Supporting information

Development of the validated LCMS bioanalysis of Trastuzumab in human plasma using selective detection method for complementarity-determining regions of monoclonal antibody: nano-surface and molecular-orientation limited (nSMOL) proteolysis

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Signature peptide prediction

The candidates of signature peptides for Trastuzumab quantitation were predicted by the ClustalW multiple alignment of 4 mAbs (Trastuzumab, Mogamulizumab, Bevacizumab, and Nivolumab) amino acid sequences. Consideration of the frequency of amino acid substitution, tryptic peptides with cyeteine or methionine, peptide amino acid number, the position of conserved cysteine residue for disulfide bonding, and the insertion or deletion of amino acid, we have selected 4 peptides of the CDR containing region for Trastuzumab quantitation. The optimized MRM transition are shown in Table S1.

Table S1. MRM conditions of predicted signature peptides.

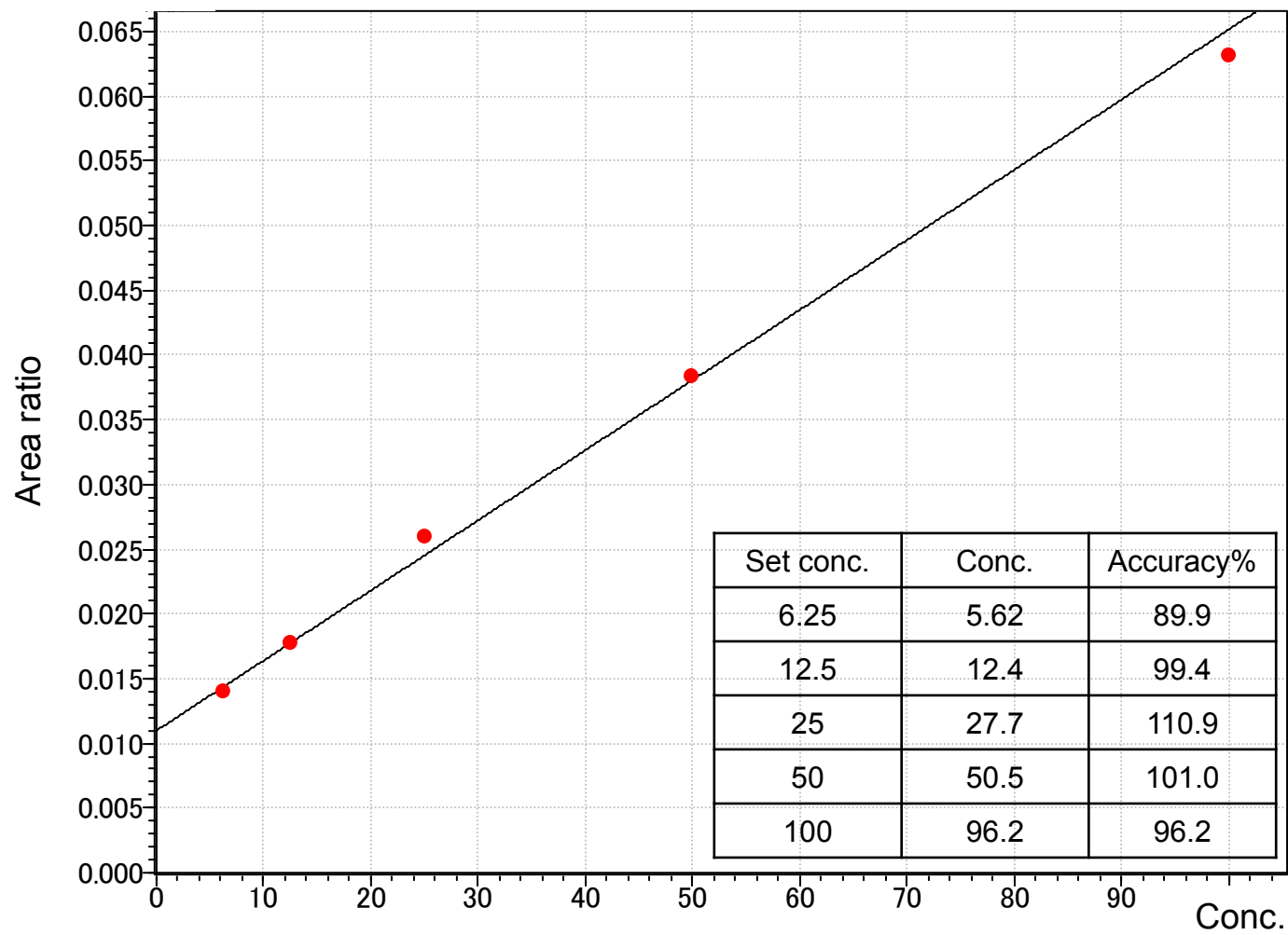| Selected peptide	Region	Optimal MRM condition				Role
		Transition mass filter [m/z]	Q1 [V]	Collision [V]	Q3 [V]	
DTYIHWVR	H-chain of CDR1	363.9→299.2 (y4 ++)	-15	-17	-24	Quantitation
		363.9→460.3 (y3 +)	-27	-17	-22	Structure
		363.9→597.3 (y4 +)	-14	-16	-20	Structure
GLEWVAR	H-chain of CDR2	415.7→660.3 (y5 +)	-16	-15	-34	Quantitation
		415.7→531.3 (y4 +)	-16	-16	-40	Structure
		415.7→345.2 (y3 +)	-16	-20	-24	Structure
FTISADTSK	H-chain of CDR2	485.15→721.3 (y7 +)	-19	-18	-38	Quantitation
		485.15→608.3 (y6 +)	-19	-20	-32	Structure
		485.15→521.2 (y5 +)	-19	-21	-26	Structure
IYPTNGYTR	H-chain of CDR2	542.8→404.7 (y7 ++)	-20	-18	-30	Quantitation
		542.8→808.4 (y7 +)	-20	-18	-28	Structure
		542.8→610.3 (y5 +)	-20	-25	-22	Structure

Interference confirmation of Trastuzumab peptide in human plasma

The predicted 4 signature peptides were analyzed by 2-fold serial dilution from 3.13 to 100 μg/ml in plasma for the interference confirmation of Trastuzumab peptides from human plasma matrix. Each calibration curve is shown in Figure S2. The calibration curve of a) DTYIHWVR (m/z 363.9→299.2), and c) FTISADTSK (m/z 485.15→721.3) have fit to linear regression, however low concentration range of Trastuzumab was interfered by plasma matrix. The curve of b) GLEWVAR (m/z 415.7→660.3) could not fit on linear regression. Peptide of d) IYPTNGYTR (m/z 542.8→404.7) was good correlation with the Trastuzumab concentration.

Figure S1. Calibration curves of Trastuzumab in plasma by MRM quantitation of the 4 signature peptides of a) DTYIHWVR (m/z 363.9→299.2), b) GLEWVAR (m/z 415.7→660.3), c) FTISADTSK (m/z 485.15→721.3), and d) IYPTNGYTR (m/z 542.8→404.7). Each range of calibration curve was shown from the low concentration level on linear regression fit within 100 ± 15% accuracy to 100 μg/ml. In the each table, back-calculated concentration and accuracy% were described.

Figure S1

a) DTYIHWVR

Set conc.	Conc.	Accuracy%
6.25	5.62	89.9
12.5	12.4	99.4
25	27.7	110.9
50	50.5	101.0
100	96.2	96.2

b) GLEWVAR

c) FTISADTSK

Set conc.	Conc.	Accuracy%
6.25	6.06	96.9
12.5	13.3	106.1
25	23.2	92.9
50	52.9	105.8
100	98.4	98.4

d) IYPTNGYTR

Set conc.	Conc.	Accuracy%
3.125	3.16	101.0
6.25	6.24	99.8
12.5	12.7	102.0
25	22.8	91.3
50	52.8	105.6
100	103	102.8
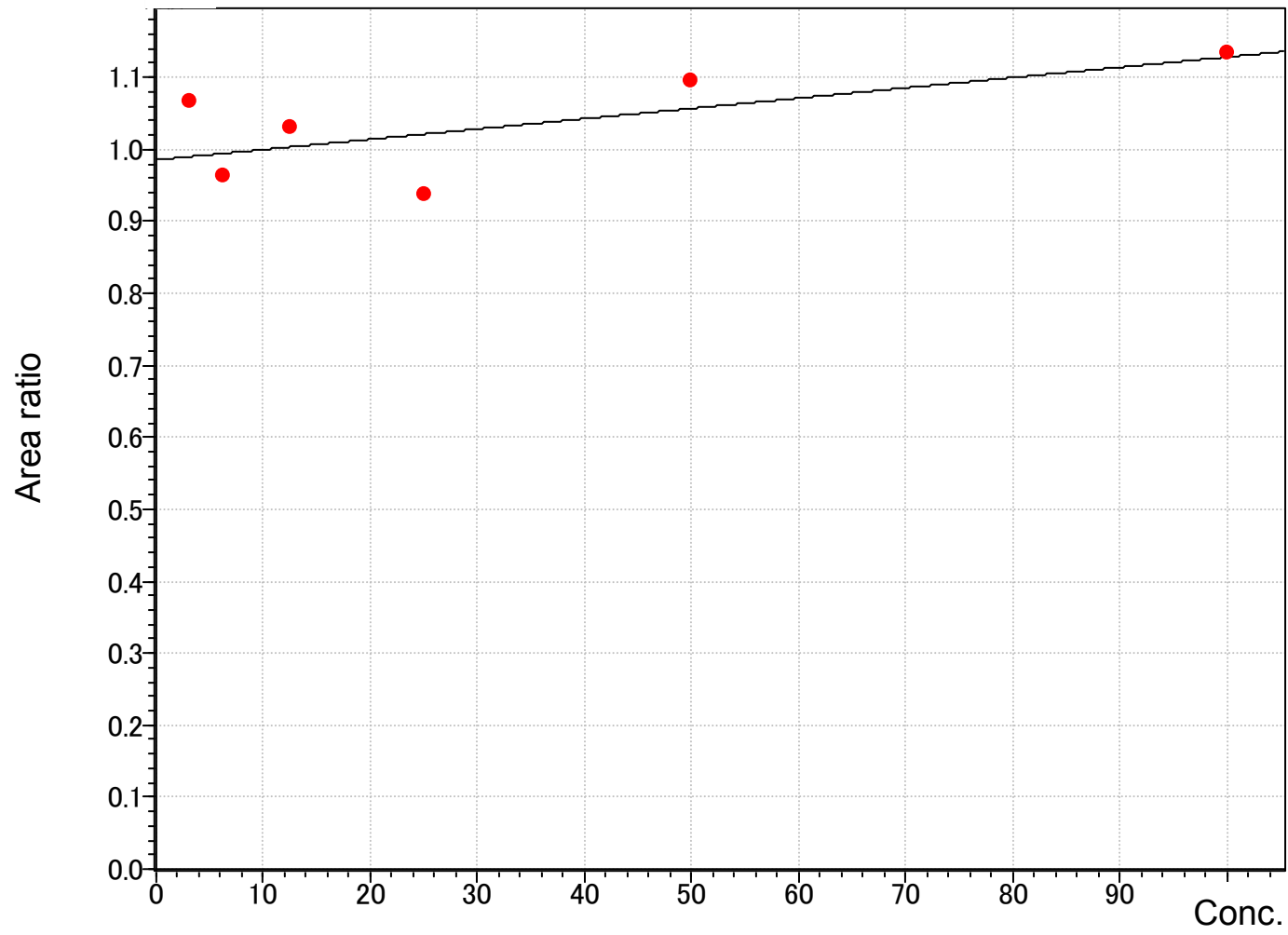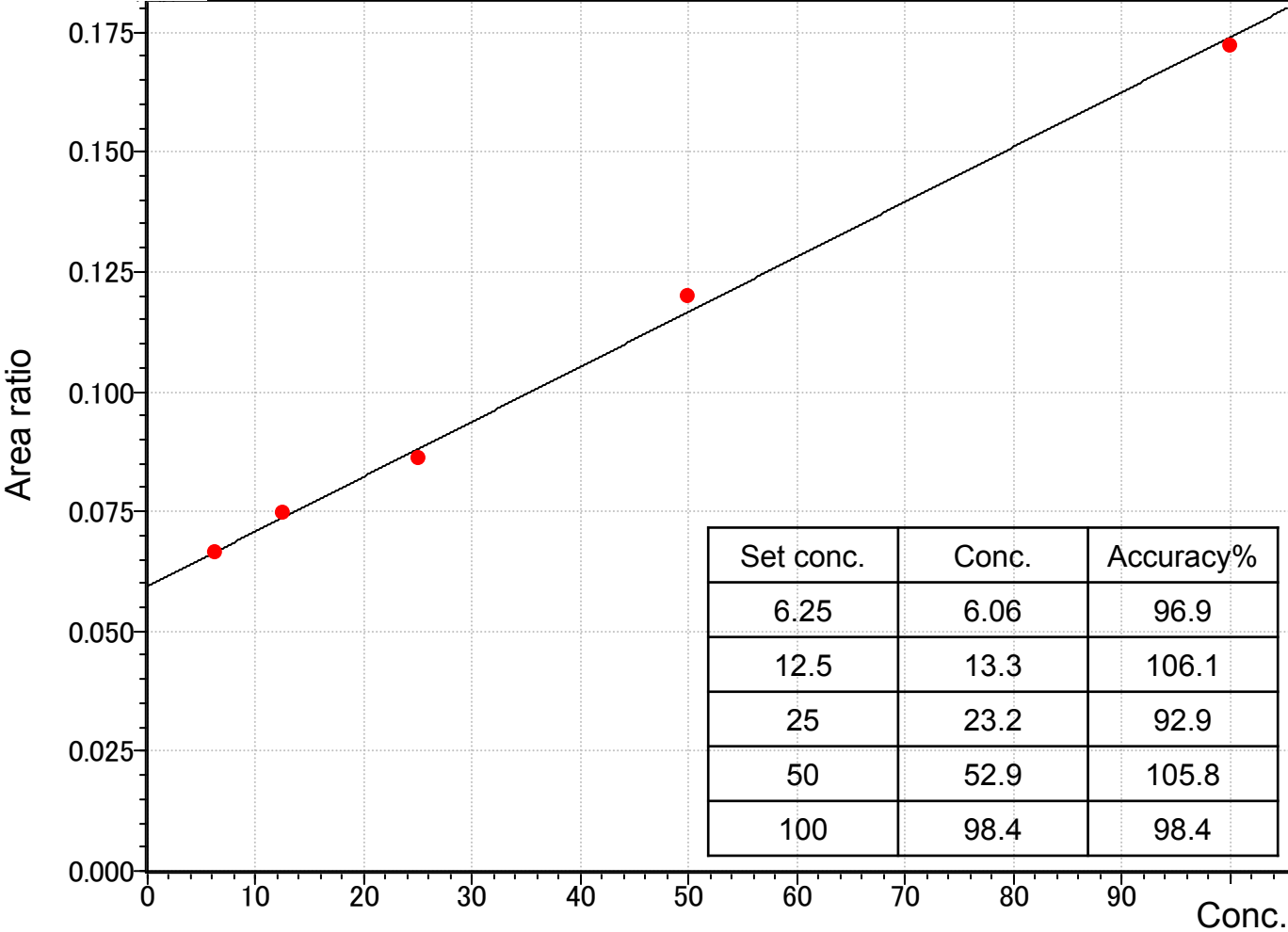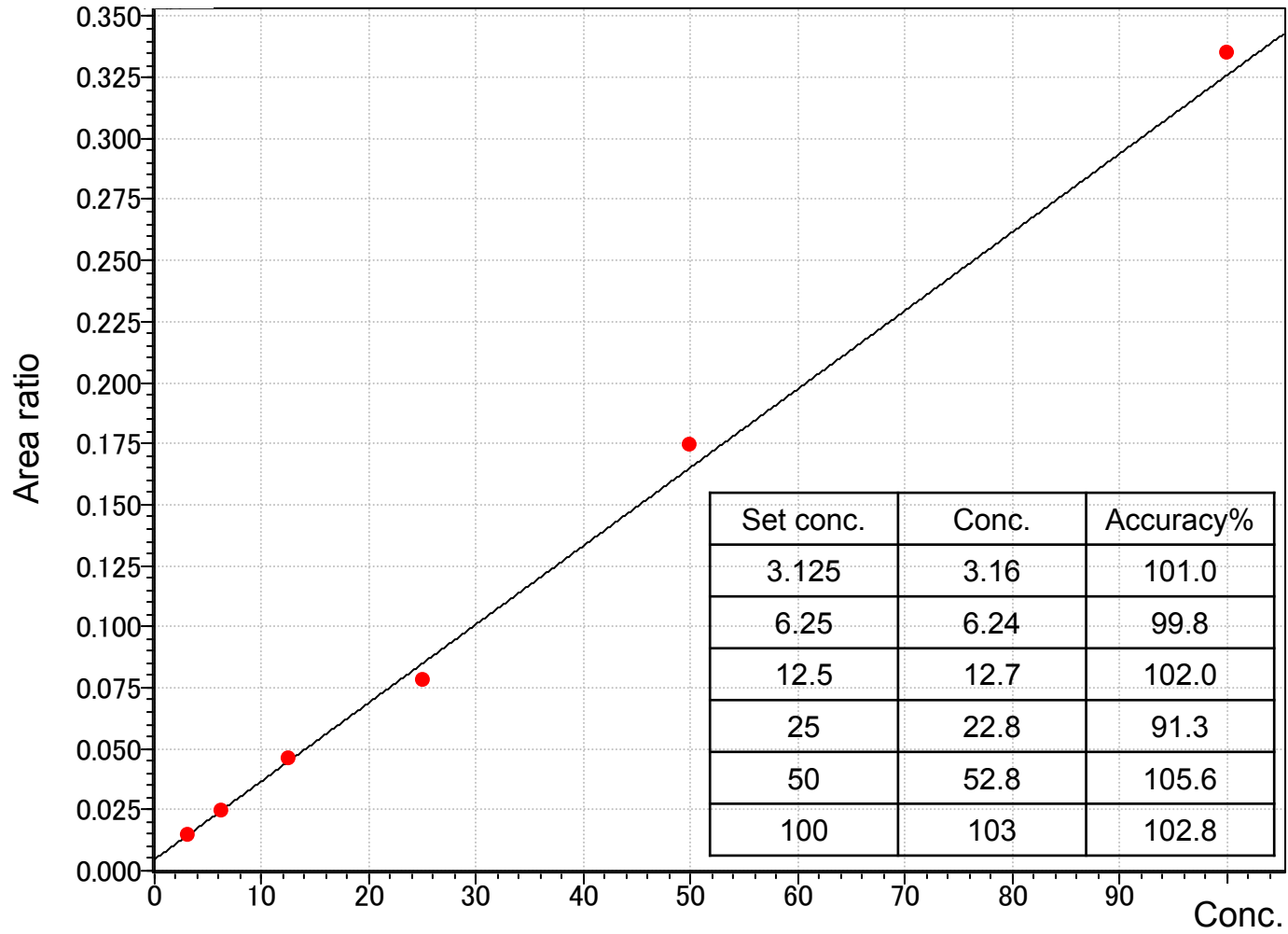
Summary of the bioanalytical LCMS validation of Trastuzumab in plama by nSMOL protocol

Table S2. Matrix effect

Analyte	Corresponding concentration (μg/ml)	Blank matrix No.	$P_{14}R$-normalized MF	Mean	SD	CV (%)
Trastuzumab	2.93	M1	0.821	0.845	0.074	8.7
		M2	0.891			
		M3	0.800			
		F1	0.882			
		F2	0.735			
		F3	0.941			
	200	M1	0.559	0.547	0.022	4.0
		M2	0.570			
		M3	0.564			
		F1	0.544			
		F2	0.527			
		F3	0.515			

Table S3. Carry-over

Compound	Run	Peak area		Peak area rate (%)
		LLOQ	Carry over sample	
Trastuzumab	1	838	ND	NC
	2	841	ND	NC
	3	901	ND	NC
$P_{14}R$	1	1593763	730	0.05

ND: Not detected

NC: Not calculated

Table S4. Calibration curve

Nominal concentration (µg/ml)	Back-calculated concentration (µg/ml)			Accuracy (%)		
	1	2	3	1	2	3
0.977	1.02	0.932	1.03	104.4	95.1	105.3
1.95	1.75	2.11	1.74	89.5	108.2	89.2
3.91	4.01	4.48	3.93	102.7	114.5	100.5
7.81	8.67	8.36	8.22	110.9	107.0	105.2
15.6	16.8	15.3	16.5	107.2	97.9	105.3
31.3	31.8	33.8	34.5	101.8	108.2	110.5
62.5	61.1	60.5	60.0	97.7	96.8	96.0
125	126	114	122	100.6	90.8	97.5
250	232	231	245	93.0	92.4	98.0

Table S5. Precision and accuracy

Run	Nominal concentration	Concentration (µg/ml)			
		0.977	2.93	23.4	200
1	Observed	0.933	2.84	24.2	209
		0.919	2.64	24.2	203
		0.955	3.04	24.3	230
		0.923	2.81	25.7	229
		0.967	2.48	24.4	226
	Mean	0.939	2.76	24.6	219
	SD	0.021	0.21	0.6	13
	CV (%)	2.2	7.6	2.6	5.7
	Accuracy (%)	96.2	94.2	104.8	109.7
2	Observed	0.759	2.44	23.7	198
		0.735	2.38	25.5	204
		0.902	2.42	24.9	206
		0.921	2.43	23.5	225
		0.952	2.22	25.0	209
	Mean	0.854	2.38	24.5	208
	SD	0.099	0.09	0.9	10
	CV (%)	11.7	3.8	3.6	4.9
	Accuracy (%)	87.4	81.2	104.6	104.2
3	Observed	0.747	2.63	23.1	205
		0.960	2.52	25.2	224
		0.743	2.56	23.3	200
		0.970	2.53	27.1	198
		0.917	2.79	25.6	205
	Mean	0.867	2.61	24.9	206
	SD	0.114	0.11	1.7	10
	CV (%)	13.1	4.3	6.7	5.0
	Accuracy (%)	88.8	89.0	106.1	103.2
	Mean (N=15)	0.887	2.58	24.6	211
	SD (N=15)	0.090	0.21	1.1	12
	CV (%)	10.2	8.2	4.4	5.6
	Accuracy (%)	90.8	88.1	105.2	105.7

Table S6. Dilution integrity

Nominal concentration (μg/ml)	Dilution factor	Observed* (μg/ml)	Mean (μg/ml)	SD (μg/ml)	CV (%)	Accuracy (%)
300	10	308	299	28	9.5	99.7
		334				
		309				
		285				
		259				

*Values of the QC samples were multiplied by the dilution factor

Table S7. Confirmation of QC sample for stability

Parameters for stability studies	Concentrations of Trastuzumab in human plasma (μg/ml)			
	2.93		200	
	Mean (μg/ml)	Accuracy (%)	Mean (μg/ml)	Accuracy (%)
Stability in plasma during freeze (-20 °C) and thaw cycles				
Cycle 5	2.87	98.1	199	99.7
Stability in plasma during freeze (-80 °C) and thaw cycles				
Cycle 5	2.97	101.5	221	110.3
Short-term stability in plasma for 4 hours at room temperature				
	2.68	91.6	192	96.2
Long-term stability in plasma for 15 days at -20 °C				
	3.03	103.5	203	101.3
Long-term stability in plasma for 15 days at -80 °C				
	2.71	92.5	207	103.3
Processed sample stability in autosampler set at 4 °C				
For 24 hours	2.78	95.0	203	101.5
For 48 hours	2.67	91.2	211	105.5