Supplementary information.

Supplementary table 1. Chain productivity.

Status	Total Heavy MiSeq	Total Light Chain MiSeq
Productive & Unproductive	12,171,283	743,129
Productive	10,785,580	624,291
Productive Reads	89%	84%

Tabulated number of productive and unproductive heavy chain sequences from MiSeq; 89% of sequences are productive (no frame-shifts, stop codons or low-quality base-pair called). Tabulated number of productive and unproductive light chain sequences from MiSeq. 84% sequences are productive.

Supplementary table 2. Relative primer promiscuity for each IGHV primer set.

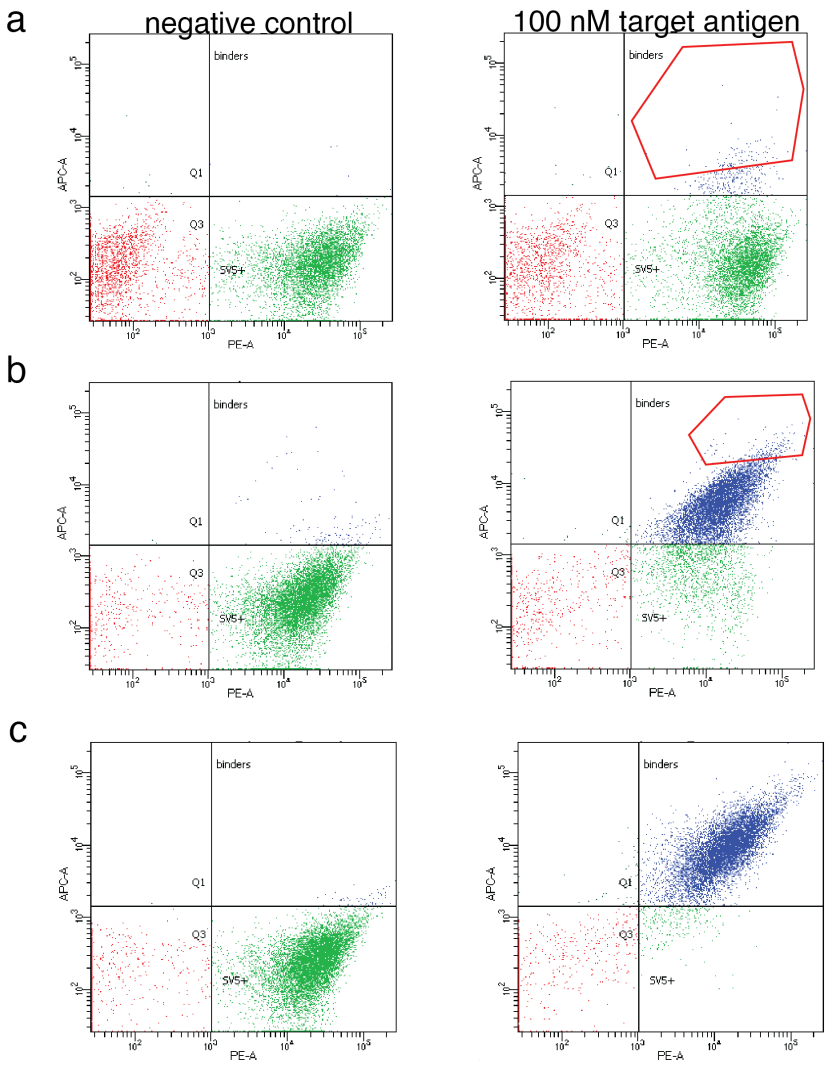
	Gene On-target	Gene Off-target	Family On-Target	Family Off-Target
IGHV1a	60	40	62	39
IGHV1b	0.2	99.8	99.4	0.6
IGHV1c	60	40	85	15
IGHV1d	6	94	67	33
IGHV2a	77	23	96	4
IGHV2b	61	39	99	1
IGHV3a	30	70	53	47
IGHV3b	22	78	60	40
IGHV3c	45	55	70	30
IGHV4	96	4	98	2
IGHV5a	31	69	31	69
IGHV6a	50	50	50	50

Supplementary table 3. Clonotype analysis.

	GM-CSF	OX40	B7-H4	CD40-L
Unique HCD3_Clonotypes Hamming ≤ 1, ≥ 2 Reads	386	234	395	394
Unique HCD3_Germline Clonotypes Hamming ≤ 1, ≥ 2 Reads	905	452	1137	913
# Reads	62689	59780	55013	54881

Number of the different HCDR3 clonotypes identified after NGS analysis of the selection campaign outputs. HCDR3 are clustered into a “Unique HCDR3 clonotype) if the Hamming distance is ≤1 and abundance > 1. Clones are considered to belong to an “Unique HCDR3-Germline clonotypes” if the Hamming distance is ≤1, they belong to a different IGHV germline and the abundance is > 1).

Supplementary Figure 1.



The yeast cells were analyzed simultaneously for: i) the phycoerythrin signal (PE), related with the display of the scFvs thanks to the detection of their V5 tag with and anti-V5 PE-conjugated antibody, ii) and allophycocyanin signal (APC) related with the detection of the biotinylated antigen by the scFvs thanks to the use of strepatavidin-APC conjugated. Four gates were generated. The “binders” gate represents double positive cells: scFvs are displayed and they bind to the target. In this gate the sort gate was established (in red). **a** Right after the subcloning of the phage selection output into yeast a sorting gate is designed corresponding to ~0.5-1% of the scFv-displaying yeast cells. **b** During the analysis of the cells after the first sort, a sorting gate is designed corresponding to ~0.5-1% of the scFv-displaying yeast cells. **c** The resulting population after two rounds of sorting show that all the displaying yeast cells bind to the specific antigen and not the negative control one.