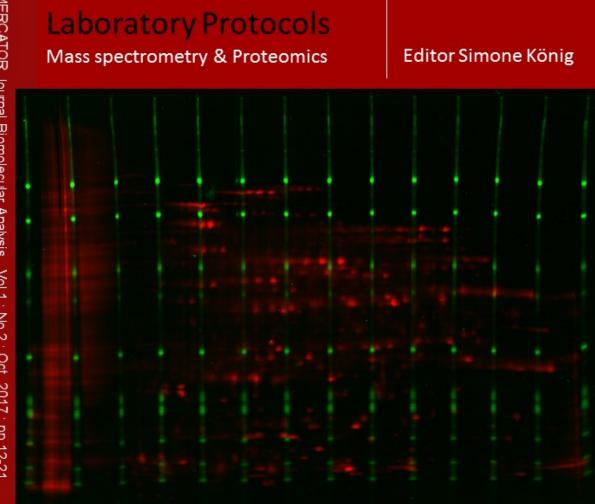


# MERCATOR Journal Biomolecular Analysis



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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<sup>2</sup>, Claudia Rössig<sup>2</sup>

<sup>1</sup> Core Unit Proteomics, Interdisciplinary Center for Clinical Research,

<sup>2</sup> 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 NH4HCO3 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.

MVVMAPRTLFLLLSGALTLTETWAGSHSMRYFSAAVSRPGRGEPRFIAMGYVDDTQFVRF 60 SP|P17693|HLAG HUMAN SP| P30455 | 1A36 HUMAN MAVMAPRTLLLLLSGALALTOTWAGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTOFVRF 60 SP|P04439|1A03\_HUMAN MAVMAPRTLLLLLSGALALTQTWAGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRF 60 SP|P16188|1A30 HUMAN MAVMAPRTLLLLLSGALALTHTWAGSHSMRYFSTSVSRPGSGEPRFIAVGYVDDTQFVRF 60 SP|P13746|1A11\_HUMAN MAVMAPRTLLLLLSGALALTOTWAGSHSMRYFYTSVSRPGRGEPRFIAVGYVDDTOFVRF 60 SP|P13746-2|1A11 HUMAN MAVMAPRTLLLLLSGALALTQTWAGSHSMRYFYTSVSRPGRGEPRFIAVGYVDDTQFVRF 60 MAVMAPRTLVLLLSGALALTOTWAGSHSMRYFSTSVSRPGRGEPRFIAVGYVDDTOFVRF 60 SP|P05534|1A24\_HUMAN \* \*\*\*\*\*\*\* \*\*\*\*\*\*\*\* SP|P17693|HLAG\_HUMAN DSDSACPRMEPRAPWVEQECPEYWEEETRNTKAHAQTDRMNLQTLRGYYNQSEASSHTLQ 120 DSDAASOKMEPRAPWIEQEGPEYWDOETRNMKAHSOTDRANLGTLRGYYNOSEDGSHTIQ 120 SP|P30455|1A36 HUMAN SP|P04439|1A03 HUMAN DSDAASQRMEPRAPWIEQEGPEYWDQETRNVKAQSQTDRVDLGTLRGYYNQSEAGSHTIQ 120 SP|P16188|1A30 HUMAN DSDAASQRMEPRAPWIEQERPEYWDQETRNVKAQSQTDRVDLGTLRGYYNQSEAGSHTIQ 120 SP|P13746|1A11\_HUMAN DSDAASORMEPRAPWIEQEGPEYWDQETRNVKAQSOTDRVDLGTLRGYYNQSEDGSHTIQ 120 SP|P13746-2|1A11 HUMAN DSDAASQRMEPRAPWIEQEGPEYWDQETRNVKAQSQTDRVDLGTLRGYYNQSEDGSHTIQ 120 SP|P05534|1A24\_HUMAN DSDAASQRMEPRAPWIEQEGPEYWDEETGKVKAHSQTDRENLRIALRYYNQSEAGSHTLQ 120 . \*\*\*-\*\*\* :-\*\*\*\*\*\*:\*\*\* \*\*\*\*::\*\* : \*\*::\*\*\*\* :\* \*\*\*\*\* WMIGCDLGSDGRLLR<mark>GYEQYAYDGKDYLALNEDLRSWTAADTAAQISK</mark>RKCEA<mark>ANV</mark>AEQR 180 SP|P17693|HLAG HUMAN SP|P30455|1A36 HUMAN IMYGCDVGPDGRFLRGYRQDAYDGKDYIALNEDLRSWTAADMAAQITKRKWEAVHAAEQR 180 SP|P04439|1A03 HUMAN IMYGCDVGSDGRFLRGYRQDAYDGKDYIALNEDLRSWTAADMAAQITKRKWEAAHEAEQL 180 SP|P16188|1A30\_HUMAN IMYGCDVGSDGRFLRGYEOHAYDGKDYIALNEDLRSWTAADMAAOITORKWEAARWAEOL 180 SP|P13746|1A11 HUMAN IMYGCDVGPDGRFLRGYRQDAYDGKDYIALNEDLRSWTAADMAAQITKRKWEAAHAAEQQ 180 SP|P13746-2|1A11 HUMAN IMYGCDVGPDGRFLRGYRQDAYDGKDYIALNEDLRSWTAADMAAQITKRKWEAAHAAEQQ 180 MMFGCDVGSDGRFLRGYHOYAYDGKDYTALKEDLRSWTAADMAAOTTKRKWEAAHVAEOO 180 SP|P05534|1A24 HUMAN ې بې بې RAYLEGTCVEWLHRYLENGKEMLQRADPPKTHVTHHPVFDYEATLRCWALGFYPAEIILT 240 SP|P17693|HLAG HUMAN SP|P30455|1A36\_HUMAN RVYLEGTCVEWLRRYLENGKETLQRTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLT 240 SP|P04439|1A03 HUMAN RAYLDGTCVEWLRRYLENGKETLQRTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLT 240 SP|P16188|1A30 HUMAN RAYLEGTCVEWLRRYLENGKETLORTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLT 240 SP|P13746|1A11 HUMAN RAYLEGRCVEWLRRYLENGKETLQRTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLT 240 SP|P13746-2|1A11 HUMAN RAYLEGRCVEWLRRYLENGKETLQRTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLT 240 SP|P05534|1A24\_HUMAN RAYLEGTCVDGLRRYLENGKETLORTDPPKTHMTHHPISDHEATLRCWALGFYPAEITLT 240 WQRDGEDQTQDVELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPEPLMLRWKQ 300 SP|P17693|HLAG\_HUMAN WORDGEDOTODTELVETRPAGDGTFOKWAAVVVPSGEEORYTCHVOHEGLPKPLTLRWEL 300 SP|P30455|1A36\_HUMAN SP|P04439|1A03 HUMAN WORDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEL 300 SP|P16188|1A30\_HUMAN WORDGEDOTODTELVETRPAGDGTFOKWAAVVVPSGEEORYTCHVOHEGLPKPLTLRWEL 300 SP|P13746|1A11 HUMAN WORDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEL 300 WORDGEDOTODTELVETRPAGDGTFOKWAAVVVPSGEEORYTCHVOHEGLPKPLTLRWEL 300 SPIP13746-211A11 HUMAN SP|P05534|1A24 HUMAN WQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEP 300 SSLPTIPIMGIVAGLVVLAAVVTGAAVAAVLWRKKSSD------- 338 SP|P17693|HLAG HUMAN SP|P30455|1A36 HUMAN SSQPTIPIVGIIAGLVLLGAVITGAVVAAVMWRRKSS-----DRKGGSYTQAASSDSAQ 354 SP|P04439|1A03 HUMAN SSQPTIPIVGIIAGLVLLGAVITGAVVAAVMWRRKSS-----DRKGGSYTQAASSDSAQ 354 SSQPTIPIVGIIAGLVLLGAVITGAVVAAVMWRRKSS-----DRKGGSYTQAASSDSAQ 354 SP|P16188|1A30\_HUMAN SSQPTIPIVGIIAGLVLLGAVITGAVVAAVMWRRKSS-----DRKGGSYTQAASSDSAQ 354 SP|P13746|1A11 HUMAN SSOPTIPIVGIIAGLVLLGAVITGAVVAAVMWRRKSSGGEGVKDRKGGSYTQAASSDSAQ 360 SP|P13746-2|1A11 HUMAN SP|P05534|1A24 HUMAN SSOPTVPIVGIIAGLVLLGAVITGAVVAAVMWRRNSS-----DRKGGSYSOAASSDSAO 354 

**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.

spiP17693|HLAG\_HUMAN

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.

. R.APWVEQEGPEYWEEETR.N [72, 88] (rank 5)
🖮 🥥 🥼 1067.9713++ (dotp 0.92)
🧿 🍌 E [y13] - 1681.6976+ (rank 5)[3]
💿 🏒 G [y10] - 1295.5539+ (rank 1)[1]
🛯 🏒 P [y9] - 1238.5324+ (rank 4)[2]
• • J, W [y6] - 849.3737+ (rank 2)[4]
J E [y5] - 663.2944+ (rank 6)[5]
🗄 🖷 🗣 🖓 👖 R.GYEQYAYDGK.D [135, 144] (rank 4)
⊟ 🧿 🦽 597.2591++ (dotp 0.82)
💿 🎢 A [y5] - 553.2617+ (rank 3)[2]
💿 🎢 Y [y4] - 482.2245+ (rank 5)[4]
💿 🎢 D [y3] - 319.1612+ (rank 6)[3]
G [v2] - 204.1343+ (rank 4)[1]
🖶 🖷 🗣 🖓 📙 K.DYLALNEDLR.S [145, 154] (rank 3)
😑 💿 🥼 611.3091++ (dotp 0.88)
🧿 🥼 A [y7] - 830.4367+ (rank 1)[2]
🛯 🏒 N [y5] - 646.3155+ (rank 3)[3]
😑 🖷 🧕 🖓 🛓 R.SWTAADTAAQISK.R [155, 167] (rank 2)
😑 💿 🥼 675.3384++ (dotp 0.85)
💿 🥂 A [y9] - 904.4734+ (rank 1)[1]
🎍 🎢 D [y8] - 833.4363+ (rank 2)[2]
🎍 🥂 [y7] - 718.4094+ (rank 3)[4]
🞍 刈 A [y6] - 617.3617+ (rank 5)[6]
🎍 🎢 A [y5] - 546.3246+ (rank 4)[3]
🖮 🞍 🖓 K.WAAVVVPSGEEQR.Y [267, 279] (rank 1)
🚊 🛥 🎝 👖 714.3675++ (dotp 0.94)
🥥 🎢 A [y11] - 1170.6113+ (rank 4)[4]
···· 🛯 🏒 V [v10] - 1099.5742+ (rank 5)[5]
···· 🛯 🏒 V [y9] - 1000.5058+ (rank 2)[2]
🎍 🎢 V [y8] - 901.4374+ (rank 3)[3]
🛯 🏒 P [y7] - 802.3690+ (rank 1)[1]

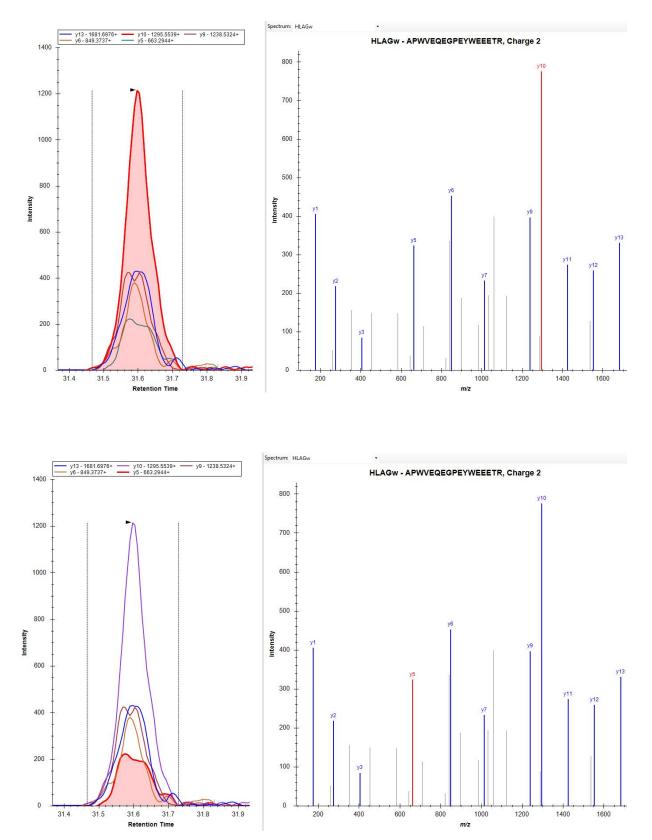
...... A [v2] - 303.1775+ (rank 6)[6]

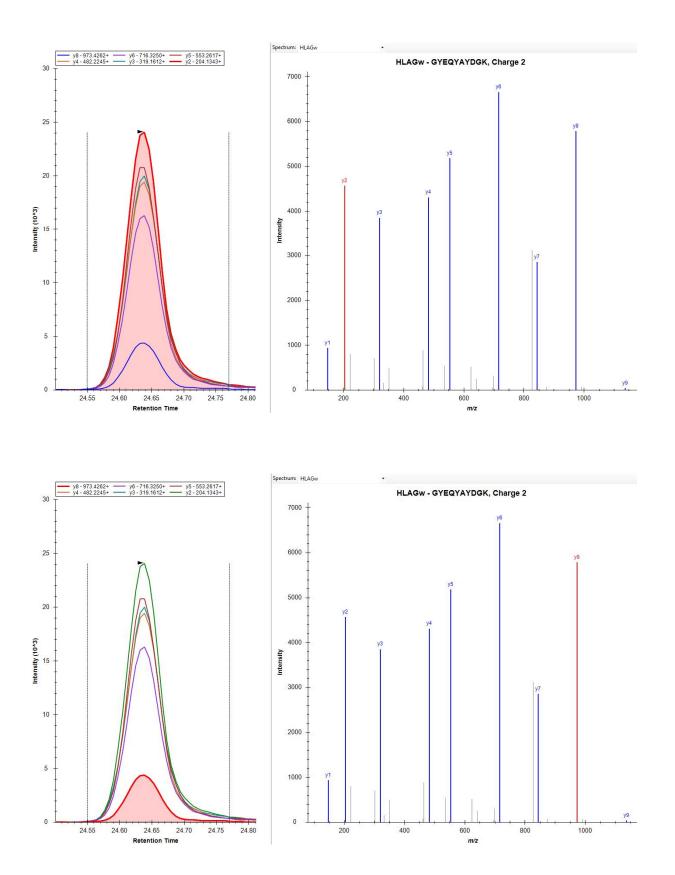
Figure 3: MRM method transferred from Skyline to MassLynx. Detection range m/z 50-2000 in 0.4 s. Five peptides are targeted via their doublycharged ions.

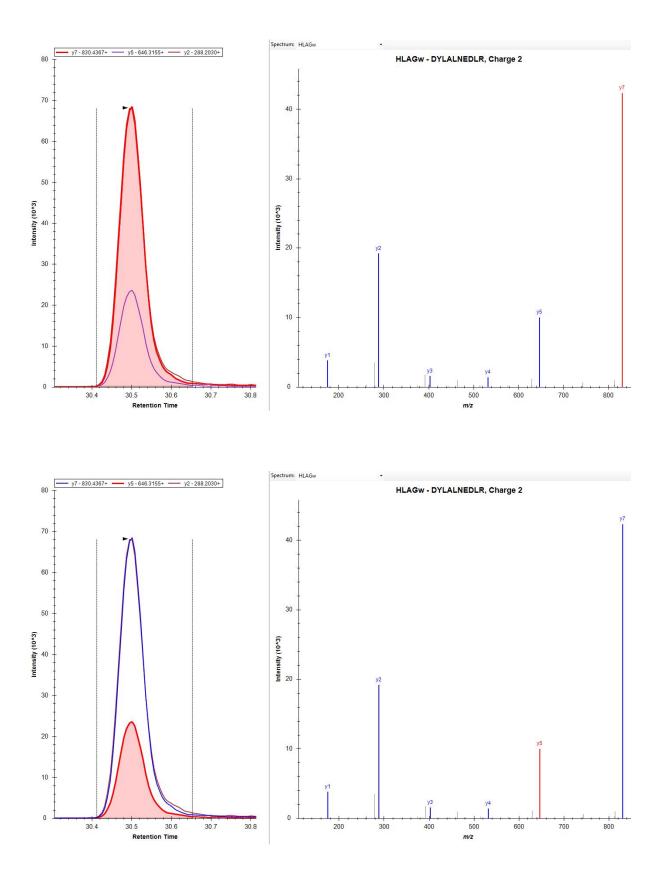
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	Use MRM sca RM Windows	n padding						
100	None	🔘 Single Isoto	pe (	🔵 Isoto	ipe Clus	ter		
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Y:\2 No		017\nano\service Mass	\roessig\h Fragm	-	Therases.	RT		
10000		land the		-	Therases.	RT		
No 1	Name	Mass	- Fragm	Start	End	BT		
No 1	Name sp[P17693]	Mass 597.2591	- Fragm 204.1343	Start 23.7	End 25.7	RT		
10000	Name spiP17693j spiP17693j	Mass 597.2591 675.3384	Fragm 204.1343 904.4734	Start 23.7 26.1	End 25.7 28.1	RT		

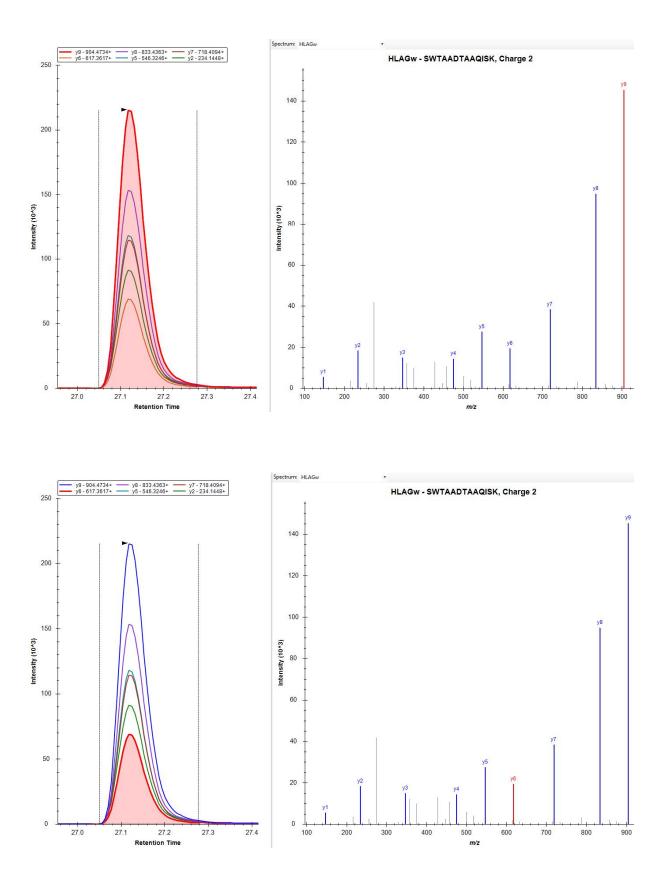
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Retentic	29.6				
Set Mas	s:		714.3675		
-> Fragm	nents:				
	802.369	1000.5058	901.4374		
	1170.6113	1099.5742	303.1775		
Trap CE	Ramp:	25	32.5		
Transfer	CE Ramp:	2	2 30		
Cone Vo	oltage:				
Target E	nhancement	t m/z:	0		

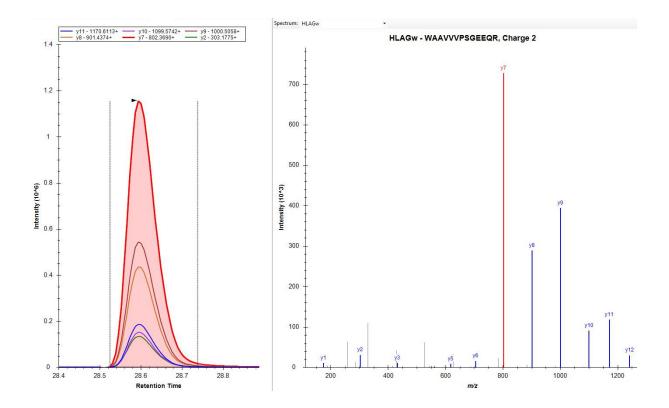
**Figure 4:** Peak shape and abundance as well as the fragment ion spectrum for 50 fmol protein digest on-column. Shown are the selected fragment ions with the highest and the lowest abundance, respectively, for each peptide from Figure 2.

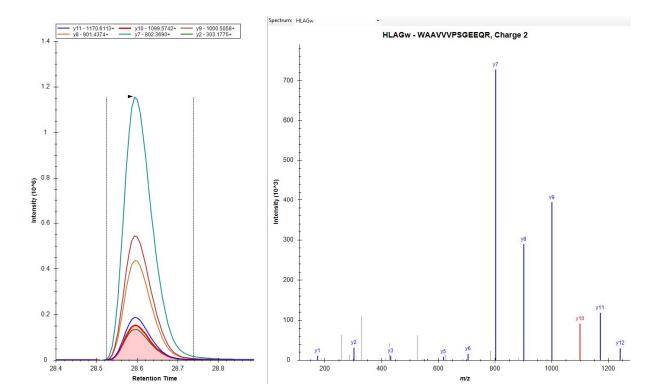












Peptide Sequence	30	50	80	100	150	200	check	250		
APWVEQEGPEYWEEETR	7513	15224	24971	30534	41588	56297	10326	200	v = 2E-05x - 13,246	
GYEQYAYDGK	185462	402615	692944	881437	1272537	1795047	357870	-	R <sup>2</sup> = 0,9674	in the second
SWTAADTAAQISK	1350546	3311347	5057404	5618869	7773784	9010694	2640142	150		
Total area HLAG	1543521	3729186	5775319	6530840	9087909	10862038	3008338	100		
Amount fmol	30	50	80	100	150	200	47	50		
						Theor 50 fr	nol	0	50	100
						SD	2,2	U		100 x 100000
						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 receptorengineered human NK cells induces upregulation of immune-inhibitory HLA-G. Oncolmmunology e1250050