

Biotherapeutic Peptide Mapping Information Independent SWATH[®] Acquisition Method

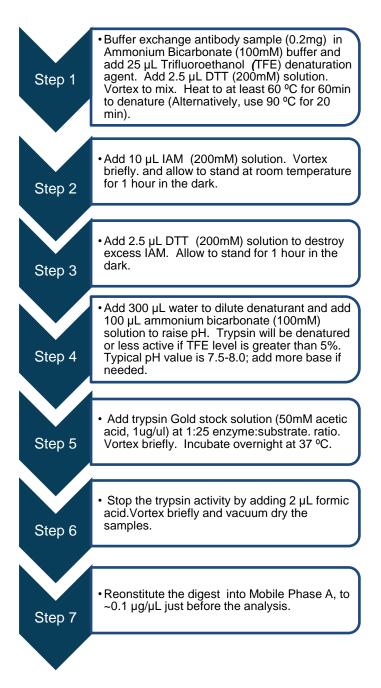
Routine biotherapeutic accurate mass peptide mapping analysis on the X500B QTOF System

Method details for the routine peptide mapping of a biotherapeutic monoclonal antibody (mAb) protein by high-resolution accurate mass analysis on the X500B QTOF System, powered by SCIEX OS Software. An information independent SWATH Acquisition method was employed to acquire highresolution MS and MS/MS level data on the digested biologic protein product.

SWATH Acquisition utilizes either fixed or variable Q1 mass isolation window, transmitting all precursor ions in the defined Q1 window through to the collision cell. Transmitted ions are fragmented and analyzed at high-resolution. The Q1 isolation window is stepped across the entire mass range, with an LC compatible cycle time, resulting in the comprehensive acquisition of high-resolution MS/MS spectra for every precursor ion in a sample. This unbiased data acquisition approach ensures data completeness is maximized, and enables detection of low abundance peptides and modifications.



A generic sample preparation strategy is shown for reduction and tryptic digestion of an antibody biotherapeutic prior to LC-MS analysis.





LC Method

Column	Waters Acquity UPLC	Waters Acquity UPLC BEH C18 Column, 130 1.7 $\mu\text{m},$ 2.1 mm X 100 mm						
Mobile Phase A	Water, 0.1% Formic ac	Water, 0.1% Formic acid						
Mobile Phase B	Acetonitrile, 0.1% Form	nic acid						
Flow rate	200 µL/min							
Column temperature	40° C							
Injection volume	10 μL, 1 μg total protei	n						
Gradient profile	Time (min)	% B						
	8.0	2						
	40.0	30						
	60.0	50						
	62.0	90						
	66.0	90						
	66.5	2						
	75.0	2						



MS Method

Suggested starting MS and MS/MS method parameters for routine SWATH based peptide mapping analysis as displayed in SCIEX OS user interface. The SWATH acquisition criteria are shown with a 50Da fixed SWATH window from 350-2000 m/z acquiring high-resolution MS/MS in each cycle. For best sequence coverage and sensitivity, the specific SWATH parameters should be optimized for the length of HPLC separation used.

沿 Generic SWATH											
Method Overview Device: X500 QTOF	Method	l duration	75 🗘 min	То	tal scan time:	1.11322 se	c				
Ion Source: TurboSpray	Estimat	ed cycles:	4042								
SWATH (TOF MSMS Scans: 33)	 Source 	and Gas Parar									
0 min - 75 min	Ion sou	rce gas 1	40 🗘 psi	Curtai	in gas	35	Temper	ature	450	\$	°C
	Ion sou	rce gas 2	40 🗘 psi	CAD o	jas	7	•				
	▼ Experin	nent swath	•								
	Polarity		Positive 💙	Au	tofill SWATH V	Vindows				X	
				Gen	Generate an initial set of SWATH windows and then use the windows to autofill the MSMS mass table						
	TOF MS TOF sta	rt mass	350 🗘 Da	Р	recursor start mass	350	Da Wind	ow width	50	Da	v
	TOF sto	p mass	2000 🗘 Da	P	recursor stop mass	2000	Da Wind	ows per cycle:	33		v
	Accumu	lation time	0.125 🗘 s	Pop	ulate the MSMS tab	le					
	TOF MS	MS			Append to existin	ng list					
	TOF sta		50 🗘 Da		Overwrite the exi	sting list					
	Accumu	lation time	0.025 🗘 s					Apply	Cancel		
	Mass T	able <u>Autofill</u>	SWATH windows								
		Precursor ion st	Precursor ion st	Decluste	eri DP	spread (V)	Collision energy (V)	CE spread (V)		
	1	350.0000	400.0000	80	0		19	15			
	2	399.0000	450.0000	80	0		22	15			
	3	449.0000	500.0000	80	0		26	15			
	4	499.0000	550.0000	80	0		29	15			
	5	549.0000	600.0000	80	0		32	15			
	6	599.0000	650.0000	80	0		35	15			
	7	649.0000	700.0000	80	0		38	15			
	8	699.0000	750.0000	80	0		41	15			
	9	749.0000	800.0000	80	0		44	15			



Batch

In the Batch setup, open the 'Automated Calibration Editor' window in order to select the use of the autocalibration function. Designate use of the 'X500 ESI Positive Calibration Solution', and then determine how often you would like the system to perform a fast, automated calibration. These short calibrations will be added automatically to your queue once you have submitted a sample batch.

🗘 - Batch	습 🎹 🗒 🚪					<u>.</u>	(O) Running		? – □ ×
	Auto-	Calibrate Plate Layout	New	Open	♥ Save	♥ Print	Manage 👻	Submit	\otimes
Untitled									
Sample Name	MS Method		LC Method		Rack code	Vial position	Data File		â
1 Intact protein	intact protein analy	sis MS	Intact_10	min	1.5mL (105 vial)	1	Intact protein file		
2									
3									
4									
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8						_			
9	Batch - Automatic C	Calibration Editor				×	1		
10									
11	Provide ion reference and	calibrant delivery settings to	o be applied aut	omatically, at t	the correct frequency duri	ng acquisition			_
12									
13	Ion reference table	X500 ESI Positive Calibratio	on Solu 💙		Edit				
14	Calibrate every	APCI Negative Calibration S	Solution sa	amples					
16		APCI Positive Calibration Sc	olution						
17	Calibrant delivery	Beta Galactosidase Digests			CDS channel 1	*			
18		Bovine Insulin							
19		CsI_ALILTLVS Peptide			ОК	Cancel			
20		ESI Positive Calibration Solu	ution		OK	cuncer			
21		Glu-fibrinopeptide B							
22		PPG Negative Calibration S	olution						
23		PPG Positive Calibration So	lution						
24		X500 ESI Negative Calibrati	ion Solution						
25		X500 ESI Positive Calibratio							
26			2						

Batch - Automatic Calibration Editor							
Provide ion reference and	لمع d calibrant delivery settings to be applied automatically, at the correct frequency during acc	quisition					
Ion reference table	X500 ESI Positive Calibration Solu 💙 Edit						
Calibrate every	3 samples						
Calibrant delivery	CDS CDS channel 1	~					
	OK	:el					



Data Processing

Process SWATH[®] biotherapeutic peptide mapping data in BioPharmaView[™] Software 2.0.

Input the protein sequence, and assign potential modifications in the 'Assay Information' window.

Project Accyl Information Packe Netwin Packet Nage Assay Information Intel Protein Protein Sequence Monipactory													
Atacy Information Image Transmission Image Transmissin Image Transmission Image Tra	CIEX)	Rituximab)								Create	Open Sa	ve Save As
says information Protein Spear Antibody Add Color: Monoidadia Potein MW: Monoidadia: Monoidadia: Monoidadia: Monoidadia: Monoidadia: Antideate: Add Color: Monoidadia: Monoidadia	ect	Assay Information	Sequenc	ce Features	Intact Prot	ein Peptide Mapp	ng						
act Protein	y Information 🔹 🕨	Protein Sequence											
Anacterite Standard Andelsen	t Protein	a second second second second second	y 🗸 🗛	dd Chain	Unmod	lified Protein MWs:							
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201-213 SSPUTRSFNRGEC Modifications Cysteine Modifications Can Replace Disulfide Bonds Disulfide Bonds - (16) Import Capor		JUL TOU NSTI	KAASATTATH	COMPROVE	INCRADE	NALPAPIENTIS	NANGUPREPUVITI	DEFORDEDITIVE		SE TEOD		TAO AL PIANAY	RITEE
Chains Type Name Position Modified MAA Applies To Workflow Usage Mass Shift From Chain To Chain To Chain Chain Chain <th></th> <th>401-450 VLDS Chain 4 Light chain 2 AA Inde 1-100 QIVLs</th> <th>DGSFFLYSKL xxes: SQSPAILSASE</th> <th>TVDKSRWC</th> <th>CRASSSV</th> <th>SVMHEALHNHYT SYIHWFQQKPGS</th> <th>OKSLSLSPG SPKPWIYATSNLAS</th> <th>GVPVRFSGSGSG</th> <th>TSYSLTI</th> <th>IRVEAE</th> <th>DAATYYC</th> <th>QQWTSNPP</th> <th>Delete Chain TFGGG</th>		401-450 VLDS Chain 4 Light chain 2 AA Inde 1-100 QIVLs	DGSFFLYSKL xxes: SQSPAILSASE	TVDKSRWC	CRASSSV	SVMHEALHNHYT SYIHWFQQKPGS	OKSLSLSPG SPKPWIYATSNLAS	GVPVRFSGSGSG	TSYSLTI	IRVEAE	DAATYYC	QQWTSNPP	Delete Chain TFGGG
Image: billing Nyme Name Position AA Popine 10 Worknow Gage Name Chain Chain Cysteine Cysteine 1 1-4 N-terminal Gin->pyro-Glu - Q Q Both -17.0265 1 1 1 2 8 2 1-4 Internial Gin->pyro-Glu - N NQR Peptide Mapping 0.9940 1 1 1 2 8 7 3 1-4 Internal Oxidation - n/a NVMCNYPRP Peptide Mapping 10.9940 3 1 2 2 1 1 3 3 12 2 1 1 3 2 1 3 3 1 2 1 3 3 1 2 1 3 3 1 2 2 1 3 3 1 4 2 2 2 3 1 4 2 2 3 <th></th> <th>401-450 VLDS Chain 4 Light chain 2 AA Inde 1-100 QIVL: 101-200 TKLE:</th> <th>DGSFFLYSKL xxes: SQSPAILSASE IKRTVAAPSVE</th> <th>TVDKSRWC</th> <th>CRASSSV</th> <th>SVMHEALHNHYT SYIHWFQQKPGS SVVCLLNNFYPR</th> <th>QKSLSLSPG SPKPWIYATSNLAS EAKVQWKVDNALQS</th> <th>SGVPVRFSGSGSG SGNSQESVTEQDS</th> <th>TSYSLTI: KDSTYSL:</th> <th>RVEAE STLTL</th> <th>DAATYYC SKADYEK</th> <th>QQWTSNPP</th> <th>Delete Chain TFGGG THQGL</th>		401-450 VLDS Chain 4 Light chain 2 AA Inde 1-100 QIVL: 101-200 TKLE:	DGSFFLYSKL xxes: SQSPAILSASE IKRTVAAPSVE	TVDKSRWC	CRASSSV	SVMHEALHNHYT SYIHWFQQKPGS SVVCLLNNFYPR	QKSLSLSPG SPKPWIYATSNLAS EAKVQWKVDNALQS	SGVPVRFSGSGSG SGNSQESVTEQDS	TSYSLTI: KDSTYSL:	RVEAE STLTL	DAATYYC SKADYEK	QQWTSNPP	Delete Chain TFGGG THQGL
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Navigate to the 'Peptide Mapping' tab complete processing parameters and to generate all peptide forms for matching.

SCIEX?	Rituximab					Create	Open Save S	Save As Close
Project	Assay Information	Sequence Features In	tact Protein Peptide Mapping					
Assay Information	Processing Parameters			Batch Pr	ocessing Para	neters		î
Intact Protein	m/z Tolerance, ppm:	± 5.0 ppm	RT Range Processing: Time Selection		ntion Time Toler		.50 min	
Characterize Standard	Minimum Score for Auto-	Validation: 3.0	Start RT: 0.00 min	Patch De	ocessing Pass	/ Eail Critoria		
Create Batch	MS/MS Matching Toleran	ce: 0.03 Da	Stop RT: 58.36 min		Area Limits:	± 1		
				Min	mum Sequence		5.0 %	
Review Results					uired Form Minin			
Peptide Mapping					ricted Form Max		20 %	
Characterize Standard	And the second second							
Create Batch	Annotated Protein Seq	uence						
Review Results	Chain 1 - Light Chain1	PRUMMPODACCUCYTUM	FQQKPGSSPKPWIYATSNLASG	VDVPFSCSCSC	TOVOLTTODU	PAPDAATVY	COOWTSNDDTFCCC	
Sustam	TKLEIKRTVAAPSVFI		LNNFYPREAKVQWKVDNALQSG					
System	SSPVTKSFNRGEC							
View Queue	Chain 2 - Heavy Chain 1							
Create Report	YYGGDWYFNVWGAGTT TYICNVNHKPSNTKVD NSTYRVVSVLTVLHQD VLDSDGSFFLYSKLTV	VTVSAASTKGPSVFPLAF KKAEPKSCDKTHTCPPCF	HWVKQTPGRGLEWIGAIYPGNG SSKSTSGGTAALGCLVKDYFP APELLGGPSVFLFPKPKDTLM PIEKTISKAKGQPREPQVYTLF ALHNHYTQKSLSLSPG	PVTVSWNSGAL	TSGVHTFPAV VDVSHEDPEV	LQSSGLYSL KFNWYVDGV	SSVVTVPSSSLGTO EVHNAKTKPREEQY	2
	Chain 3 - Heavy Chain 2	OTTANOOR & COMPROVING	UNUYORDODOT PRICE TYDON	DESTROY		TA VMOT POT	TOPDONIVYONDO	1
	YYGGDWYFNVWGAGTT TYICNVNHKPSNTKVD NSTYRVVSVLTVLHQD	VTVSAASTKGPSVFPLAF KKAEPKSCDKTHTCPPCF	HHWVKQTPGRGLEWIGAIYPGNG SSKSTSGGTAALGCLVKDYFP APELLGGPSVFLFPPKPKDTLM PIEKTISKAKGQPREPQVYTLF SIHMVYFOKTISPC	PVTVSWNSGAL	TSGVHTFPAV VDVSHEDPEV	LQSSGLYSL KFNWYVDGV	SSVVTVPSSSLGTC EVHNAKTKPREEQY	2
		DRSK#QQGWYESCSYIME	ADMANTIQUEDEDEG					
	Chain 4 - Light chain 2	FKVTMTCRASSSVSVIH	FQQKPGSSPKPWIYATSNLASG	VPVRFSGSGSG	TSYSLTISRV	FAFDAATYY	COOWTSNPPTFGGG	
			LNNFYPREAKVQWKVDNALQSG					
	Peptide Mapping							
	Cysteine Alkylation: Iodoa	cetamide 💙 Ma	ximum Number of Combined Moo	difications per Pep	tide 4			
	Digest Agent: Trypsi	n 💙 Ma	ximum Missed Cleavages: 4 💙					
	Peptides 🗸 Re	duced Protein Form Seque	nce coverage of 0 Matched peptides	= 0.0 %			Filter	est
	Chains Peptide A	A Index Sequence	Modifications	Disulfide Mo		Charge X	IC Area	tion 🍵
	1 1.4 T1	1-18 QIVLSQSPAILSAS		Bonds Ma	ss m/z 23.9993	charge 7	Time	
	2 1,4 T8-11		DEQLK Carbamidomethyl@26(133)		94.3570			
	3 1,4 T8-11	108-148 TVAAPSVFIFPPSI	DEQLK Deamidated@*, Oxidation@	45	53.3305		-	-
			DEQLK Carbamidomethyl@26(133)	·	93.3730		-	-
	5 1,4 T8-11 6 1,4 T8-10	and the second se	DEQLK Oxidation@* DEQLK Carbamidomethyl@26(133)		52.3465 85.0456		-	-
			DEQLK Carbamidomethyl@26(133)		70.0347			-
			DEQLK Deamidated@*, Deamidate		29.0081			- 0
	9 1,4 T8-10	108-144 TVAAPSVFIFPPS	DEQLK Carbamidomethyl@26(133)	40	84.0616		2	
			DEQLK Carbamidomethyl@26(133)		69.0507		-	-
	11 1,4 T8-10	and the second se	DEQLK Deamidated@*, Oxidation@	1.1643	28.0241		-	•
	12 1,4 T8-10 13 1,4 T8-10		DEQLK Carbamidomethyl@26(133) DEQLK Deamidated@*, Deamidate	1	54.0398 13.0132		-	
	14 1,4 T8-10		DEQLK Carbamidomethyl@26(133)					
	15 1,4 T8-10		DEQLK Oxidation@*, Oxidation@*		27.0401		-	
	16 1,4 T8-10	108-144 TVAAPSVFIFPPS	DEQLK Carbamidomethyl@26(133)	40	53.0558		-	
	17 1,4 T8-11		DEQLK Oxidation@*, Oxidation@*	110.55	68.3414		-	
	18 1,4 T8-10		DEQLK Deamidated@*, Oxidation@	1 1 2 2 2	12.0292			
	19 1,4 T8-11		DEQLK Carbamidomethyl@26(133)		09.3679			-
	20 1,4 T8-11 21 1,4 T8-12		DEQLK Carbamidomethyl@26(133) DEQLK Deamidated@*, Oxidation(95.3410 86.2763		-	
	21 1,4 10-12 22 1,4 T8-12		DEQLK Carbamidomethyl@26(133)					
	23 1,4 T8-12		DEQLK Deamidated@*, Deamidate	12.00	71.2654			
	24 1,4 T8-12	and the second sec	DEQLK Carbamidomethyl@26(133)		26.3188			-
A ? !	25 1,4 T8-12		DEQLK Oxidation@*, Oxidation@*		85.2923			
Settings Help About	26 1.4 T8-12	108-168 TVAAPSVEIEPPSI	DEOLK. Carbamidomethyl@26(133)	67	11.3079			



Navigate to the 'Settings' icon and review your global 'Peptide Mapping Settings'

BioPharmaView Settings		-		X	J
Custom Modifications Intact Protein Settings	Peptide Mapping Settings				
Peptide Mapping Settings	Maximum Charge State:	10]		
	Minimum Peptide Length:	3]		
	Peptide Deconvolution Tolerance:	10.00	ppm		
	XIC m/z Width:	0.025	Da		
	Number of TOFMS Spectra to Combine:	3	± scans		
	Recalibration:	Autom	iatic 💿 Manual		
	Number of MRM Transitions to Export:	3]		
	Chromatogram Peaks Labeling				
	Label Matching Tolerance:	0.10	Minutes		
	Display Labels For:	Auto-Vali	dated Matches 💙		
				Reset to Default	
				OK Cancel	

Data extraction, including peptide matching can be performed in minutes, on either a single datafile, or on multiple samples using the batch processing function. Review your peptide mapping results in the BioPharmaView Software window. Full sequence coverage of matched peptides can be viewed by clicking 'View Sequence'. Peptide matches can be reviewed in the 'Peptide Results' window. For each selected peptide, corresponding TOF-MS raw spectrum (lower left) and high-resolution, annotated MS/MS spectrum (lower right) are shown for easy confirmation.



- O X BioPharmaView SCIEX) × Rituximab E Close Project Characterize Standard for Peptide Mapping Assay Information 20160713-Ritu R SWATH01.wiff2 Sample # 1 💙 Experiment # 1 💙 Intact Protein Processing Parameters **RT Range Processing** Characterize Standard m/z Tolerance, ppm: ± 5.0 Automatic ppm **Create Batch** Minimum Score for Auto-Validation: 3.0 Time Selection - to - min **Review Results** MS/MS Matching Tolerance: 0.03 Da Peptide Mapping ጭ 🔅 🚺 BPC/TIC/XIC Graph BPC from 201607 13-Ritu R SWATH01.wiff2 (sample 1) - 20160713-Ritu R SWATH. Experiment 1. +SWATH TOF MS (400 - 1500) Create Batch 6e5 2,3,740 2,3.112 2,3,732 27.88 2,3.T21 1.4.T1-2*2.3.T36 1.4.T1* 2.3.T5 35.87 36.68 2,3.T24-25* 2,3.T10 **Review Results** 2,3.T4* 2,3.T19 2,3.T12* 2.3.T16 2,3.T20* 4e5 1,4.T8-12 2,3.T8 2,3.T8 14.56 16.56 1,4.T9* 1,4.T9* 30.72 2.3.T13* 1,4.T12 23.70 35.87 40.64 2e5 System E I 31.95 44.13 20.04 51.64 55.01 View Queue 0e0 52 54 14 26 28 32 34 38 40 44 Create Report Peptide Results Matched Unmatched ٥ Filter P...99.1 % View Sequ Update Assay I Theoretical Observed Error Modifications Disulfide Bonds Charge XIC Area RT Sequence Score Mono m/z Mono m/z (PPM) 178 20.02 VDNALOSGNSOESVTEODSK 534,7476 534,7466 3.5976 179 -2.0 5.445 4 448.2779 0.718 180 19.48 TISK 448.2766 3.0 1 2.5574 ቤ 🔅 ቤ 🔅 🗖 TOF MS MS/MS Graph Graph Fragments TOF MS (400 from 20160713-Ri. +MS/MS (50 - 1500) from 20160713-Ritu_R_SWA 100% 1 +SWATH Mono m/z: 712.6587 from 19.90 to 20.15 m min, (699 - 725 Da. 100% *712.6587 (3) 712.9933 (3) 707.3192 (3) 707.6563 (3) *708.3221 (1) 713.3268 (3) tensity tensity 1-b. *72.0808 (1) 1-0₄ 1-0₆ 400.1821641.3242 50% 50% 1-y₇ 806.3872 713.6621 (3) 713.9970 (3) 1022.4606 *1312. 1024.4684 (1) *1312.5730 (1) 1.L u. 0% 蓉 ? L 712 714 717 200 400 600 1000 1200 716 Settings Help About m/z. Da m/z. Da All Matched Peptides Auto-Validated Used for IDs Selected Peptides Chain 1 - Light Chain1 Sequence Coverage 100.0 % OIVLSOSPAILSASPGEKVINTCRASSSVSYIHNFOOKPGSSPKPMIYATSNLASGVPVRFSGSGSGTSYSLTISRVEAE DAATYICOOMTSNPPIFGGGTKLEIRRTVAAFSVFIFPSDDOLKSGTASVVCLLINFYPREAKVOMKVINALOSGNSOE SYTEODSKDFISLSSITLISKADIFERNIVAGEVTHGGLSSVTHSSINGEG Chain 2 - Heavy Chain 1 Sequence Coverage 98.7 % niani - Heny Chani I Jegueire Confege John // VOILOOPAELUKKAGASVIMSCKASCYTTEYNMHWYKOTPGRGLENIGALYPGNGDTSYNOKFKGKATLTADKSSSTAY MLISLISEDSAVYYCARSTYYCGHVIFNWIGAGTTYTVSAASTKOPSVFILAPSSKATGGTAALGCLVKDVFPEPUYT SMNSGALTSGVHIFPAVLOSSCI SVLISSVTVTVSSSLGGTYI TONNKENSMTKOKKARPKSCHKTIFCPCPAPELU Gesvfilpppkphtplikistrevitovusheddevkinnyvtogvenkaktkpregonistikuvsvijouduko KYKCKVSNALADEIENTISKACOPREGOVTIDPSRDLINVSSLCJUKGYPSOLAVENSOCPENNIKTP VLDSDGSFFLYSKLTVDKSRWOOGNVFSCSVMHEALHNHYTOKSLSLSPG Chain 3 - Heavy Chain 2 Sequence Coverage 98.7 % JAN 3 - ORANY (JAM 2 - SAQUAROE COVERIGE 98.7 % OUI_OOPCAELDE JAN 2 - SAQUAROE COVERIGE 98.7 % MOLSSIJEEDBAVYYCARSTYNGGRYTFWINGAGTTYTVSAASTKOFSVEPLAFSKRESGOTAALCCIVEDYFEPEVYV SMISGALISGUSTFEPAVLGSGISTJELSSVYVT9SSIJGOYI I GVONHREDBIYDVAHKEDSHIVDVAHKABFKSCHAFHTOFPEAPELIG GFSVELFPEVRKNTIMISKTEVTC-VVVDHEDPEVRFWYVDGVEVHAKHKPREEQYNSTXRVVSVLFVLHODMLMG KEYKCKVSNKAKLAPEIRETIKFKACOPREPOVTHEBSRDILTNOVSIJCIVKGPYPSDIAVEWESMGOPENNYKTPP VLDSDGSFFLYSKLTVDKSRWQGNVFSCSVHHEALHNHYTQKSLSLSPG Chain 4 - Light chain 2 Sequence Coverage 100.0 % OIVLSOSPAILSASPGEKVTWTCRASSSVSYIHWFQOKPGSSPKPWIYATSNLASGVPVRPSGSGSGTEYSLAISRVEAE DAATYYCQOWTSNPPTFGGGTKLEIKRTVAAPSVFIPPSDEOLKSGTASVVCLLNNFYPREAKVOWKVDNALQSGNSOE SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHGGLSSPVTKSPNRGEC Displaying 106 unique peptides

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