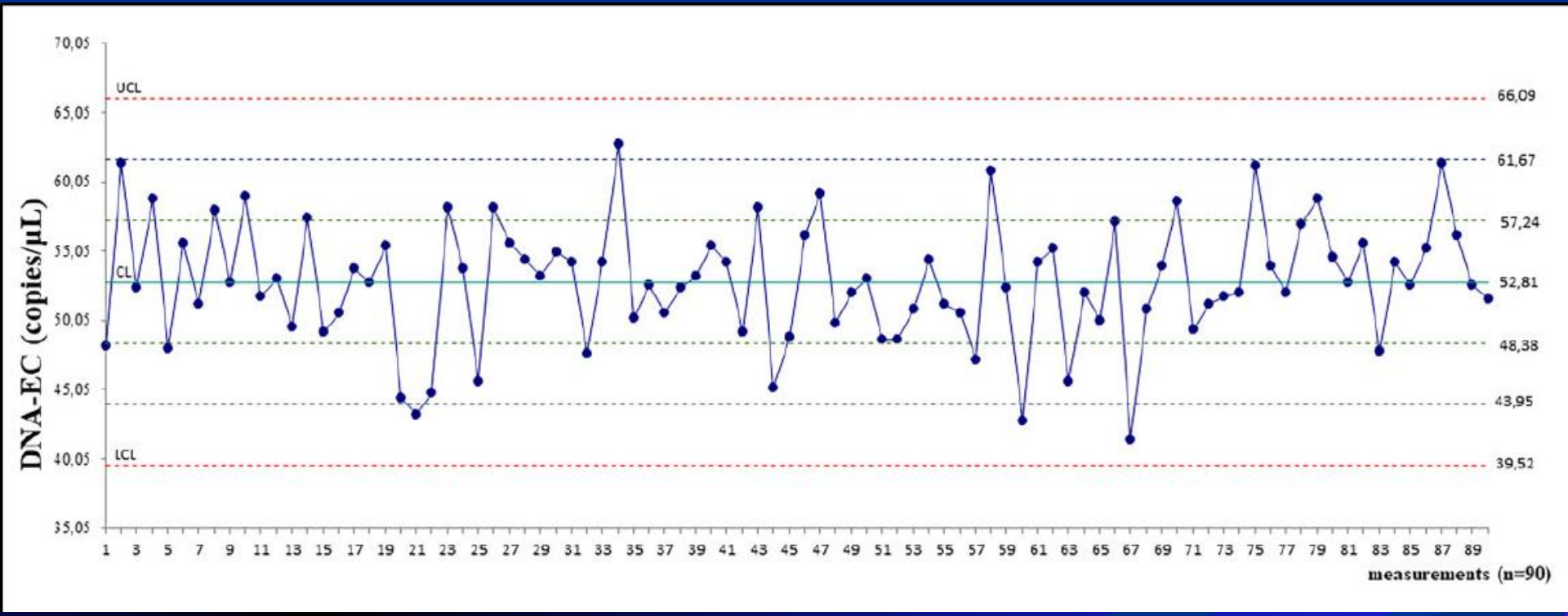

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Table 2. Direct comparison between RT-ddPCR and RT-qPCR for *ARFL*, *ARV7*, *PSA*, and *PSMA* transcripts using EpCAM-positive CTC fractions (n = 90).

	RT-ddPCR	
RT-qPCR	Positive	Negative
ARFL		
Positive	65	8
Negative	6	11
Total	71	19
Concordance	76/90 (80.8%), $P^a < 0.001$, k = 0.514	
ARV7		
Positive	13	2
Negative	15	60
Total	28	62
Concordance	73/90 (81.1%), $P^a < 0.001$, k = 0.495	
PSA		
Positive	35	9
Negative	6	38
Total	41	47
Concordance	73/88 (83.0%), $P^a < 0.001$, k = 0.659	
PSMA		
Positive	28	6
Negative	12	41
Total	40	47
Concordance	69/87 (79.3%), $P^a < 0.001$, k = 0.579	

[a]Fisher exact test.

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Conclusions

6-plex RT-ddPCR assay was highly sensitive, specific, and reproducible,

Simultaneous and absolute quantification of 5 gene transcripts in minute amounts of CTC-derived cDNA

Direct comparison with RT-qPCR for the same markers in the same clinical samples revealed RT-ddPCR to have superior diagnostic sensitivity

Zavridou et al, Clinical Chemistry, 2022

Thank you for your attention!!!