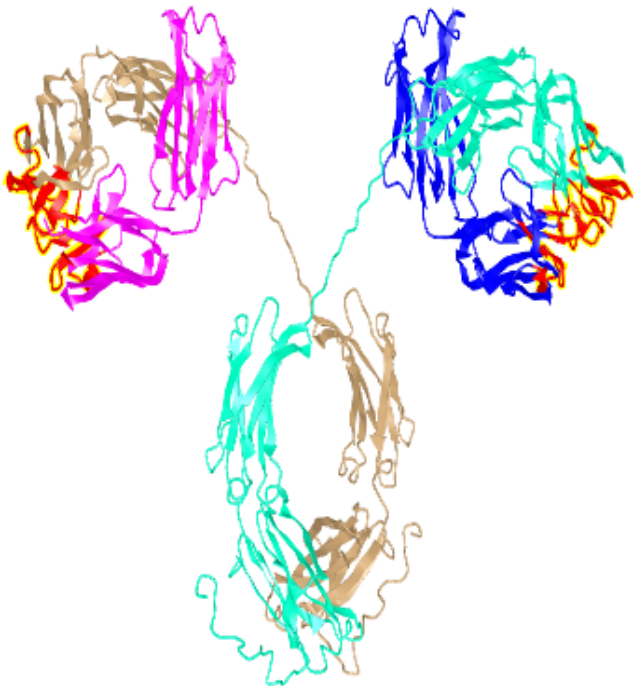
PEMBROLIZUMAB METHOD VALIDATION IN HUMAN SERUM: COMPARISON OF TRIPLE QUADRUPOLE AND HIGH RESOLUTION INSTRUMENT.

23/11/2019/EBF-Barcelona

Jordane BIARC

charles river

AIM OF THE PROJECT



1. Develop a generic method for the quantification of therapeutic antibodies by mass spectrometry in human matrices.
2. The study has been performed with one therapeutic antibody, Pembrolizumab, an anti-PD1 compound.
3. Performance of several commercial tools / automated platforms have been tested including digestion and capture kits and are compared to in-house protocols.
4. Further validation has been performed in human serum on a Sciex API 6500+.
5. Method performance has been evaluated on a High Resolution instrument Triple TOF (Sciex).

DEVELOPMENT

PROTOCOL OVERVIEW

Antibody: Pembrolizumab

No Affinity capture	CR-STN	Proteinworks®	Smart digest®
Sample volume	50 µL	50 µL	50 µL
Matrix	Human plasma	Human plasma	Human plasma
Digestion	Denaturation/reduction/alkylation/digestion	Denaturation/reduction/alkylation/digestion	Denaturation/digestion
Peptide cleanup (SPE)	HLB®	HLB®	HLB®

Affinity capture protein A/L	CR-STN	IA-SmartDigest®	Assay MAP Bravo®
Sample volume	10 µL	10 µL	10 µL
Matrix	Human plasma	Human plasma	Human plasma
Digestion	Reduction/alkylation/digestion	Denaturation/digestion	Reduction/alkylation/digestion
Peptide cleanup (SPE)	HLB®	HLB®	HLB®/C18®

PROTOCOL RESULTS

Human results-Pembrolizumab-1-200 µg/mL (No capture) or 0.25-200 µg/mL (capture).

Protocols	**No capture**			**With capture**	
Parameters	CR-STN	**Proteinworks®**	**SmartDigest®**	CR-STN	**AssayMAPbravo®**
Protocol	No enrichment	No enrichment	No enrichment	Protein L	Protein A
LLOQ (µg/mL)	1	1	1	0.25	0.25
Volume Human plasma	50 µL	50 µL	50 µL	10 µL	10 µL
Preparation Time	1 day	1 day	0.5 day	1 day	1 day
Samples/day	~100 (15 min run)	~48 (30 min run)	~100 (15 min run)	100 (15 min run)	~100 (15 min run)
Additional cost	4 €/sample	8 €/sample	8 €/sample	8 €/sample	13 €/sample

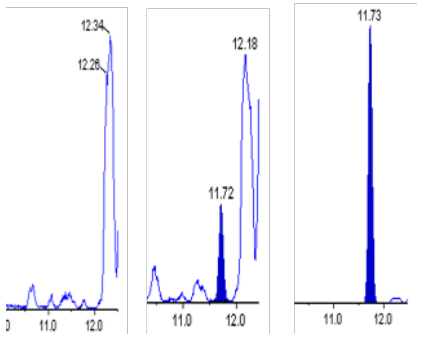
PROTOCOL RESULTS

Human results-Pembrolizumab-1-200 µg/mL (No capture) or 0.25-200 µg/mL (capture).

No capture

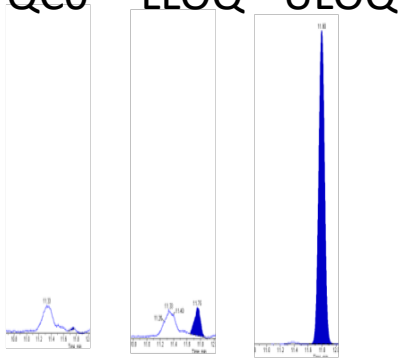
CR-STN

QC0 LLOQ ULOQ



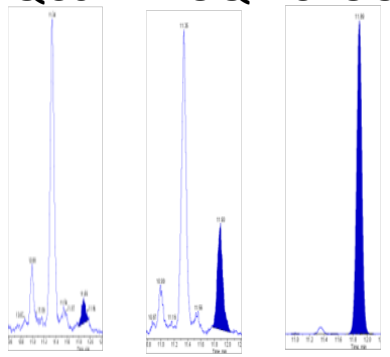
Proteinworks®

QC0 LLOQ ULOQ



SmartDigest®

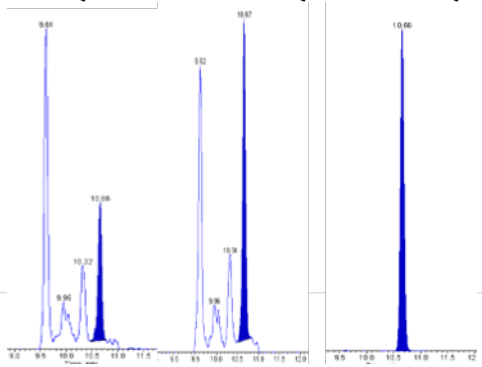
QC0 LLOQ ULOQ



With capture

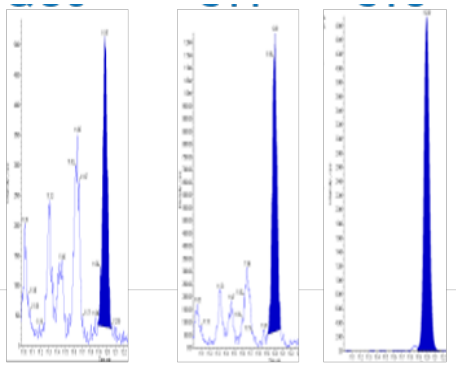
CR-STN (Protein L)

QC0 LLOQ ULOQ



Assay MAP bravo® (Protein A)

QC0 LLOQ ULOQ



charles river

CONCLUSIONS

Evaluation of several protocols for quantification of anti-PD1 antibodies.

No capture	Antibody	Atlanbio	Waters	Thermo	
No capture (LLOQ-Human)	Pembrolizumab	1 ug/mL	1 ug/mL	1 ug/mL	
	Nivolumab	5-10 ug/mL			
Capture	**Antibody**	**Atlanbio**		**Thermo**	**Agilent**
Capture (LLOQ-Human)	Pembrolizumab	>1 ug/Ml (A) 1 µg/mL (L)			1 ug/mL
	Nivolumab				2-3 ug/mL

Protocols without capture:

- Generic for all antibodies and matrices (total measurement without ADA influence).
- Allows good performance and not expensive. Its is a good solution if you have enough sample.

Protocols with capture:

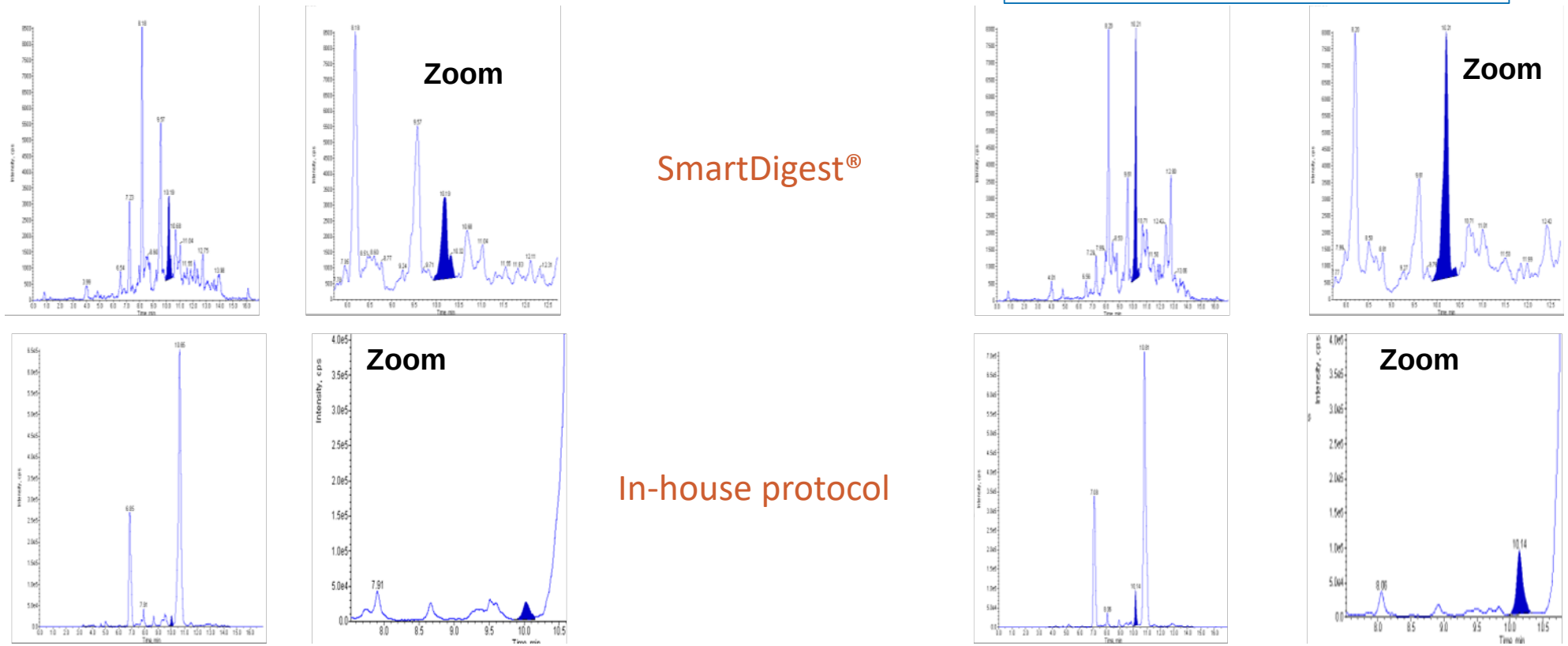
- Generic for all antibodies and all matrices (total measurement with possible ADA influence).
- Good performance with high capacity tips+automation and capture with protein L).
- Improvement of sensitivity (especially with low sensitive peptides) but in our specific case it increases of non selectivity.

DIGESTION AND SELECTIVITY

Comparison in-house and Smartdigest® protocol in individual matrix

QC00-INDIVIDUAL MATRIX

QC LLOQ-INDIVIDUAL MATRIX



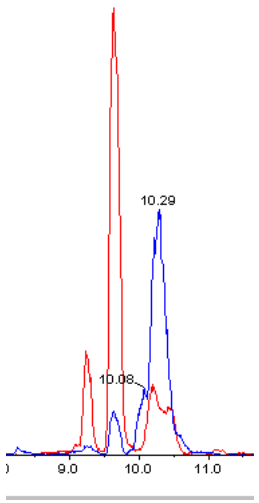
The choice of digestion protocol is of utmost importance for selectivity and sensitivity. Even though SmartDigest® preparation time is shorter, background noise was more important for this antibody and in-house method was chosen for full validation.

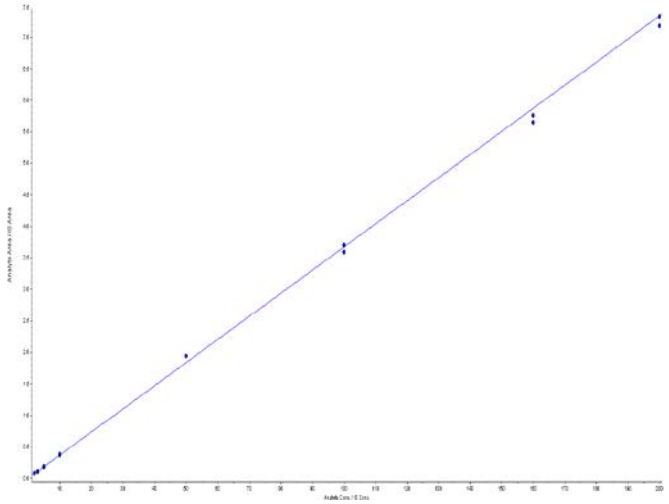
charles river

VALIDATION: TRIPLE QUADRIPOLE

PERFORMANCE AND SELECTIVITY

Calibration curve 2 to 200 µg/mL in human serum- Anova on LIMS Watson 7.6 (Internal standard: labelled surrogate peptide)





Nominal Conc.	QCLLOQ 1.00 µg/mL	QCLLOQ 2.00 µg/mL	QCLOW 3.00 µg/mL	QCLOW 6.00 µg/mL	QCMED 100 µg/mL	QCHIGH 160 µg/mL
Mean Observed Conc.	0.947	2.13	2.82	6.35	99.3	160
%DEV	-5.30	6.50	-6.00	5.83	-0.70	0.00
Between Run Precision (%CV)	0.00	N/A	6.62	N/A	5.44	6.25
Within Run Precision (%CV)	5.97	N/A	3.56	N/A	4.60	4.28
Total Variation (%CV)	5.54	N/A	7.51	N/A	7.12	7.57
n	18	6	18	6	24	24
Number of Runs	3	1	3	1	4	4

Selectivity (LLOQ 1 µg/mL)

H serum Batch	QC0/0/QC LLOQ	Results
Matrix 1	5.45%	Pass
Matrix 2	8.75%	Pass
Matrix 3	28.21%	Failed
Matrix 4	17.27%	Pass
Matrix 5	37.92%	Failed
Matrix 6	30.34%	Failed

Selectivity (LLOQ 2 µg/mL)

H serum Batch	QC0/0/QC LLOQ	Results	QC0/0/QCLLOQ IS (%)	Results
Matrix 1	3.52%	Pass	0.07%	Pass
Matrix 2	3.39%	Pass	0.12%	Pass
Matrix 3	4.83%	Pass	0.09%	Pass
Matrix 4	4.06%	Pass	0.12%	Pass
Matrix 5	7.16%	Pass	0.13%	Pass
Matrix 6	3.12%	Pass	0.12%	Pass

charles river

STABILITY

Stability short term

Hour	SIM1 RT 3 µg/mL	%DEV	Mean (%DEV)	SIM2 RT 160 µg/mL	%DEV	Mean (%DEV)
24	3.23	7.67	2.78 (-7.33)	160	0.00	159 (-0.63)
24	2.63	-12.33		160	0.00	
24	2.62	-12.67		154	-3.75	
24	2.88	-4.00		164	2.50	
24	2.61	-13.00		163	1.88	
24	2.69	-10.33		154	-3.75	

Stability of extracted samples

Hours	SOE01 3.00 µg/mL	%DEV	Mean (%DEV)	SOE02 160 µg/mL	%DEV	Mean (%DEV)
24	3.07	2.33	3.01 (0.33)	164	2.50	170 (6.25)
24	2.86	-4.67		166	3.75	
24	3.03	1.00		167	4.38	
24	3.13	4.33		181	13.13	
24	3.02	0.67		170	6.25	
24	2.97	-1.00		169	5.63	

Freeze/thaw

Cycles	FTS1 3C -20 3.00 µg/mL	%DEV	Mean (%DEV)	FTS1 3C -70 3.00 µg/mL	%DEV	Mean (%DEV)	FTS2 3C -20 160 µg/mL	%DEV	Mean (%DEV)	FTS2 3C -70 160 µg/mL	%DEV	Mean (%DEV)
3	3.13	4.33	3.18 (6.00)	3.23	7.67	3.13 (4.33)	183	14.38	169 (5.63)	154	-3.75	180 (12.50)
3	3.17	5.67		3.40	13.33		162	1.25		177	10.63	
3	3.30	10.00		3.19	6.33		157	-1.88		195	21.88	
3	3.10	3.33		3.02	0.67		171	6.88		188	17.50	
3	3.09	3.00		2.87	-4.33		173	8.13		185	15.63	
3	3.29	9.67		3.09	3.00		167	4.38		181	13.13	

RECOVERY/MATRIX EFFECT

Recovery

H serum 6203 Batch	REF01 6µg/ml	REC01 6µg/ml	REF02 100µg/ml	REC02 100µg/ml	REF03 160µg/ml	REC03 160µg/ml
	Peak area ratio Pembrolizumab surrogate peptide / IS					
Mean	0.1763	0.1692	2.8010	2.8543	4.3980	4.5701
SD	0.0016	0.0078	0.0579	0.1333	0.0473	0.0993
%CV	0.93	4.59	2.07	4.67	1.07	2.17
n	3	3	3	3	3	3
Extraction recovery (%)	95.97		101.91		103.91	
%Diff. between lowest and highest			7.94			

Matrix effect

H Serum Batch	IS normalized matrix factor 6µg/ml	IS normalized matrix factor 160µg/ml
	Peak area ratio Pembrolizumab surrogate peptide / IS	
Mean	1.26	1.55
SD	0.01	0.02
%CV	1.10	1.49
n	8	8

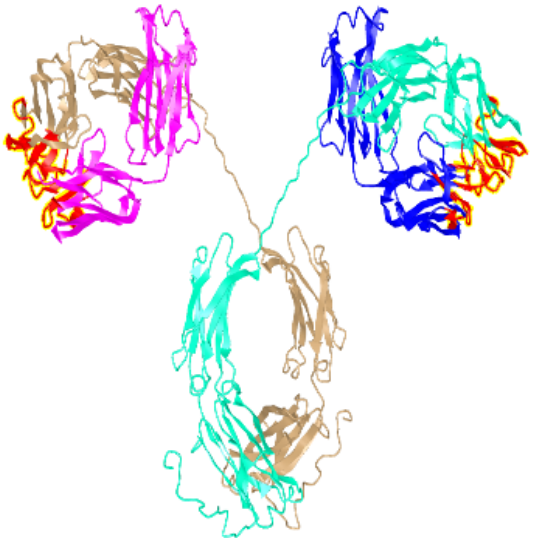
PERFORMANCE ON HIGH RES. INSTRUMENT

HIGH RESOLUTION INSTRUMENT: TRIPLE-TOF 6600 (SCIEX)

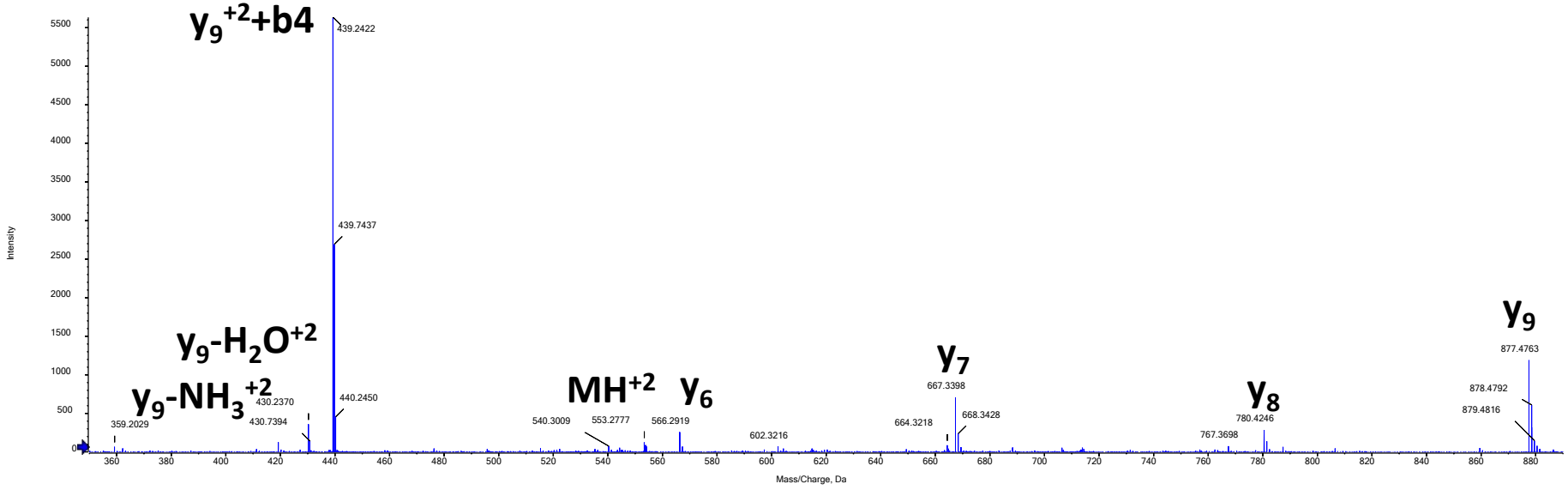
- Acquisition of a Triple-TOF 6600 from Sciex.
- Will support Toxicokinetic (TK) / Pharmacokinetic (PK) evaluation of both small and large molecules.
- Comparison of method performance Triple Quadripole 6500+/ Triple-TOF on the pembrolizumab method (same calibration curve)
- Focus on main benefits and disadvantages known (Type of experiments, sensitivity, dynamic range, selectivity, robustness, softwares and data processing).

TOF-MS SPECTRA OF SURROGATE PEPTIDE

Sequence confirmation



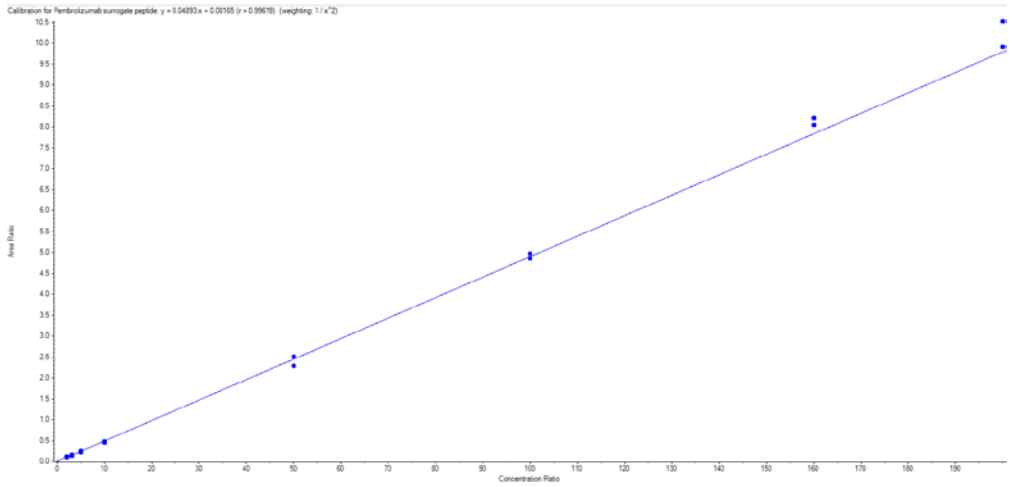
Peptide surrogate choice into CDR region



charles river

METHOD PERFORMANCE

Calibration curve 2 to 200 µg/mL in human serum- Anova on LIMS Watson 7.6



Nominal Conc.	QCLLOQ 2.00 µg/mL	QCLOW 6.00 µg/mL	QCMED 100 µg/mL	QCHIGH 160 µg/mL
Mean Observed Conc.	2.07	5.70	97.9	159
%DEV	3.50	-5.00	-2.10	-0.63
Between Run Precision (%CV)	3.72	0.77	3.90	2.89
Within Run Precision (%CV)	2.23	1.34	3.57	4.30
Total Variation (%CV)	4.34	1.54	5.29	5.18
n	18	18	18	18
Number of Runs	3	3	3	3

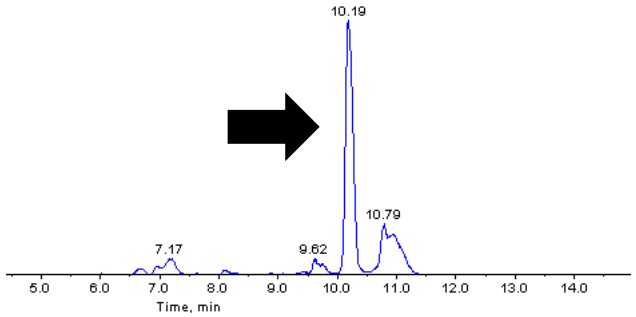
Performance very similar to triple-quadripole with possibility to keep same dynamic range

charles river

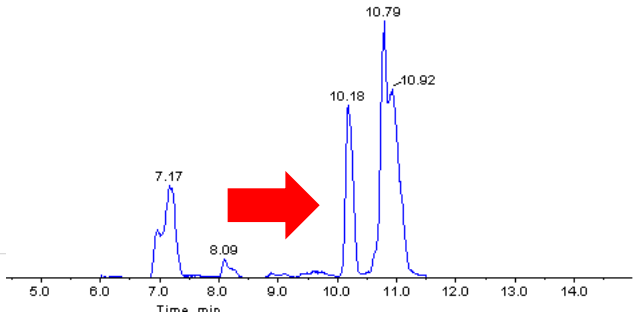
CHROMATOGRAPHY

Triple-Quadripole 6500+

TIC (surrogate peptide+IS)

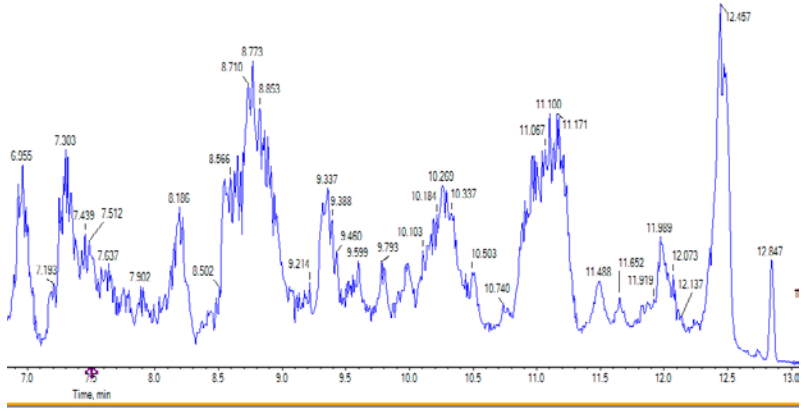


XIC surrogate peptide

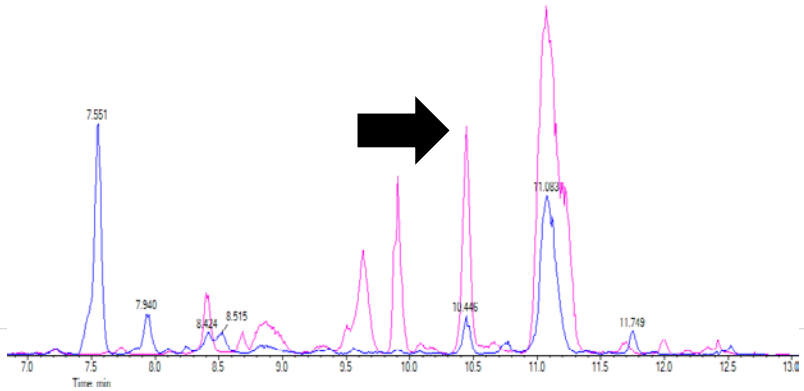


Triple-TOF 6600

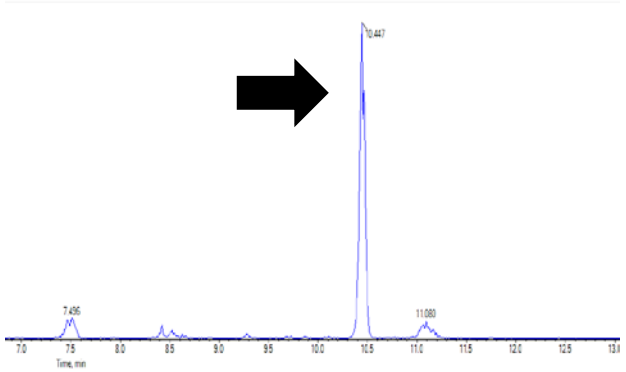
TIC TOF-MS+product ion (surrogate peptide+IS)

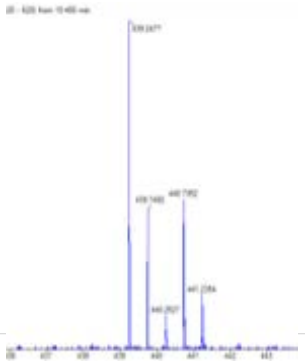


TIC product ion (surrogate peptide+IS)



XIC product ion (surrogate peptide)



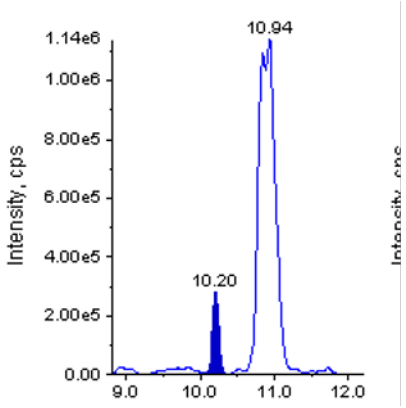


charles river

SENSITIVITY/SELECTIVITY

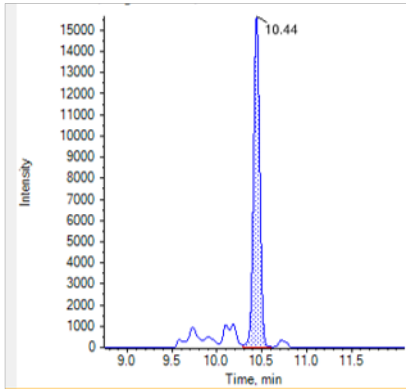
Triple-Quadripole 6500+

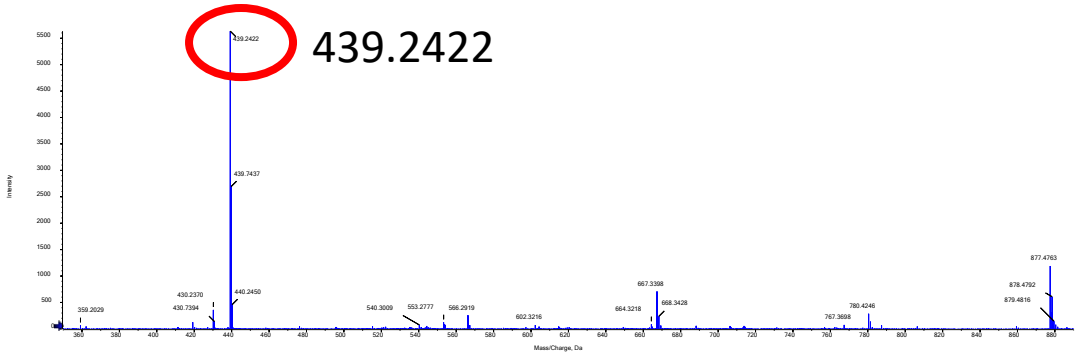
S/N=12 (6500+)

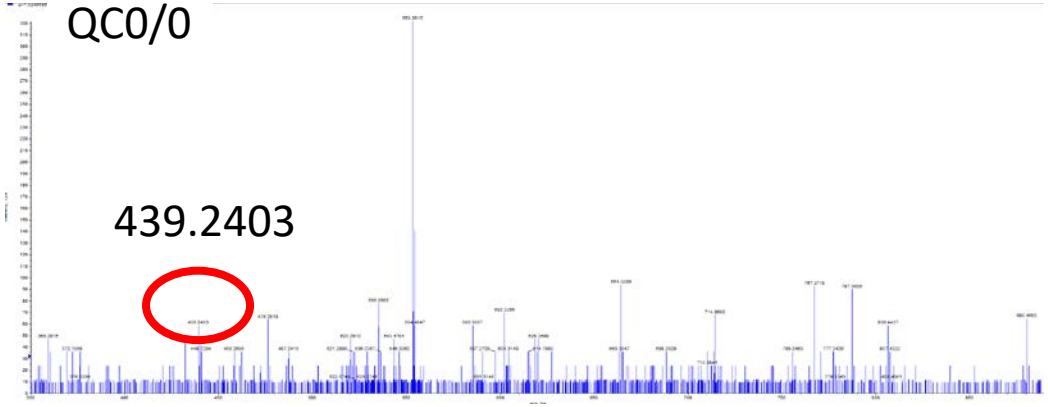


Triple-TOF 6600

S/N=12 (Triple-TOF)







Selectivity (LLOQ 1 µg/mL)

H serum Batch	QC0/0/QC LLOQ-Triple-quadripole	QC0/0/QC LLOQ-Triple-TOF
Matrix 1	5.45%	9.0%
Matrix 2	8.75%	2.6%
Matrix 3	28.21%	4.2%
Matrix 4	17.27%	9.2%
Matrix 5	37.92%	32.1%
Matrix 6	30.34%	6.3%

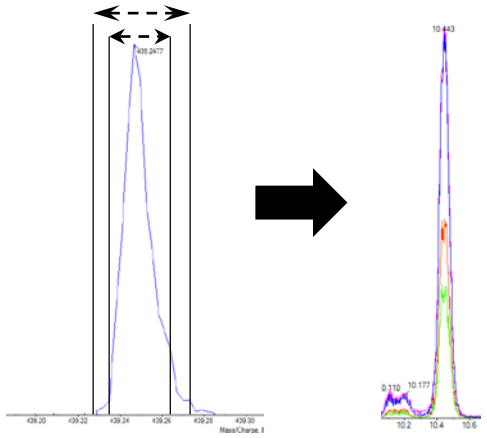
High resolution mass precision improves selectivity

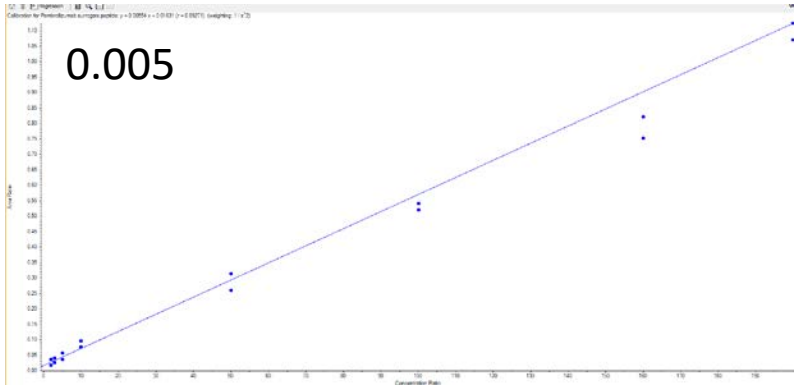
charles river

DATA PROCESSING

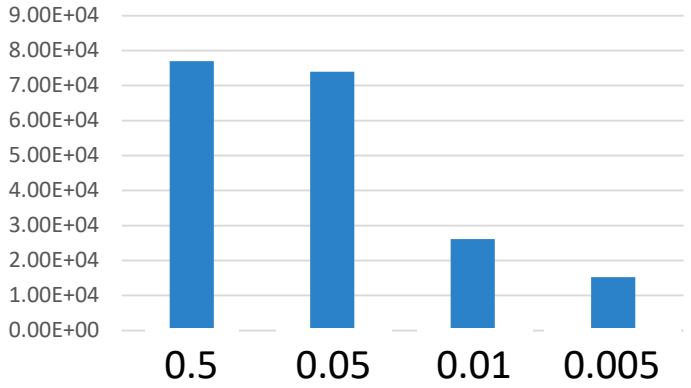
Extraction windows

windows





Peak areas STD1



Accuracy calibration curves

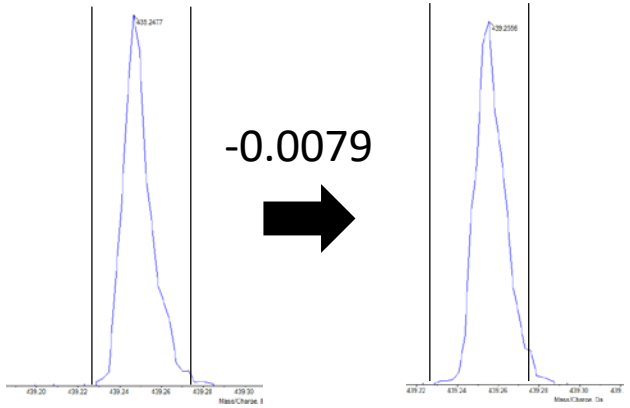
	Extraction windows			
	0.5	0.05	0.01	0.005
STD1	94.14	95.15	**148.56**	**178.47**
STD2	94.79	94.10	**127.41**	**141.24**
STD3	94.49	92.71	**135.97**	**144.06**
STD4	93.35	92.50	**139.37**	**143.50**
STD5	95.23	94.96	108.53	107.36
STD6	99.94	99.93	96.11	94.72
STD7	101.56	102.14	89.2	90.83
STD8	101.70	101.88	98.22	100.05

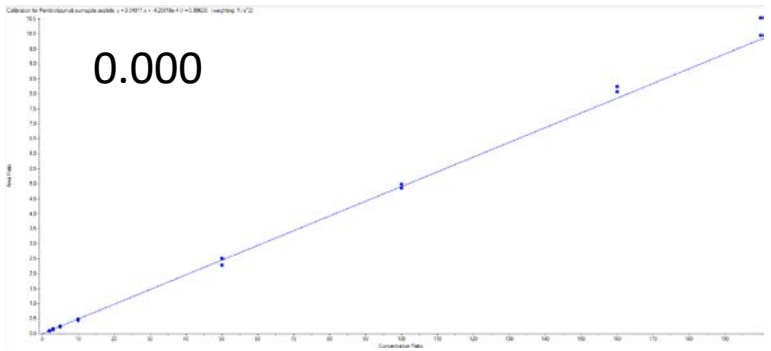
The choice of extraction windows influences method performance.

charles river

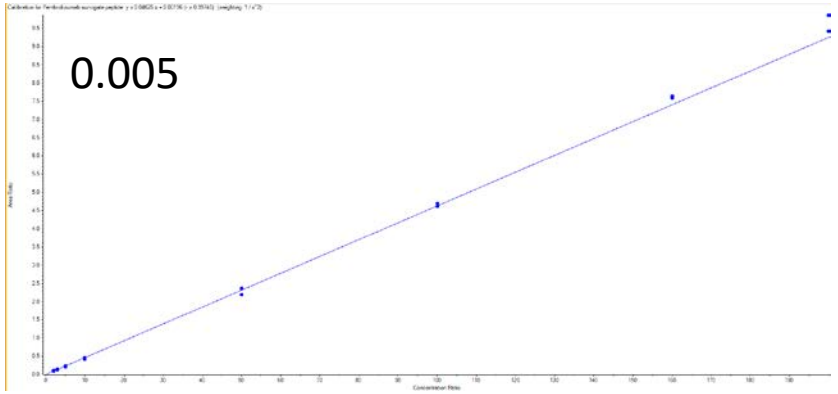
DATA PROCESSING

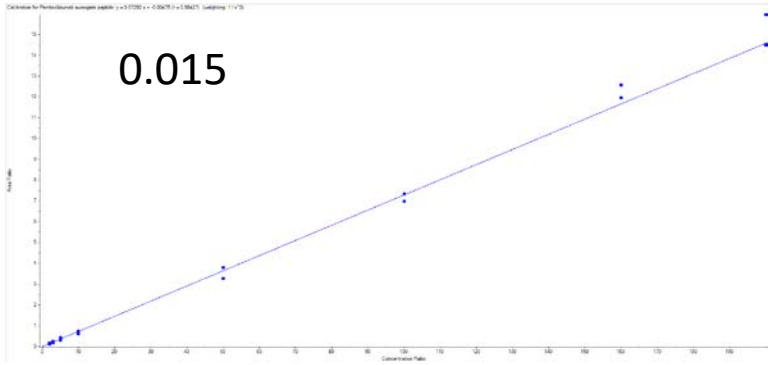
Instrument calibration





Calibration error				
	0.000	0.005	0.01	0.015
STD1	95.15	94.40	92.55	**76.65**
STD2	94.10	93.30	91.79	**82.61**
STD3	92.71	91.84	90.41	**82.59**
STD4	92.50	91.40	89.83	**84.29**
STD5	94.96	94.38	93.48	89.87
STD6	99.93	99.68	99.17	95.76
STD7	102.14	102.52	102.78	102.57
STD8	101.88	101.81	101.32	99.40





The calibration of the instrument influences method performance.

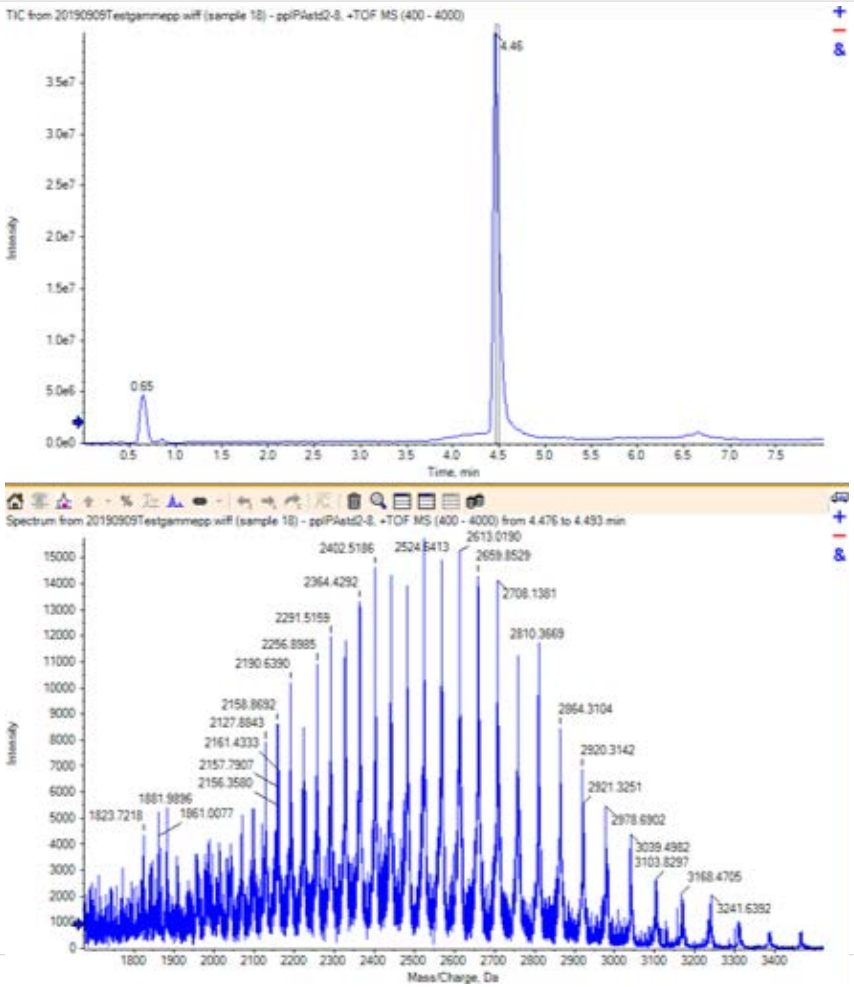
charles river

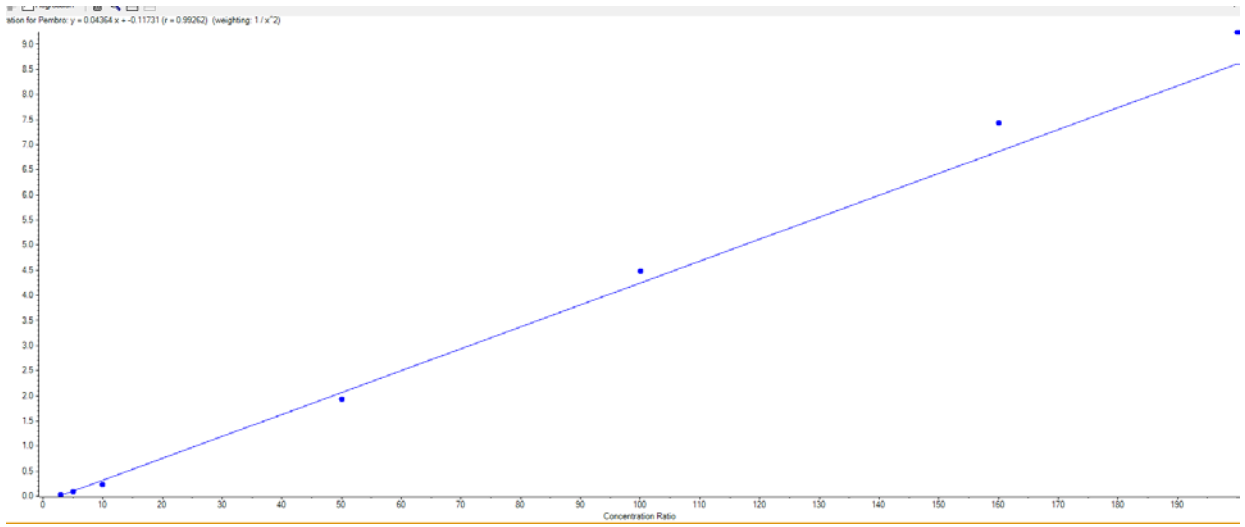
CONCLUSIONS

1. DEVELOPMENT AND VALIDATION OF PEMBROLIZUMAB IN HUMAN SERUM ON TRIPLE-QUADRIPOLE
2. SAME METHOD ON TRIPLE-TOF SHOWED SIMILAR SENSITIVITY AND IMPROVED SELECTIVITY MOST OF THE TIME
3. HIGH RESOLUTION INSTRUMENT ALLOWS RAPID CONFIRMATION OF PEPTIDE SEQUENCE AND INTERFERENCES
4. HIGH RESOLUTION INSTRUMENT NEEDS PERFECT CALIBRATION AND DATA PROCESSING CARE

NEXT STEPS: INTACT MODE

Calibration curve 3 to 200 µg/mL in solvent





charles river

ACKNOWLEDGMENTS

CHARLES RIVER-
SAINT NAZAIRE

Philippe Couerbe

Mark Warren

Benedicte Picot

Christelle Maccaigne

Ronan Pommereuil

Serge Desmoulin

Shuai Wu

Alexander Schwahn

Yanaelle Le Pan

Xavier Rodriguez

Valerie Guilloteau

Denise Scherb

Kerstin Pohl

Yannick Thiriet

THE SOLUTION FOR SMALL & LARGE MOLECULES