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Mass spectrometry & Proteomics

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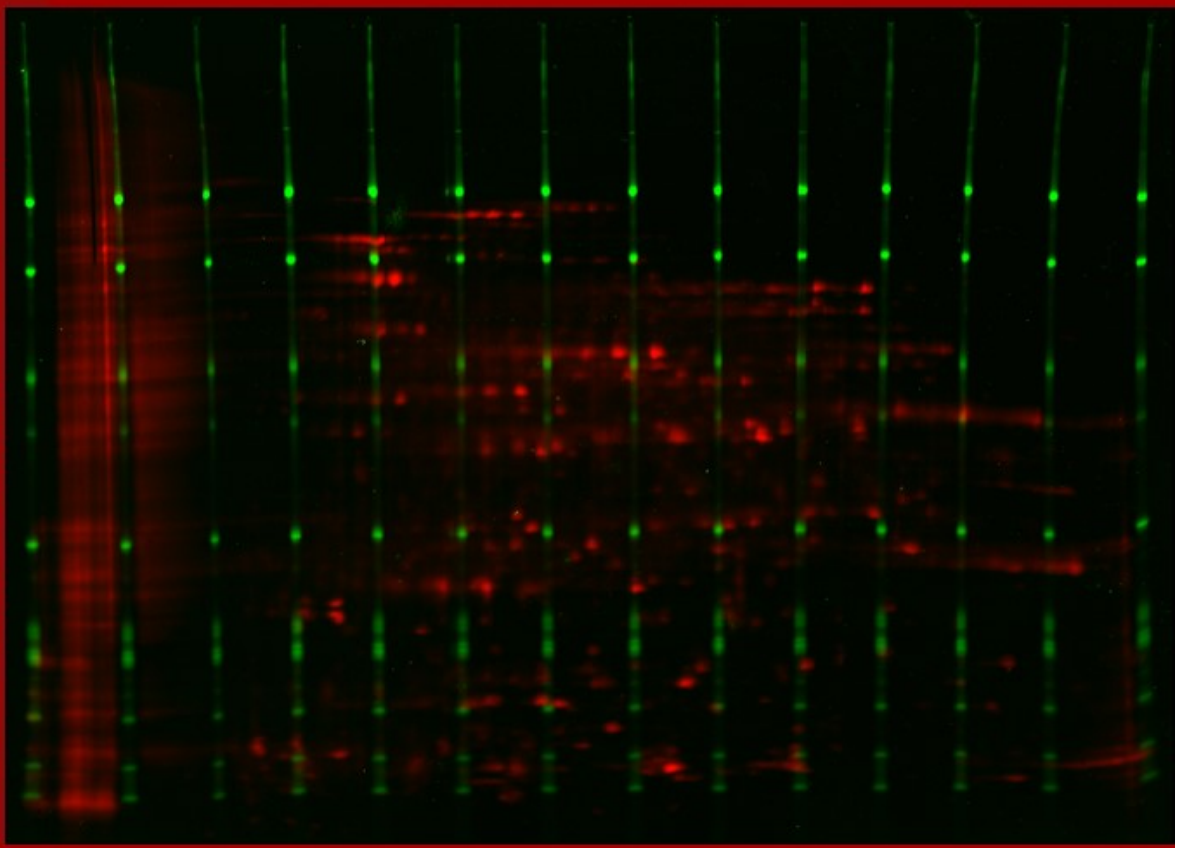

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Germany
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Edited by Simone König
CUP/IZKF, Röntgenstr. 21, 48149 Münster
koenigs@uni-muenster.de
Ph. +49-251-8357164

Cover image
Mercator gel (run by D. Ackermann at CUP) representing the award-winning CoFGE technology for standardized gel electrophoresis

Protocol

Pseudo-MRM method for the selective detection of human HLA-G

Simone König[1], Christian Spurny[2], Claudia Rössig[2]*

[1] Core Unit Proteomics, Interdisciplinary Center for Clinical Research,

[2] University Children's Hospital Münster, Pediatric Hematology & Oncology, Germany

*Corresponding author: koenigs@uni-muenster.de

Abstract

A pseudo-MRM method using high-resolution mass spectrometry is presented for the specific detection of HLA-G against a background of homologous proteins. The method relies on three unique and two unspecific tryptic peptides. The latter are used to monitor the level of background proteins in comparison to the desired HLA-G. Instrument response is linear in the range from 30 to 200 fmol.

Introduction

Histocompatibility antigen, class I, G, also known as human leukocyte antigen G (HLA-G) binds to three inhibitory receptors on immune cells leading to direct inhibitory effects in effector cells and to the expansion of myeloid suppressor cells, which together suppresses immune response. HLA-G is expressed at the placental barrier and serves to protect the fetus from maternal rejection. It can also be expressed by tumor cells to allow them to evade immune recognition. Thus, HLA-G can act as an immune checkpoint molecule. (for review see [1])
HLA-G was found in Ewing sarcomas in response to NK cell therapy in *in vivo* models [2]. It may contribute to resistance to immune targeting and thus is a potential target for more effective immunotherapeutic combination regimens in this and other cancers. In order to measure the protein in the context of experimental studies that assess the functional role of HLA-G in cancer, we developed a sensitive mass spectrometry (MS)-based method for its specific detection. The difficulty of specifically detecting HLA-G is that it has high similarities to other HLA molecules. High-resolution MS was used in a targeted pseudo multireaction-monitoring (MRM) approach. Tryptic peptides were selected which distinguished HLA-G from highly homologous proteins such as HLA-A (Figure 1). For the selection of these target compounds other considerations additionally applied such as the response to the detection method and the absence of peptide modifications; methionine and cysteine containing peptides were thus avoided.

Experimental

Recombinant human HLA-G (0.4 mg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH8.0, 50% glycerol) was obtained from Cusabio (sequence in Figure 1, no signal peptide). 10 µl were digested by adding 4 µl trypsin solution (0.02 µg/µl trypsin in buffer (50 mM NH_4HCO_3 containing 10% acetomitrile)) and 10 µl of the buffer. Digestion proceeded at 37°C overnight. The peptide solution was adjusted to 100 fmol/µl for reversed-phase liquid chromatography coupled to tandem mass spectrometry (MS). Experiments were performed using Synapt G2 Si ion mobility mass spectrometer with M-Class UPLC (Waters Corp.) with a 30 min gradient (solvent system 100% water versus 100% acetonitrile, both containing 0.1% formic acid; trap column V/M Symmetry C18 100 Å 5 µm, 180 µm x 20 mm; reversed phase column HSS T3 1.8 µm 75 µm x 200 mm; 0.5 to 2 µl injection volume). The MRM method was set up using Skyline software (vs. 3.6.0.10162, University of Washington) by training it on three subsequent 0.5 µl injections run with data-independent acquisition. Peptides (Figure 2) were selected and the MRM method (Figure 3) exported to the driver software MassLynx 4.1. Figure 4 illustrates the response of all peptides obtained when measuring 50 fmol digest on-column. It is important to consider the ion intensities of several fragments when evaluating unknowns. They should match the presented spectra in their overall relation; if they do not, the assignment may be incorrect. This effect is often noted when measuring at the detection limit. Singular low-mass ions may suggest the presence of a particular peptide, but they are rather the result of background sequencing. The calibration curve (Figure 5) was determined with HLA-G unique peptides only.

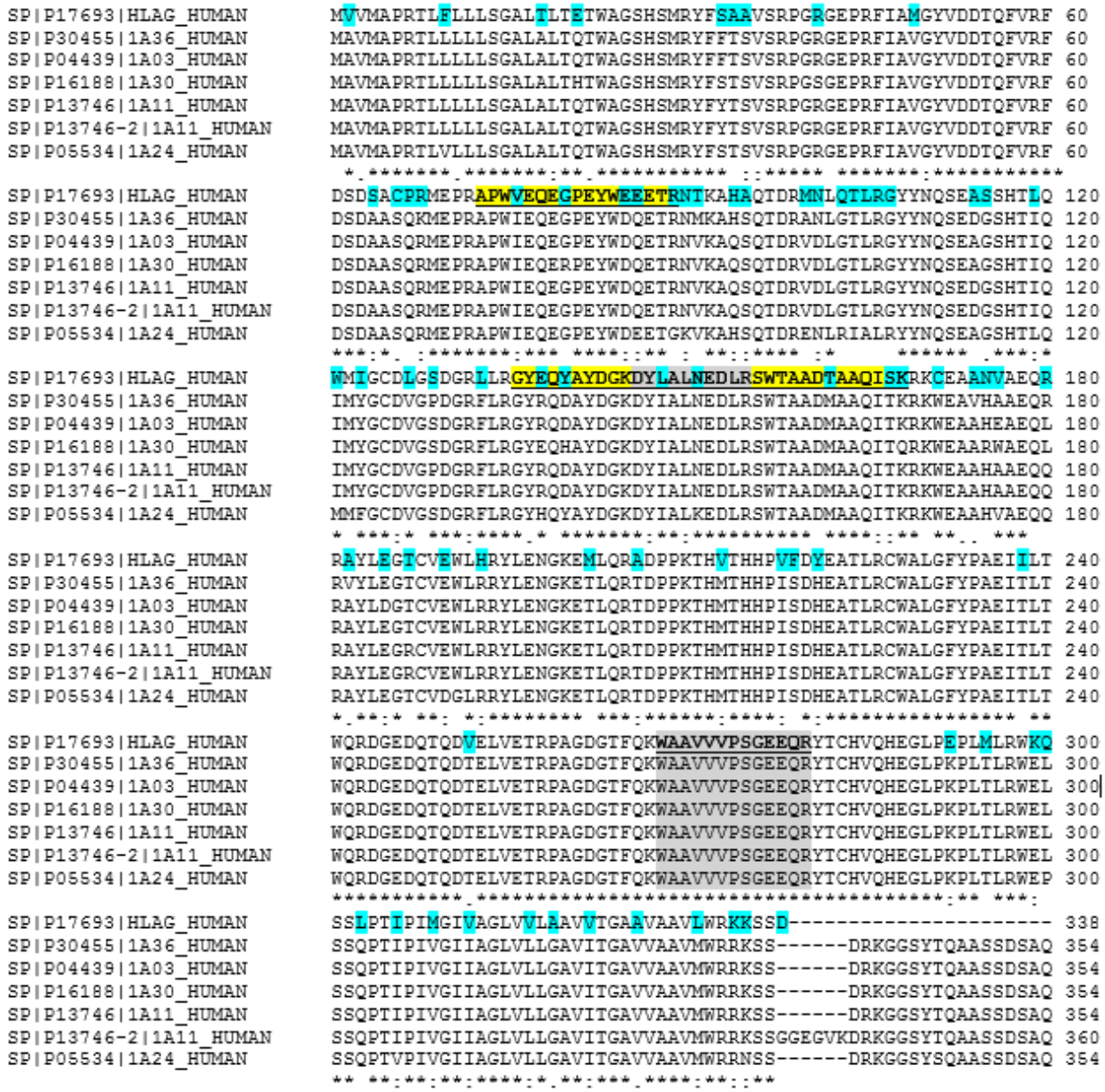


Figure 1: Clustal O(1.2.4) multiple sequence alignment of HLA-G following Blast search on Uniprot.org. Target peptides are underlined. Amino acid (AA) residues which differ in HLA-G are marked in blue. One peptide is identical in all the proteins and is used as a background marker (WAAVVVPSGEEQR). Another peptide (DYLALNEDLR) partially serves the same purpose. It differs in two AA, but isoleucine and leucine have the same residue mass and cannot be distinguished. The change N to K is only present in one of the six homologous proteins shown (1A24) and can thus only exclude that particular protein. The three specific peptides are marked in yellow. The signal peptide is the sequence AA 1-24.

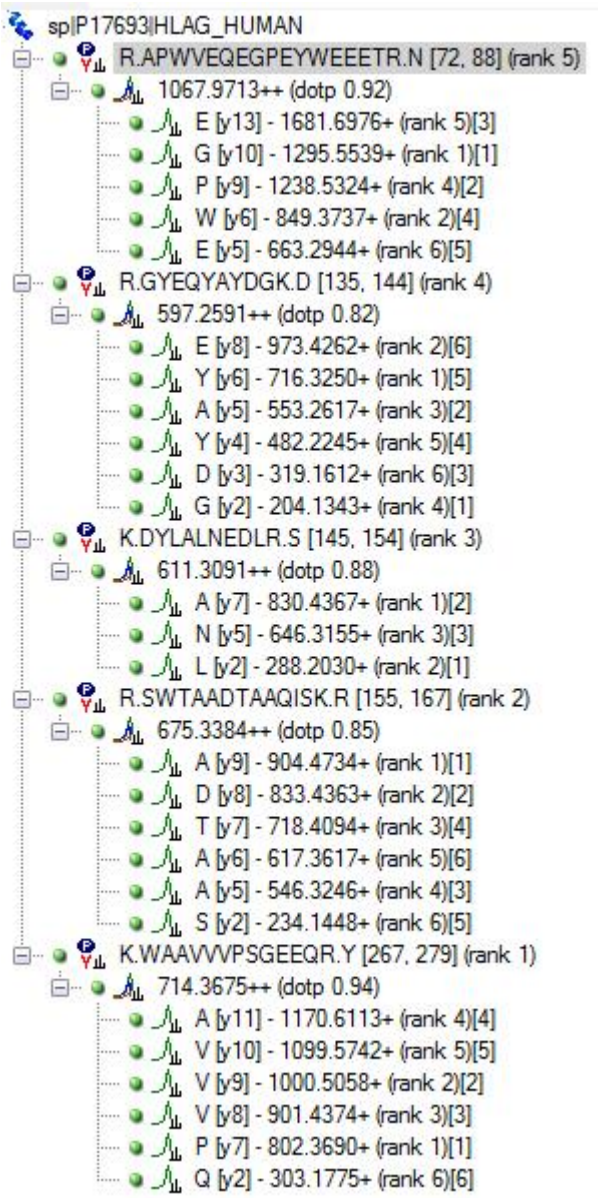


Figure 2: Target peptides and their fragment ions for MRM as displayed in Skyline. They were selected according to their response in the mass spectrometer from three subsequent measurements of 50 fmol of HLA-G digest.

Figure 3: MRM method transferred from Skyline to MassLynx. Detection range *m/z* 50-2000 in 0.4 s. Five peptides are targeted via their doubly-charged ions.

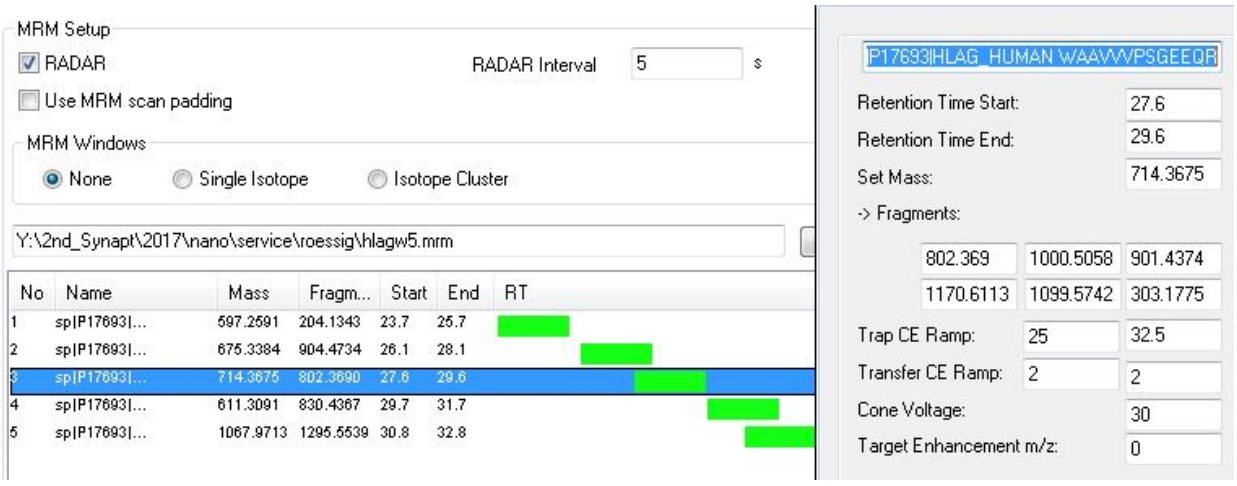
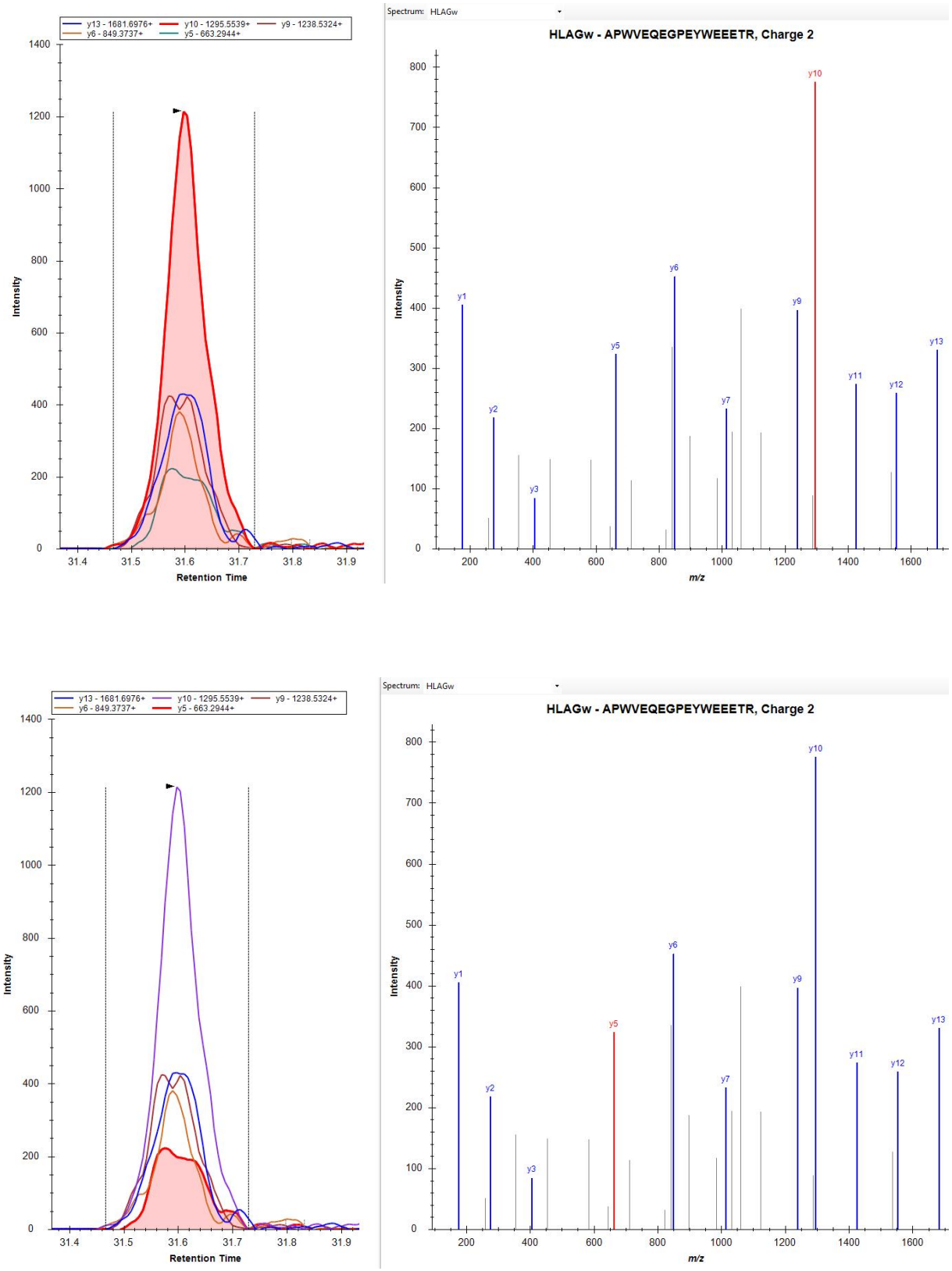
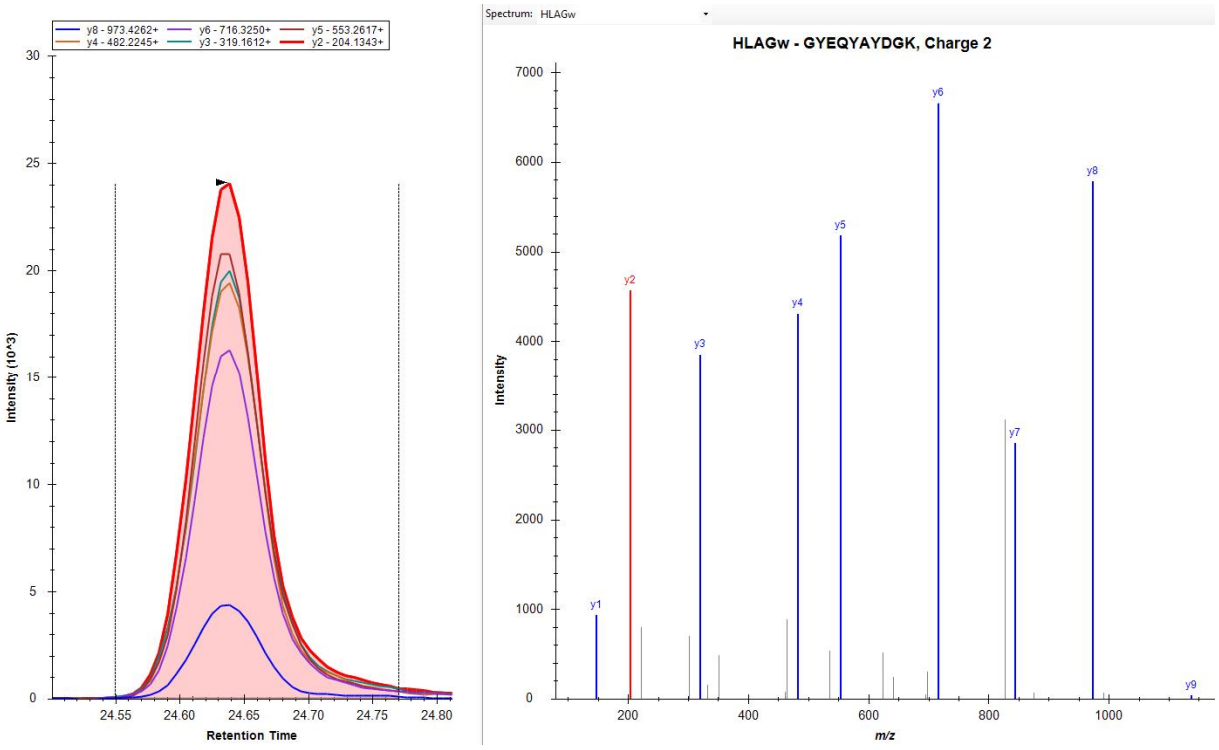


Figure 4: Peak shape and abundance as well as the fragment ion spectrum for 50 fmol protein digest on-column. Shown are the selected fragment ions with the highest and the lowest abundance, respectively, for each peptide from Figure 2.

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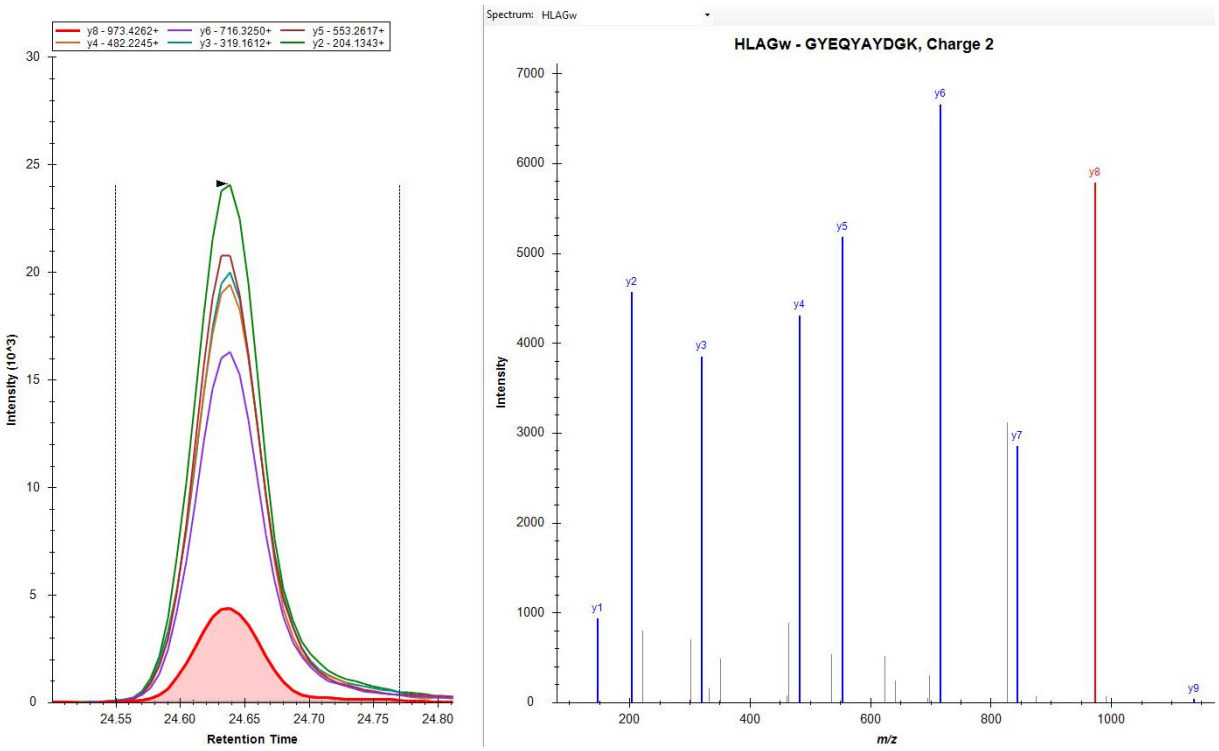
y6 - 716.3250+
y5 - 553.2617+
y4 - 482.2245+
y3 - 319.1612+
y2 - 204.1343+
Intensity (10^3)
Retention Time
Spectrum: HLAGw
HLAGw - GYEQYAYDGK, Charge 2
Intensity
m/z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HLAGw - GYEQYAYDGK, Charge 2
Intensity
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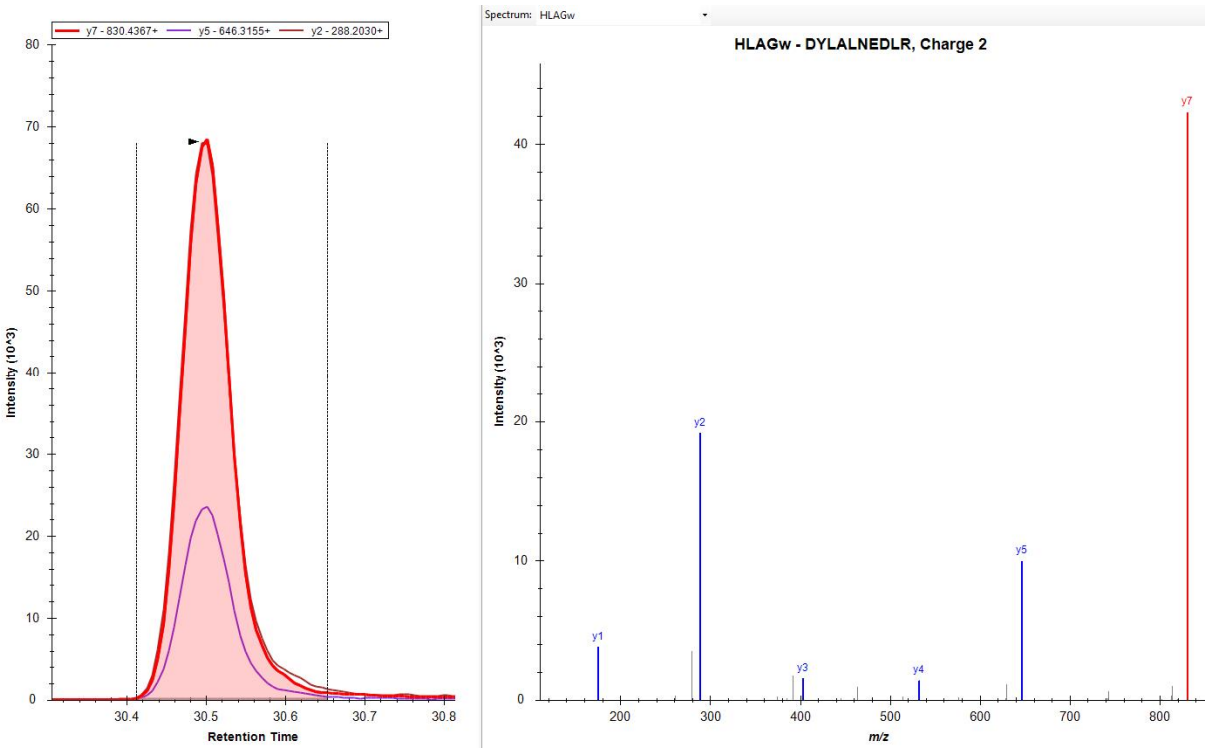
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Intensity (10^3)
Retention Time
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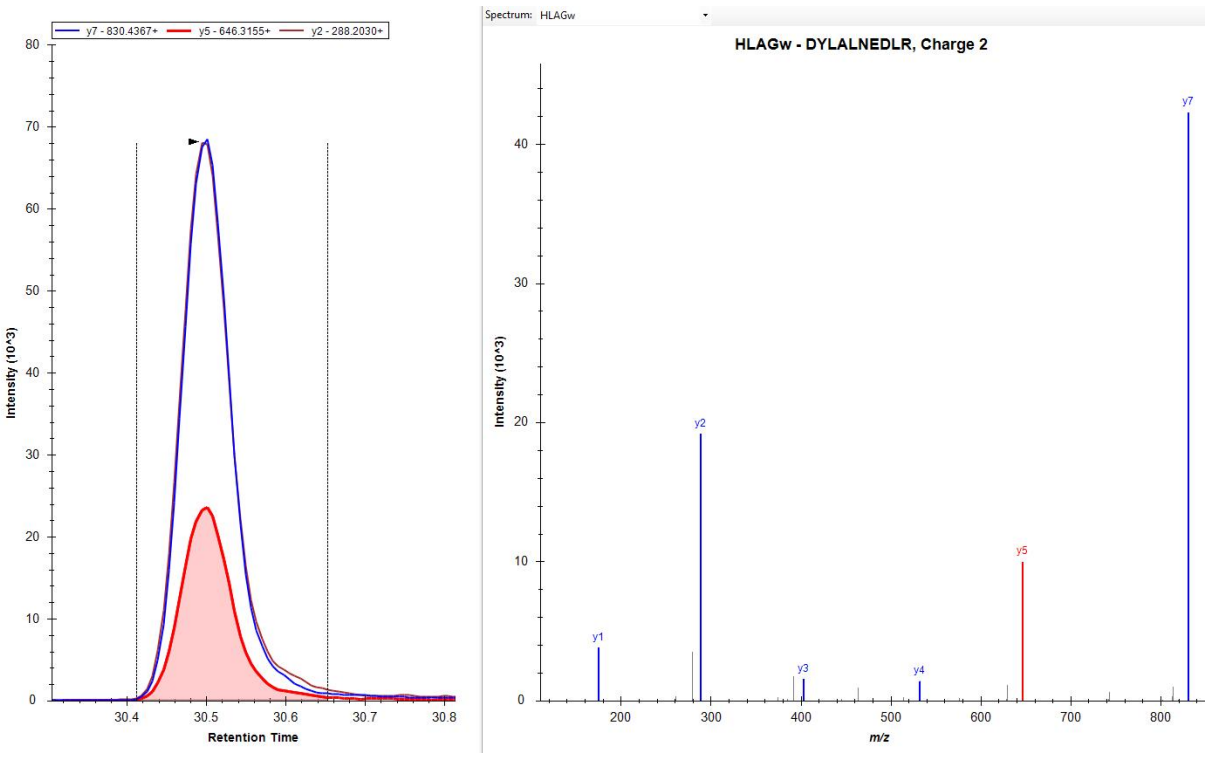
HLAGw - DYLALNEDLR, Charge 2
y7
y2
y5
y1
y3
y4
m/z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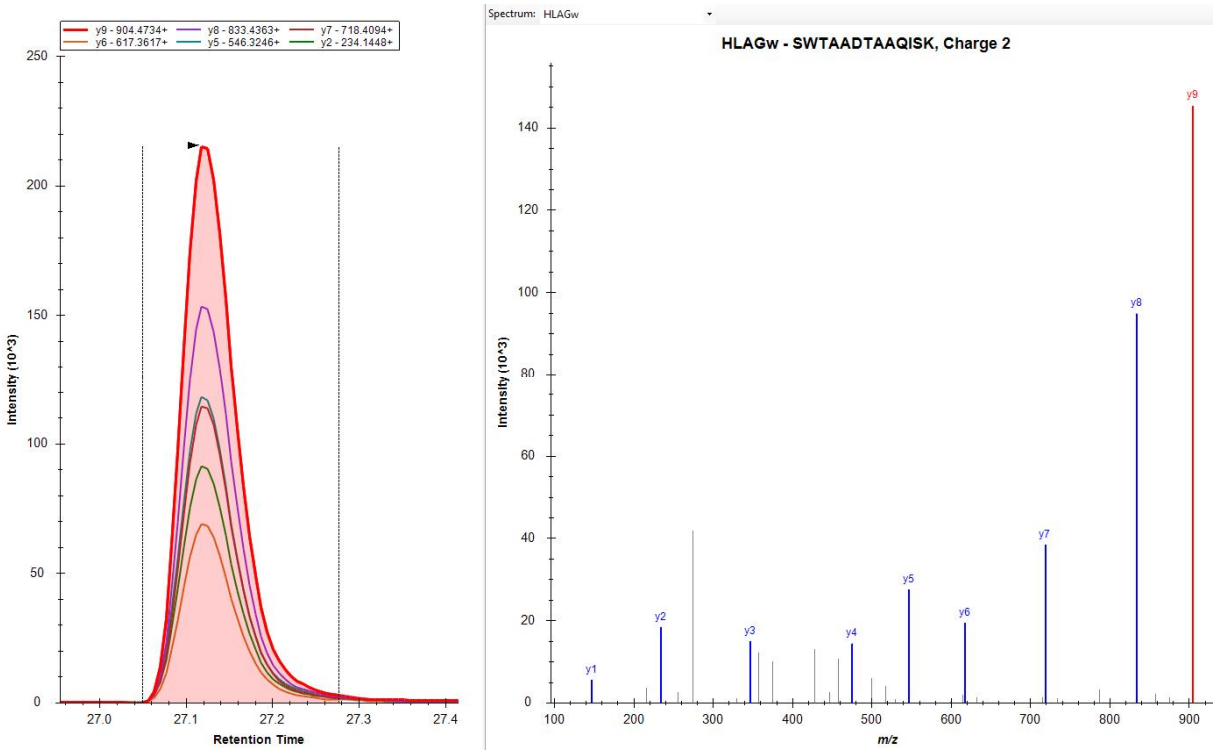
Spectrum: HLAGw
HLAGw - DYLALNEDLR, Charge 2
y7
y2
y5
y1
y3
y4
m/z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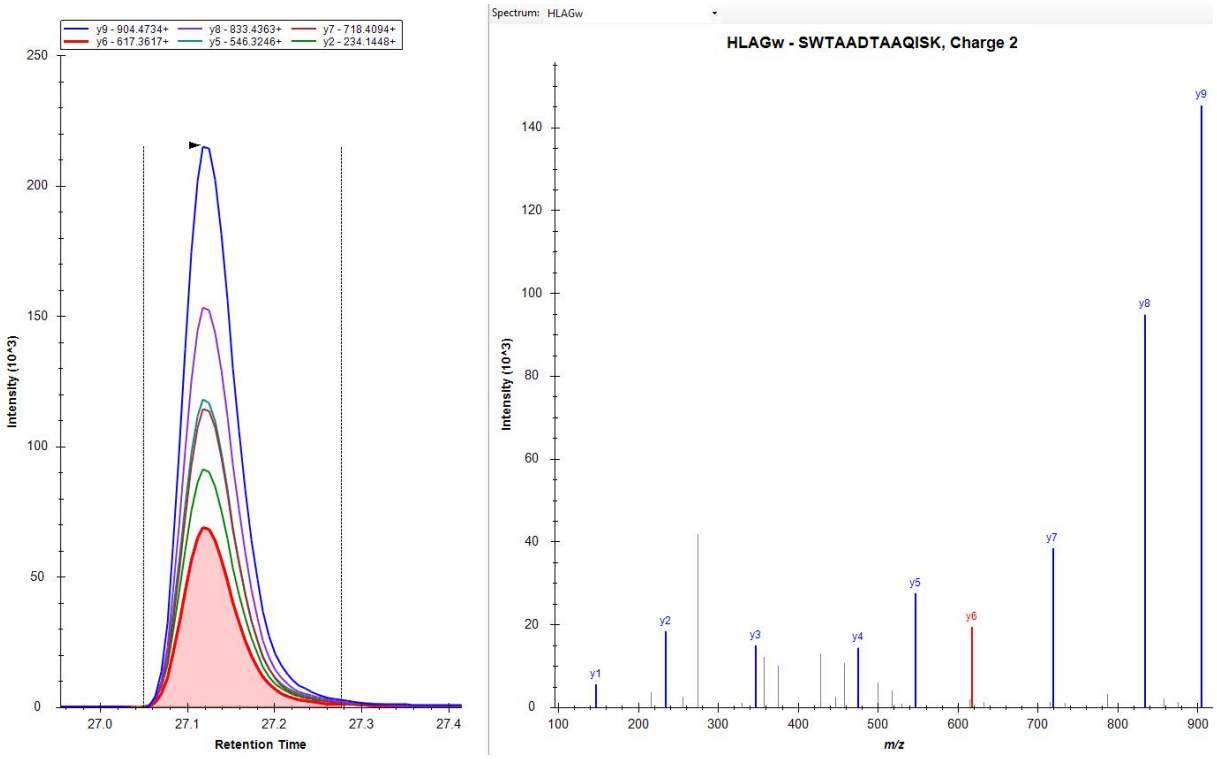
y8 - 833.4363+
y7 - 718.4094+
y6 - 617.3617+
y5 - 546.3246+
y2 - 234.1448+
Intensity (10^3)
Retention Time
Spectrum: HLAGw
HLAGw - SWTAADTAAQISK, Charge 2
m/z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y8 - 833.4363+
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y5 - 546.3246+
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Intensity (10^3)
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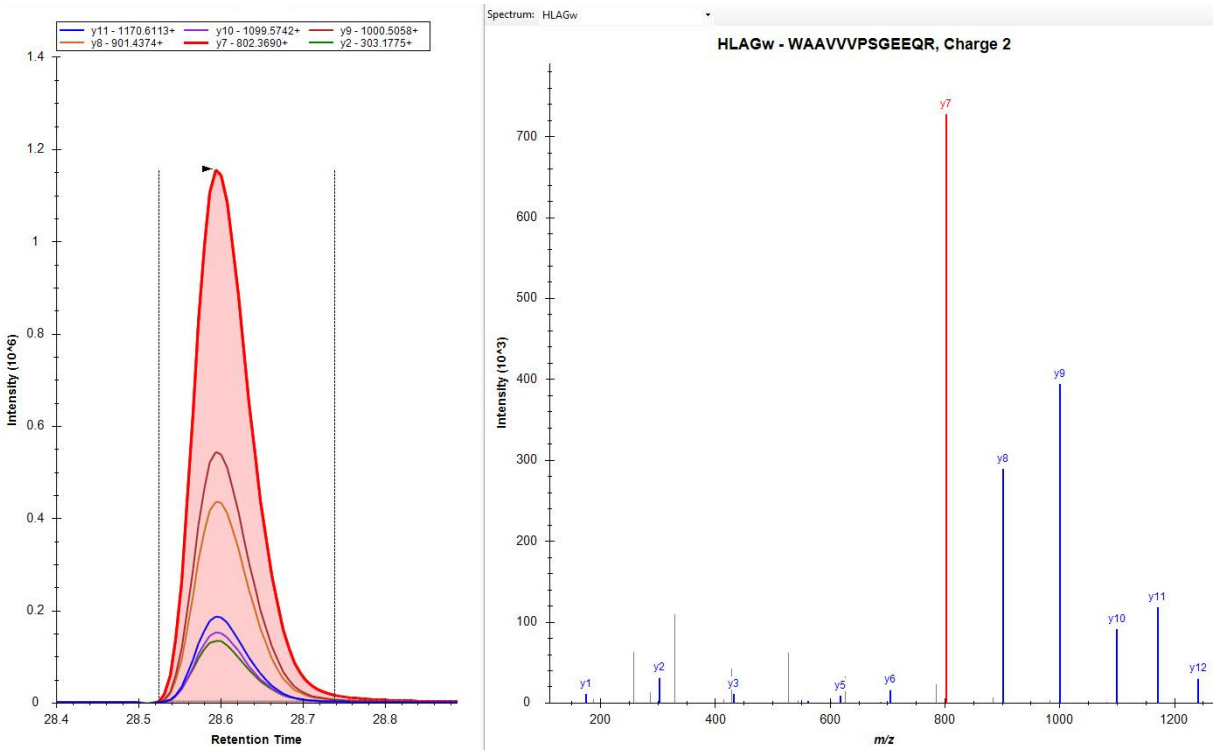
m/z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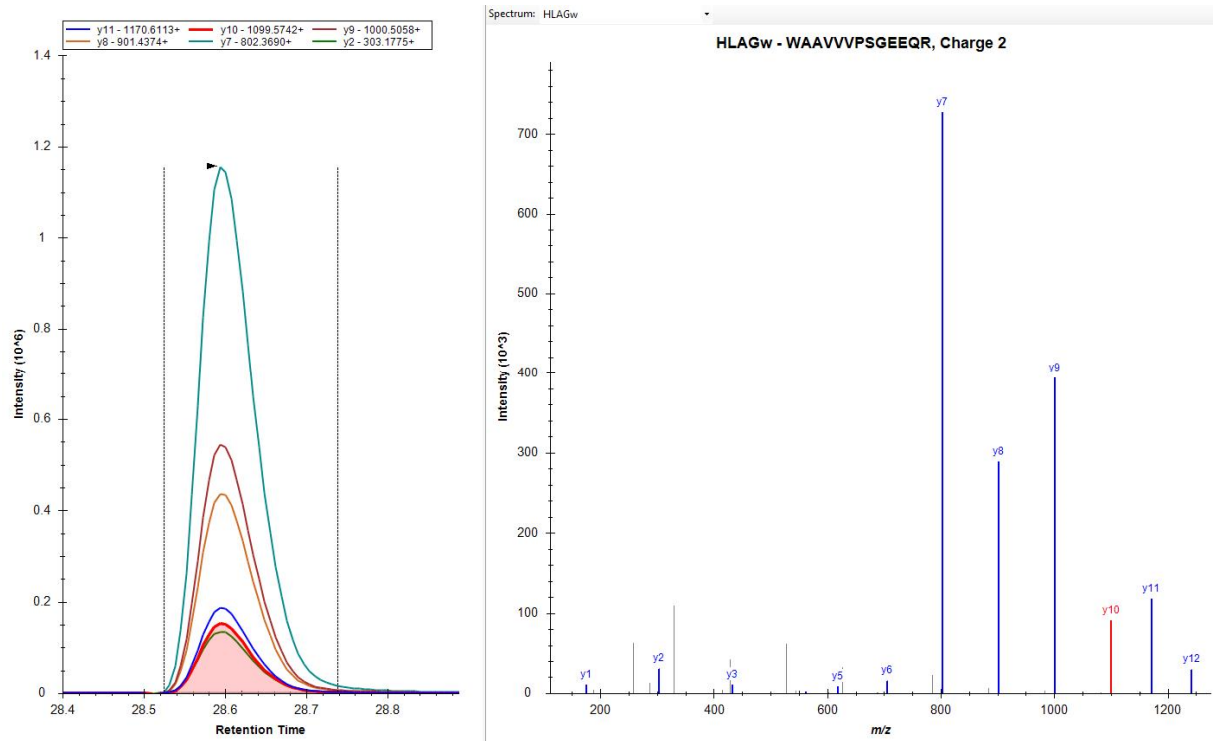
HLAGw - WAAVVVPSGEEQR, Charge 2
Intensity (10^3)
m/z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y9 - 1000.5058+
y8 - 901.4374+
y7 - 802.3690+
y2 - 303.1775+
Intensity (10^6)
Retention Time
Spectrum: HLAGw
HLAGw - WAAVVVPSGEEQR, Charge 2
Intensity (10^3)
m/z

Peptide Sequence	30	50	80	100	150	200	check
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GYEQYAYDGK	185462	402615	692944	881437	1272537	1795047	357870
SWTAADTAAQISK	1350546	3311347	5057404	5618869	7773784	9010694	2640142
Total area HLAG	**1543521**	**3729186**	**5775319**	**6530840**	**9087909**	**10862038**	**3008338**
Amount fmol	**30**	**50**	**80**	**100**	**150**	**200**	47
						Theor 50 fmol	
						SD	2,2
						RSD	4,4

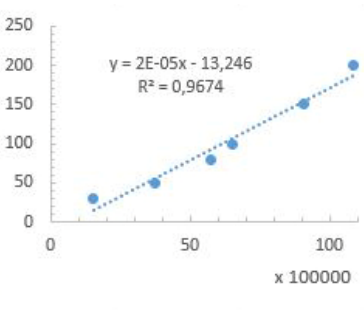

Figure 5: Calibration with unique peptides, 30-200 fmol on-column.

Conclusion

A pseudo-MRM for the specific detection of HLA-G is presented. It can be used to quantify the protein at low fmol levels in a background of homologous proteins such as HLA-A.

Acknowledgements

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References

[1] Carosella ED, Rouas-Freiss N, Tronik-Le Roux D, Moreau P, LeMaoult J, 2015, HLA-G: An immune checkpoint molecule. Adv Immunol 127:33-144

[2] Kailayangiri S, Altvater B, Spurny C, Jamitzky S, Schelhaas S, Jacobs AH, Wiek C, Roellecke K, Hanenberg H, Hartmann W, Wiendl H, Pankratz S, Meltzer J, Farwick N, Greune L, Fluegge M, Rossig C, 2016, Targeting Ewing sarcoma with activated and GD2-specific chimeric antigen receptor-engineered human NK cells induces upregulation of immune-inhibitory HLA-G. OncoImmunology e1250050