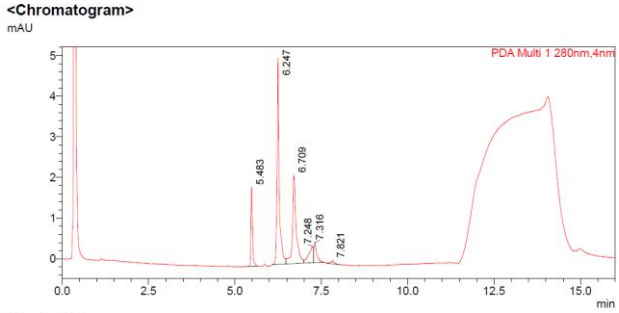
Preparation of SG3249 antibody-drug conjugates

Conjugate A: Herceptin-SG3249 (ConjA)

Antibody (15 mg, 100 nanomoles) was diluted into 13.5 mL of a reduction buffer containing 10 mM sodium borate pH 8.4, 2.5 mM EDTA and a final antibody concentration of 1.11 mg/mL. A 10 mM solution of TCEP was added (1.5 molar equivalent/antibody, 150 nanomoles, 15 μL) and the reduction mixture was heated at +37 °C for 1.5 hours in an incubator. After cooling down to room temperature, SG3249 was added as a DMSO solution (5 molar equivalent/antibody, 500 nanomoles, in 1.5 mL DMSO). The solution was mixed for 1.25 hours at room temperature, then the conjugation was quenched by addition of *N*-acetyl cysteine (1 micromole, 100 μL at 10 mM), and injected into an AKTA™ Pure FPLC using a GE Healthcare HiLoad™ 26/600 column packed with Superdex 200 PG, eluting with 2.6 mL/min of sterile-filtered phosphate-buffered saline (PBS). Fractions corresponding to **ConjA** monomer peak were pooled, concentrated using a 15mL Amicon Ultracell 50KDa MWCO spin filter, analysed and sterile-filtered.

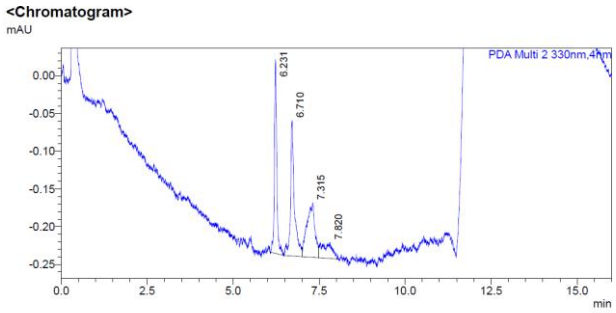
UHPLC analysis on a Shimadzu Prominence system using a Phenomenex Aeris 3.6u XB-C18 150 x 2.1 mm column eluting with a gradient of water and acetonitrile on a reduced sample of **ConjA** at 280 nm and 330 nm (SG3249 specific) shows a mixture of light and heavy chains attached to several molecules of SG3249, consistent with a drug-per-antibody ratio (DAR) of 2.51 molecules of SG3249 per antibody.



<Peak Table>
PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	5.483	6273	1912	12.198
2	6.247	21831	4987	42.449
3	6.709	16126	2168	31.356
4	7.248	4004	398	7.786
5	7.316	2640	493	5.134
6	7.821	554	79	1.077
Total		51428	10037	100.000

Fig A. UHPLC of reduced ConjA at 280 nm

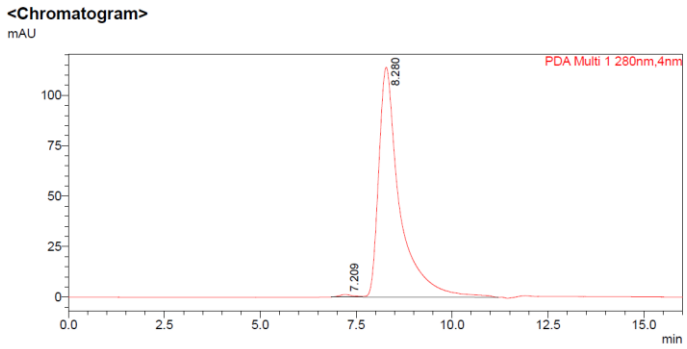


<Peak Table>
PDA Ch2 330nm

Peak#	Ret. Time	Area	Height	Area%
1	6.231	1227	255	28.986
2	6.710	1399	178	33.039
3	7.315	1183	72	27.930
4	7.820	425	20	10.045
Total		4234	525	100.000

Fig B. UHPLC of reduced ConjA at 330 nm

UHPLC analysis on a Shimadzu Prominence system using a Phenomenex Yarra 3u SEC-3000 300 mm x 4.60 mm column eluting with sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (*v/v*) on a sample of **ConjA** at 280 nm shows a monomer purity of over 99% with no impurity detected. UHPLC SEC analysis gives a concentration of final **ConjA** at 1.44 mg/mL in 9.0 mL, obtained mass of **ConjA** is 12.97 mg (86% yield).



<Peak Table>
PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	7.209	29288	1169	0.673
2	8.280	4324563	113849	99.327
Total		4353852	115018	100.000

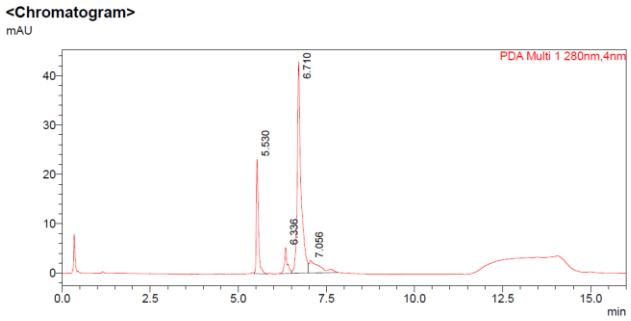
Fig C. UHPLC SEC of ConjA at 280 nm

Conjugate B: site-specific Herceptin-SG3249 (ConjB)

A 50 mM solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in water was added (40 molar equivalent/antibody, 24 micromoles, 480 µL) to a 20 mL solution of antibody (90 mg, 600 nanomoles) in reduction buffer containing phosphate-buffered saline pH 7.4 (PBS) and 1 mM ethylenediaminetetraacetic acid (EDTA) and a final antibody concentration of 4.5 mg/mL. The reduction mixture was heated at +37 °C for 2.5 hours in an incubator with gentle agitation. After cooling to room temperature, the reduced antibody was buffer exchanged, *via* dialysis using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) into a reoxidation buffer containing PBS and 1 mM EDTA (2 x 4 L at +4 °C with stirring) to remove the excess reducing agent. A 50 mM solution of dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 12 micromoles, 240 µL) in DMSO was added and the reoxidation mixture was allowed to react for 4 hours at room temperature with gentle agitation at an antibody concentration of ~ 2.7 mg/mL (or until full reoxidation of the cysteine thiols to reform the inter-chain cysteine disulfides is observed by UHPLC). The reoxidation mixture was then sterile-filtered and diluted in a conjugation buffer containing PBS

and 1 mM EDTA for a final antibody concentration of 1.0 mg/mL. SG3249 was added as a DMSO solution (10 molar equivalent/antibody, 1.0 micromole, in 1.5 mL DMSO) to 13.5 mL of this reoxidised antibody solution (15 mg, 100 nanomoles) for a 10% (*v/v*) final DMSO concentration. The solution was mixed for 1.5 hours at room temperature, then the conjugation was quenched by addition of *N*-acetyl L-cysteine (4 micromoles, 400 µL at 10 mM), concentrated, and injected into an AKTA™ Pure FPLC using a GE Healthcare HiLoad™ 26/600 column packed with Superdex 200 PG, eluting with 2.6 mL/min of sterile-filtered phosphate-buffered saline (PBS). Fractions corresponding to **ConjB** monomer peak were pooled, concentrated using a 15mL Amicon Ultracell 50KDa MWCO spin filter, analysed and sterile-filtered.

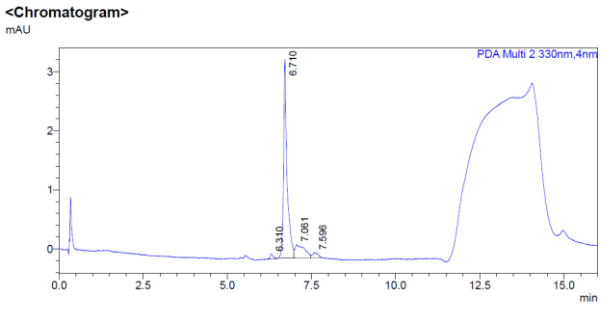
UHPLC analysis on a Shimadzu Prominence system using a Phenomenex Aeris 3.6u XB-C18 150 x 2.1 mm column eluting with a gradient of water and acetonitrile on a reduced sample of **ConjB** at 280 nm and 330 nm (SG3249 specific) shows unconjugated light chains and a mixture of unconjugated heavy chains and heavy chains attached to a single molecule of SG3249, consistent with a drug-per-antibody ratio (DAR) of 1.81 molecules of SG3249 per antibody.



<Peak Table>
PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	5.530	97083	22886	19.415
2	6.336	29824	5156	5.964
3	6.710	319263	42702	63.846
4	7.056	53880	2499	10.775
Total		500049	73243	100.000

Fig D. UHPLC of reduced ConjB at 280 nm

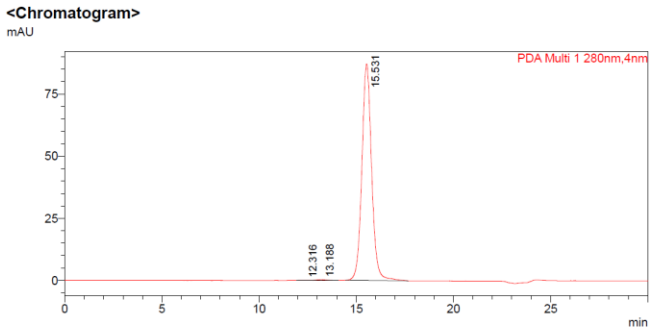


<Peak Table>
PDA Ch2 330nm

Peak#	Ret. Time	Area	Height	Area%
1	6.310	480	79	1.588
2	6.710	24264	3351	80.180
3	7.061	4450	226	14.704
4	7.596	1068	87	3.528
Total		30262	3744	100.000

Fig E. UHPLC of reduced ConjB at 330 nm

UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel G3000SWXL 5 µm 7.8 x 300 mm column (with a 7 µm 6.0 x 40 mm guard column) eluting with sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (*v/v*) on a sample of **ConjB** at 280 nm shows a monomer purity of over 99% with no impurity detected. UHPLC SEC analysis gives a concentration of final **ConjB** at 1.57 mg/mL in 8.5 mL, obtained mass of **ConjB** is 13.34 mg (89% yield).



<Peak Table>
PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	12.316	1972	71	0.065
2	13.188	12898	311	0.422
3	15.531	3041007	87111	99.513
Total		3055876	87493	100.000

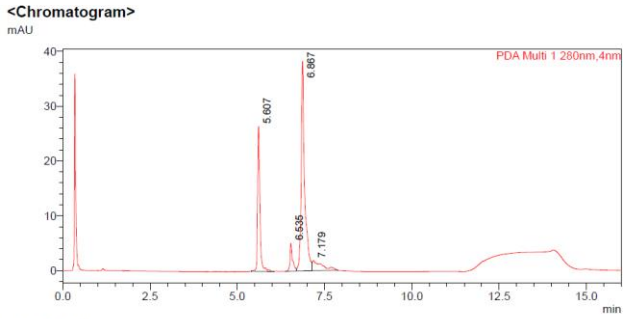
Fig F. UHPLC SEC of ConjB at 280 nm

Conjugate C: non-binding IgG-SG3249 (ConjC)

A 50 mM solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in water was added (40 molar equivalent/antibody, 24 micromoles, 480 µL) to a 20 mL solution of antibody (90 mg, 600 nanomoles) in reduction buffer containing phosphate-buffered saline pH 7.4 (PBS) and 1 mM ethylenediaminetetraacetic acid (EDTA) and a final antibody concentration of 4.5 mg/mL. The reduction mixture was heated at +37 °C for 2.5 hours in an incubator with gentle agitation. After cooling to room temperature, the reduced antibody was buffer exchanged, *via* dialysis using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific) into a reoxidation buffer containing PBS and 1 mM EDTA (2 x 4 L at +4 °C with stirring) to remove the excess reducing agent. A 50 mM solution of dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 12 micromoles, 240 µL) in DMSO was added and the reoxidation mixture was allowed to react for 4 hours at room temperature with gentle agitation at an antibody concentration of ~ 2.8 mg/mL (or until full reoxidation of the cysteine thiols to reform the inter-chain cysteine disulfides is observed by UHPLC). The reoxidation mixture was then sterile-filtered and diluted in a conjugation buffer containing PBS and 1 mM EDTA for a final antibody concentration of 1.0 mg/mL. SG3249 was added as a DMSO solution (10 molar equivalent/antibody, 1.0 micromole, in 1.5 mL DMSO) to 13.5 mL of this reoxidised antibody solution (15 mg, 100 nanomoles) for a 10% (*v/v*) final DMSO concentration. The solution was mixed for 1.5 hours at room temperature, then the conjugation was quenched by addition of *N*-acetyl L-cysteine (4 micromoles, 400 µL at 10 mM), concentrated, and injected into an AKTA™ Pure FPLC using a GE Healthcare HiLoad™ 26/600 column packed with Superdex 200 PG, eluting with 2.6 mL/min of sterile-filtered phosphate-buffered saline (PBS).

Fractions corresponding to **ConjC** monomer peak were pooled, concentrated using a 15mL Amicon Ultracell 50KDa MWCO spin filter, analysed and sterile-filtered.

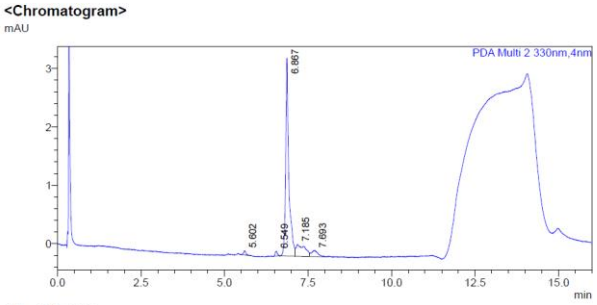
UHPLC analysis on a Shimadzu Prominence system using a Phenomenex Aeris 3.6u XB-C18 150 x 2.1 mm column eluting with a gradient of water and acetonitrile on a reduced sample of **ConjC** at 280 nm and 330 nm (SG3249 specific) shows unconjugated light chains and a mixture of unconjugated heavy chains and heavy chains attached to a single molecule of SG3249, consistent with a drug-per-antibody ratio (DAR) of 1.79 molecules of SG3249 per antibody.



<Peak Table>
PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	5.607	121124	26232	27.168
2	6.535	25518	5030	5.724
3	6.867	261800	38193	58.722
4	7.179	37389	1869	8.386
Total		445831	71324	100.000

Fig G. UHPLC of reduced ConjC at 280 nm



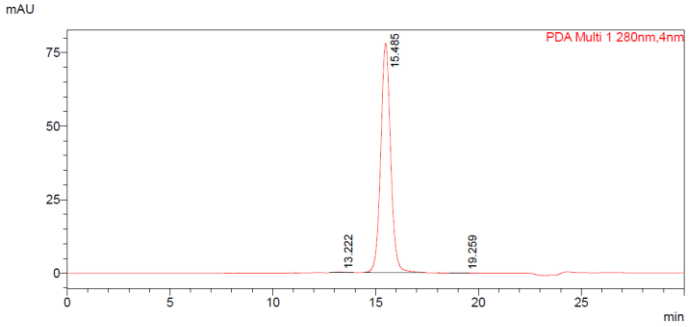
<Peak Table>
PDA Ch2 330nm

Peak#	Ret. Time	Area	Height	Area%
1	5.602	354	71	1.255
2	6.549	372	80	1.321
3	6.867	22296	3380	79.150
4	7.185	3743	200	13.286
5	7.693	1405	104	4.987
Total		28170	3835	100.000

Fig H. UHPLC of reduced ConjC at 330 nm

UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel G3000SWXL 5 µm 7.8 x 300 mm column (with a 7 µm 6.0 x 40 mm guard column) eluting with sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (*v/v*) on a sample of **ConjC** at 280 nm shows a monomer purity of over 99% with no impurity detected. UHPLC SEC analysis gives a concentration of final **ConjC** at 1.43 mg/mL in 9.1 mL, obtained mass of **ConjC** is 13.02 mg (87% yield).

<Chromatogram>



<Peak Table>

PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	13.222	6345	192	0.243
2	15.485	2605614	77975	99.716
3	19.259	1069	37	0.041
Total		2613028	78204	100.000

Fig I. UHPLC SEC of ConjC at 280 nm