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Quantification of Human Papillomavirus (HPV) Antigens in the Nonavalent Vaccine GARDASIL 9

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Abstract

Quantification and identity testing are an important part of vaccine substance and product batch release. Traditional methods can be non-selective, time consuming to develop, and may have poor multiplexing capabilities. GARDASIL 9 is a nonavalent vaccine composed of nine different HPV L1 protein antigens that provide broad protection against HPV subtypes responsible for increased cervical cancer and genital wart risk. In this work, a complete sample preparation workflow (including adjuvant desorption, tryptic digestion, and LC-MS/MS quantitative monitoring) has been leveraged to measure GARDASIL 9 components via the surrogate peptide method. We were able to simultaneously quantify all nine HPV strain specific L1 proteins with high reproducibility in one LC-MS/MS method over the concentration range of 10–200 µg/mL.

Benefits

- ACQUITY UPLC I-Class PLUS and Xevo TQ-S micro allowed for highly reproducible quantification of all nine HPV L1 proteins in one method with a total analysis time of seven minutes

- ACQUITY PREMIER Peptide BEH C$_{18}$ Columns enable faster column passivation and increased peak area of peptides

- A rapid, simple protocol to effectively remove vaccine adjuvant prior to analysis

Introduction

GARDASIL 9 is a nonavalent vaccine composed of HPV L1 protein antigens which form virus like particles (VLPs). These antigens protect against nine HPV strains which account for 80–90% of cervical cancers and 90% of genital warts.[1,2] An important step in the vaccine production process is quality control of vaccine substance and product batches. Batch release testing includes sterility, potency, purity, antigen quantification, and identity to name a few. Traditional methods for quantification and identity testing include total protein assays and ELISA. These assays have limitations which include non-specificity (or unknown specificity or cross reactivity), are time consuming to develop, and have poor multiplexing capabilities. There is therefore an opportunity to leverage mass spectrometry as a tool for both antigen quantification and identity testing. Mass spectrometry allows for the simultaneous and selective determination and quantification of type specific L1 proteins. In this work, we have developed a complete sample preparation workflow encompassing desorption of HPV L1 proteins from aluminum adjuvant, and tryptic digestion of

proteins for the quantification of GARDASIL 9 HPV L1 antigens via the surrogate peptide method. This workflow, including mass spectrometric analysis, enabled the highly reproducible quantification of these peptides over the range of 10–200 µg/mL which easily covers the expected HPV L1 protein concentration range of 40–120 µg/mL in the GARDASIL 9 vaccine product.

Experimental

Sample Preparation

Preparation of Samples for Peptide Mapping

Each HPV L1 protein was diluted to 750 µg/mL (30 µg) and denatured with *Rapi*Gest SF surfactant (Waters, P/N 186001861 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186001861-rapigest-sf-1-mg--5-pk.html>), followed by reduction with dithiothreitol and alkylation with iodoacetamide. Samples were digested with trypsin (Promega, P/N V5111) using a 1:10 trypsin:protein ratio (w/w) and quenched with formic acid. Peptide mapping using a LC-MS[E] method was performed by injecting 10 µL of sample onto an ACQUITY UPLC I-Class PLUS System coupled with a Xevo G2-XS QTof Mass Spectrometer.

Preparation of Samples for Method Validation Studies

Two microliters of blanks, standards, quality control, or vaccine product samples were added to a 16 µL aqueous solution of 100 µg/mL bovine serum albumin (Sigma, P/N 05470), respectively. 2 µL of internal standard was added to all samples except blanks. 20 µL of 0.1% (w/v) *Rapi*Gest SF surfactant (Waters, P/N 186001861 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186001861-rapigest-sf-1-mg--5-pk.html>) prepared in 100 mM Tris HCl, pH 10.0 was added to each sample. Samples were incubated for 10 minutes at 80 °C, then centrifuged for 5 minutes at 2000 x g to pellet adjuvant. 32 µL of sample was carefully removed from the pelleted adjuvant and neutralized to pH 8.0 with 1.2 µL of 10% hydrochloric acid (v/v). Samples were digested with 0.3 µg of trypsin (Promega, P/N V5111), (1:10 trypsin:protein ratio) at 37 °C overnight. The digestion reaction was quenched and *Rapi*Gest was precipitated with 2 µL of 20% formic acid in water (v/v) for 15 minutes at 37 °C. Samples were centrifuged at 1800 x g for 15 minutes at 10 °C to pellet *Rapi*Gest. 30 µL of each sample was transferred to a QuanRecovery 96-well plate and 5 µL was injected onto an ACQUITY UPLC I-Class PLUS System coupled to a Xevo TQ-S micro Mass Spectrometer for LC-MS/MS analysis.

Peptide Mapping LC-MS/MS Method Conditions

LC system:	ACQUITY UPLC I-Class PLUS (Fixed Loop)
MS system:	Xevo G2-XS QTof Mass Spectrometer, ESI+
Sample plates:	QuanRecovery with MaxPeak High Performance Surfaces Vials
Column(s):	ACQUITY UPLC PREMIER Peptide BEH C_{18}, 300 Å, 1.7 µm, 2.1 x 100 mm
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Flow rate:	0.2 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient:	1–35% B in 50 minutes at 0.2 mL/min followed by column wash and equilibration
Ionization mode:	ESI+
Capillary voltage:	1.2 kV
Cone voltage:	40 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Cone gas flow:	20 L/Hr

Desolvation gas flow:	600 L/Hr
Informatics system for data collection and processing:	UNIFI (v.1.9.4)

Method Validation – LC-MS/MS Method Conditions

LC system:	ACQUITY UPLC I-Class PLUS (Fixed Loop)
MS system:	Waters Xevo TQ-S micro Mass Spectrometer, ESI+
Sample plates:	QuanRecovery with MaxPeak High Performance Surfaces 96-well plates
Column(s):	ACQUITY UPLC PREMIER Peptide BEH C_{18}, 300 Å, 1.7 µm, 2.1 x 100 mm
Column temp.:	60 ^{o}C
Sample temp.:	10 ^{o}C
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient:	10–15% B in 1 minute, 15–30% B in 3 minutes followed by column wash and equilibration. 7-minute run time.
Ionization mode:	ESI+

Capillary voltage:	1.2 kV
Cone voltage:	20 V
Source temp.:	150 °C
Desolvation temp.:	600 °C
Cone gas flow:	100 L/Hr
Desolvation gas flow:	1000 L/Hr
MS data collection:	MassLynx v4.2
Informatics for data processing:	TargetLynx XS v4.2

Results and Discussion

Peptide Mapping for the Identification of Surrogate Peptides

Characterization of HPV L1 proteins via a peptide mapping approach helps to select peptide candidates for quantitation and may also identify the presence of protein backbone modifications such as deamidation and oxidation. The complete workflow for protein analysis and quantification has been described previously for Fc containing proteins in application note 720006969EN < https://www.waters.com/webassets/cms/library/docs/720006969en.pdf> and is used here with minor modifications.[3] Peptide mapping experiments were performed for all nine HPV L1 proteins present in GARDASIL 9. As an example, shown in Figure 1, excellent fragmentation data confirms the HPV 18 peptide FSLDLDQYPLGR. This peptide was chosen as a surrogate of HPV 18 on the basis of intensity, low likelihood of modifications, and quantitative reproducibility. MRM transitions for each surrogate peptide were identified and optimized via the MassLynx-Skyline interface workflow.[4]
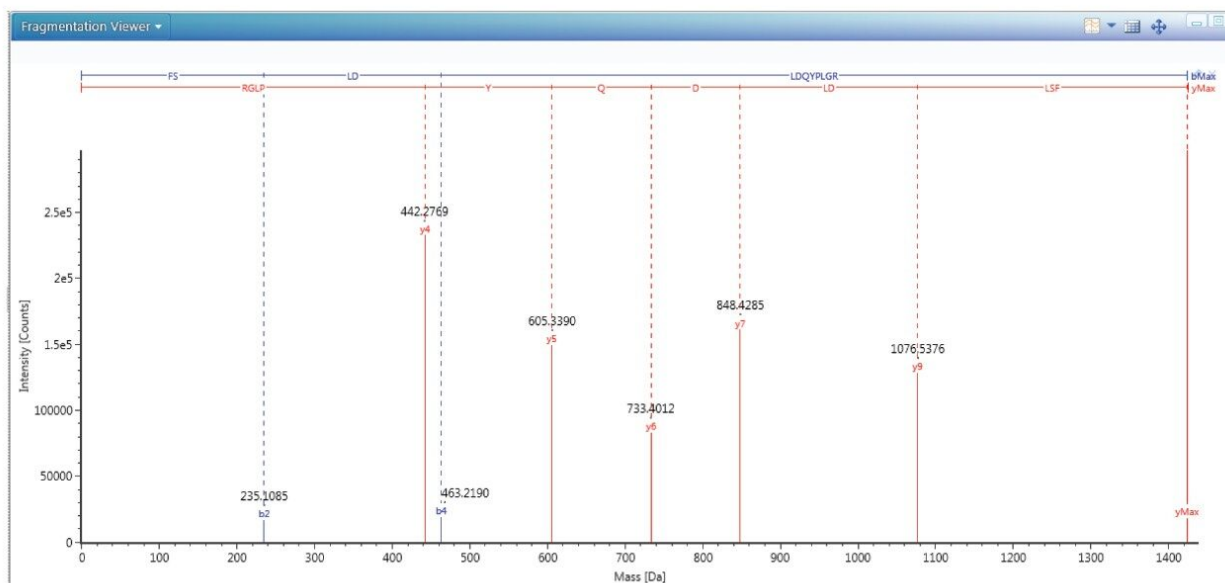
Figure 1. Eight primary b and y fragments of the HPV 18 FSLDLDQYPLGR peptide were observed in MS^E high-energy fragmentation spectrum.

LC-MS/MS Optimization

Briefly, after surrogate peptides are identified via peptide mapping experiments, retention times of these peptides are identified by monitoring precursor masses of each peptide using the optimized chromatographic method. The most intense charge state for each peptide precursor is chosen and all b and y ion product ions for the selected peptide are comprehensively examined to identify the most intense MRM transitions. The top MRM transitions are further optimized for collision energy and a final MS method is generated with these optimal parameters. MRM transitions used for quantification can be viewed in Table 1.

MS parameters				
Peptide	Precursor (*m/z*)	Product (*m/z*)	Cone (V)	Collision energy (eV)
HPV 6 - FSSELDQYPLGR	706.35	848.43	35	25
HPV 11 - FSSELDQFPLGR	698.35	832.43	35	22
HPV 16 - AGAVGENVPDDLYIK	780.90	863.45	35	25
HPV 18 - FSLDLDQYPLGR	712.36	605.34	35	22
HPV 31 - SGTVGESVPTDLYIK	783.41	849.47	35	28
HPV 33 - AGTLGEAVPDDLYIK	781.41	1033.56	35	25
HPV 45 - FSSDLDQYPLGR	699.34	848.43	35	25
HPV 52 - LLTVGHPYFSIK	458.93	631.35	35	12
HPV 58 - LGEAVPDDLYIK	666.86	863.45	35	18

Table 1. MRM transitions and mass spectrometry parameters for nine HPV L1 protein surrogate peptides.

Column selection is an important part of the LC method development process. The ACQUITY PREMIER Peptide BEH C$_{18}$ 300 Å Column utilizing HPS (High Performance Surfaces) Technology was found to be optimal for the quantification of HPV L1 proteins. In Figure 2, a comparison of a standard column and a PREMIER Column is shown.[5] Both columns were passivated with 10 injections of a high concentration sample for passivation, followed by 5 analyte injections. The PREMIER Column with MaxPeak HPS Technology enabled faster column passivation and provided a 2x increase in peak area over the standard column. This peak area increase allows lower limits of quantification and enhances reproducibility of peptide measurements.
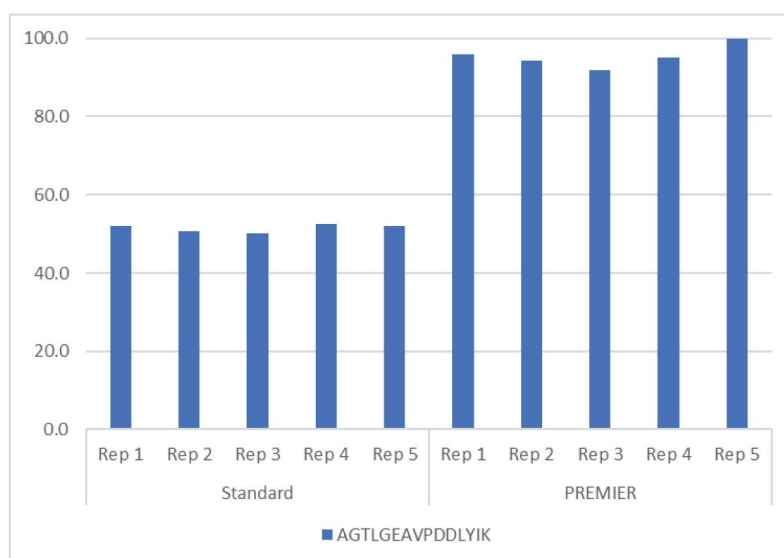
Figure 2. Columns were passivated with 10 injections of a highly concentrated standard, followed by five test injections. Faster passivation and a two-fold increase in peptide peak area was achieved using an ACQUITY PREMIER Peptide Column over a standard ACQUITY Peptide Column.

Adjuvant Desorption

Efficient adjuvant desorption is key to the success of a quantitative measurement of antigen concentration in adjuvanted vaccine products. Proteins can bind to aluminum adjuvants via electrostatic forces, hydrophobic interactions, hydrogen exchange via Van der Waals forces, and ligand exchange.[6] Ligand exchange provides the tightest binding of proteins and results from interactions between surface hydroxyl groups of adjuvant and protein antigen phosphate groups. GARDASIL 9 is adjuvanted to the proprietary amorphous aluminum hydroxyphosphate sulfate (AAHS) which has surface hydroxyl and phosphate groups.[7] This vaccine is formulated at approximately pH 7.0 and AAHS has a point of zero charge at this pH.[8] The antigen HPV L1 protein VLPs have isoelectric points ranging from 8.1 to 8.6 and are therefore positively charged in the formulated vaccine product.[9,10]

We have experimentally determined that surfactants and high pH are needed in order to disrupt this adsorption, completely desorb proteins from the AAHS adjuvant, as well as begin to dissociate the VLPs themselves.[11] We have used the MS-friendly surfactant *Rapi*Gest to denature the HPV L1 proteins which was required to enable complete desorption. Our adjuvant desorption protocol also utilizes high pH to fully switch

the charge of the HPV L1 proteins from positive to negative. Shown in Figure 3, pH 10.0 achieved the highest level of protein desorption from the adjuvant. Important to note, very little protein desorption was achieved at pH 8.0 where the proteins are expected to be positively charged. This result indicates that electrostatic repulsion was required to adequately disrupt binding and lead to protein desorption.
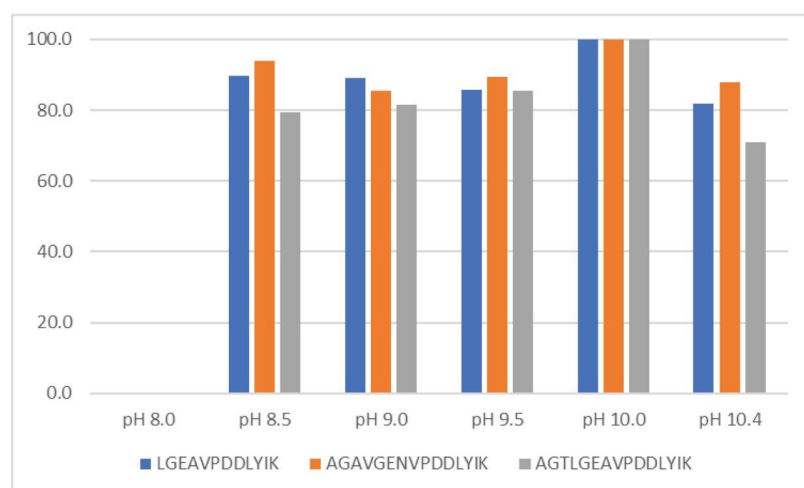

Figure 3. Increase in the pH of TRIS HCl buffer was required to achieve complete protein desorption from the amorphous aluminum hydroxyphosphate sulfate adjuvant. Complete desorption was observed at pH 10.0.

Digestion Optimization

Complete digestion of proteins is essential for accurate, sensitive, and reproducible quantification via the surrogate peptide method. In order to ensure complete digestion, digestion parameters such as enzyme:substrate ratio and digestion time must be examined and optimized. To measure digestion completeness, the peptide FSLDLDQYPLGR and the peptide EKFSLDLDQYPLGR with one missed cleavage were monitored and quantified.

The ratio of trypsin to protein was optimized based on the overnight digestion results at various enzyme:substrate ratios. The results of these experiments are shown in Figure 4, panel A. At the trypsin:protein ratio of 1:10 and 1:5, the maximum intensity of peptide FSLDLDQYPLGR and the minimum intensity peptide EKFSLDLDQYPLGR were obtained, suggesting a more complete digestion of the HPV L1 proteins was achieved. A 1:10 ratio was chosen to use in the rest of the experiments to reduce the autolysis of the trypsin due to the need of high concentrations. Shown in figure 4, panel B, digestion time was evaluated.

A digestion time of 8 hours to overnight resulted in the most complete digestion of the HPV L1 proteins. Overnight digestion was chosen due to completeness of digestion and workday convenience.
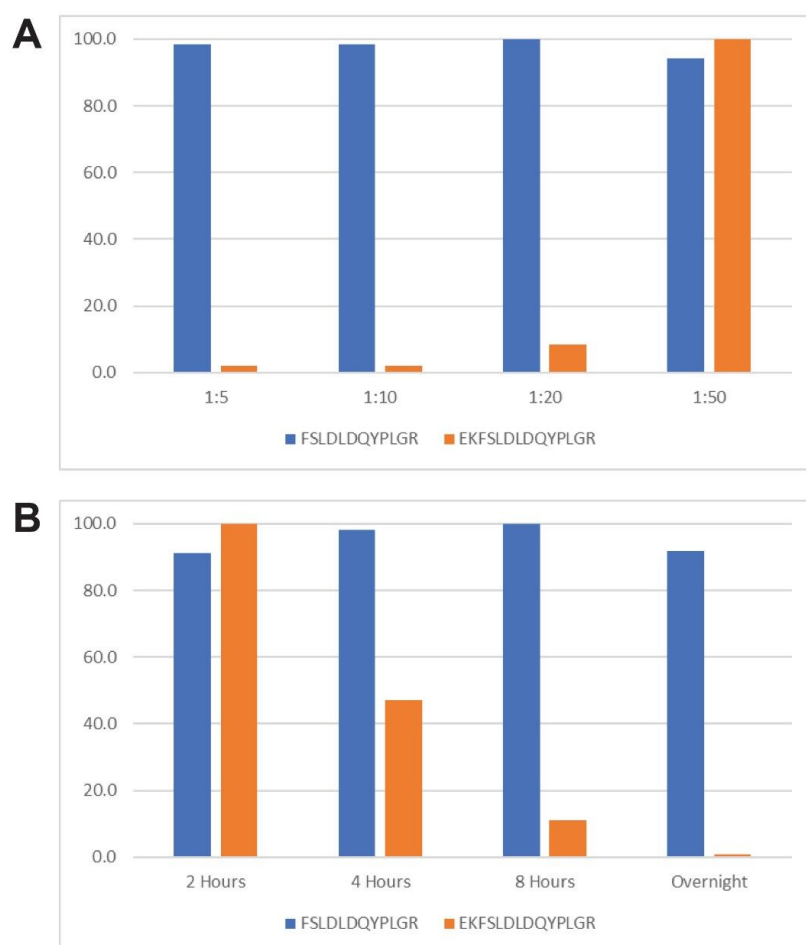

Figure 4. The peptides FSLDLDQYPLGR and EKFSLDLDQYPLGR (1 missed cleavage) were monitored for peak area using different trypsin:protein ratios for tryptic digestion. A 1:10 trypsin:protein ratio was chosen due to its high peak area and low amount of missed cleavages (Panel A). These same peptides were monitored over a digestion time course of 2 hours to overnight (~16 hours). Overnight incubation with 1:10 trypsin:protein was required to achieve complete digestion (Panel B).

Method Validation Results

Standard curves, QCs, and vaccine product were prepared over three days to validate inter- and intra-day

assay precision and accuracy. Calibration curves were linear for HPV 16 over the range of 20–200 µg/mL. The remaining HPV proteins were linear over the range of 10–100 µg/mL (Table 2). Low, medium, and high QCs were prepared, and the CVs for the accuracy and precision of QCs were <8% (Table 3). Shown in Table 4, measurement of all nine HPV L1 proteins was highly reproducible over three preparations and three days of measurements with CVs <8%. In most cases, LC-MS/MS measurements in this study agreed with the labelled values in general, although some values were higher. These results indicate that this assay is fit for purpose for the quantification of HPV L1 proteins in GARDASIL 9.

Calibration curve statistics				
Peptide	Statistics	Day 1	Day 2	Day 3
HPV 16 - AGAVGENVPDDLYIK	Linear fit (R^2)	0.996	0.996	0.997
	% Accuracy range	97.3–104.9	96.1–104.1	96.9–102.9
HPV 18 - FSLDLDQYPLGR	Linear Fit (R^2)	0.995	0.994	0.991
	% Accuracy range	94.3–106.7	96.9–103.0	94.7–110.4

Table 2. Standard curve statistics for HPV 16 and 18 strains over three days of method validation preparations.

QC statistics						
Peptide	QC level	Statistics	Day 1 (N=6)	Day 2 (N=6)	Day 3 (N=6)	Inter-day (N=18)
HPV 16 AGAVGENVPDDLYIK	LQC (60 µg/mL)	% Accuracy	106.1	104.0	105.0	105.0
		% RSD	4.0	2.7	3.9	3.5
	MQC (100 µg/mL)	% Accuracy	102.4	107.4	102.8	104.2
		% RSD	6.5	6.4	2.9	5.7
	HQC (160 µg/mL)	% Accuracy	103.1	105.8	105.9	104.9
		% RSD	4.3	3.4	3.7	3.8
HPV 18 FSLDLDQYPLGR	LQC (30 µg/mL)	% Accuracy	103.4	107.3	108.6	106.4
		% RSD	3.5	3.5	7.1	5.2
	MQC (50 µg/mL)	% Accuracy	101.5	107.6	101.0	103.3
		% RSD	5.1	5.4	1.8	5.2
	HQC (80 µg/mL)	% Accuracy	102.8	103.6	96.6	101.0
		% RSD	5.6	3.4	6.1	5.7

Table 3. QC quantitative performance for HPV 16 and 18 strains over three days of method validation preparations.

Vaccine product quantification			
Peptide	Expected concentration (μg/mL)	Measured concentration (μg/mL)	Measured concentration %RSD (n=18)
HPV 6 - FSSELDQYPLGR	60	72.7	5.9
HPV 11 - FSSELDQFPLGR	80	96.5	5.3
HPV 16 - AGAVGENVPDDLYIK	120	198.3	3.7
HPV 18 - FSLDLDQYPLGR	80	74.2	7.4
HPV 31 - SGTVGESVPTDLYIK	40	67.5	4.3
HPV 33 - AGTLGEAVPDDLYIK	40	64.2	4.7
HPV 45 - FSSDLDQYPLGR	40	43.5	5.5
HPV 52 - LLTVGHPYFSIK	40	64.4	5.2
HPV 58 - LGEAVPDDLYIK	40	64.2	4.4

Table 4. Measurement of all HPV L1 protein antigens present in GARDASIL 9 vaccine product.

Conclusion

A mass spectrometry assay for the simultaneous quantification of HPV L1 protein antigens in GARDASIL 9 was developed. Combined with optimized sample preparation, proteins were quantified by LC-MS/MS with high accuracy and precision.

· Peptide mapping experiments were used to characterize HPV L1 proteins and identify a list of surrogate peptides for quantification

· MassLynx Skyline Interface was used to rapidly develop an MRM assay for nine surrogate peptides

· ACQUITY PREMIER Columns enabled rapid column passivation and increased peptide peak area, allowing for more sensitive and reproducible peptide measurements

· Efficient adjuvant desorption and optimized tryptic digestion were necessary for accurate and reproducible quantification

· GARDASIL 9 HPV L1 proteins were quantified over the range of 10–200 μg/mL with high precision and accuracy

References

1. Clifford GM, Smith JS, Aguado T, Franceschi S. Comparison of HPV Type Distribution in High-Grade Cervical Lesions and Cervical Cancer: A Meta-Analysis. *Br J Cancer*. 2003;89(1):101–5.

2. NIH National Cancer Institute, Human Papillomavirus (HPV) Vaccines. Accessed October 2020 from https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents < https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents> .

3. Dunning CM, Wrona MD. Developing a Quantitative Surrogate Peptide Assay – Peptide mapping through MRM Optimization for Measuring Dulaglutide in a Rat PK Study. Waters Application Note 720006969EN <https://www.waters.com/webassets/cms/library/docs/720006969en.pdf> .

4. Tanna N, Dunning CM, Molloy B. MassLynx-Skyline Interface (MSI) – A New Automated Tool to Streamline MRM Method Development and Optimization for Large Molecule Quantification. Waters Application Note 720006813EN <https://www.waters.com/webassets/cms/library/docs/720006813en.pdf > .

5. Lauber M, Walter TH, Gilar M, DeLano M, Boissel C, Smith K, Birdsall R, Rainville P, Belanger J, Wyndham K. Low Adsorption HPLC Columns Based on MaxPeak High Performance Surfaces. Waters White Paper 720006930EN <https://www.waters.com/webassets/cms/library/docs/720006930en.pdf> .

6. Hem SL, Hogenesch H. Relationship Between Physical and Chemical Properties of Aluminum-Containing Adjuvants and Immunopotentiation. *Expert Rev Vaccines*. 2007;6(5):685–98.

7. Food and Drug Administration GARDASIL 9 Package Insert. Accessed October 2020 from https://www.fda.gov/media/90064/download <https://www.fda.gov/media/90064/download> .

8. Caulfield MJ, Shi L, Wang S, Wang B, Tobery TW, Mach H, Ahl PL, Cannon JL, Cook JC, Heinrichs JH, Sitrin RD. Effect of Alternative Aluminum Adjuvants on the Absorption and Immunogenicity of HPV16 L1 VLPs in Mice. *Hum Vaccin.* 2007;3(4):139–45.

9. Wibom C, Department of Virology, Umeå University, pI Predictor. Accessed October 2020 from http://www.acc.umu.se/~wibom/virology/cgi-bin/pI_predictor.cgi < http://www.acc.umu.se/~wibom/virology/cgi-bin/pI_predictor.cgi> .

10. Mistry N, Wibom C, Evander M. Cutaneous and Mucosal Human Papillomaviruses Differ in Net Surface Charge, Potential Impact on Tropism. *Virol J.* 2008;5:118.

11. McCarthy MP, White WI, Palmer-Hill F, Koenig S, Suzich JA. Quantitative Disassembly and Reassembly of Human Papillomavirus Type 11 Viruslike Particles *In Vitro*. *Virol J.* 1998;72(1):32–41.

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