

SUPPLEMENTARY INFORMATION

A high throughput bispecific antibody discovery pipeline

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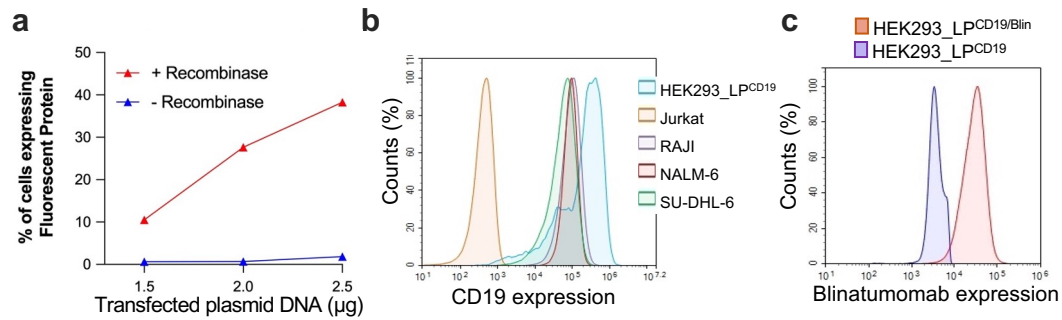
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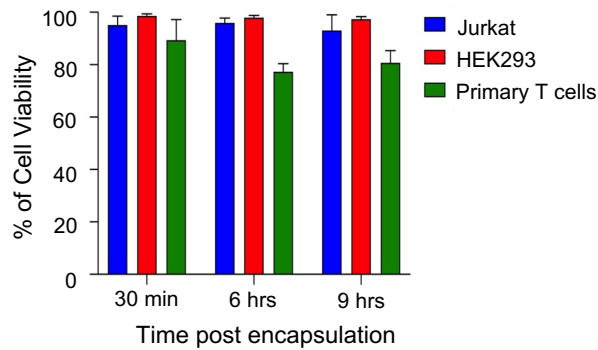
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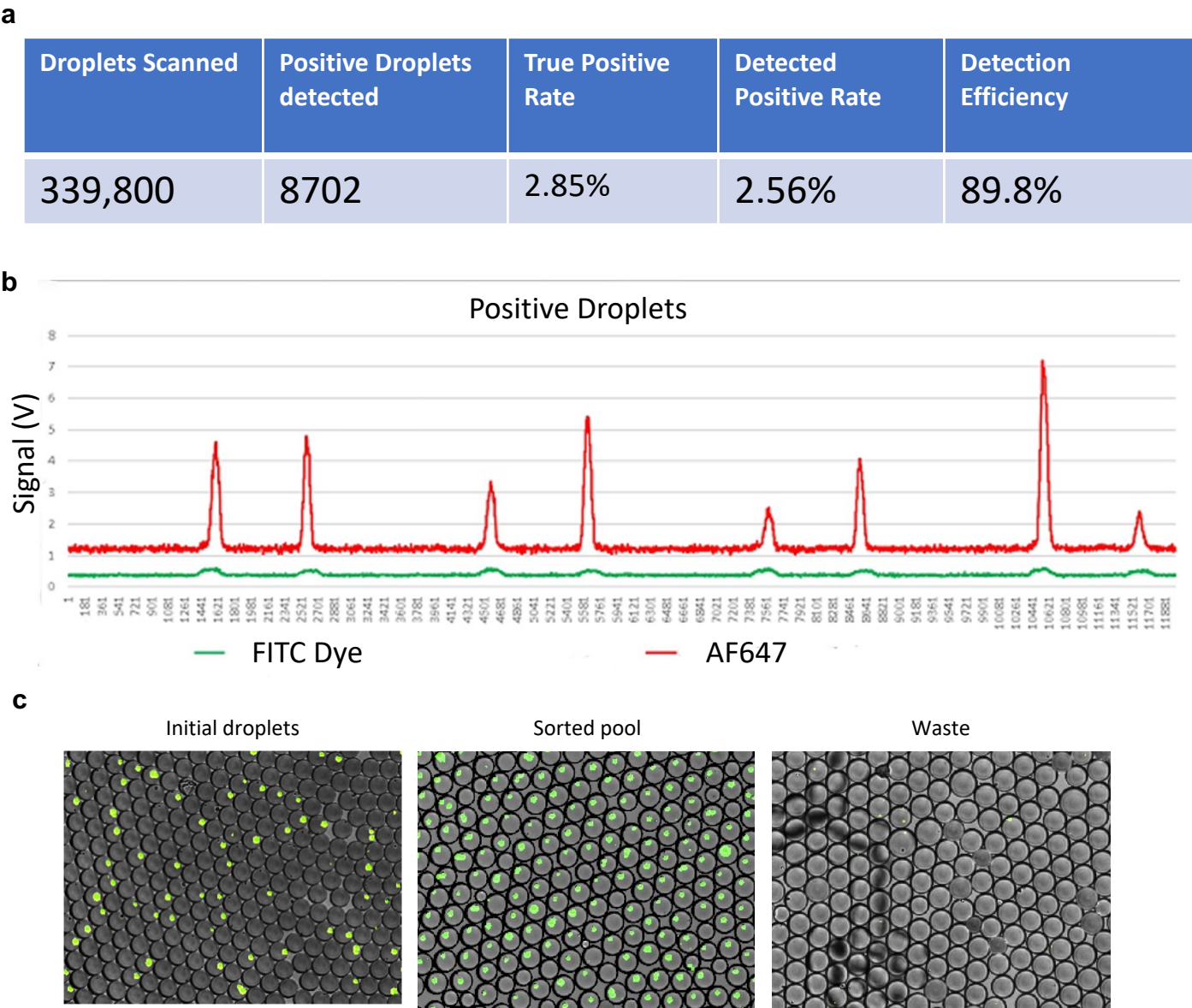
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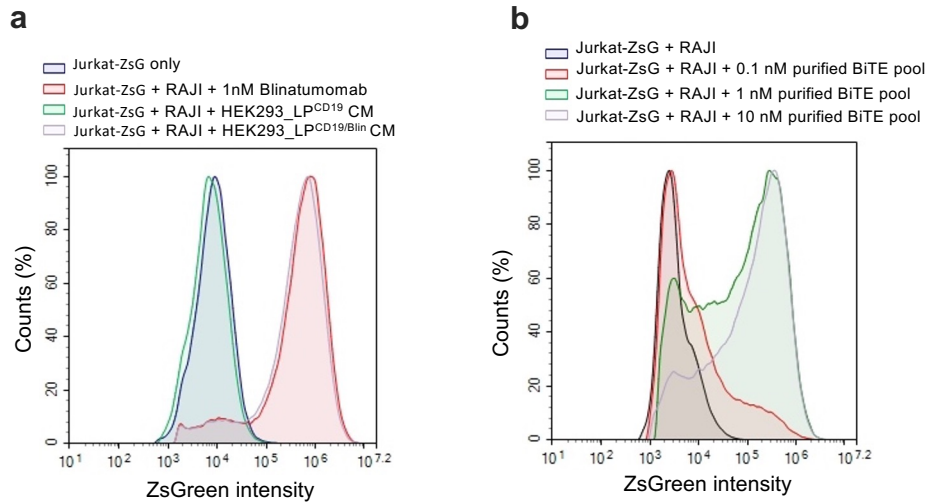
Supplementary Fig. 1. Establishment of a HEK293 cell line expressing CD19 (target) and a positive CD19xCD3 BiTE (Blinatumomab). (a) Line graph showing the recombina-se-dependent integration efficiency at the landing pad of the HEK293 cells, using a mCherry-encoding donor plasmid as a test material. (b) Flow cytometry profiling of a selected clone showing moderate to high CD19 (“HEK293_L^{CD19}”), compared to a few lymphoma lines (RAJI, NALM-6 and SU-DHL-6); Jurkat served as a negative control. (c) Flow cytometry validation of HEK293_L^{CD19/Blin} cells with a CD19xCD3 BiTE (Blinatumomab) that is integrated into the landing pad. The expression of Blinatumomab (6xHis-tagged) was validated using the cell staining with an anti-6xHis antibody.



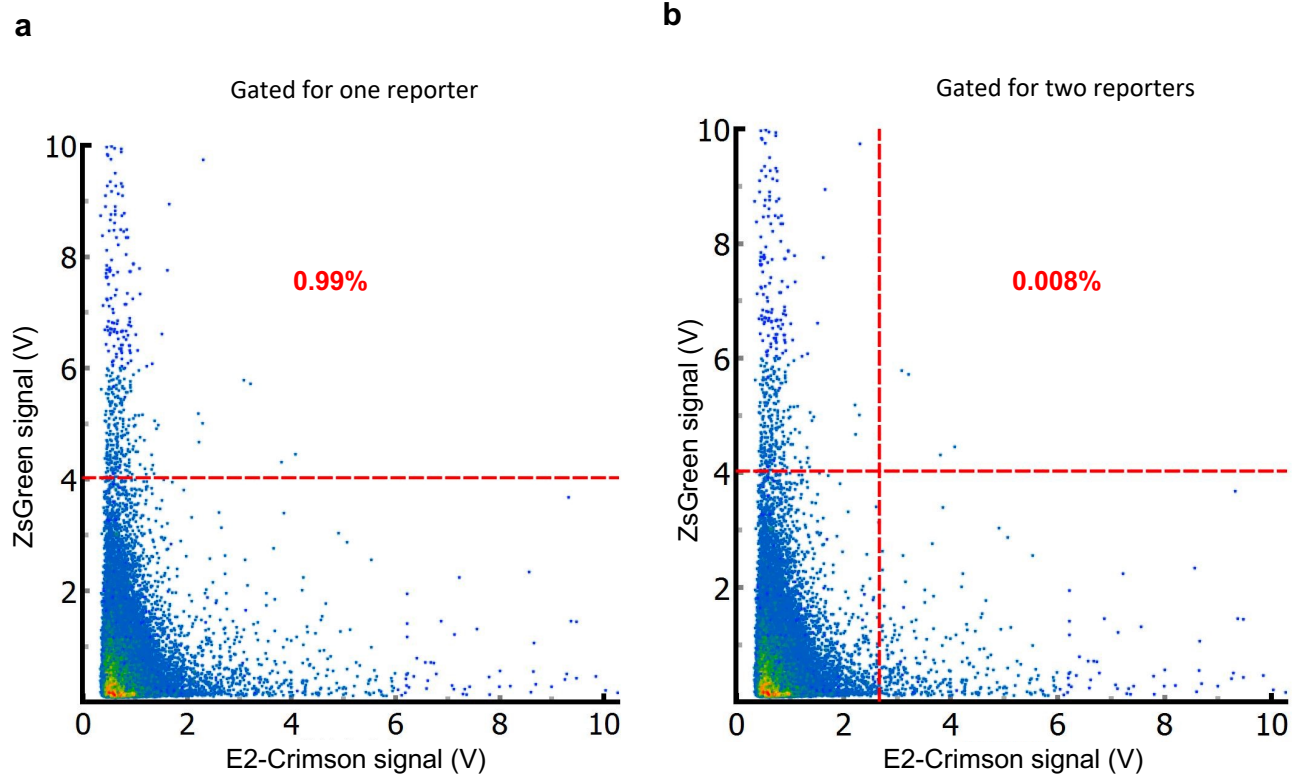
Supplementary Fig. 2. The viability profile of a few human cell types over a duration that is typical of screening assays conducted on single cell discovery platform. Cells are respectively encapsulated in 250-pL droplets and incubated at 37°C under 5% CO₂. Droplets are de-emulsified to recover cells in bulk for subsequent 7AAD staining followed by flow cytometry. Jurkat (E6.1), a human leukemia T cell line; HEK293, an immortalized embryonic kidney epithelial cell line; primary T cells: enriched from human PBMCs. Values of mean from $n = 2$ encapsulation experiments are presented as bar graphs with $mean \pm SD$ error bars.



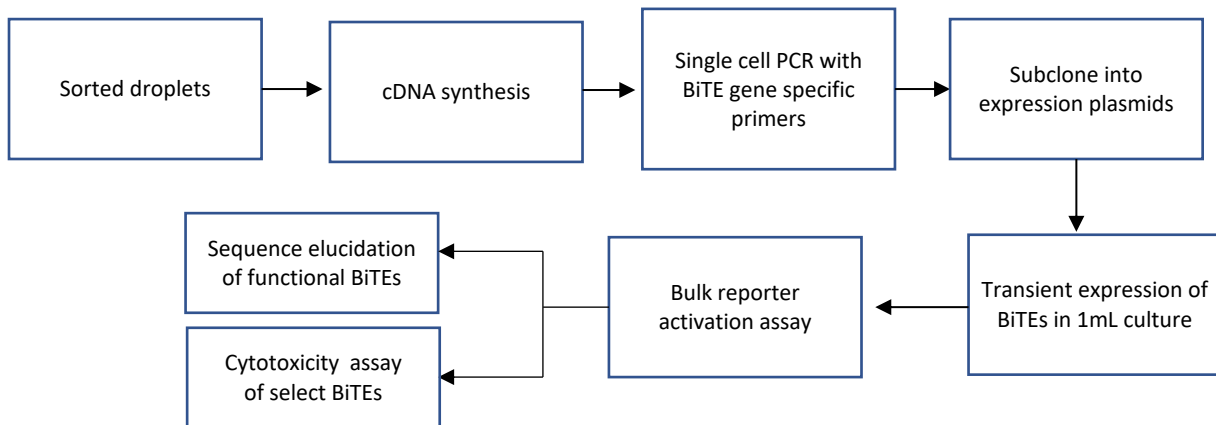
Supplementary Fig. 3. (a) Detection efficiency of our single cell discovery platform. The detection efficiency for intra-droplet fluorescent particles is approx. 90%. This performance metric is determined by an experiment conducted using FITC dyed droplets allowing the detection of empty droplets, spiked with FC AF647 category 3 calibration beads to simulate cells. Beads are spiked into droplets to achieve a calculated positive rate of 3% (estimated percentage of droplets containing beads). Microscope imaging was then used to verify the actual positive rate to be 2.85%. The system then scanned 339,800 droplets and detected 8702 positive droplets using a threshold set at 3 times the Signal-to-Noise (SNR), which is a positive rate of 2.56%. We then divided the system's detected positive rate with the true positive rate to estimate the detection efficiency (89.8%). This experiment was triplicated ($n = 3$). **(b) Representative PMT detection of positive droplets.** **(c) Microscopic images for droplets before sorting (left), sorted pool(center), and waste pool(right).** Green, GFP reporter signal in activated individual reporter T cells (Jurkat) inside droplets (bright field image). Scale bar, 200 μm .



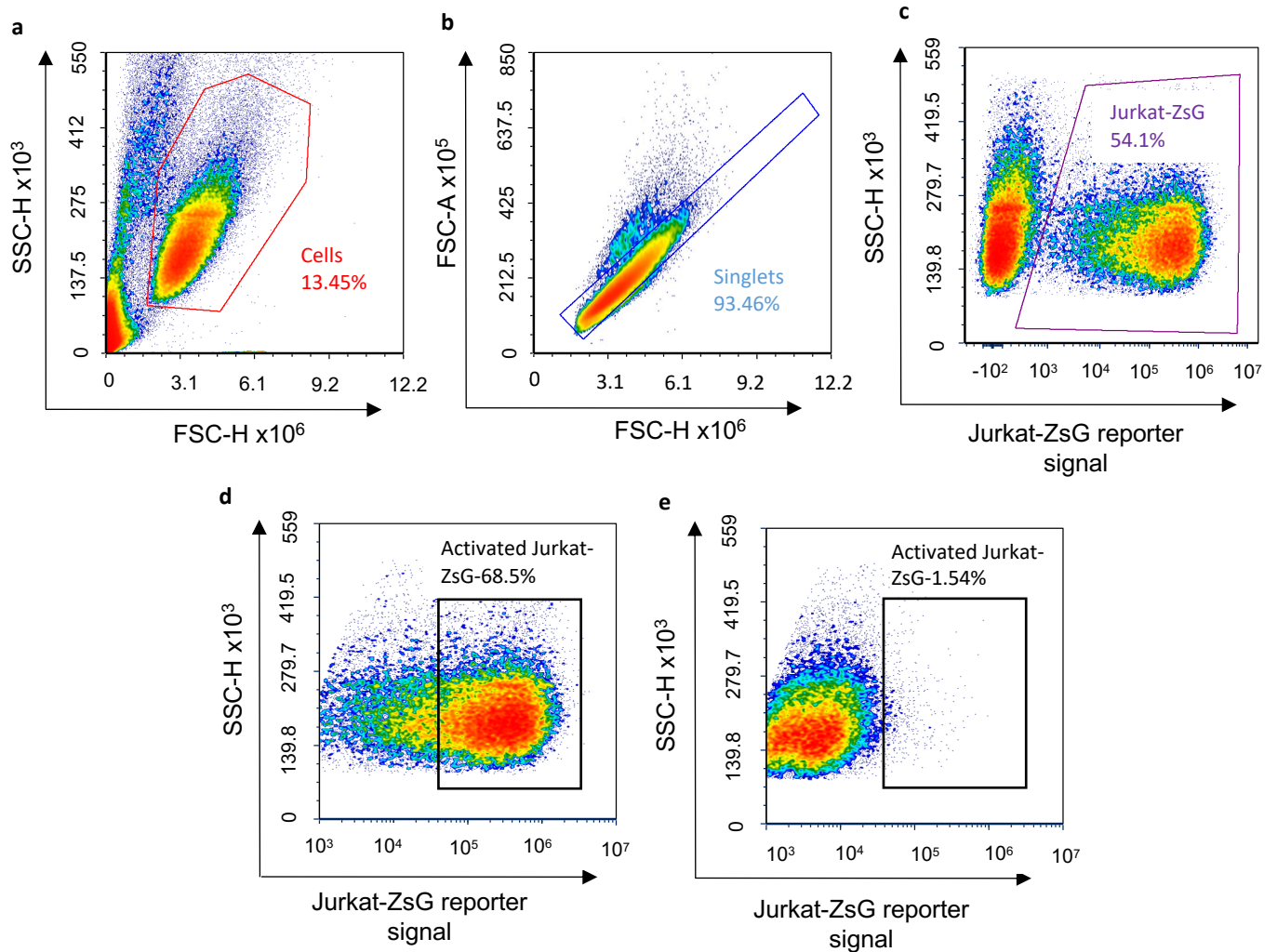
Supplementary Fig. 4. Functional validation of secretion of Blinatumomab and BiTE library by assay of cell culture supernatants. (a) Blinatumomab containing cell culture supernatants (CM) mixed with a co-culture of Jurkat-ZsG and Raji cells for 24 hrs activated Jurkat-ZsG reporter cells. Media containing 1nM recombinant Blinatumomab is used as positive control. **(b)** Jurkat-ZsG reporter cells were co-cultured with HEK293^{CD19} for 24 hours in the presence of purified BiTE pool (0.1, 1 and 10 nM respectively) from the day-3 condition medium of the library cell culture. Reporter activation was measured using flow cytometry.



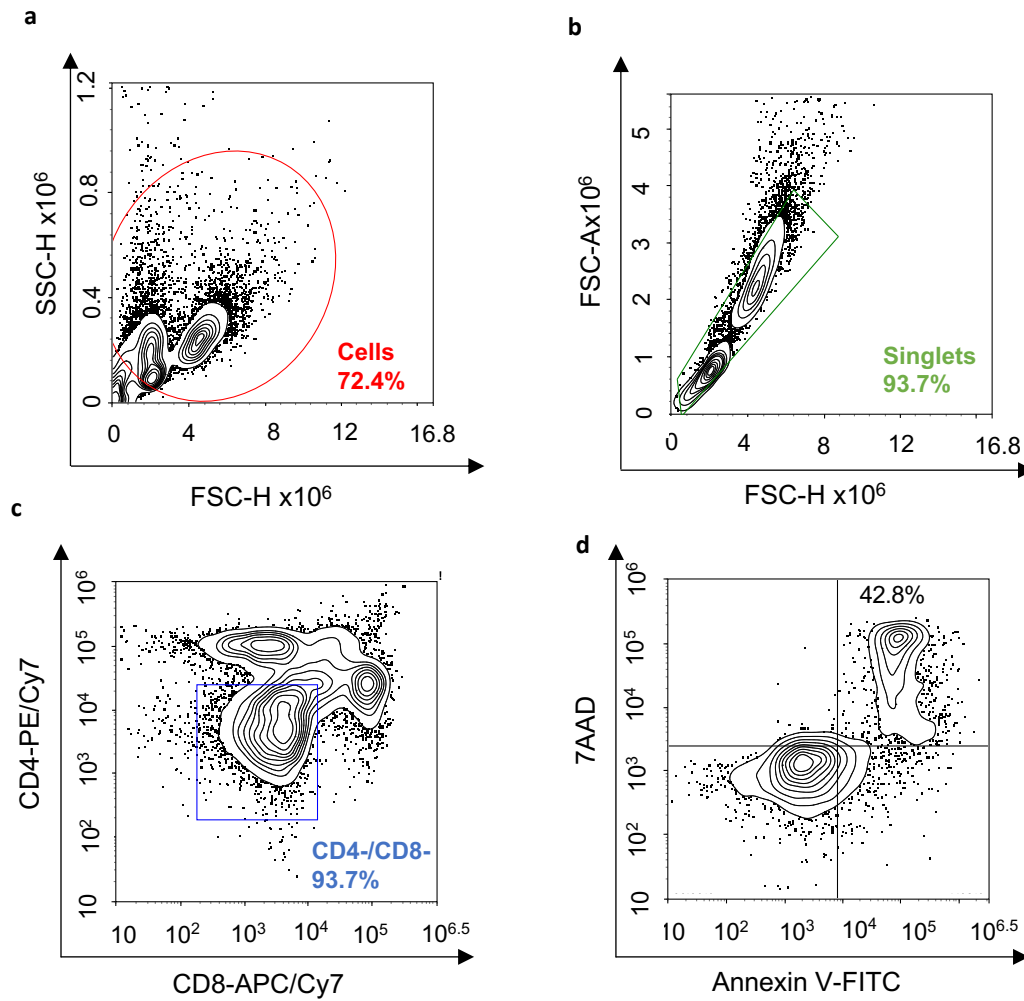
Supplementary Fig. 5. Representation of false positivity rate from detection modules of the single cell discovery platform (a) False positive rate when only ZsGreen reporter signal is used. **(b)** False positive rate when both ZsGreen and E2-Crimson signal are used.



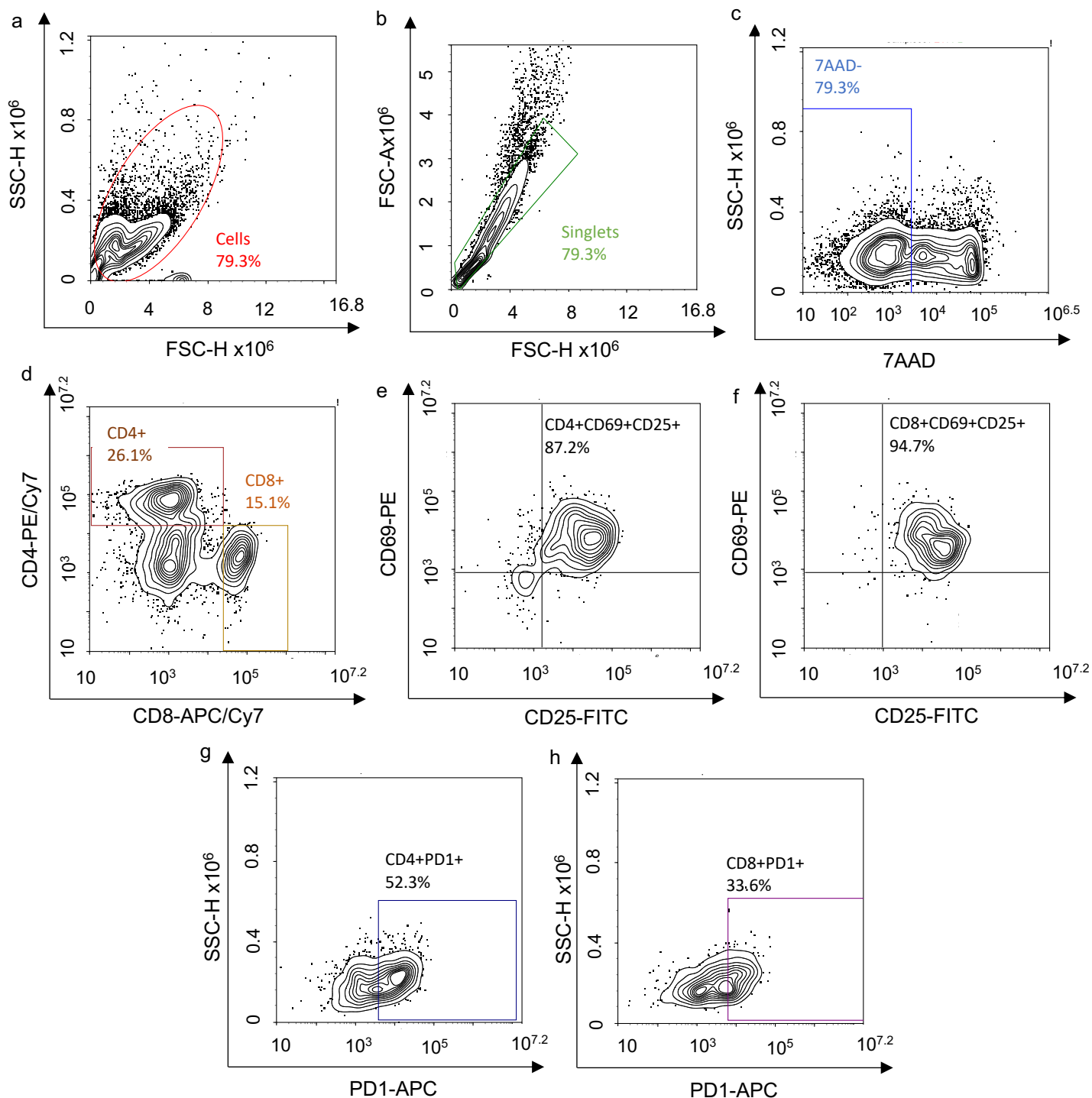
Supplementary Fig. 6. Flowchart illustrating downstream processes after sorting.



Supplementary Fig. 7. Flow cytometry gating strategy for bulk reporter activation assay. BiTE clones were expressed in Expi293FTM cells and conditioned media from Day 3 expression was used in a coculture of Raji and Jurkat ZsG cells. 24 hrs after co-culture, ZsGreen protein expression from Jurkat-ZsG cells was analyzed by flow cytometry. Representative gating strategy for identifying population of activated Jurkat-ZsG cells is shown for the case where Raji and Jurkat-ZsG cells were co-incubated with Blinatumomab conditioned medium. **(a)** Cell debris was first excluded based on FSC-H/SSC-H gating. **(b)** Singlets were isolated with FSC-H/FSC-A gates. **(c)** Raji cells were then excluded based on basal ZsG expression from Jurkat-ZsG reporter cells. **(d)** Percentage of activated reporter cells (*bottom left*) were identified by gating for Jurkat-ZsG cells expressing ZsGreen over basal level. **(e)** A negative control consisting of Jurkat-ZsG and Raji cells co-incubated with media from mock transfected Expi293FTM cell line was used to identify basal Jurkat-ZsG fluorescence.



Supplementary Fig. 8. Flow cytometry gating strategy for in-vitro cytotoxicity assay. PanT cells isolated from donor PBMCs were co-incubated with Raji cells in presence of purified BiTE clones at different concentrations. 48 hrs after co-culture, cytotoxicity was assessed by flow cytometry. Gating strategy for the flow cytometric analysis is shown for a representative case where panT cells and Raji cells are co-incubated with recombinant Blinatumomab (used as positive control at 0.125 nM). **(a)** Cell debris was excluded with FSC-H/SSC-H gating. **(b)** Singlets were identified using gates for FSC-A/FSC-H. **(c)** Raji cells were identified as a non-T cell population (double negative CD4-CD8- population). **(d)** Annexin V+ and 7AAD+ cells were identified from Raji cell population (*top right quadrant*).



Supplementary Fig. 9. Flow cytometry gating strategy for in-vitro cytotoxicity assay. PanT cells isolated from donor PBMCs were isolated from co-incubated with Raji cells in presence of purified BiTE clones at different concentrations. 48 hrs after co-culture, activation was assessed by flow cytometry. Gating strategy for the flow cytometric analysis is shown for a representative case where panT cells and Raji cells are co-incubated with recombinant Blinatumomab (used as positive control at 0.125 nM). **(a)** Cell debris was excluded with FSC-H/SSC-H gating. **(b)** Singlets were identified using gates for FSC-A/FSC-H. **(c)** 7AAD- population was used to gate for live cells **(d)** CD4+ and CD8+ cells were identified. **(e)** Percentage of CD4+CD69+CD25+ cells were identified by subgating for CD69+ and CD25+ from the CD4+ population. **(f)** Percentage of CD8+CD69+CD25+ cells were identified by subgating for CD69+ and CD25+ from the CD8+ population **(g)** Percentage of CD4+PD1+ population was obtained by subgating for PD1+ from the CD4+ population **(h)** Percentage of CD8+PD1+ population was obtained by subgating for PD1+ from the CD8+ population.

aCD19 scFv	Affinity (nM)	Epitope of binding	CDRH3 length	CDRH3-NNW randomized sequences/scFv	VH/VL orientation
4G7	8.4 nM	Conformationally similar epitope with FMC63, partially overlapping yet distinct from B43.	12	216	2
FMC63	0.4 nM	Discontinuous epitope partially overlapping with B43.	12	216	2
Juno 241	NA	NA	16	288	2
B43	1.5 nM	Loop 216-224, Loop 155-166 on CD19	15	270	1
Total Number of aCD19 variants					1710

Supplementary Table 1. aCD19 scFvs and their characteristics such as affinity, epitope and CDRH3 lengths.

The diversity generated in CD19 scFvs by performing site scanning mutagenesis on CDRH3 residues and altering relative orientation of VH-VL regions is also shown.

aCD3 scFv	Affinity (nM)	Epitope of binding	CDRH3/ Randomization	VH/VL orientation
OKT3	0.5 nM	Discontinuous epitope on CD3ε, middle of extracellular domain near a charged patch	No randomization of CDRH3 residues	2
L2K	110 nM	Overlapping epitope with OKT3		2
TRX4	0.09 nM	NA		2
h38E4.v1	1 nM	N-terminal portion of CD3ε extracellular domain		1
Total Number of aCD3 variants				7

Supplementary Table 2. aCD3 scFvs and their characteristics such as affinity, epitope of binding. Diversity in aCD3 scFvs was generated by altering relative orientation of VH-VL regions.

scFv connecting linker	Sequence	Characteristics
Linker 1	GGGGS	Flexible, uncharged, short
Linker 2	GGGGSGGS	Flexible uncharged, long
Linker 3	AEAAAKA	Rigid, charged, short
Linker 4	AEAAAKEAAKA	Rigid, charged, long

Supplementary Table 3. scFv interconnecting linkers chosen for library generation.

Linkers were varied based on flexibility, charge and length.

Theoretical library diversity			
Total number of aCD19 variants	Total number of aCD3 variants	Number of Linkers	Theoretical number of library variants
1,710	7	4	47,880

Supplementary Table 4. Theoretical library diversity of BiTE library calculated as product of number of aCD19 variants, number of aCD3 variants and number of linkers.

	4G7	B43	FMC63	JUNO241
OKT3	9	9	2	8
L2K	15	7	2	10
TRX4	9	8	4	10
h38E4.v1	2	3	0	1

Supplementary Table 5: Distribution of CD19 and CD3 scFv functional pairs in the sorted candidates

scFv sublibrary sequences	Orientation	Amino acid sequence	Target
OKT3-VH-Linker-VL	VH-VL	QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIG YINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCAR YYDDHYCLDYWGQGTTVTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAI MSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLAGVPA HFRGSGSGTSSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEIK	CD3 scFvs
L2K-VH-linker-VL	VH-VL	QVQLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIG YINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCAR YYDDHYCLDYWGQGTTVTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAI MSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPY RFSGSGSGTSSYSLTISSMEAEDAATYYCQQWSSNPFTFGAGTKLEIK	
hu38E4.v1_VH-linker-VL	VH-VL	QVQLQQSGAEVKKPGASVKVSCKASGFTFTSYIHWVRQAPGQGLEWIG WIYPENDNTKYNEKFKDRVITITADTSTSTAYLELSSLRSEDVAVYYCAR DGYSRYFYFDYWGQGTTVTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPDS LAVSLGERATINCKSSQSLNSRTRKNYLAWYQQKPGQSPKLLIYWTST RKSGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCKQSFILRTFGQGTK LEIK	
TRX4_VH-Linker-VL	VH-VL	QVQLQQGGGLVQPGGSLRLSCAASGFTFSSFPMAWVRQAPGKGLEWVST ISTSGGRYYRDSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKF RQYSGGFYDWGQGTTVTVSSVEGGSGGSGGSGGSGGVDDIQLTQPNVS TSLGSTVKLSCTLSSGNIENNYVHWYQLYEGRSPTTMIYDDDKRPDGP DRFSGSIDRSSNSAFLTIHNVAIEDEAIYFCHSYVSSFNVFGGGKLEIK	
OKT3-VL-Linker-VH	VL-VH	DIQLTQSPAISASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYD TSKLAGVPAHFRGSGSGTSSYSLTISGMEAEDAATYYCQQWSSNPFTFG SGTKLEIKVEGGSGGSGGSGGSGGVDDQVQLQQSGAELARPGASVKMSCK ASGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLT DKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTVTVSS	

hu38E4.v1_VL-linker-VH	VL-VH	DIQLTQSPDSLAVSLGERATINCKSSQSLLNSRTRKNYLAWYQQKPGQS PKLLIYWTSTRKSGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCKQSF ILRTFGQGTKLEIKVEGGSGGSGGSGGSGGVDQVQLQQSGAEVKKPGAS VKVSCKASGFTFTSYIHWVRQAPGQGLEWIGWIYPENDNTKYNEKFKD RVTITADTSTSTAYLELSSLRSEDVAVYYCARDGYSRYYFDYWGQGTTV TVSS	CD3 scFvs
TRX4-VL-Linker-VH	VL-VH	DIQLTQPNVSTSLGSTVKLSCTLSSGNIENNYVHWYQLYEGRSPTTMI YDDDKRPDGPDRFSGSIDRSSNSAFLTIHNVAIEDEAIYFCHSYVSSF NVFGGGTKLEIKVEGGSGGSGGSGGSGGVDQVQLQQGGGLVQPGGSLRL SCAASGFTFSSFPMAWVRQAPGKGLEWVSTISTSGGRYYRDSVKGRFT ISRDNKNTLYLQMNSLRAEDTAVYYCAKFRQYSGGFDYWGQGTTVTVS S	
Juno-241-VH-linker-VL	VH-VL	QVQLQQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVS GISWNSGSIGYADSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCAR DQGYHYDSEAHAFDIWGQTTVTVSSGGGGSGGGSGGGGSDIQLTQS PSSLSASVGDRVTVCQASQDISNYLNWYQQKPGRAPKLLIYDASNKA GVPSRFSGGSGTDFTLTISSLQPEDFATYYCQQSYSTPQAYTFGQGTK LEIK	CD19 scFvs
4G7-VH-linker-VL	VH-VL	QVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIG YINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCAR GTYYYGSRVFDYWGQGTTVTVSSGGGGSGGGSGGGGSDIQLTQAAPSI PVTSGESVVISCRSSKSLNSNGNTYLYWFLQRPQSPQLLIYRMSNLA SGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPTFTFGAGTKL EIK	
FMC63-VH-Linker-VL	VH-VL	QVQLQQSGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLG VIWGSETTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDETAIYYCAKH YYYGGSYAMDYWGQGTTVTVSSGGGGSGGGSGGGGSDIQLTQTTSSLS ASLGDRVTISCRASQDISKYLWYQQKPDGTVKLLIYHTSRLHSGVPSR FSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEIK	

B43-CD19-VL-linker-VH	VL-VH	DIQLTQSPASLAVSLGQRATISCKASQSVVDYDGDSYLNWYQQIPGQPPK LLIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTED PWTFGGGTKLEIKGGGSGGGGSGGGGSQVQLQQSGAELVRPGSSVKIS CKASGYAFSSYWMNWVKRPGQGLEWIGQIWPBGDGTNYNGKFKGKATL TADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGT TVSS	CD19 scFvs
Juno-241-VL-linker-VH	VL-VH	DIQLTQSPSSLSASVGDRVTVTCQASQDISNYLNWYQQKPGRAPKLLIY DASNVKAGVPSRFSGGSGTDFTLTISLQPEDFATYYCQQSYSTPQAY TFGQGTKLEIKGGGSGGGGSGGGGSQVQLQQSGGLVQPGSLRLSCA ASGFTFDDYAMHWVRQAPGKGLEWVSGISWNSGSIGYADSVKGRFTISR DNAKNSLYLQMNSLRAEDTAVYYCARDQGYHYYDSAEHAFDIWGQGT TVSS	
4G7-VL-linker-VH	VL-VH	DIQLTQAAPSIPVTPGESVSISCRSSKSLNSNGNTYLYWFLQRPQGSP QLLIYRMSNLASGVDPDRFSGSGSGTAFTLRISRVEAEDVGYYCMQHLE YPFTFGAGTKLEIKGGGSGGGGSGGGGSQVQLQQSGPELIKPGASVKM SCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDGTKYNEKFKGKAT LTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGT TVSS	
FMC63-VL-linker VH	VL-VH	DIQLTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIY HTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTF GGGTKLEIKGGGSGGGGSGGGGSQVQLQQSGPGLVAPSQSLSVTCTVS GVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNALSKSLTIIDNS KSQVFLKMNSLTDDTAIYYCAKHYYYGGSYAMDYWGQGT TVSS	

Blinatumomab® sequence used in HEK293_LP^{CD19/Blin} cell lines	CD19_{VL-VH}- CD3_{VH-VL}	DIQLTQSPASLAVSLGQRATISCKASQSVDDYDGD SYLNWYQQIPGQPPK LLIYDASNLVSGIPPRFSGSGSGTDFTLNHPVEKVDAAATYHCQQSTED PWTFGGGKLEIKGGGGSGGGSGGGGSQVQLQQSGAELVRPGSSVKIS CKASGYAFSSYWMNWVKRPGQGLEWIGQIWPGDGDTNYNGKFKGKATL TADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGT TVTVSSGGGGSQVQLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQ RPGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTS EDSAVYYCARYYDDHYCLDYWGQGT TVTVSSVEGGSGGSGGSGGSGVD DIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYD TSKVASGVPYRFSGSGSGTSYSLTISSEAEADAATYYCQQWSSNPLTFG AGTKLEIKHHHHHH	CD19xCD3 BiTE
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Supplementary Table 6: Amino acid sequences of CD3 and CD19 scFv sublibraries. N-terminal sequence of all VH domains was normalized to QVQLQQ and C-terminal domains of all VH sequences were normalized to TVTVSS. N-terminal sequence of all VL domains was normalized to DIQLTQ and C-terminal sequence of all VL domains was normalized to TKLEIK. The Blinatumomab sequence used for generation of control cell line HEK293_LP^{CD19/Blin} is also shown.

CDRH3_NNW_Primer	Primer sequence 5'-3'
4G7_VH_NNW_1	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGANNWACCTACTACTACGGCAGCCGGGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_2	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCNNWTACTACTACGGCAGCCGGGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_3	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACNNWTACTACTACGGCAGCCGGGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_4	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACNNWTACGGCAGCCGGGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_5	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACNNWGGCAGCCGGGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_6	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACNNWAGCCGGGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_7	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACGNNWCGGGTGTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_8	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACGGCAGCNNWGTGTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_9	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACGGCAGCCGNNWTTTCGATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_10	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACGGCAGCCGGGTGNNWGTATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_11	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACGGCAGCCGGGTGTTTCNNWTATTATTGGGGCCAGGGAACCACCG
4G7_VH_NNW_12	CCTGACCTCTGAGGACAGCGCCGTGTACTATTGTGCCAGAGGCACCTACTACTACGGCAGCCGGGTGTTTCGATNNWTGGGGCCAGGGAACCACCG
FMC63_VH_NNW_1	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGNNWTACTACTACGGCGGCAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_2	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACNNWTACTACTACGGCGGCAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_3	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACNNWTACGGCGGCAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_4	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACNNWGGCGGCAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_5	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACNNWGGCAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_6	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGNNWAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_7	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCNNWTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_8	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCAGCNNWGCATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_9	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCAGCTACNNWATGGATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_10	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCAGCTACGCCNNWGTATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_11	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGNNWTATTATTGGGGCCAGGGCACCACCGT
FMC63_VH_NNW_12	CCTGCAGACCGACGACACCGCCATCTACTACTGCGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGGATTATTGGGGCCAGGGCACCACCGT
Juno241_VH_NNW_1	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGANNWAGGGCTACCACTACTACGACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_2	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATNNWGGCTACCACTACTACGACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_3	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGNNWTACCACTACTACGACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_4	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCANNWCACTACTACGACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_5	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACNNWTACTACTACGACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_6	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCACNNWTACGACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_7	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACNNWACTCTGCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_8	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACNNWCTGCGGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_9	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACNNWCCGAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_10	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTNNWAGCAGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_11	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTGCCNNWACGCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_12	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTGCCAGNNWCGCTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_13	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTGCCGAGCAGCANNWTTCGATATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_14	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTGCCGAGCAGCGCANNWGTATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_15	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTGCCGAGCAGCGCTTCNNWATCTGGGGCCAGGGAACCACCGT
Juno241_VH_NNW_16	CCTGAGAGCCGAGGACACCGCCGTGTACTACTGTGCCAGAGATCAGGGCTACCCTACTACTACGACTCTGCCGAGCAGCGCTTCGATNNWTGGGGCCAGGGAACCACCGT
B43_VH_NNW_1	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGANNWGAACCACCACCGTGGGCAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_2	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGNNWACCACCACCGTGGGCAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT

B43_VH_NNW_3	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAANNWACCACCGTGGGCAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_4	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCNWACCCTGGGCAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_5	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCNWGTGGGCAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_6	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCNWGGCAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_7	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGNNWAGATATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_8	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCNNWTATTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_9	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGANNWTACTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_10	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGATATTNNWTACGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_11	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGATATTACNNWGCCATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_12	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGATATTACTACNNWATGGACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_13	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGATATTACTACGCCNNWACTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_14	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGATATTACTACGCCATGNNWTACTGGGGCCAGGGCACCACCGT
B43_VH_NNW_15	CCTGGCCAGCGAAGATAGCGCCGTGTACTTCTGTGCCAGACGGGAAACCACCACCGTGGGCAGATATTACTACGCCATGGACNNWTGGGGCCAGGGCACCACCGT

Supplementary Table 7: Primer sequences encoding NNW codons at each CDRH3 residue of CD19 scFvs

Oligo name	Oligo sequence	Decription
oBiTE_21	AGATCCTCCGCCTCCCTTGATTTCAGCTTGGT	GGGGS,VL-Cterm-Rev
oBiTE_22	AGATCCACCAGATCCTCCGCCTCCCTTGATTTCAGCTTGGT	GGGSGGS,VL-Cterm-Rev
oBiTE_23	AGCTTTAGCAGCGGCTTCAGCCTTGATTTCAGCTTGGT	AEAAKA,VL-Cterm-Rev
oBiTE_24	AGCTTTGGCAGCAGCTTCTTTAGCGCGGCTTCAGCCTTGATTTCAGCTTGGT	AEAAKEAAKA,VL-Cterm-Rev
oBiTE_25	GGAGGCGGAGGATCTCAGGTTTCAGCTGCAGCAG	GGGGS,VH-Nterm,Fwd
oBiTE_26	GGAGGCGGAGGATCTGGTGGATCTCAGGTTTCAGCTGCAGCAG	GGGSGGS,VH-Nterm,Fwd
oBiTE_27	GCTGAAGCCGCTGCTAAAGCTCAGGTTTCAGCTGCAGCAG	AEAAKA,VH-Nterm,Fwd
oBiTE_28	GCTGAAGCCGCCGCTAAAGAAGCTGCTGCCAAAGCTCAGGTTTCAGCTGCAGCAG	AEAAKEAAKA,VH-Nterm,Fwd
oBiTE_29	GGAGGCGGAGGATCTGACATCCAGCTGACACAG	GGGGS,VL-Nterm, Fwd
oBiTE_30	GGAGGCGGAGGATCTGGTGGATCTGACATCCAGCTGACACAG	GGGSGGS,VL-Nterm,Fwd
oBiTE_31	GCTGAAGCCGCTGCTAAAGCTGACATCCAGCTGACACAG	AEAAKA,VL-Nterm,Fwd
oBiTE_32	GCTGAAGCCGCCGCTAAAGAAGCTGCTGCCAAAGCTGACATCCAGCTGACACAG	AEAAKEAAKA,VL-Nterm,Fwd
oBiTE_33	AGATCCTCCGCCTCCAGAAGAAACGGTCACGGT	GGGGS,VH-Cterm,Rev
oBiTE_34	AGATCCACCAGATCCTCCGCCTCCAGAAGAAACGGTCACGGT	GGGSGGS,VH-Cterm,Rev
oBiTE_35	AGCTTTAGCAGCGGCTTCAGCAGAAGAAACGGTCACGGT	AEAAKA,VH-Cterm,Rev
oBiTE_36	AGCTTTGGCAGCAGCTTCTTTAGCGCGGCTTCAGCAGAAGAAACGGTCACGGT	AEAAKEAAKA,VH-Cterm,Rev
oBiTE_37	ATCATCCTGTTTCTGGTGGCCACAGCCACAGGCGCCTATGCTCAGGTTTCAGCTGCAGCAG	SS-VH_Nterm
oBiTE_38	ATCATCCTGTTTCTGGTGGCCACAGCCACAGGCGCCTATGCTGACATCCAGCTGACACAG	SS-VL-Nterm
oBiTE_39	CTTTAACAGAGAGAAGTTCGTGGCATGGTGATGGTGGTGGTGAGAAGAAACGGTCACGGT	VH_Cterm_P2A
oBiTE_40	CTTTAACAGAGAGAAGTTCGTGGCATGGTGATGGTGGTGGTGCTTGATTTCAGCTTGGT	VL_Cterm_P2A

Supplementary Table 8: Primer sequences used for assembly of scFv sequences from CD19-NNW-scFv sublibrary, scFv sequences from CD3-scFV-sublibrary, and linker oligos