Electronic Supplementary Material

Site-specific fluorescent labeling of antibodies and diabodies using SpyTag/SpyCatcher system for *in vivo* optical imaging

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Supplementary Methods

Cloning

Expression plasmids were cloned using standard PCR methods and Gibson assembly [27]. To generate pFUSEss-CHIg-Nimotuzumab-hG1-SpyTag and pFUSEss-CHIg-MBP-hG1-SpyTag plasmids, we first we introduced SpyTag into the pFUSEss-CHIg-hG1 plasmid (Invivogen) at the C-terminus of the Fc domain to generate pFUSEss-CHIg-hG1-SpyTag plasmid. Nimotuzumab and anti-MBP VH domains were then introduced at the N-terminus of CH1 of the pFUSEss-CHIg-hG1-SpyTag plasmid to generate pFUSEss-CHIg-Nimotuzumab-hG1-SpyTag (See Elctonic Supplementary Material (ESM): SEQ:01) and pFUSEss-CHIg-Anti-MBP-hG1-SpyTag (see ESM SEQ:02) plasmids, respectively. To generate pFUSEss-CLIg-Nimotuzumab-hG1 and pFUSEss-CLIg-Anti-MBP-hG1 plasmids, nimotuzumab and anti-MBP VL domain were introduced at the N-terminus of CL of pFUSEss-CLIg-hG1-hk plasmid (Invivogen) respectively.

We used the previously reported pCW-SpyCatcher-His_6 [17] to clone the anti-HER3-diabody and anti-MBP-diabody. To generate pCW-anti-HER3-diabody-SpyCatcher-His_6 (see ESM SEQ:03) and pCW-anti-MBP-diabody-SpyCatcher-His_6 (see ESM SEQ:04) plasmids, the anti-HER3-diabody and anti-MBP-diabody were PCR amplified from pCW-anti-HER3-Fab and pCW-anti-MBP-Fab plasmids [28], respectively, using overlap extension primers TGS157 and KA3R primers [17]. The PCR product was cloned into *Sac1/Xho1*-digested pCW-SpyCatcher-His_6 plasmid using Gibson assembly.

Expression and purification of antibodies

Nimotuzumab-SpyTag and anti-MBP-SpyTag were expressed using the Gibco™ Expi293™

Expression System (Life Technologies, Catalog Number: A14635), according to the manufacturer's protocol. Briefly, one day before transfection, Expi293F cells were diluted to $2x10^6$ cells/ml in Expi293 Expression Medium (Life Technologies). On the day of transfection, 30 µg of plasmid DNA (1:1 ratio) was complexed with 80 µL ExpiFectamine™ 293 reagent. The complexed DNA was then transferred to $7.5x10^7$ cells (final cell density of $2.5x10^6$ cells/ml). The next day, Enhancer 1 and Enhancer 2 were added to the media to bring the final volume up to 30 ml. Cells were cultured for 6-7 days. Cells were spun down and supernatant was collected and filtered through a 0.45-micron membrane filter (Minisart, Sartorius Stedim). Protein A binding buffer (Sodium Phosphate 20 mM, 0.15 M NaCl, pH 7.2) was added to the supernatant and the antibody-SpyTag was purified by GE Healthcare AKTA FPLC system using HiTrap MabSelect column (GE healthcare). The antibody-SpyTag was eluted using IgG elution buffer (Fisher Scientific) and neutralized with Neutralization Buffer (1M Tris-HCl pH 9.0). Antibody-SpyTag was dialyzed overnight with phosphate-buffered saline (PBS) and concentrated using a 30K MWCO filter (Millipore). Fragments were filter sterilized and stored at -80°C.

Expression and purification of diabody-SpyCatcher fusions

Anti-HER3 diabody-SpyCatcher and anti-MBP diabody-SpyCatcher expression plasmids have a pelB sequence for mediating its secretion into the periplasmic space of *E. coli.* Plasmids were electroporated into RosettaTM (DE3) competent *E. coli* cells (Novagen) and cultured on LB agar plates containing carbenicillian (100 µg/m) and chloramphenicol (34 µg/ml). Single colonies were picked and cultured overnight in Instant TB media (Novagen) for 20 hrs at 30°C with shaking (250 RPM). Diabody-SpyCatcher fusions were purified with the AKTA FPLC system (GE Healthcare) using HiTrap Protein L column (GE healthcare) as described previously [17]. Briefly,

the cell pellet was collected by centrifugation and re-suspended in Protein L Binding buffer (Sodium Phosphate 20 mM, 0.15 M NaCl, pH 8.0). The cell pellet was lysed using a Cell Disruptor (Constant System LTD. USA) set at 35 Kpsi. The cell lysis solution was centrifuged at 12,000 x g for 20 min. The supernatant was collected and filtered through a 0.45-micron membrane filter (Minisart, Sartorius Stedim) and loaded onto a HiTrap Protein L column using Akta Prime system (GE Healthcare). Diabodies were eluted using IgG elution buffer (Fisher Scientific) and neutralized with neutralization buffer (1M Tris-HCl pH 9.0). Purified diabodies were dialyzed overnight in PBS and concentrated using 10K MWCO filter. Diabodies were filter sterilized and stored at -80°C.

Ligation of antibody-SpyTag with SpyCatcher-IRDye800CW and diabody-Catcher with SpyTag-IRDye800CW

Nimotuzumab-SpyTag and anti-MBP-SpyTag (10 µM) were ligated to SpyCatcher-IRDye800CW (30 µM) for 3 h at room temperature in the presence of phosphate-citrate buffer, pH 7, as described by Alam, et al. [17] and Zakeri, et al. [15]. Ligated products were named to reflect orientation the orientation of the SpyTag and SpyCatcher. For example, antibody-SpyCatcher reacted with SpyTag-IRDye800CW was labeled antibody-SpyCatcher/SpyTag-IRDy800CW. Nimotuzumab-SpyTag/ SpyCatcher-IRDye800CW and anti-MBP-SpyTag/SpyCatcher -IRDye800CW were purified using Protein A chromatography to remove unligated SpyCatcher-IRDye800CW. Anti-HER3 diabody-SpyCatcher and anti-MBP diabody-SpyCatcher (10 µM) were ligated to SpyTag-IRDye800CW (30 µM) using the same protocol described previously [17]. Diabody-SpyCatcher/SpyTag-IRDye800CW products were filtered through 10 kDa MWCO concentrator (Millipore). The filtration was repeated 4 times with PBS to remove unligated SpyTag-IRDye800CW.

Antibody-SpyTag/SpyCatcher-IRDye800CW and diabody-SpyCatcher/SpyTag-IRDye800CW were filter sterilized using Millipore Ultrafree MC Centrifugal Filter Device. The concentration was measured using the formula: Protein Conc (mg/ml) = (A280-(0.030A780))/εProtein × MWprotein × dilution factor. 0.03 is a correction factor for the absorbance of the IRDye800CW at 280 nm (equal to 3.0% of its absorbance at 780 nm). $\varepsilon_{Protein}$ is the molar extinction coefficient for protein. $MW_{protein}$ is the molecular weight of the protein. Dilution factor is the dilution of the labeled protein prior to measurement by spectrophotometer. The number of IRDye800CW molecules on the antibody or diabody were calculated using the formula: IRDye800CW/protein = $(A_{789}/\varepsilon_{IR})/(A_{280} - (0.03 \times A_{778})/\varepsilon_{Protein})$, where ε_{IR} is the molar extinction coefficient of IRDye800CW and $\varepsilon_{Protein}$ is the molar extinction coefficients of the antibody or diabody.

SDS-PAGE analysis

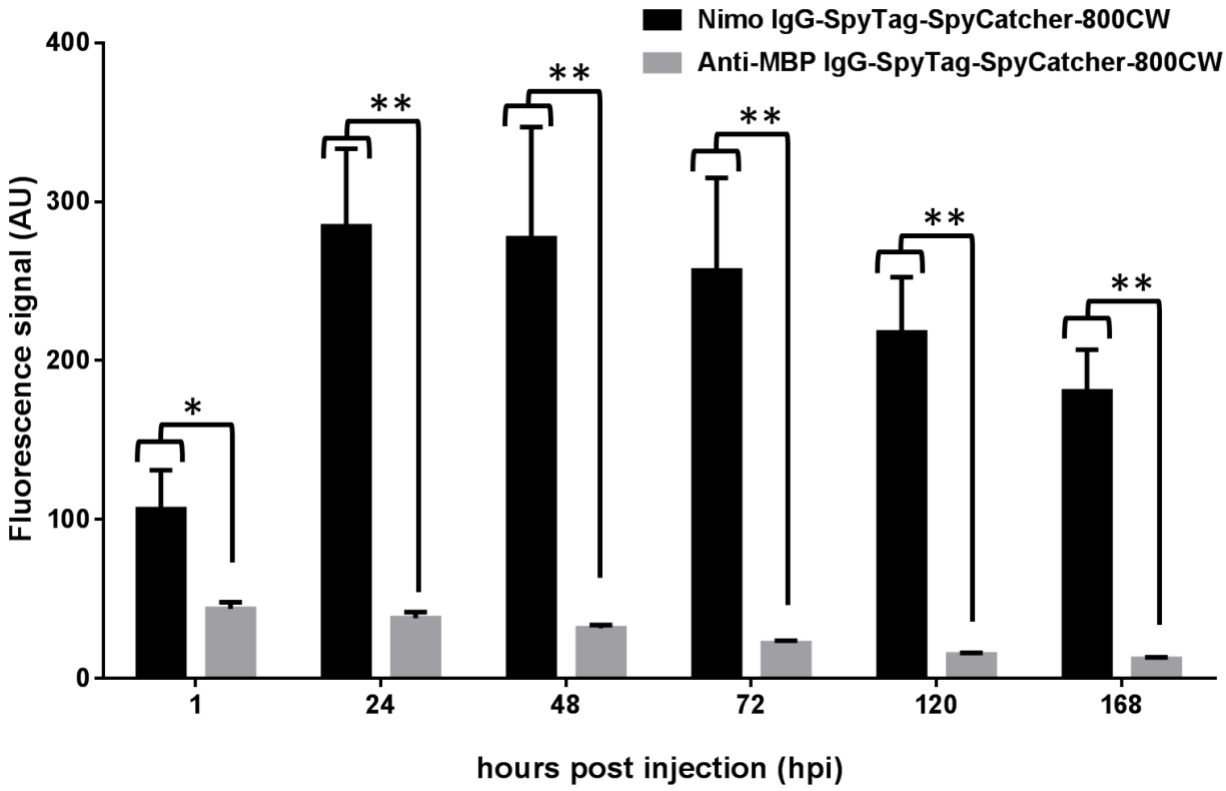
Purified IRDye800CW labeled and unlabeled antibodies and diabodies were resolved under reducing or non-reducing conditions using a precast BioRad 4–15% gel (BioRad, cat # 56-1084) with a BioRad PowerPac™ Cell. Gels were stained with coomassie blue. After destaining, protein bands were visualized by BioRad GelDoc XR^+ system. Unstained SDS-PAGE gels were scanned using the Odyssey Infrared Imaging system (LI-COR Bioscience) and images processed using the Odyssey 3.0.16 application software (LI-COR Bioscience).

Biolayer interferometry

Kinetic analyses were performed using a ForteBio OctetRed 384 instrument, according to the manufacturer's protocols. SpyCatcher/SpyTag Nimotuzumab constructs were immobilized to anti-human IgG Fc-capture sensors (ForteBio) and its interaction with the recombinant hEGFR (R&D system) analyte was measured. For the SpyCatcher/SpyTag anti-HER3 diabody,

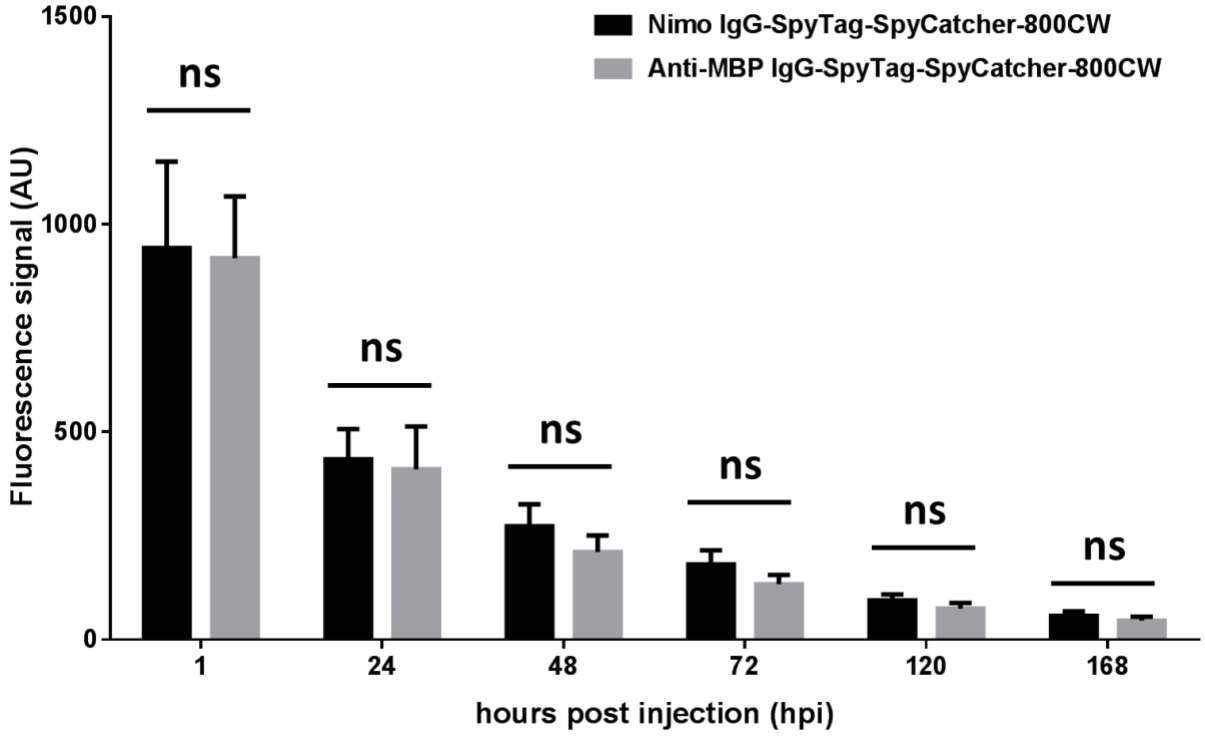
recombinant Fc-hHER3 (R&D system) was immobilized to anti-human IgG Fc-capture sensors (ForteBio) and its interaction with the diabody analyte was measured. The unlabeled anti-HER3 diabody was immobilized to amine reactive generation 2 (ARG2) sensors (ForteBio) and its interaction with the recombinant Fc-hHER3 (R&D system) analyte was measured. Antibodies and fragments were immobilized to sensors by dipping the sensor in a 384-well tilted-bottom plate, containing 50 µL of 10 - 12 µg/ml of antibody or fragment . Association rates (k_{on}) were monitored for 2-5 min and dissociation rates (k_{off}) were monitored for 10 min. Binding reactions were performed at 30°C in PBS. Data was collected with Octet Data Acquisition version 8.1 (ForteBio) and globally fit to 1:1 binding model using Octet Data Analysis version 7.1 (ForteBio).

Supplementary Figure S1



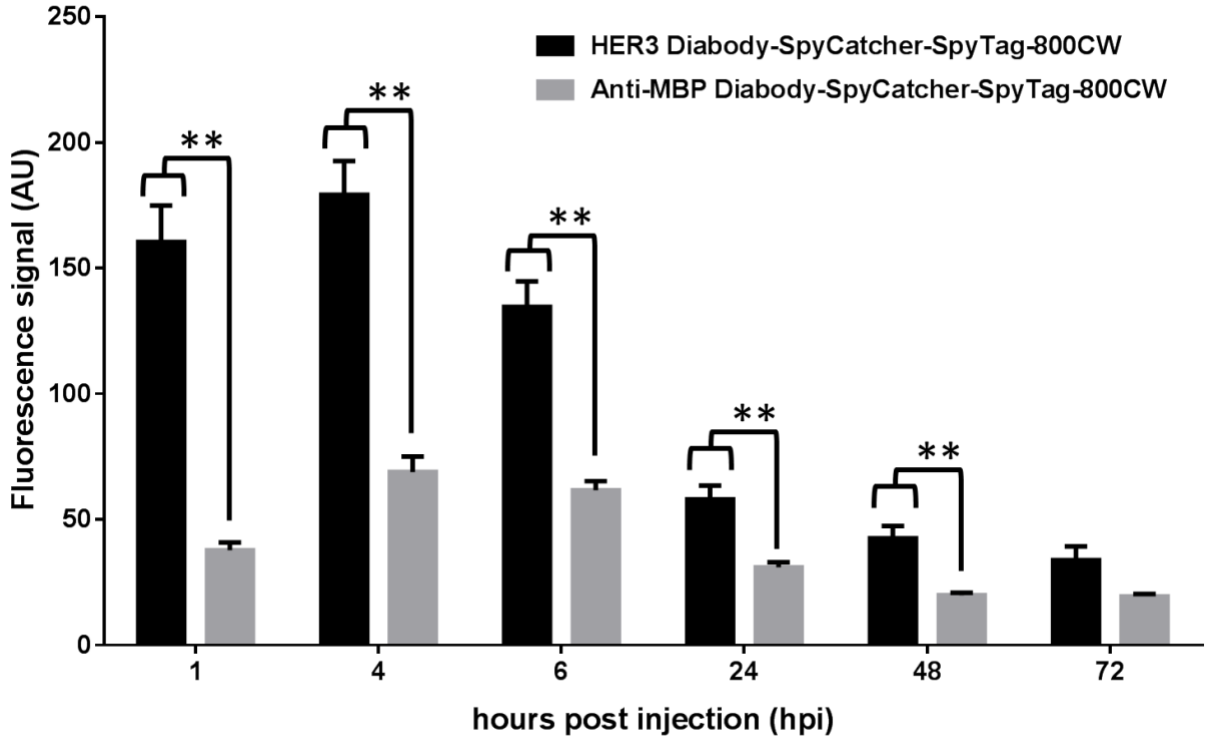
Supplementary Figure S1: In vivo xenograft biodistribution analysis of nimotuzumab IgG-SpyTag-Catcher-800CW and control anti-MBP IgG-SpyTag-Catcher-800CW in mice bearing A431 xenografts. Mean fluorescent signal (arbitrary units) for nimotuzumab IgG-SpyTag-Catcher-800CW (black), and control anti-MBP IgG-SpyTag-Catcher-800CW (gray) in A431 xenografts at 1, 24, 48, 72, 120, and 168 hours post injection (hpi). ** = p value < 0.01, AU represents arbitrary units. Error bars represent standard deviation.

Supplementary Figure S2



Supplementary Figure S2: In vivo liver biodistribution analysis of nimotuzumab IgG-SpyTag-Catcher-800CW and control anti-MBP IgG-SpyTag-Catcher-800CW in mice bearing A431 xenografts. Mean fluorescent signal (arbitrary units) for nimotuzumab IgG-SpyTag-Catcher-800CW (black), and control anti-MBP IgG-SpyTag-Catcher-800CW (gray) in liver at 1, 24, 48, 72, 120, and 168 hours post injection (hpi). ns = p value > 0.05, AU represents arbitrary units. Error bars represent standard deviation.

Supplementary Figure S3



Supplementary Figure S3: In vivo xenograft biodistribution analysis of anti-HER3 diabody-spycatcher-spytag-800CW and control anti-MBP diabody-spycatcher-spytag-800CW in mice bearing FaDu xenografts. Mean fluorescent signal (arbitrary units) for anti-HER3 diabody-spycatcher-spytag-800CW (black), and control anti-MBP diabody-spycatcher-spytag-800CW (gray) in FaDu xenograft at 1, 4, 6, 24, 48, and 72 hours post injection (hpi). ** = p value < 0.01, AU represents arbitrary units. Error bars represent standard deviation.

Supplementary sequences

Supplementary SEQ:01

Amino Acid sequence of Nimotuzumab-IgG1-SpyTag:

Heavy Chain-SpyTag (VH-CH1-Hinge-Fc-GGS-SpyTag):

QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIYWVRQAPGQGLEWIGGINPTSGGSNFNEKF
KTRVTITADESSTTAYMELSSLRSEDTAFYFCTRQGLWFDSDGRGFDFWGQGTTVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV
PSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG
KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GKGGSAHIVMVDAYKPTK

Light chain (VL-CL):

DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTYLDWYQQTPGKAPKLLIYKVSNRFSGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQITRRTVAAPSVFIFPPSDEQLK
SGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK
VYACEVTHQGLSSPVTKSFNRGEC

Supplementary SEQ:02

Amino Acid sequence of anti-MBP-IgG1-SpyTag:

Heavy Chain-SpyTag (VH-CH1-Hinge-Fc-GGS-SpyTag):

EISEVQLVESGGGLVQPGGSLRLSCAASGFNFSSSSIHWVRQAPGKGLEWVASISSSYGYTYYAD
SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARTVRGSKKPYFSGWAMDYWGQGTLVTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPGKGGSAHIVMVDAYKPTK

Light Chain (VL-CL):

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGVPSRFSGSR
SGTDFTLTISSLQPEDFATYYCQQSSYSLITFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVV
CLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEV
THQGLSSPVTKSFNRGEC

Supplementary SEQ:03

Amino Acid sequence of Anti-HER3-Diabody-SpyCatcher-His_6:

SDIQMTQSPSSLSASVGDRVTITCRASQGISNYLAWYQQKPGKAPKLLIYAASSLQSGVPSRFSGS
RSGTDFTLTISSLQPEDFATYYCQQYGWLPLTFGQGTKVEIKGGGGSEISEVQLVESGGGLVQPG
GSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNT
LYLQMNSLRAEDTAVYYCARTDPYSLGGYYFDYWGQGTLVTVSSGGSMVDTLSGLSSEQGQSG
DMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAA
PDGYEVATAITFTVNEQGQVTVNGKATKGDAHIGGSHHHHHH

Supplementary SEQ:04

Amino Acid sequence of Anti-MBP-Diabody-SpyCatcher-His_6:

SDIQMTQSPSSLSDIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASS
LYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSSYSLITFGQGTKVEIKGGGGSEISEVQLV
ESGGGLVQPGGSLRLSCAASGFNFSSSSIHWVRQAPGKGLEWVASISSSYGYTYYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYYCARTVRGSKKPYFSGWAMDYWGQGTLVTVSSGGSMV
DTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQVKDF
YLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHIGGSHHHHHH