Supplementary Figures and Data

Table S1. Potency of BFP receptor binding and redirected T cell killing of tumor cells

BFP	CD3 binding IC_{50} of SP34-PE (nM)	HLA binding EC_{50} (nM)	Tumor cell killing with non-activated T cells and exogenous antigen peptide EC_{50} (nM)[a]	Tumor cell killing with pre-activated T cells and endogenous antigen peptide EC_{50} (nM)[b]
NY-ESO-1 TCR-IgG	17	10	Inactive	n.d.[c], Inactive
NY-ESO-1 TCR-Fab	130	1.4	0.1 (90%)	n.d., 20 (90%)
NY-ESO-1 TCR-scFv	230±30[d]	2.5	7.0 (75%)	n.d., 200 (50%)
MAGE-A3 TCR-IgG	5.7	13	Inactive	Inactive, 0.6 (60%)
MAGE-A3 TCR-Fab	130	15	0.7 (100%)	0.7 (95%), 0.6 (100%)
MAGE-A3 TCR-scFv	110	14	2.1 (100%)	2.0 (95%), 1.0 (100%)

[a]NY-ESO-$1_{157-165}$ SLLMWITQC or MAGE-$A3_{168-176}$ EVDPIGHLY peptide was pre-incubated for 2 hours on Saos-2 or HCT116 cells, respectively, prior to the addition of T cells and BFP.
[b]T cells preactivated with anti-CD3/anti-CD28 and cultured with IL-2 were added with the BFPs onto HCT116 (left number) or A375 (right number) tumor cells. Numbers in parentheses indicates the percent tumor cell killing.
[c]n.d. = not done
[d]Average of 2 separate experiments

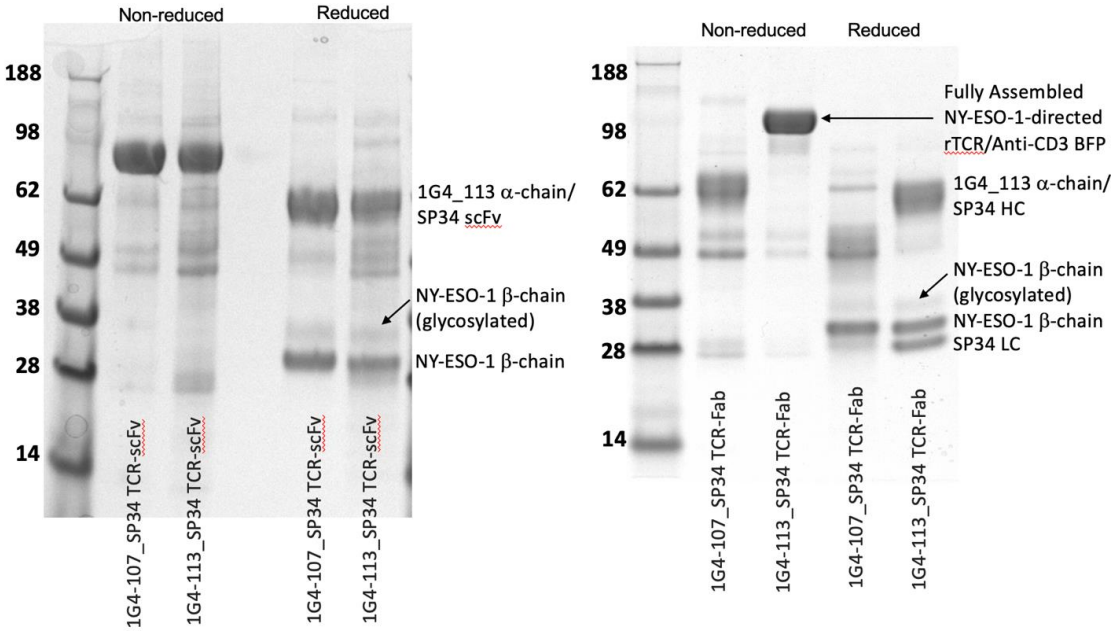

Figure S1. SDS-PAGE analysis of the NY-ESO-1 directed TCR-scFv constructs (left) and TCR-Fab constructs (right). Only the 1G4_113 molecules were used for further studies. Reduction was performed by adding 1 mM DTT prior to heating the samples and loading them onto the gels.

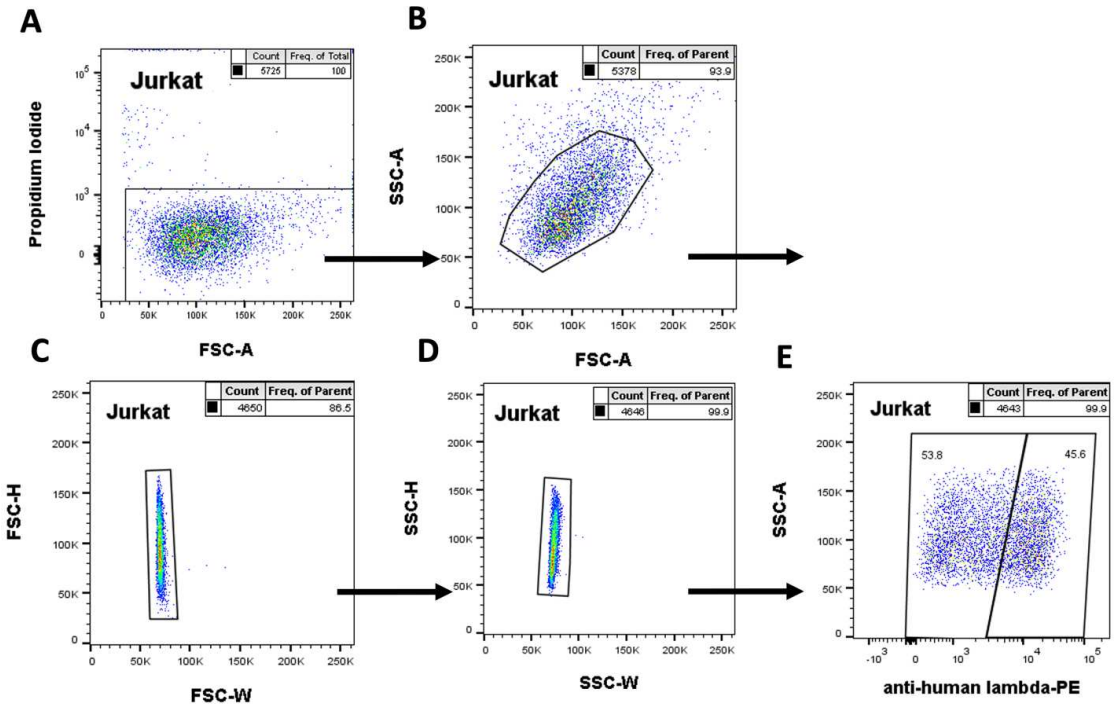

Figure S2. Gating Strategy for SP34-PE, TCR-IgG and TCR-Fab binding to CD3+ Jurkat cells. Jurkat cells were centrifuged and incubated for 45 minutes with rTCR/anti-CD3 BFPs. After washing, cells were stained with anti-human lambda-PE and incubated for 45 minutes. Propidium iodide was used as the live/dead cell discriminator. Acquisition was performed on a Becton Dickinson Fortessa using BD FACSDiva software v8.0.1. Analysis was performed using FlowJo v10.7.1. Cells were initially gated based on A: FSC-A vs Propidium Iodide to exclude dead cells. B: FSC-A vs SSC-A to identify cells based on light scattering. C and D: FSC-W vs FSC-H and SSC-W vs SSC-H for doublet discrimination. E: anti-human lambda-PE vs SSC-A was used to detect binding of the BFPs positivity while SP34-PE was directly labeled. Example shown uses the NY-ESO-1-directed TCR-IgG.

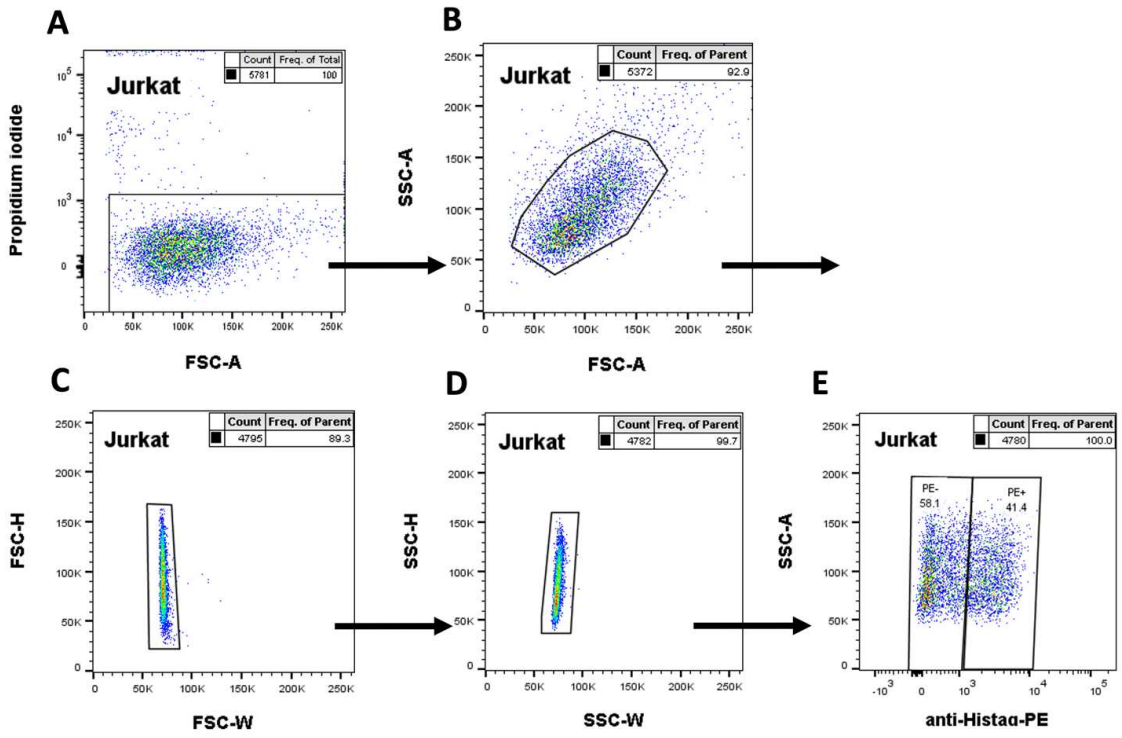

Figure S3. Gating Strategy for TCR-scFv binding to CD3+ Jurkat cells. Jurkat cells were centrifuged and incubated for 45 minutes with TCR BsAbs. After washing, cells were stained with anti-Histag-PE and incubated for 45 minutes. Propidium iodide was used as the live/dead cell discriminator. Acquisition was performed on a Becton Dickinson Fortessa using BD FACSDiva software v8.0.1. Analysis was performed using FlowJo v10.7.1. Cells were initially gated based on A: FSC-A vs Propidium Iodide to exclude dead cells. B: FSC-A vs SSC-A to identify the distribution of cells based on light scatter. C and D: FSC-W vs FSC-H and SSC-W vs SSC-H for doublet discrimination. E: anti-Histag-PE vs SSC-A for determination of positivity. Example shown uses the NY-ESO-1-directed TCR-scFv.

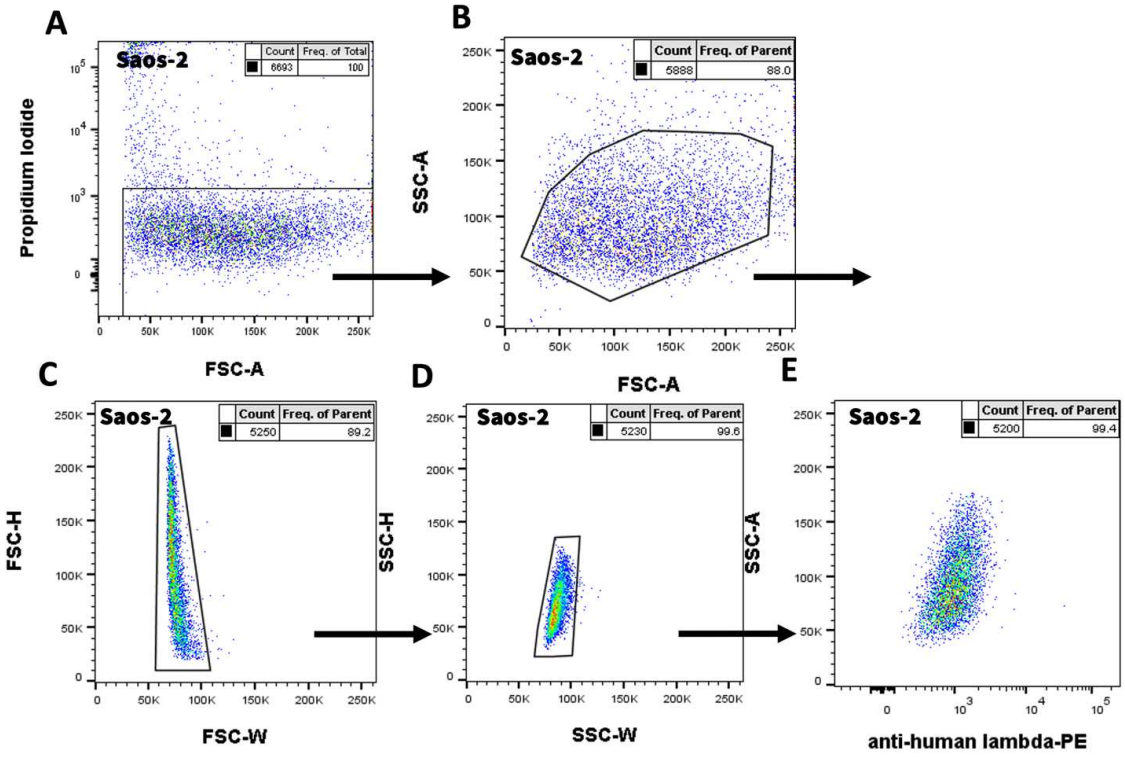

Figure S4. Gating Strategy for TCR-IgG and TCR-Fab binding to HLA-A2+ Saos-2 cells preloaded with NY-ESO-1$_{157-165}$ SLLMWITQC peptide. Saos-2 cells were pulsed with peptide for 3 hrs., centrifuged and incubated for 45 minutes with TCR BsAbs. After washing, cells were stained with anti-human lambda-PE and incubated for 45 minutes. Propidium iodide was used as the live/dead discriminator. Acquisition was performed on a Becton Dickinson Fortessa using BD diva software v8.0.1. Analysis was performed using FlowJo v10.7.1. Cells were initially gated based on A: FSC-A vs Propidium Iodide to exclude dead cells. B: FSC-A vs SSC-A to identify the distribution of cells based on light scatter. C and D: FSC-H vs FSC-W and SSC-H vs SSC-W for doublet discrimination. E: anti-human lambda-PE vs SSC-A for determination of positivity for TCR-IgG and TCR-Fab BFPs.

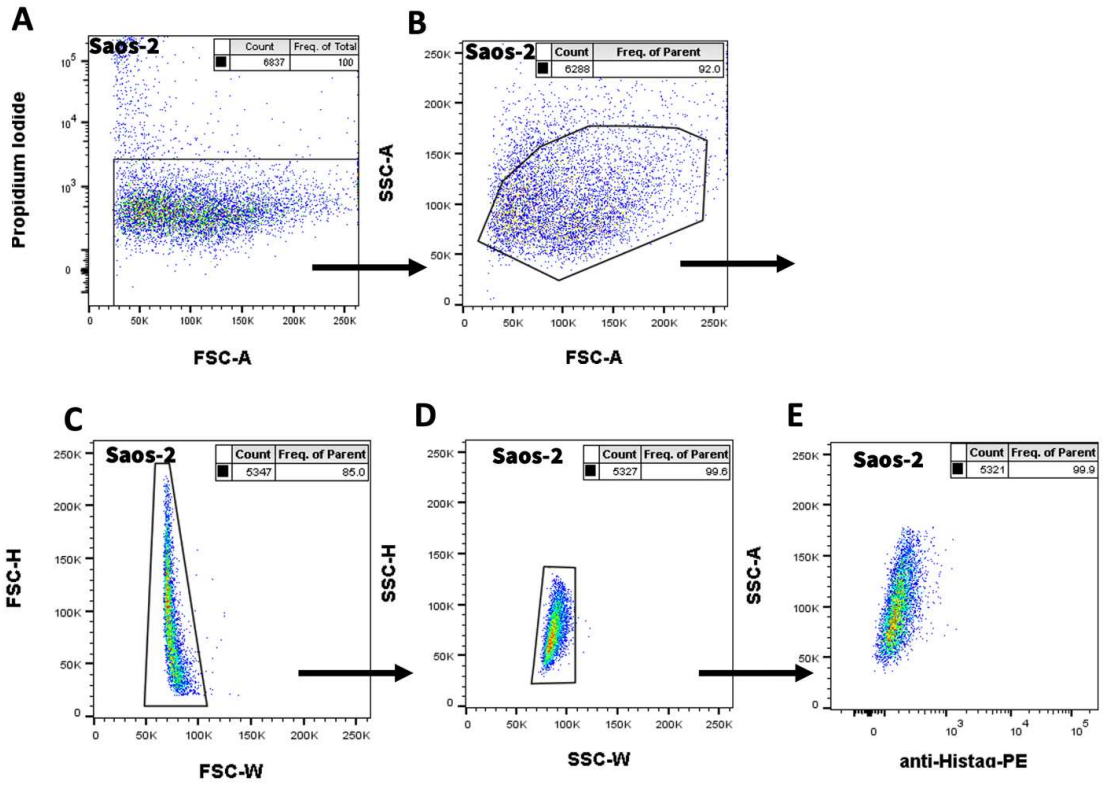

Figure S5. Gating Strategy for TCR-scFv binding to HLA-A2+ Saos-2 cells preloaded with NY-ESO-1$_{157\text{-}165}$ SLLMWITQC peptide. Saos2 cells were pulsed with peptide for 3 hrs., centrifuged and incubated for 45 minutes with TCR BsAbs. After washing, cells were stained with anti-Histag-PE and incubated for 45 minutes. Propidium iodide was used as the live/dead discriminator. Acquisition was performed on a Becton Dickinson Fortessa using BD diva software v8.0.1. Analysis was performed using FlowJo v10.7.1. Cells were initially gated based on A: FSC-A vs Propidium Iodide to exclude dead cells. B: FSC-A vs SSC-A to identify the distribution of cells based on light scatter. C and D: FSC-H vs FSC-W and SSC-H vs SSC-W for doublet discrimination. E: anti-Histag-PE vs SSC-A for determination of positivity.

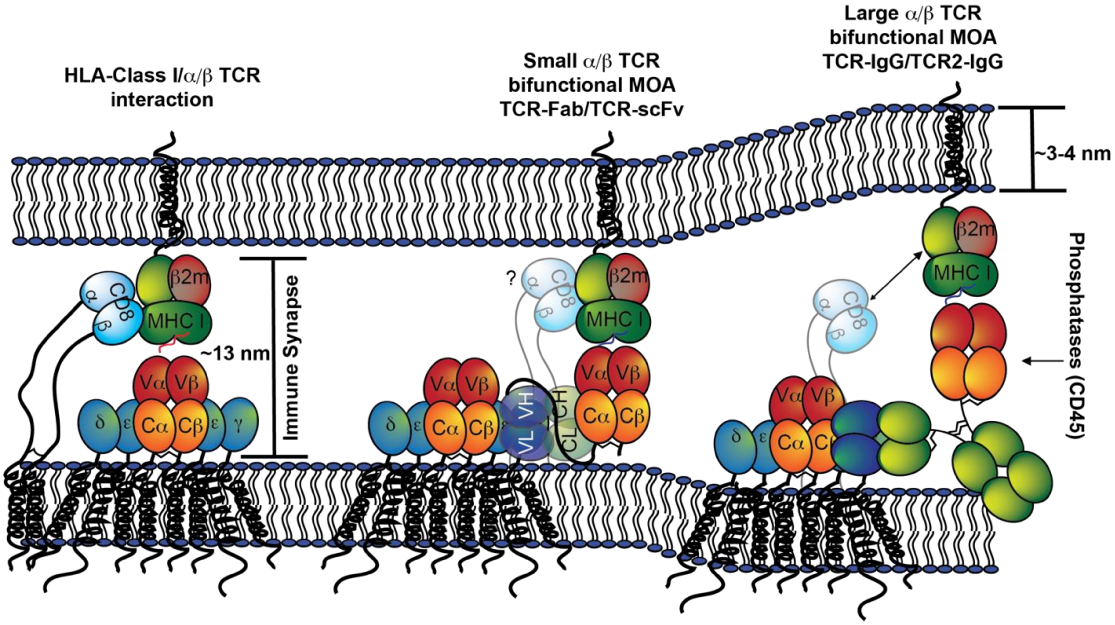

Figure S6. Schematic diagram depicting hypothetical challenges for the use of large and/or complex rTCR/Anti-CD3 BFPs. The natural TCR/MHC/CD8 complex formation is depicted on the left side of the diagram including the synaptic distance bridged by the TCR/MHC-peptide interaction as well as the CD8-coreceptor interaction. The rTCR/Anti-CD3 TCR-Fab and TCR-scFv molecules are depicted in the center of the diagram enabling similarly tight synapse formation, while the larger rTCR/Anti-CD3 BFPs exemplified by the TCR-IgG on the right-side of the diagram do not. The potential challenge of co-localizing the TCR-coreceptor CD8 is clear based on the diagram if the synaptic distance is too large.

Recombinant T cell Receptor/Anti-CD3 Bifunctional Protein Sequences

>MAGE-A3 TCR-scFv

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>MAGE-A3 TCR-Fab

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>MAGE-A3 TCR-IgG

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>NY-ESO-1 TCR-scFv

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SLSPGK

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>2TCR.1-Fab
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NCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDGTYIPTFGRG
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SIIPEDTFFPSPESSCGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVAR
IRSKYNNYATYYADSVKDRFTISRDDSQSLLYLQMNNLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTLVTVSAASTKGPSVFPL
APCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKR
VESKYG

QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEA
IYFCALWYSNLWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN
NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
YFCASSYLGNTGELFFGEGSRLTVLEDLKNVFPPEVAVFEPSKAEISRTQKATLVCLATGFYPPHVELSWWVNGKEVHDGVCTDPQ
PLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

>2TCR.2-Fab
HHHHHHHHGSQEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYI
AASQPGDSATYLCAVRPLLDGTYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCV
LDMRSMDFKSNSAVAWSNKSDFTCANAFNNSIIPEDTFFPSPESSCGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPKGSLKL
SCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSQSLLYLQMNNLKTEDTAMYYCVRHGNFGN
SYVSWFAYWGQGTLVTVSAASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
VVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGGGGGSGGGGSGGGGSGGGGSQEVTQIPAALSVPEGENLVLNCSFTDSAIY
NLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDGTYIPTFGRGTSLIVHPYIQ
NPDPAVYQLRDSKSSDKFVCLFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSDFTCANAFNNSIIPED
TFFPSPESSC

QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEA
IYFCALWYSNLWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN
NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
YFCASSYLGNTGELFFGEGSRLTVLEDLKNVFPPEVAVFEPSKAEISRTQKATLVCLATGFYPPHVELSWWVNGKEVHDGVCTDPQ
PLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

>2TCR.3-Fab
HHHHHHHHGSEVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDD
SQSLLYLQMNNLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTLVTVSAASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPV
TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGGGGSGGGGSGGGGSGGGGS
QEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSAT
YLCAVRPLLDGTYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKS
NSAVAWSNKSDFTCANAFNNSIIPEDTFFPSPESSCGGGGSGGGGSGGGGSGGGGSQEVTQIPAALSVPEGENLVLNCSFTDSAIY
NLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDGTYIPTFGRGTSLIVHPYIQ
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SPESSC

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IYFCALWYSNLWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN
NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
YFCASSYLGNTGELFFGEGSRLTVLEDLKNVFPPEVAVFEPSKAEISRTQKATLVCLATGFYPPHVELSWWVNGKEVHDGVCTDPQ
PLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

>2TCR.1-IgG
QEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSAT
YLCAVRPLLDGTYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKS
NSAVAWSNKSDFTCANAFNNSIIPEDTFFPSPESSCGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPKGSLKLSCAASGFTFN
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QGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVADYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLASVVTVPSSSLG
TQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRRPRVYTLPPSREEMTKNQVSLV
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSVLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTINQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLMSDGSFFLASKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSGGGGSQEVTQIPAALS
VPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDG
TYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSD
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NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
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PLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

>2TCR.2-IgG
EVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSQSLLYLQMN
NLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTLVTVSAASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGGGGSGGGGSGGGGSGGGGSQEVTQIPAALS
VPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDG
TYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSD
FTCANAFNNSIIPEDTFFPSPEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRRPRVYTLPPSREEMTKNQVSLV
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DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVL
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TTPPVLMSDGSFFLASKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGGGSGGGGSGGGGSGGGGSQEVTQIPAALS
VPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDG

TYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSD
FTCANAFNNSIIPEDTFFPSPEPKSC

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NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
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PLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

>2TCR.3-IgG
EVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSQSLLYLQMN
NLKTEDTAMYYCVRHGNFGNSYVSWFAYWGQGTLVTVSAASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGGGGSGGGGSGGGGSGGGGSQEVTQIPAALS
VPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLLDG
TYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSD
FTCANAFNNSIIPEDTFFPSPEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRRPRVYTLPPSREEMTKNQVSLV
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QEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLITPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSAT
YLCAVRPLLDGTYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKFVCLFTDFDSQINVSQSKDSDVYITDKCVLDMRSMDFKS
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DPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
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SLSPGK

QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEA
IYFCALWYSNLWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN
NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
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>2TCR.4-IgG
QEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLISPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSAT
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NSAVAWSNKSDFTCANAFNNSIIPEDTFFPSPEKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPETCVVVDVSHED
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EMTKNQVSLVCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSVLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
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AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYG

QEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLITPWQREQTSGRLNASLDKSSGRSTLYIAASQPGDSAT
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DPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
EEMTDNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLMSDGSFFLASKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEA
IYFCALWYSNLWVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSN
NKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC

GVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVAIQTTDRGEVPNGYNVSRSTIEDFPLRLLSAAPSQTSV
YFCASSYLGNTGELFFGEGSRLTVLEDLKNVFPPEVAVFEPSKAEISRTQKATLVCLATGFYPPHVELSWWVNGKEVHDGVCTDPQ
PLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADC