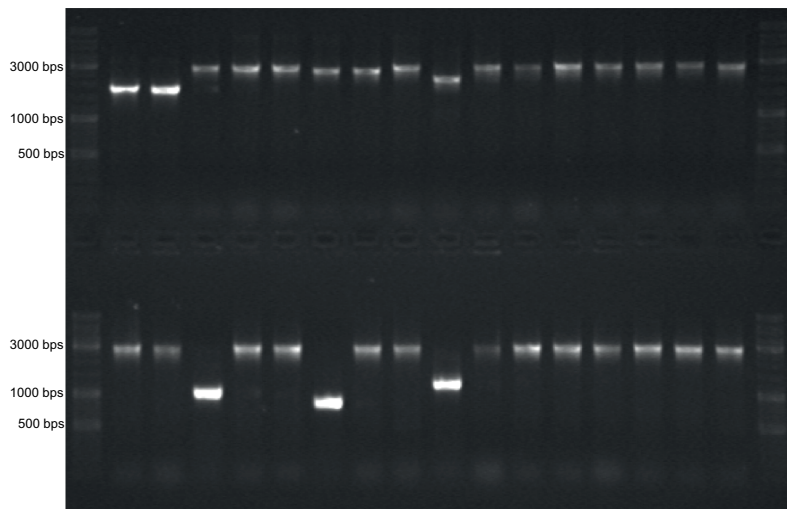


Supplementary figure 1. Detailed view of the unit operations for restriction based (left) and head-to-tail SPC (right). A liquid handler performed operations inside the light grey boxes. Green operations were bead captures, pink operations were carried out on heating block and other operations on the liquid handlers are shown as blue boxes. Yellow operations were done manually.



Supplementary figure 2. Comparison head-to-tail SPC and Gibson assembly. The same four DNA fragments were assembled with both head-to-tail SPC (upper row) and Gibson cloning (lower row).



Supplementary figure 3. Bi-specific antibodies. Small affinity proteins were fused to the C-terminal of full-length IgG by 3 different linker lengths. Both the affinity proteins and the linkers were inserted as synthetic oligos.

Supplementary table 1. Detailed view of the DNA sequence near the bead and terminal ends for the applied restriction enzymes. Restriction sites are highlighted in bold font.

NotI Bead---CTCGAG**GCGGCCG**CNNN---assembled construct---NNNG**GCGGCCG**CACGACTC  
 AscI Bead---CTCGAG**GCGGCCG**CNNN---assembled construct---NNNG**GCGGCCG**CCACGACTC  
 NheI Bead---CTCGAG**GCTAGC**NNN---assembled construct---NNNG**GCTAGC**CGACTC  
 EcoRI Bead---CTCGAG**GAATTC**NNN---assembled construct---NNNG**GAATTC**CGACTC  
 BamHI Bead---CTCGAG**GGATCC**NNN---assembled construct---NNNG**GGATCC**ACGACTC  
 BsiWI Bead---CTCGAG**CGTACG**NNN---assembled construct---NNNG**CGTACG**CGACTC  
 AatII Bead---CTCGAG**GACGTC**NNN---assembled construct---NNNG**GACGTC**ATGCAT

Supplementary table 2. Detailed list of constructs assembled with head-to-tail SPC.

Construct name	Bricks assembled	Backbone brick length (bp)	Insert type	Insert brick 1 length (bp)	Insert 2 brick (bp)	Insert brick 3 (bp)	Total length	Colonies picked per construct	Correct (%)
pHis2_GFP11	2	2815	Oligo	93			2857	6	100
pHis2_EGFP	2	2815	PCR	762			3526	12	100
pHis2_mCherry	2	2815	PCR	753			3517	16	71
pHis2_GFP_pZEE	2	257	PCR	2854			3060	48	96
2180012_2180013pA#8c	2	378	PCR	5691			6014	5	100
2250322_2250323pA#8c	2	324	PCR	5548			5819	6	100
3270055_3270054pA#8c	2	225	PCR	5435			5609	9	11
HerceptinCH_pcDNA3.3_HumiraVH	2	6424	PCR	406			6781	8	88
HerceptinCH_pcDNA3.3_OmnitargVH	2	6424	PCR	400			6775	8	100
HerceptinCL_pOptivec_HumiraVL	2	4750	PCR	384			5050	8	100
HerceptinCL_pOptivec_OmnitargVL	2	4750	PCR	384			5050	8	100
96 different single chains to full length antibodies	2	6478 or 4798	PCR	400 or 364			6830 or 5114	4	87
16 variants of failed expression vectors	2	5075	Oligo	180			5207	4	98
8 proteins drug tagged with GFP 11	2	5549 to 6633	Oligo	90			5627 to 6741	12	99
pcDNA3.3_HerceptinH_GFP11	2	6847	Oligo	134			6931	8	100
pOptivec_HerceptinH_GFP11	2	5134	Oligo	134			5218	8	100
pcDNA3.3_Herceptin_18_Binding protein_GFP11	2	7066	Oligo	104			7168	8	88
pcDNA3.3_HER2 signal peptide	2	5431	Oligo	111			5491	48	35
pHis2_VEGF_EGFP	3	2815	PCR	393	762		3889	6	83
pHis2_VEGF_mCherry	3	2815	PCR	393	753		3880	16	84
pHis2_mCherry_EGFP	3	2815	PCR	750	762		4246	28	79
Bi-specific antibodies	3	6847	Oligo	114	192		7102	8**	44**
pHis2_VEGF_AG_mCherry	4	2815	PCR	364	1389	753	5239	16	81
pHis2	4	1533	PCR	894	448	757	3535	4	75
2250323pA#8c	4	4126	PCR	998	527	393	5947	4	75
pHis2 (kanamycin)	4	1046	PCR	998	527	757	3053	4	75

\*\*With PAGE purified

Supplementary table 3. Estimated importance of assembly properties for selected cloning applications. Up to three plusses were given to a property based on how beneficial it is for the application.

Assembly properties

Application	Multiple inserts	Sequence independent	Synthetic oligo
Fusion proteins	+++	++	+/-
Biosensor	+/-	+	+/-
Expression vectors	+/-	++	+++
Mammalian surface expression	+/-	++	+
scFv to full length IgG	+/-	+	+/-
Bi-specific proteins with variable linkers	+++	+++	+++
Protein tagging with peptide	+/-	++	+++