TITLE: **Unraveling the dynamic protein expression levels of Androgen Receptor Variants in Prostate Cancer**

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CONTRACTING ORGANIZATION: University of Minnesota, Minneapolis, MN

REPORT DATE: May 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
May 2021	Annual	01Apr2020-31Mar2021

4. TITLE AND SUBTITLE

Unraveling the dynamic protein expression levels of Androgen Receptor Variants in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-20-1-0070

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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OFFICE OF SPONSORED PROJECTS
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer patients are often given androgen deprivation therapies (ADT) that block the function of the signaling protein, androgen receptor (AR). While effective initially, tumors typically transform into a more aggressive disease known as castration resistant prostate cancer (CRPC) while on ADT. One major cause of resistance in CRPC is thought to be the emergence of Androgen Receptor variants (AR-Vs) at the genomic level. AR-Vs are altered forms of AR that are not sensitive to these drugs. This proposal aims to develop a high-throughput platform that can identify at the protein level which AR-Vs are present in clinical samples including tumor tissues and Circulating Tumor Cells (CTCs). Recent evidence from this grant suggests that there are three novel AR-Vs expressed at the protein level which was not previously known in PCa cell lines and in LuCaP Patient derived xenografts. Our results indicates that the presence of AR-Vs might contribute to the ADT resistance and provides rational to further our investigation and identify if these variants are involved mechanistically in drug resistance and open avenues to develop new treatments approaches.

15. SUBJECT TERMS
Prostate Cancer (PCa), AR splice variants (AR-Vs), metastatic Castration Resistance Prostate Cancer (mCRPC), Single Cell, Proteomics, Targeted Mass Spectrometry

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer in men in the United States. Androgen Receptor (AR) dysregulation is one of the main drivers in PCa becoming the main target for treatment. If a patient has aggressive PCa, typical upfront therapy involves Androgen Deprivation hormonal Therapy (ADT)[2,3]. While effective initially, tumors typically progress to a more aggressive disease known as metastatic Castration Resistance Prostate Cancer (mCRPC)[3,4] and the administration of second generation ADT therapies including abiraterone, and enzalutamide[2] are the most common line of treatment. With the increased disease management using these drugs, more potent tumor phenotypes have begun to emerge and either lose AR signaling altogether or AR splice variants (AR-Vs)[2,4] genomics expression develops. We rationalized that this resistance is associated with the emergence expression of AR-Vs leading to drug resistance in mCRPC[4-7]. Our objectives are to 1.) Develop a targeted mass spectrometry-based platform to evaluate and measure if the AR splice are translated to protein expression, 2.) Determine the dynamic mRNA and protein expression of these variants and if they are being expressed in cell lines and clinical tumor and 3.) Evaluate if these variants are expressed in single and cluster circulating tumor cells using single cell proteomics technologies. The success of these work will significantly contribute to disease management and help inform clinicians the adequate treatment to prevent resistance and unnecessary drug administration.

KEYWORDS

Prostate Cancer (PCa); Androgen Deprivation hormonal Therapy (ADT); AR splice variants (AR-Vs) metastatic Castration Resistance Prostate Cancer (mCRPC); Enzalutamide; Abiraterone; Single Cell; Proteomics; Targeted Mass Spectrometry.

ACCOMPLISHMENTS

What were the major goals of the project?

Within the first year of this DOD application, the major goals for the Drake were to:

Major Task #1:

1. Finalize the ARvT-MS platform using the CMSP core (University of Minnesota).
2. Finalize the ARvT-MS platform using the Dr. MacCoss Lab Collaboration (University of Washington, Seattle).

Major Task #2:

3. Measure temporal dynamic protein expression of AR-Vs during anti-hormonal therapy.

What was accomplished under these goals?

Major Task #1:

1. **Refine and Finalize the ARvT-MS platform.** To assess the dynamic protein expression of AR-Vs in prostate cancer samples, we employed a targeted mass spectrometry-based proteomics approach. We identified and designed sequences that are specific to each variant and commercially synthesized these peptides. We used these peptides to calibrate the mass spectrometer (MS) so that when clinical samples are evaluated, we can identify AR-Vs in high throughput without antibody enrichment steps. We finished running a library of 54 peptides that identifies all the AR-Vs. On our initial analysis, we identify that all the peptides' parameters cannot be analyzed in one mass spectrometer inclusion list method. Therefore, we prioritize the analysis by narrowing down the list of peptides based on the ability to detect these peptides in PDX tumor samples. Out of the 54 peptides identifying 17 AR-Vs plus AR-FL, we moved forward with a total of 9 peptides standards that includes two known AR protein isoforms: AR-FL_1, AR-FL_2, AR-V7_2, AR-V7_3 and five novel isoforms: AR-V2, AR-V5, AR-V6, AR-V12 and AR-V23. These standards were distributed equally to two different MS core facilities. Both Institutions, CMSP core (University of Minnesota, Twin Cities) and the Dr. MaCoss Lab Collaboration (University of Washington, Seattle) use two different instruments so we can validate our approach and that these peptides are reproducible and detectable in different locations with different technical handling individuals. We had achieved our goal and we have been able to observe

several AR-Vs protein expressions in tumor samples of 5 novel ARVs that has not been observed previously.

2. **Subtask 1-4: Perform stability assay on the remaining peptides, perform Limit of Detection (LOD) and Limit of Quantification (LOQ) assays on the remaining peptides for relative peptide quantification, and measure peptide recovery by spiking in the synthetic peptides prior to sample processing into negative AR-Vs cells.** We rely on synthetic peptides standards to be able to run stability assay, limit of detection, limit of quantification and peptide recovery. Currently, MilliporeSigma the company that we use to generate the synthetic peptides sent us bad quality peptides. This is not normal, MilliporeSigma aqua peptides are typically of great quality and reproducible. We were expecting new batch of peptides in March of 2020. The new batch of peptides were ready but when MilliporeSigma were about to ship these peptides, the University of Minnesota was shut down due to a National emergency of COVID-19 pandemic. These peptides were held back in storage and were not delivered until August 2020. These peptides were again distributed to both institutions UMN and UW on August 25th. After both institutions received these peptides, they started to perform the analysis and previous mentioned assays. After a couple of months running and optimizing these peptides, they came to the conclusion that these peptides have high levels of polymer contamination which led to column and instrument clogging. Several of the peptides were of very low quality. I initiated and conducted a meeting with between both institutions and MilliporeSigma to present our challenges working with their synthesized peptides.

   To eliminate the peptide standards contamination, MilliporeSigma proposed to perform a peptide clean-up and re-send the same peptides. They did send the peptides cleaned and I distributed the peptides again to both institutions. Both Institutions came back indicating that these cleaned peptides are again of poor quality. I communicated back with MilliporeSigma and we concluded that they will re-synthesize new peptides and deliver immediately so we can proceed with our studies. These is very unusual circumstance, and we are working forcefully to proceed with our studies timely.

Major Task 2:

3. **Measure temporal dynamic protein expression of AR-Vs during anti-hormonal therapy.** We are currently working on transiently silencing AR FL and evaluating the dynamic expression of the ARVs. To measure the knock down efficiency, we developed a qRT-PCR based assay that measures absolute mRNA copy number per nanogram of RNA using gene-block standards. We have completed the analysis of three AR-Vs (AR-V7, AR-V5 and AR-V12) from 22Rv1and we have four remaining probes that are on working progress currently (AR-V2, AR-V6 and AR-V23 and AR-FL).

Major Task 3:

4. **Evaluate the AR-Vs expression in single cells from PCa cell lines**. We have begun to assess the proteome expression from PCa cell lines in single cells. In order to start testing the single cell protocol, we first started to optimize the platform using single cell sorted from PCa cell lines. These cell lines are sorted and ready to proceed with MPOP-SCOPE assay. We will start working on it in the month of May-June of 2021.

Major Task 4:

5. **Evaluate the variable protein expression of AR-Vs in CTCs.** After establishing the single cell proteomics pipeline, we will proceed with the single cell proteomics from CTCs.

- **What opportunities for training and professional development has the project provided?**
  - Dr. Zoi Sychev in the Drake Lab was able to attend a virtual conference at America Society Clinical Chemistry and presented preliminary data of her work from this proposal.
  - Dr. Zoi Sychev had the opportunity to present her work in conjunction with Dr. Scott Dehm Lab and Dr. Justin Drake lab.

- **How were the results disseminated to communities of interest?**

    - To date, we have presented some aspects of our work to conferences including the Prostate Cancer Foundation Retreat and Society for Basic Urologic Research Annual Meeting in 2020. Currently, the data presented includes identification of AR-Vs on cell lines and xenograft tumors. No single cell proteomics data has been generated and matured for it to be ready for dissemination to the community.

- **What do you plan to do during the next reporting period to accomplish the goals?**
    - We will continue our work on finalizing the limit of quantification and limit of detection methods on the newly synthesized peptides. We plan to have data finalized and complete Milestone 3 within 6 months and milestone 4 the remaining time of this project.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

1. In **major task 1** of research specific tasks, we proposed to evaluate all the variants in a single mass spectrometry inclusion list. However, we determined that the mass spectrometer capacity is not more than nine peptides per run so, we narrowed down the list from 17 AR isoforms to 9 AR isoforms. We plan to follow the next 8 AR isoforms once we have accomplished our first round of tasks.

2. Due to the National emergency of COVID-19 pandemic, this has substantially delayed our work from all major task and milestones for at least 6-8 months. In addition, the issue with the peptide standard synthesis, has delayed our advancement even further which has significantly delayed us from accomplishing our proposed goals, however we are confident that we can move forward now that the country and schools are partially open, and we can go to the lab and perform the in-lab experimental work.

Actual or anticipated problems or delays and actions or plans to resolve them

1. Since the Universities and lab has been partially open, we have been able to continue our work and communications with different core facilities to proceed with the mass spectrometry runs.

Changes that had a significant impact on expenditures

None of the changes described above will result in >25% change in budget allocation

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

PRODUCTS
Publications, conference papers, and presentations

Journal publications.
Nothing to Report.

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Academy of Clinical Chemistry virtual conference 2020 and Society for Basic Urologic Research (SBUR) 2020

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Dr. Justin Drake
Project Role:	Principal Investigator (PI)
Researcher Identifier (e.g. ORCID ID):	0000-0002-8329-7748
Nearest person month worked:	
Contribution to Project:	PI
Funding Support:	

Name:	Dr. Michael MacCoss
Project Role:	Principal Investigator (PI)
Researcher Identifier (e.g. ORCID ID):	0000-0003-1853-0256
Nearest person month worked:	
Contribution to Project:	PI
Funding Support:	

Name:	Dr. Zoi Sychev
Project Role:	Post-doctoral researcher
Researcher Identifier (e.g. ORCID ID):	0000-0002-9830-5245
Nearest person month worked:	12
Contribution to Project:	Post-doctoral researcher
Funding Support:	

Name:	Dr. Eric Huang
Project Role:	Senior Research Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0002-8745-0025
Nearest person month worked:	4
Contribution to Project:	Research scientist
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

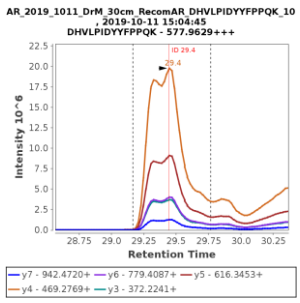
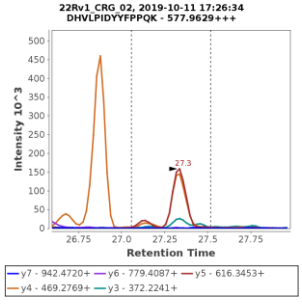
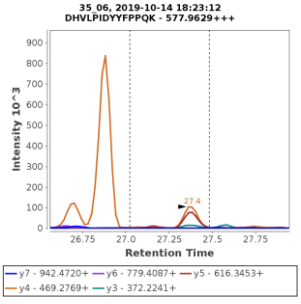
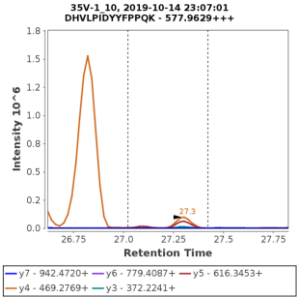
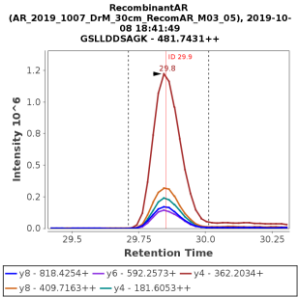
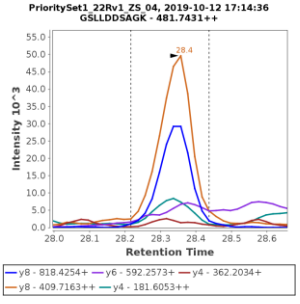
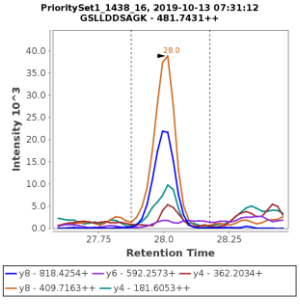
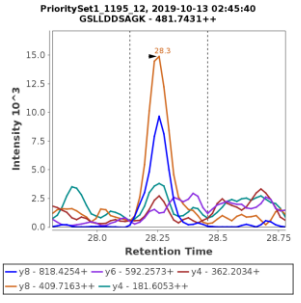
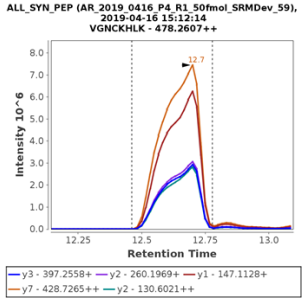
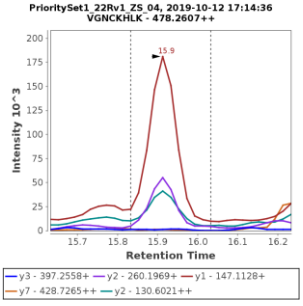
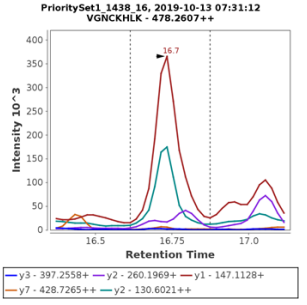
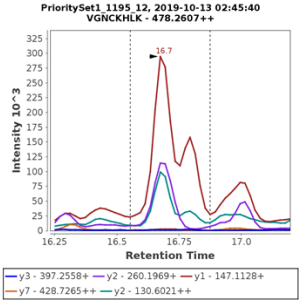
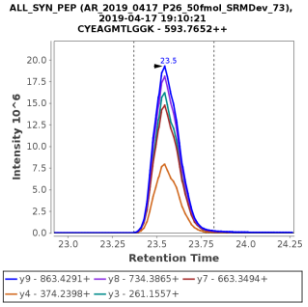
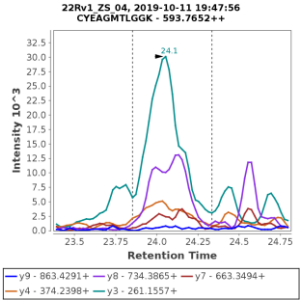
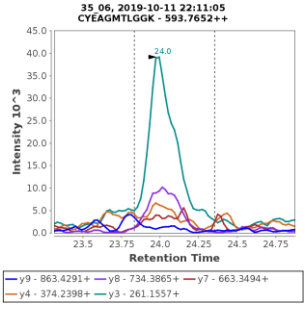
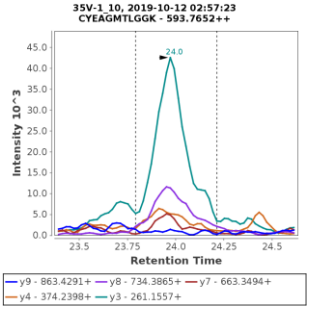
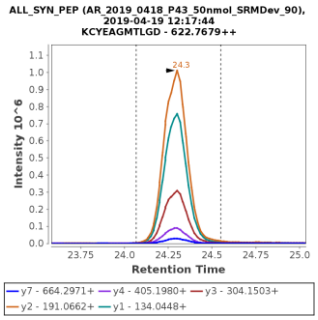
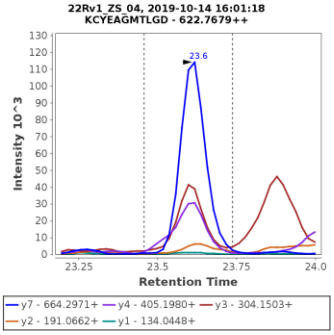
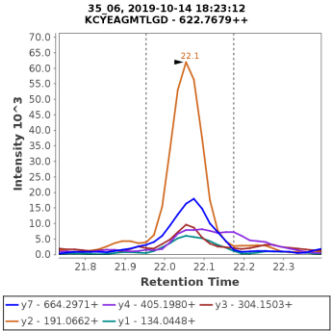
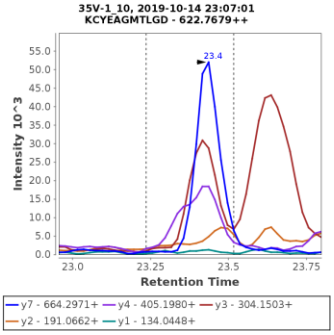
What other organizations were involved as partners?
Nothing to Report.

SPECIAL REPORTING REQUIREMENT
Nothing to report

COLLABORATIVE AWARDS:
Nothing to Report.

APPENDICES

- Figure 1

	Synthetic Peptide	22RV1	PDX Intact	PDX Castrated
AR-FL_1				
AR-FL_2				
AR-V7_1				
AR-V2				
AR-V5				

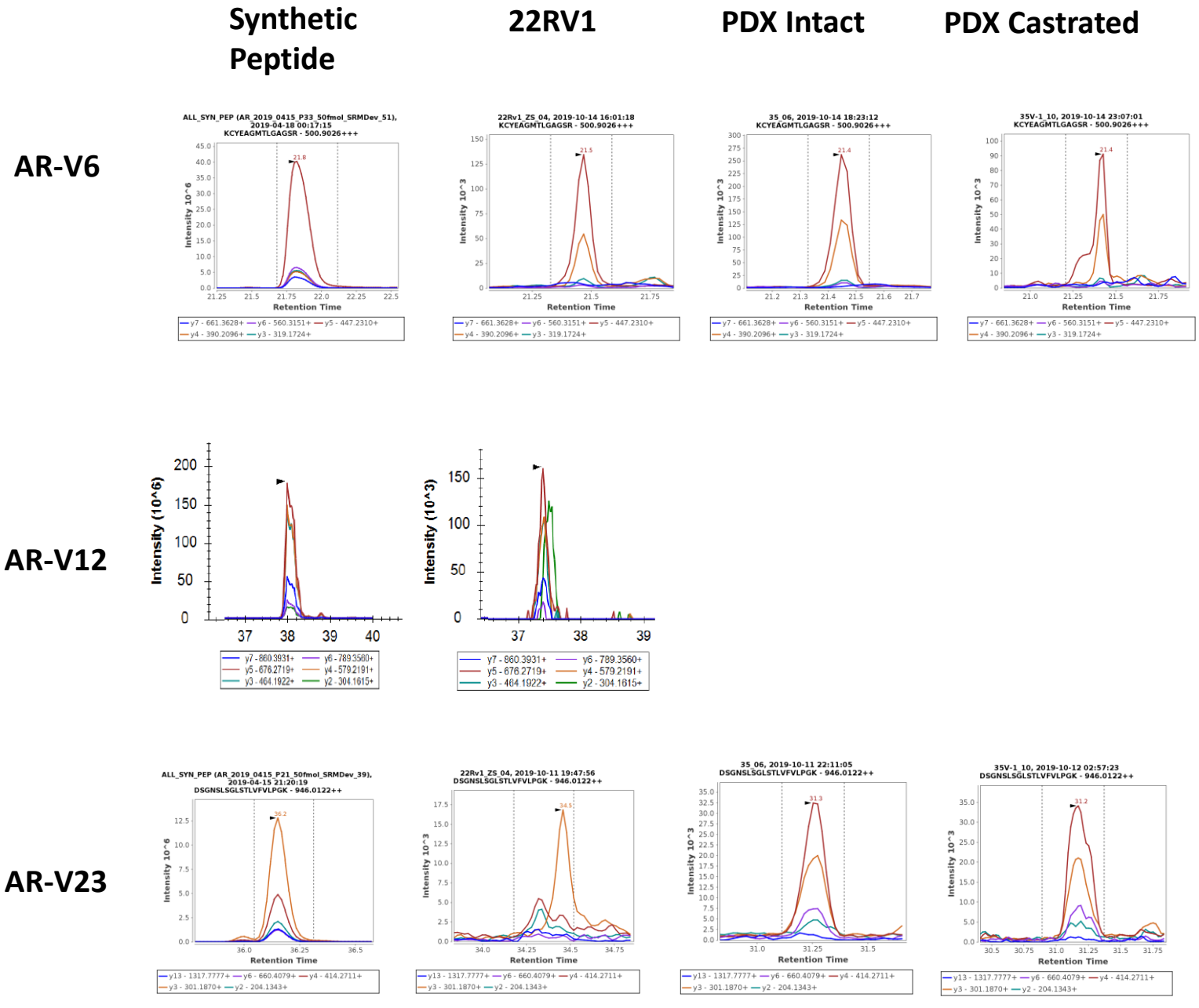


Figure 1. Compendia of AR-Vs using ARvT-MS platform. Commercially synthetic AR peptides and endogenous AR peptides were measured using ARvT-MS from 22Rv1 whole cell lysates and LuCap PDX tumors (y-axis shows the peptide relative abundance and x-axis shows the retention time). Here we show multiple columns that indicates the sample used and the rows shows the AR-Vs measured from ARvT-MS library. The box on the bottom of each graph represents the y-ions measured for each peptide, known as transitions. The retention time of each transition is on the apex of the most abundant transition and it is expected that the retention time fluctuates +/- 5 min compared to the synthetic peptides (standard).