Demonstrating Analytical Similarity of Trastuzumab Biosimilar HLX02 to Herceptin® with a Panel of Sensitive and Orthogonal Methods Including a Novel FcγRIIIa Affinity Chromatography Technology

Running Header: HLX02 is highly similar to Herceptin® and particularly more similar to the batches with high FcγRIIIa affinity

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# Equally contributed;

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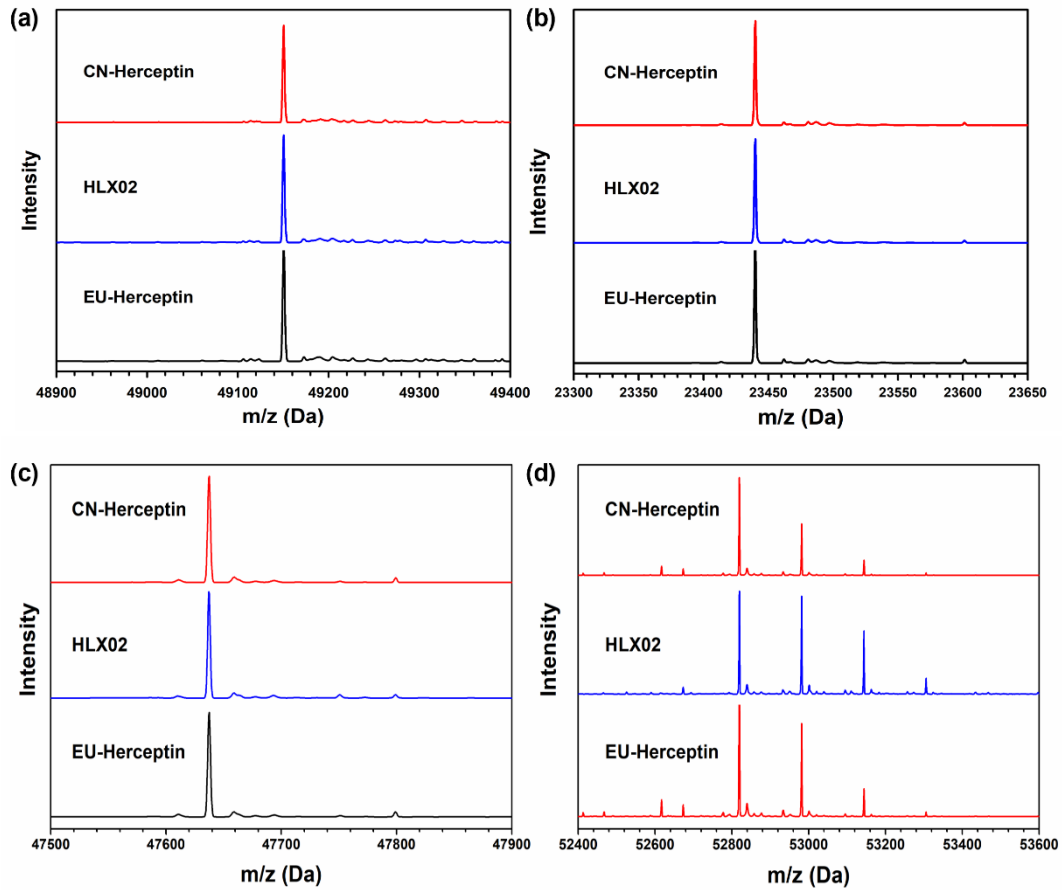
Supplemental Figure 1. MS spectra for (a) reduced and deglycosylated HC, (b) reduced LC, (c) Fab and (d) Fc of representative HLX02, CN-Herceptin® and EU-Herceptin®.
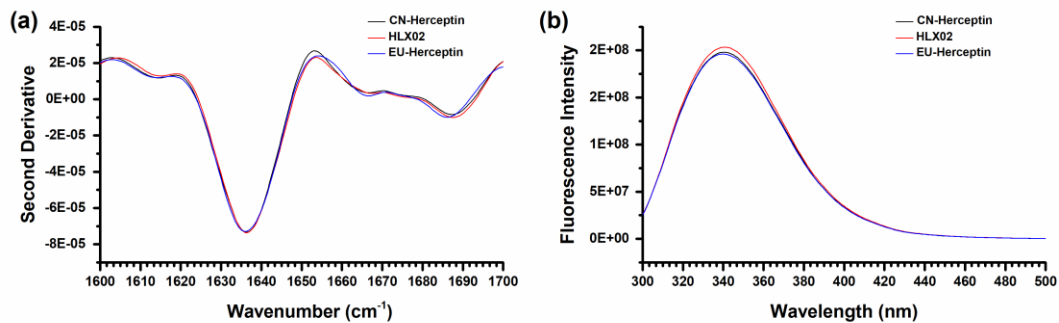
Supplemental Figure 2. Higher-order structure of representative HLX02, CN-Herceptin® and EU-Herceptin®. (a) FTIR spectra, (b) FLR spectra.
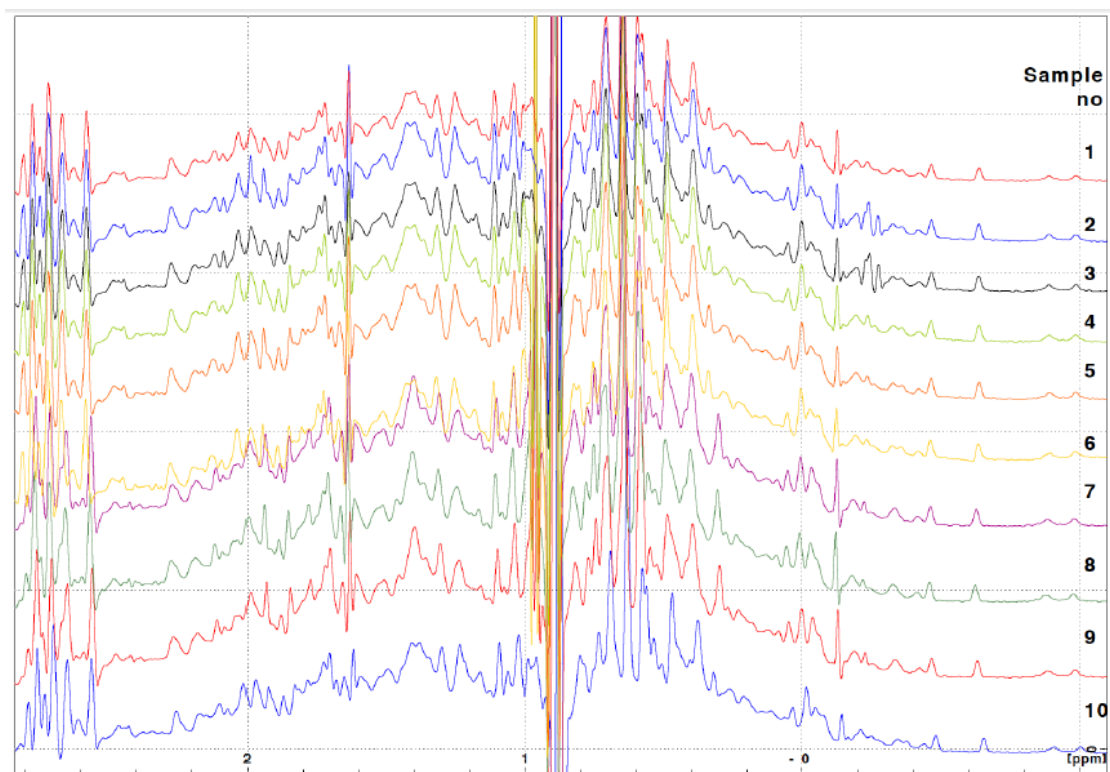
Supplemental Figure 3. Expansion of the methyl regions from 1D proton (1H) NMR spectra of representative HLX02, CN-Herceptin® and EU-Herceptin® at 303 K. All Spectra display highly similar peak patterns in terms of chemical shift position, line-widths and relative intensities.

Sample 1: HLX02 Batch No. H20180803

Sample 2: HLX02 Batch No. H20180804

Sample 3: HLX02 Batch No. H20180805

Sample 4: EU-Herceptin Batch No. H4544H03

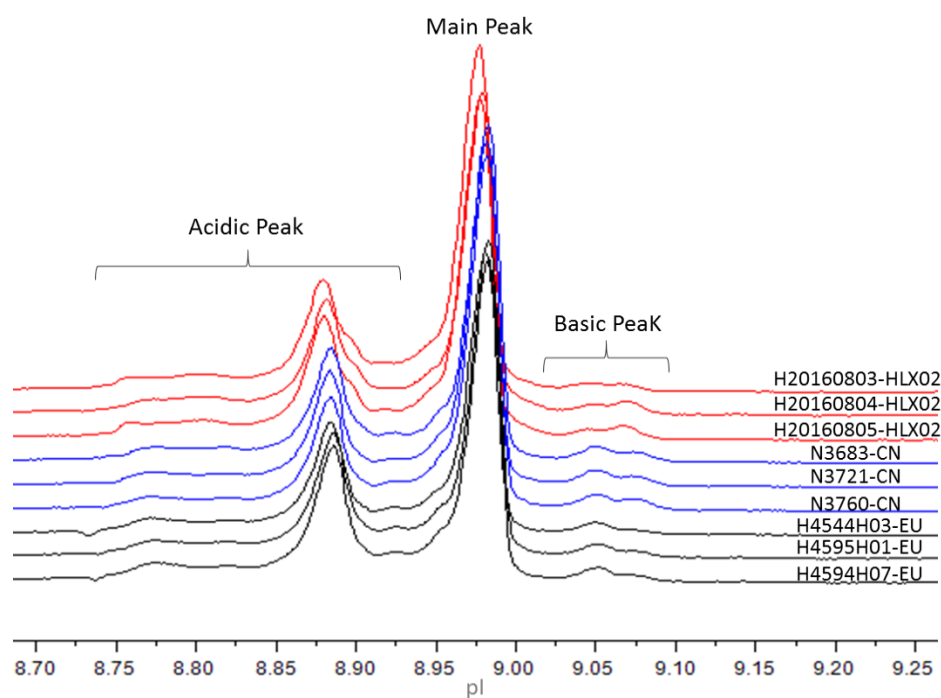Sample 5: EU-Herceptin Batch No. H4595H01

Sample 6: EU-Herceptin Batch No. H4594H07

Sample 7: CN-Herceptin Batch No. N3772

Sample 8: CN-Herceptin Batch No. N3760
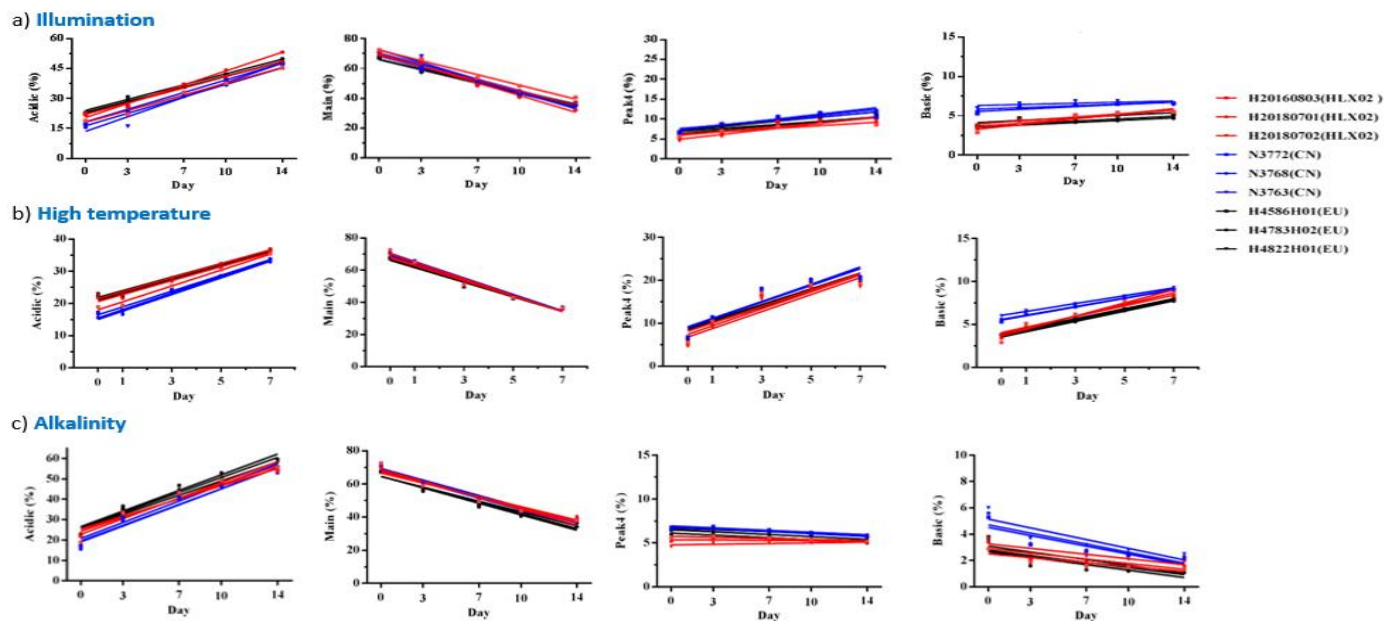
Sample 9: CN-Herceptin Batch No.N3768

Sample 10: Reference Standard, Batch No. H20160804-RM03

Supplemental Figure 4. Overlaid icIEF profiles of representative HLX02, CN-Herceptin® and EU-Herceptin®.

Supplemental Figure 5. Comparison of the CEX forced degradation trends under (a) illumination, (b) higher temperature, and (c) alkalinity for HLX02, CN-Herceptin® and EU-Herceptin®.
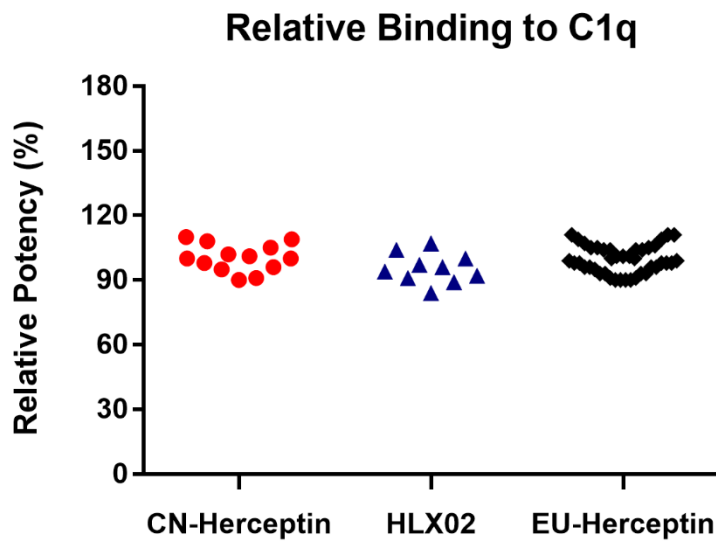
Supplemental Figure 6. C1q binding similarity assessment results of HLX02, CN-Herceptin® and EU-Herceptin®.

Supplemental Table 1 The lot number and expiration date of the reference product and HLX02.

Source	Lot code	Expire date
CN-Herceptin®	N3683	2017.08.24
	N3687	2017.09.18
	N3698	2017.11.06
	N3703	2017.12.06
	N3714	2018.01.23
	N3721	2018.03.02
	N3735	2018.04.07
	N3739	2018.06.03
	N3760	2018.09.04
	N3763	2018.09.08
	N3768	2018.11.19
	N3772	2018.12.13
	N7074B04	2018.06
EU-Herceptin®	H4544H03	2018.11
	H4586H01	2019.05
	H4594H07	2019.06
	H4595H01	2019.06
	H4597H04	2019.04.30
	H4611H04	2019.07
	H4618H05	2019.08
	H4619H07	2019.04.30
	H4620H05	2019.08
	H4634H01	2019.09
	H4638H02	2019.04.30
	H4643H01	2019.04.30
	H4723H02	2020.05.31
	H4741H02	2018.02.16
	H4756H04	2018.02.16
	H4783H02	2020.1
	N6001H03	2020.08
	H4804H01	2020.11
	H4822H01	2021.02
	H4823H01	2021.02
	N2002H02	2021.01
	N3006H03	2021.02
	N3007H01	2021.03
	N6001H06	2020.08
	N7185H01	2020.11
	N7195H03	2020.12

	H20160805	2020.08.29
	H20160101	2020.01.26
	H20160402	2020.03.31
	H20170101	2021.01.18
	H20170402	2021.04.27
HLX02	H20180701	2022.07.16
	H20180702	2022.07.29
	H20180803	2022.08.03
	H20180804	2022.08.14
	H20180805	2022.08.20

Supplemental Table 2 Temperature, sampling time points and inspection items for the forced degradation studies.

Forced degradation	Temperature	Time points	Methods
Higher temperature	50±2 ℃	Day 0, 1, 3, 5, 7	SEC-HPLC, CEX-HPLC , NR CE-SDS , Binding, Peptide mapping by LC-MS
Light exposure (4500±500 lux)	25±2 ℃	Day 0, 3, 7, 10, 14	
Control (dark)		Day 0, 3, 7, 10, 14	
Strong acid (pH 4)		Day 0, 3, 7, 10, 14	
Strong base (pH 10)		Day 0, 3, 7, 10, 14	
Continuous shaking (1000 rpm)		Day 0, 3, 7, 10, 14	
Strong oxidizer (1.0% tBHP)	2-8 ℃	Hour 0, 3, 8, 24, 32	SEC-HPLC, CEX-HPLC , NR CE-SDS , Binding, Peptide mapping by LC-MS

Supplemental Table 3 The percentage of post translational modifications (PTMs) in HLX02, CN-Herceptin® and EU-Herceptin®

Sites of PTMs	Percentage of PTMs (%)		
	HLX02	CN-Herceptin®	EU-Herceptin®
Deamidation N (LC:N30)	6.37±0.58	5.30±0.46	6.43±0.29
PyroE HC N-term (HC: E1)	0.53 ±0.06	0.87 ±0.06	0.90 ±0.10
Deamidation N (HC:N55)	1.47 ±0.12	1.50 ±0.00	1.50 ±0.00
Isomer D (HC:D102)	3.73 ±0.35	3.30 ±0.26	2.93 ±0.15
Oxidation M (HC:M255)	0.87 ±0.06	2.40 ±3.12	0.80±0.00
Deamidation N (HC:N318)	2.37 ±0.06	2.33 ±0.06	2.30 ±0.00
Deamidation N (HC:N387/392)	1.80 ±0.10	1.90 ±0.00	1.87 ±0.06
-Lysine HC:C-Term (HC: K450)	98.40 ±0.44	99.47 ±0.06	99.63 ±0.06

Supplemental Table 4 Peptides containing disulfide bonds identified in HLX02, CN-Herceptin® and EU-Herceptin®.

Peptide Sequence*	Serial number of peptides	Theory molecular weight (Da)	HLX02								CN-Herceptin®				EU-Herceptin®					
			H20160803		H20160804		H20160805		N3683		N3721		N3760		H4544H03		H4595H01		H4594H07	
			Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)	Measured molecular weight (Da)	Mass error (ppm)
VIITCR=SGTDFTLTISSL QPEDFATYYCQQHYTTPPL TFGQGTK	LC:T02-LC:T08	4819.2422	4819.2407	-0.3	4819.2407	-0.3	4819.2456	0.7	4819.2456	0.7	4819.2305	-2.4	4819.2363	-1.2	4819.2407	-0.3	4819.2388	-0.7	4819.2329	-1.9
SGTASVVCLLNNFYPR=V YACEVTHQGLSSPVTK	LC:T12-LC:T19	3555.749	3555.7456	-1	3555.7532	1.2	3555.7502	0.3	3555.7522	0.9	3555.7446	-1.2	3555.7566	2.1	3555.7483	-0.2	3555.7598	3	3555.7542	1.5
GEC=SCDK	LC:T21-HC:T20	756.2418	756.2455	4.9	756.2424	0.8	756.244	2.9	756.246	5.6	756.243	1.6	756.2451	4.4	756.244	2.9	756.2451	4.4	756.2451	4.4
LSCAASGFNIK=AEDTAV YYCSR	HC:T02-HC:T11	2384.0776	2384.0793	0.7	2384.085	3.1	2384.0828	2.2	2384.0869	3.9	2384.0825	2.1	2384.0864	3.7	2384.085	3.1	2384.0818	1.8	2384.0818	1.8
STSGGTAALGCLVK=DYF PEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHK	HC:T14-HC:T15	7389.6489	7389.6304	-2.5	7389.6313	-2.4	7389.6304	-2.5	7389.6167	-4.4	7389.6177	-4.2	7389.6309	-2.4	7389.6118	-5	7389.6382	-1.4	7389.6357	-1.8
THTCPPCPAPELLGGPSVF LFPPK=THTCPPCPAPELL GGPSVFLFPPK**	HC:T21-HC:T21	5004.4878	5004.4814	-1.3	5004.48	-1.6	5004.4907	0.6	5004.4805	-1.5	5004.48	-1.6	5004.4883	0.1	5004.4614	-5.3	5004.4917	0.8	5004.4858	-0.4
TPEVTCVVVDVSHEDPEV K=CK	HC:T24-HC:T31	2328.0977	2328.1028	2.2	2328.1038	2.6	2328.1025	2.1	2328.104	2.7	2328.103	2.3	2328.1045	2.9	2328.1011	1.5	2328.1016	1.7	2328.1047	3
NQVSLTCLVK=WQQGNV FSCSVMHEALHNHYTQK	HC:T39-HC:T44	3844.8235	3844.8257	0.6	3844.8289	1.4	3844.8135	-2.6	3844.821	-0.7	3844.8274	1	3844.8362	3.3	3844.8254	0.5	3844.8281	1.2	3844.8289	1.4

* "=" represent disulfide bonds

** HC–HC interchain disulfide bonds

Supplemental Table 5 CEX similarity assessment results of HLX02, CN-Herceptin® and EU-Herceptin®.

Sample	Acid variants % mean (range)	Main peak % mean (range)	Basic variants % mean (range)	Peak 4 % mean (range)
CN-Herceptin®	17.1 (15.6-18.8)	71.0 (69.6-72.5)	5.2 (4.4-6.1)	6.7 (6.3-6.9)
EU-Herceptin®	21.4 (17.9-23.3)	68.2 (66.3-70.5)	4.0 (3.4-5.4)	6.4 (3.4-5.4)
HLX02	22.3 (18.7-24.9)	68.2 (64.2-73.6)	4.1 (2.9-5.4)	5.4 (4.4-6.3)
Similarity interval	12.5-27.5	63.7-74.5	2.0-6.8	5.9-7.1

Supplemental Table 6 The percentage of molecular weight variants identified by SEC-UV/MALS analysis.

Sample	Aggregate % mean (range)	Aggregate Molecular weight (KD)	Monomer % mean (range)	Monomer Molecular weight (KD)
CN-Herceptin®	0.5 (0.3-0.6)	287.5-299.6	99.5 (99.4-99.6)	144.8-145.0
EU-Herceptin®	0.5 (0.4-0.5)	282.9-285.7	99.5 (99.4-99.6)	144.8-145.1
HLX02	0.3 (0.2-0.4)	279.6-308.6	99.7 (99.5-99.7)	144.7
Similarity interval	0.2-0.8	Visual Similar	99.2-99.8	Visual Similar

Supplemental Table 7 Percentage of Monomer, Dimer and Higher Oligomers based on Absorbance Data of AUC Measurements

Sample	Monomer %	Dimer%	c>dim %
HLX02	97.3-98.5	0.4-1.2	1.1-1.5
EU-Herceptin®	97.9-98.5	0.8-1.2	0.7-1.1
CN-Herceptin®	97.7-98.4	0.8-1.2	0.8-1.2

Supplemental Table 8 The purity level identified by nonreduced and reduced CE-SDS.

Sample	Nonreduced	Reduced	
	Monomer % mean (range)	Unglycosylated HC % mean (range)	(HC+LC) % mean (range)
CN-Herceptin®	97.0 (96.5-97.6)	0.8 (0.7-0.9)	98.5 (98.1-98.7)
EU-Herceptin®	96.9 (96.2-97.5)	0.7 (0.6-0.8)	98.7 (98.5-98.9)
HLX02	96.9 (96.5-97.1)	0.3 (0.3-0.4)	99.0 (98.7-99.2)
Similarity interval	96.0-97.8	0.4-1.0	98.1-99.3

Supplemental Table 9 Particle analysis results of HLX02, CN-Herceptin® and EU-Herceptin®.

Method / Sample	MFI Particles (Counts/mL)		DLS
	ECD $\geqslant$ 10 μm	ECD $\geqslant$ 25 μm	d.nm
HLX02	20-186	0-37	3.6-3.7
EU-Herceptin®	88-112	9-14	3.7-3.8
CN-Herceptin®	100-658	7-25	3.6-3.7

Supplemental Table 10 The concentration of bioprocess residuals in HLX02, CN-Herceptin® and EU-Herceptin®.

Sample	Lot code	DNA (pg/mg)	HCP (ppm)	Protein A (ppm)
HLX02	H20160803	<0.05	<2	<0.5
	H20160804	<0.05	<2	<0.5
	H20160805	<0.05	<2	<0.5
	H20170101	<0.05	<2	<0.5
	H20170402	<0.05	<2	<0.5
	H20180701	<0.05	<2	<0.5
	H20180702	<0.05	<2	<0.5
	H20180803	<0.05	<2	<0.5
	H20180804	<0.04	<2	<0.5

	H20180805	<0.05	<2	<0.5
CN-Herceptin®	N3683	<0.04	<2	<0.5
	N3687	<0.04	<2	<0.5
	N3698	<0.04	<2	<0.5
	N3703	<0.04	<2	<0.5
	N3714	<0.04	<2	<0.5
	N3721	<0.04	<2	1
	N3735	<0.04	<2	<0.5
	N3739	<0.04	<2	<0.5
	N3760	<0.04	<2	<0.5
	N3763	<0.04	<2	<0.5
	N3768	<0.04	<2	<0.5
	N3772	<0.04	<2	<0.5
	N7074B04	<0.04	<2	<0.5
EU-Herceptin®	H4544H03	<0.04	<2	<0.5
	H4595H01	<0.04	<2	<0.5
	H4586H01	<0.04	<2	<0.5
	H4594H07	<0.04	<2	<0.5
	H4620H05	<0.04	<2	<0.5
	H4618H05	<0.05	<2	<0.5
	H4634H01	<0.04	<2	<0.5
	H4611H04	<0.04	<2	<0.5
	H4597H04	<0.04	<2	<0.5
	H4643H01	<0.04	<2	<0.5
	H4619H07	<0.04	<2	<0.5
	H4638H02	<0.04	<2	<0.5
	H4723H02	<0.04	<2	<0.5
	H4756H04	<0.04	<2	<0.5
	H4741H02	<0.04	<2	<0.5
	H4783H02	<0.04	<2	<0.5
	H4804H01	<0.04	<2	<0.5
	N6001H06	<0.04	<2	<0.5
	H4823H01	<0.04	<2	<0.5
	H4822H01	<0.04	<2	<0.5
	N2002H02	<0.04	<2	<0.5
	N3006H03	<0.04	<2	<0.5
	N7185H01	<0.04	<2	<0.5
	N6001H03	<0.04	<2	<0.5
	N7195H03	<0.04	<2	<0.5
	N3007H01	<0.05	<2	<0.5

Supplemental Table 11 %Glycans of HLX02, CN-Herceptin® and EU-Herceptin®.

Sample	G0F%[*] mean (range)	Man%[§] mean (range)	Sialylation%[†] mean (range)	Gal%[‡] mean (range)	Afuc%[**] mean (range)	NANA mol/mol mean (range)	NGNA mol/mol mean (range)
HLX02	48.3 (41.8-51.4)	2.8 (2.3-3.3)	2.8 (2.4-3.7)	40.3 (37.3-46.6)	5.2 (4.2-6.4)	0.105 (0.087-0.137)	ND (ND-0.001)
CN-Herceptin®	55.4 (40.8-66.5)	3.4 (2.3-4.6)	1.2 (0.8-1.9)	33.8 (23.9-47.8)	6.1 (4.8-8.1)	0.043 (0.033-0.058)	0.003 (0.002-0.003)
EU-Herceptin®	54.7 (41.2-70.1)	1.9 (1.4-3.3)	1.2 (0.9-1.8)	36.2 (21.8-48.9)	6.0 (4.6-7.6)	0.046 (0.028-0.061)	0.004 (0.002-0.005)
Similarity interval	23.7-86.1	0-5.1	0-2.4	6.3-64.5	2.5-9.7	0.013-0.076	0.001-0.006

* represent G0F, G0F-GN, G0FB type N-glycans

§ represent Man5, Man6, Man7, Man8, Man9 type N-glycans

† represent G1FS1, G1FS1-GN, G2FS1, G2FS2 type N-glycans

‡ represent G1, G1', G1F, G1F', G1F-GN, G1FB, G2, G2F type N-glycans

** represent G0, G0-GN,G1, G1', G2 type N-glycans