Electronic Supplementary Material (ESI) for Chemical Science.

Supplemental Information

Targeting trimeric transmembrane domain 5 of oncogenic latent membrane protein 1 using a computationally designed peptide

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Materials and Methods

Rosetta design

The homotrimer of TMD5 was homology modeled according to a reasonable NMR coiled-coil structure (PDB ID 1AQ5) and validated via experiments previously[1, 2]. The anti-TMD5 peptide was designed using the Rosetta molecular modelling package[3]. The previously built model of TMD5 trimeric complex was used as the template and chain A was defined as the origin for the design of anti-TMD5[2]. The hydrogen-bonding network between the aspartic acid residues at position 150 was determined to be a driving force for the trimerization of the TMD5 peptides, thus the aspartic acid was retained in the anti-TM5 peptide design. The identities for all remaining anti-TMD5 sequence positions located at the trimeric interface were allowed to vary, while alternate side-chain conformations were sampled for the wild-type TMD5 peptides in order to search for a lower energy structure. A version of the Rosetta energy function with a dampened Lennard-Jones repulsion potential was used[4]. 50 designs were generated, and an anti-TMD5 sequence with the lowest score was selected for characterization based on the RosettaDesign energy function[5-8].

Molecular dynamics simulations

The homotrimeric TMD5 and the heterotrimer (one anti-TMD5 helix with lowest score in Rosetta and two wild type TMD5 helixes) were embedded in a1,2-dimyristoyl-sn-glycero-3-phosphocholine(DMPC) bilayer and solvated in a 62×62×78 $Å^3$ box filled with TIP3P water molecules via the CHARMM-GUI membrane builder protocol[9]. 150 mM KCl was added to neutralize the system and mimic the physiological concentration. The protein, lipids, and ions were described by the CHARMM36 force field[10, 11] and all MD simulations were performed by the NAMD2.12 program package[12].
The models with ~30,000 atoms were simulated under the NPT ensemble with a pressure of 1 atm at 310.15 K. The Nosé-Hoover Langevin piston method[13, 14] was applied for the pressure control and the Langevin thermostat for the temperature coupling. Long-range electrostatic interactions were treated by the particle mesh Ewald (PME) algorithm[15]. Non-bonded interactions were switched off at 10 ~ 12 Å. Periodic boundary conditions were applied in all directions and the time step was set as 2 fs. After a staged equilibration with a gradual decrease in harmonic constraints that act on heavy lipid and protein atoms only, further non-constrained relaxations were run for 100 ns for analysis.

Peptide synthesis

TMD5, Coumarin-tagged TMD5, anti-TMD5, FITC-tagged anti-TMD5, anti-TMD5 scramble, and FITC-tagged anti-TMD5 scramble were prepared by standard microwave-assisted solid-phase synthesis. The purity and identity of these peptide were confirmed by HPLC (>95%) and mass spectrometry (see Appendix Data). The concentrations of anti-TMD5/anti-TMD5 scramble and TMD5 were determined by absorbance at 280 nm using the extinction coefficient of 34490 $M^{-1}cm^{-1}$ and 6990 $M^{-1}cm^{-1}$, respectively. The concentration of coumarin labeled TMD5 peptide was determined by absorbance at 400 nm using an extinction coefficient of 39300 $M^{-1}cm^{-1}$. The concentration of FITC-tagged anti-TMD5/FITC-tagged anti-TMD5 scramble was determined by absorbance at 495 nm using an extinction coefficient of 84000 $M^{-1}cm^{-1}$.
Peptide Sequence:

TMD5	**KKKK-WQLLAFFLAFFLDLILLIIALYL-KKKK**
Coumarin-tagged TMD5	coumarin-**GKKKK-WQLLAFFLAFFLDLILLIIALYL-KKKK**
anti-TMD5	**KKKK-WWKLWYFLVWFLDLIILILLLWW-KKKK**
FITC-tagged anti-TMD5	FITC-**GKKKK-WWKLWYFLVWFLDLIILILLLWW-KKKK**
anti-TMD5 scramble	**KKKK-VFLWLWLIWIFIDWKLWLLYWLL-KKKK**
FITC-tagged anti-TMD5 scramble	FITC-**GKKKK-VFLWLWLIWIFIDWKLWLLYWLL-KKKK**

Fluorescence dequenching assay

100 μL of coumarin-tagged TMD5 peptide (50 nM) solution (50 mM HEPES, 150 μM C14 betaine, pH = 7.4) with the indicated concentration of unmodified anti-TMD5/anti-TMD5 scramble or NSC 259242 was pipetted into black 96-well plates in triplicate. Samples were mixed and allowed to sit at room temperature in the dark overnight to reach equilibrium and then excited at 360 nm and emission was read at 430 nm using a SYNERGY H1 Micro-plate Reader (BioTek Instruments, Carlsbad, CA, USA). The relative fluorescence intensity was normalized by TMD-5 fluorescence in the absence of TMD-5 inhibitor.

FRET assay

Fluorescence resonance energy transfer (FRET) experiments were conducted under room temperature in a 2×10 mm quartz cell (Starna Cells**,** Atascadero, CA, USA) on a Cary Eclipse spectrofluorimeter (Agilent Technologies, Santa Clara, CA, USA). 50 nM (7-hydroxy-3-carboxyamide coumarin)-labeled TMD5 peptide in 20 mM HEPES, pH = 7.4 and 1.0 mM C14 betaine micelles (or 1.0 mM bicelles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and dihexanoylphosphatidylcholine (DHPC), q = 0.3) buffer was titrated with increasing concentrations of FITC-tagged anti-

TMD5 or FITC-tagged anti-TMD5 scramble. 415 nm was chosen as the excitation. Emission at 400-600 nm was recorded. Samples containing the same amount of fluorescence acceptor (FITC-tagged anti-TMD5/ FITC-tagged anti-TMD5 scramble) served as a reference. The net FRET signals (total signal - reference signal) were used. The quenching of fluorescence signal of coumarin-labeled TMD5 peptide was analyzed by the equation, $\log (F_0/F-1) = -\log K_d + n \times \log ([\text{FITC-tagged anti-TMD5}])$.

Dominant-negative ToxR assay

Plasmids for this assay, pTox7 and pTox6, and the competent *E. coli* strain, FHK12, were kindly provided by Dr. Langosh, Technische Universität München, Germany. The pTox7 plasmid was modified by insertion of a single base (T) after the BamH1 site to keep the proper reading frame for the designed transmembrane sequences [16, 17]. TMD5-ToxR (pTox7-TMD5), anti-TMD5-ToxR* (pTox6-TMD5), anti-TMD5 scramble-ToxR* (pTox6-TMD5 scramble) and poly Leu-ToxR* (pTox6-poly Leu) constructs were made as described previously [16, 17].

ToxR construct (200 ng) and ToxR* construct (200 ng) were transformed into *E. coli* strain FHK12 by heat shock method. 50 µL of each transformation was used to inoculate, in triplicate, 5 mL of Luria-Bertani broth containing chloramphenicol (35 µg/mL), kanamycin (33 µg/mL) and 0.0025% arabinose. Cultures were incubated with shaking at 37 °C for 20 h and β-galactosidase activity was measured using a Beckman Coulter DTX 880 plate reader (Beckman Coulter, CA, USA). Briefly, 5 µL of culture was transferred to the wells of a Costar 3596 polystyrene 96-well plate (Corning, NY, USA) containing 100 µL Z buffer/chloroform (1% β-mercaptoethanol, 10% chloroform, 89% A buffer: 1 M sodium phosphate, 10 mM KCl, 1 mM MgSO4 and pH 7.0). Cells were lysed by addition of 50 µL Z buffer/ SDS (1.6% w/v sodium dodecyl sulfate in Z buffer) and shaking at 28 °C for 10 min. 50 µL Z buffer/ *o*-nitro phenyl galactoside (ONPG, 0.4% w/v in Z buffer) was added and β-galactosidase activity was measured by monitoring the reaction at 405 nm for a period of 20 min at 28°C. Miller units were calculated using the following equation: Miller units = $(OD_{405\ nm}/\text{min})/OD_{600\ nm} \times 1000$.

NF-κB assay

B lymphoblastoid 721 cell line NF-κB reporter cell was constructed previously [18], where the firefly luciferase gene was placed under the control of the NF-κB transcriptional response element. B721 NF-κB reporter cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (50 unit/mL) and streptomycin (50 µg/mL). Cells were seeded at a density of 1×10^4 cells/well in 96-well plates (100 µL/well). After overnight incubation, different concentrations of peptide were added to the cells. Following 24 h of treatment, the NF-κB activity was detected by Steady-Glo Luciferase Assay System (Promega, Madison, MI, USA) according to manufacturer's instructions. Briefly, 75 µL Steady-Glo Luciferase Assay reagent was added to each well and incubated at room temperature for 15 min. The luminescence was subsequently measured.

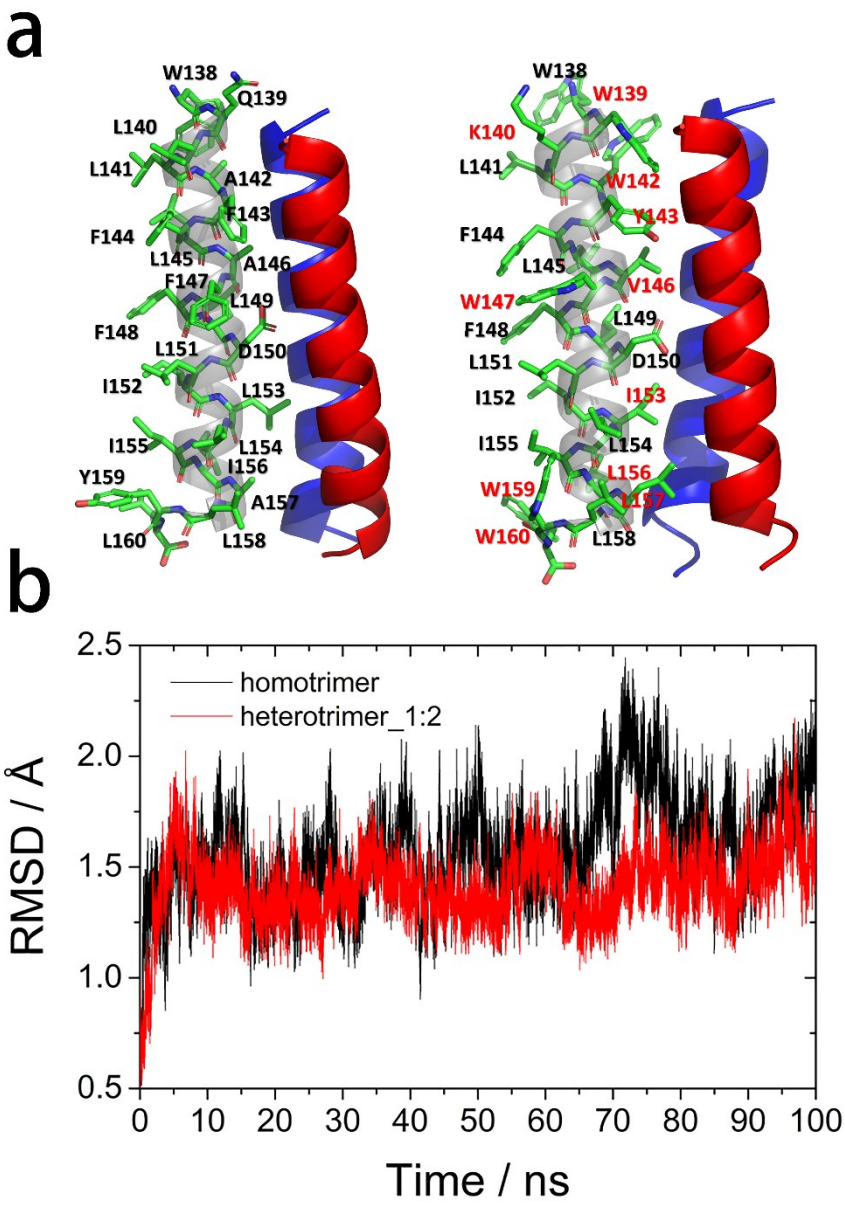

Fig. S1.Computational models for homotrimer and heterotrimer: (a) Sequences of the TMD5 and anti-TMD5 in a trimeric conformation. The variant residues in anti-TMD5 were labelled in red. (b) Backbone RMSD of homotrimer (black) and heterotrimer_1:2 (one anti-TMD5 and two TMD5s) (red) in 100 ns equilibrium molecular dynamics simulations. Both systems reached a stable state within 10 ns.

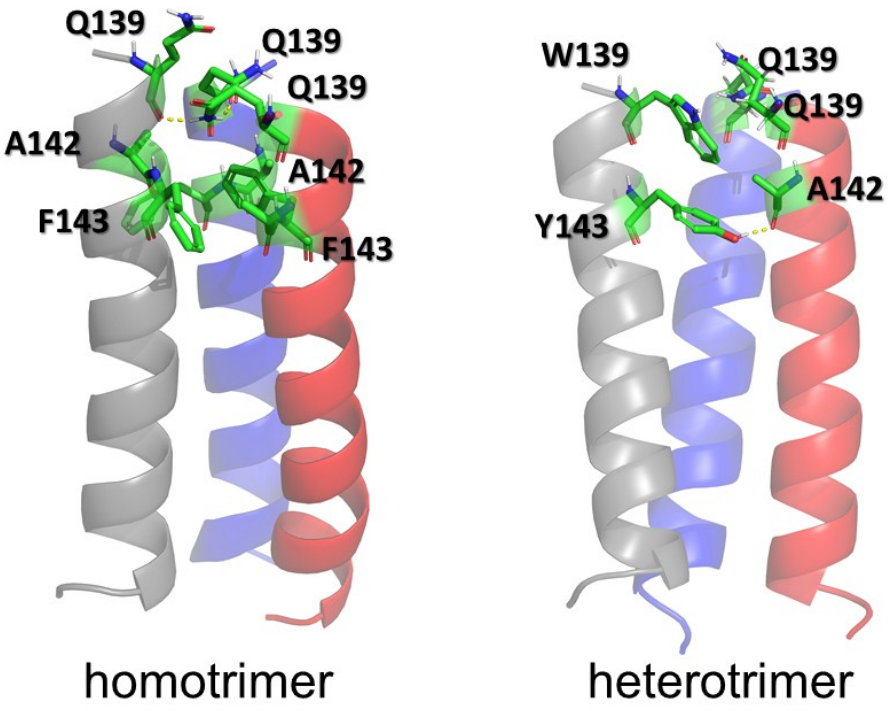

Fig. S2. Key residues stabilizing the oligomerization in the trimer.

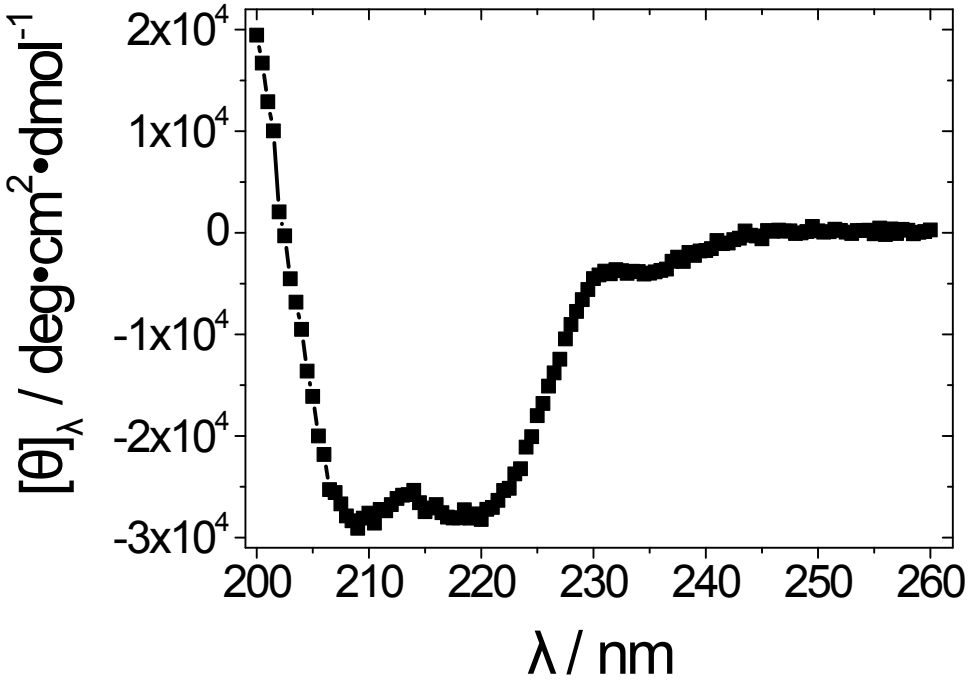

Fig. S3. Circular dichroism spectrum of anti-TMD5 in C14 betaine micelles showing an α-helical secondary structure. CD measurements were performed on a Jasco J-1500 CD spectrophotometer at room temperature. Anti-TMD5 peptides were co-dissolved in 2,2,2-trifluoroethanol (Sigma-Aldrich, St. Louis, MO, USA) and C14 betaine (3-(*N,N*-dimethylmyristyl-ammonio)propanesulfonate; Sigma-Aldrich, St. Louis, MO, USA). The organic solvent was removed under reduced pressure to generate a thin film of peptide/detergent mixture, which was then dissolved in 100 mM HEPES buffer (pH = 7.4). The final concentration of C14 betaine was 1 mM in the samples. Far UV CD spectra (200 – 260 nm) were acquired using a cell path length of 0.1 cm and the results were expressed as mean residue ellipticity $[\theta]_\lambda$ in deg·cm^2·dmol^{-1} at a given wavelength λ (nm) using the relation: $[\theta]_\lambda = \theta_\lambda \times M_o / (10 \times c \times l)$, where θ_λ is the observed ellipticity in millidegrees at wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), and l is the path length (cm). It should be noted that each observed θ_λ of the protein was corrected for the contribution of the buffer. Data were not collected below 200 nm due to the high voltage and background noise from the C14 Betaine buffer. The α-helical content of peptide was calculated according to equation[19]: α-Helix (%) = − ($[\theta]_{222\ nm}$ + 2340) / 30300 × 100.

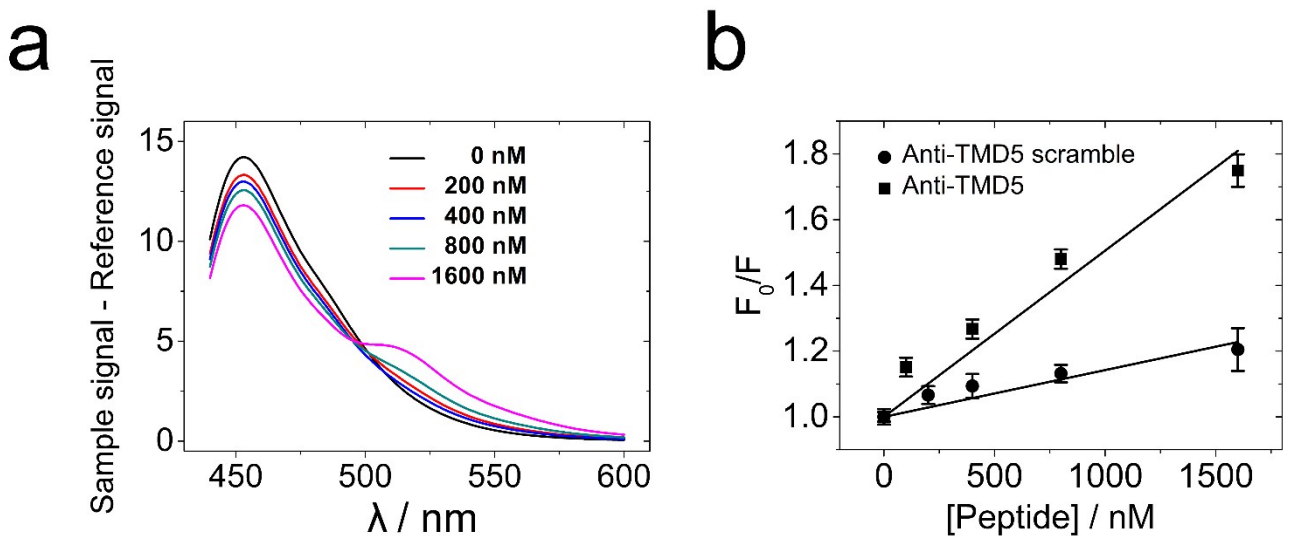

Fig. S4. FRET assay: (a) Fluorescence emission scans of coumarin-tagged TMD5 (50 nM) in the presence of different concentrations of FITC−tagged anti-TMD5 scramble in 20 mM HEPES, pH = 7.4, containing 1.0 mM C14 betaine at room temperature. λ_{ex} was set at 415 nm to selectively excite the coumarin. λ_{ex}, excitation wavelength; (b) Stern-Volmer quenching plots of coumarin labelled TMD5 fluorescence by anti-TMD5 scramble or anti-TMD5 binding.The Stern-Volmer constant of anti-TMD5 scramble ($0.14 \pm 0.02\ \mu M^{-1}$) is much weaker than that of anti-TMD5 ($0.58 \pm 0.04\ \mu M^{-1}$).

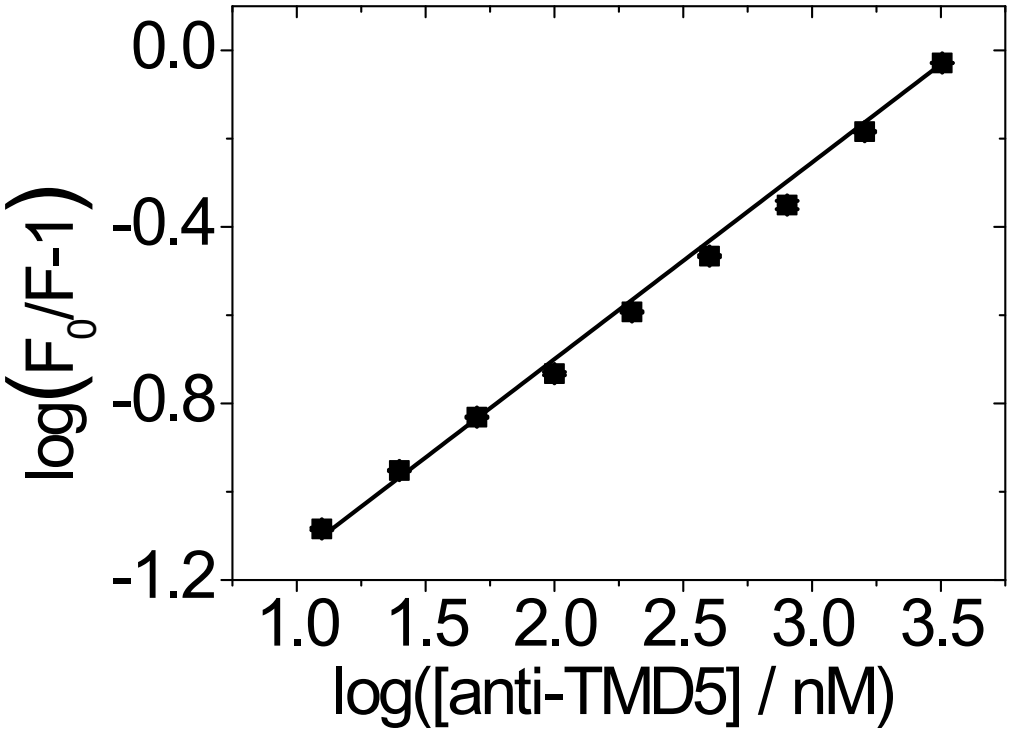

Fig. S5. The quenching of fluorescence signal from coumarin-labeled TMD5 peptide by FITC-tagged anti-TMD5 in 20 mM HEPES, pH = 7.4, containing 1.0 mM bicelles composed of POPC and DHPC (q = 0.3). The data analyzed by the equation, log (F_0/F-1) = -logK_d + n×log([anti-TMD5]). A stoichiometry n = 0.44 ± 0.03 and an apparent K_d of 40.0 ± 6.7 nM were obtained.

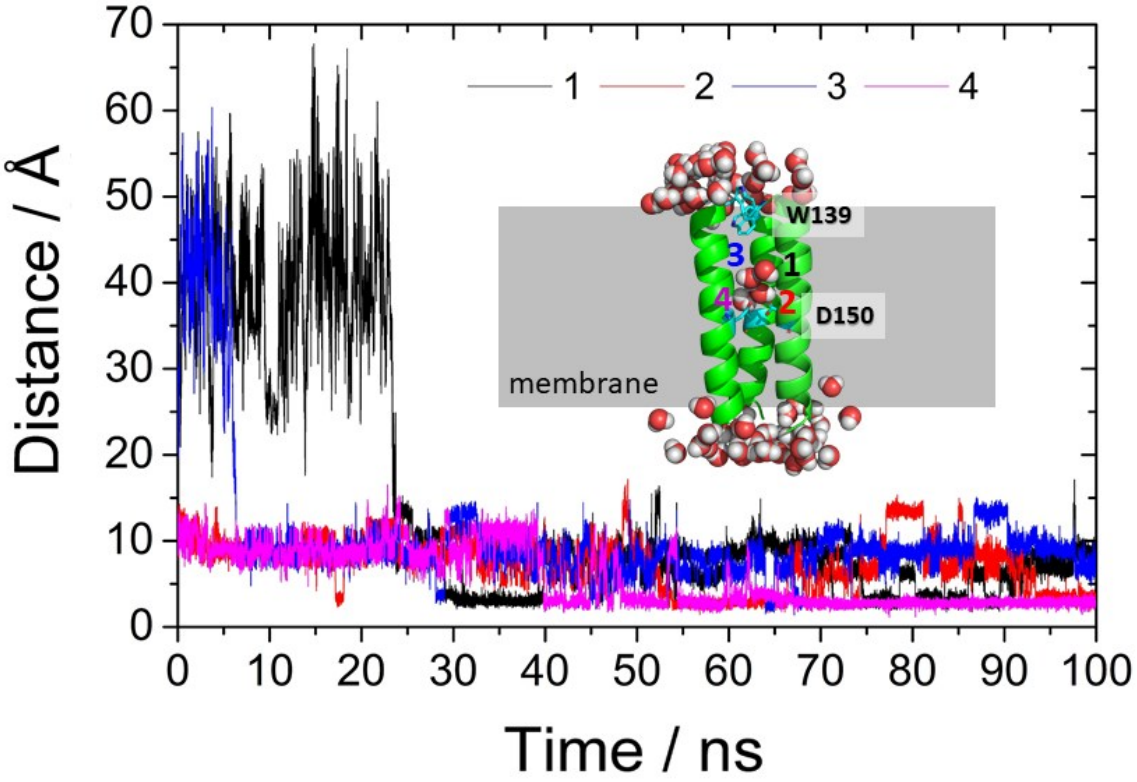

Fig. S6. Locations of four water molecules in heterotrimer_2:1 (two anti-TMD5s and one TMD5). The distance between the oxygen of each water molecule and the center of mass of the Cα of heterotrimer_2:1 were calculated and represented by black, red, blue, and magenta lines, respectively. The location of four water molecules in heterotrimer_2:1 was displayed and numbered with 1, 2, 3, and 4. W139 and D150 were represented in cyan sticks.

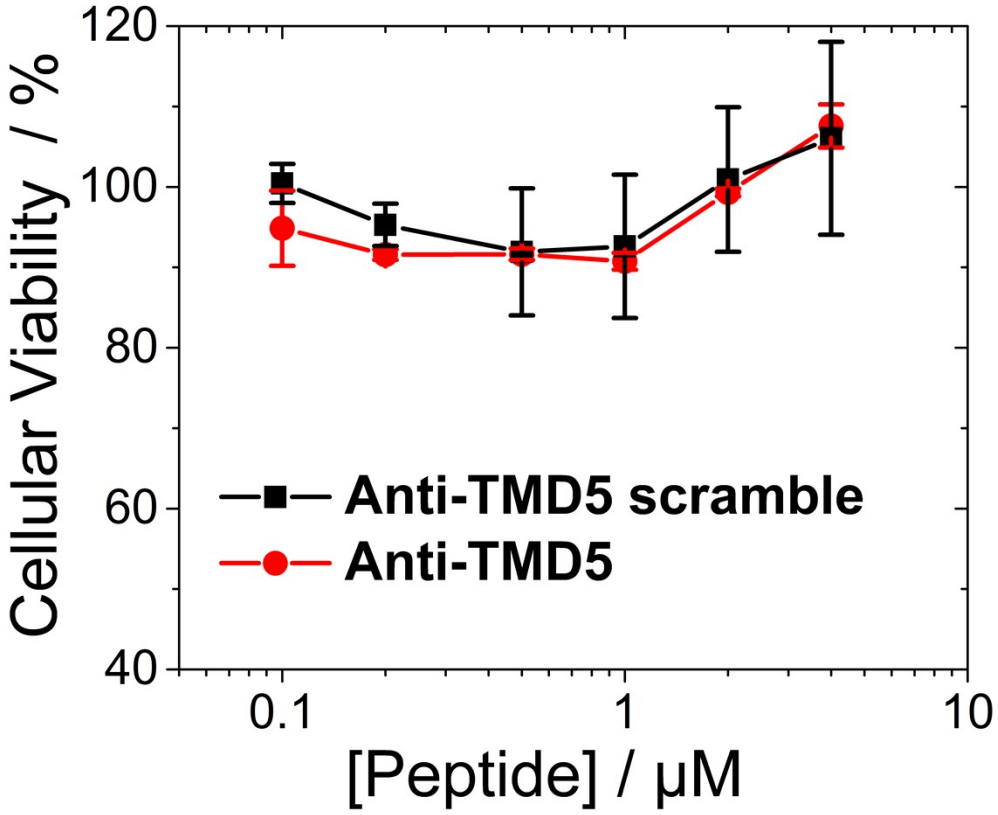

Fig. S7. Cell viability assay. B721 cells were stimulated for indicated concentrations of anti-TMD5 and anti-TMD5 scramble. Higher concentrations (> 4 μM) were not tested because of their poor solubility. TMD5/anti-TMD5 stock solutions were prepared as following: They were dissolved in 2,2,2-trifluoroethanol. The organic solvent was removed under reduced pressure to generate a thin film of peptide, which was then dissolved in 20 mM HEPES buffer (pH=7.4) containing micelle (C14 betaine) or bicelles (POPC/DHPC or DMPC/DHPC). Following 24 h treatment, 20 μL of Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) was added. After further incubation at 37 °C for 1–2 h, the absorbance at 450 nm was measured on a SYNERGY H1 Micro-plate Reader (BioTek Instruments, Carlsbad, CA, USA) and 620 nm was chosen as the reference wavelength. The $A_{450\ nm}$-$A_{620\ nm}$ for the control group was set as 100%.

References

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Appendix data:
HPLC and mass spectrometry characterizations of the synthesized peptides

a. TMD5

b. Coumarin labeled TMD5

c. Anti-TMD5

d. FITC-labeled anti-TMD5

e. Anti-TMD5 scramble

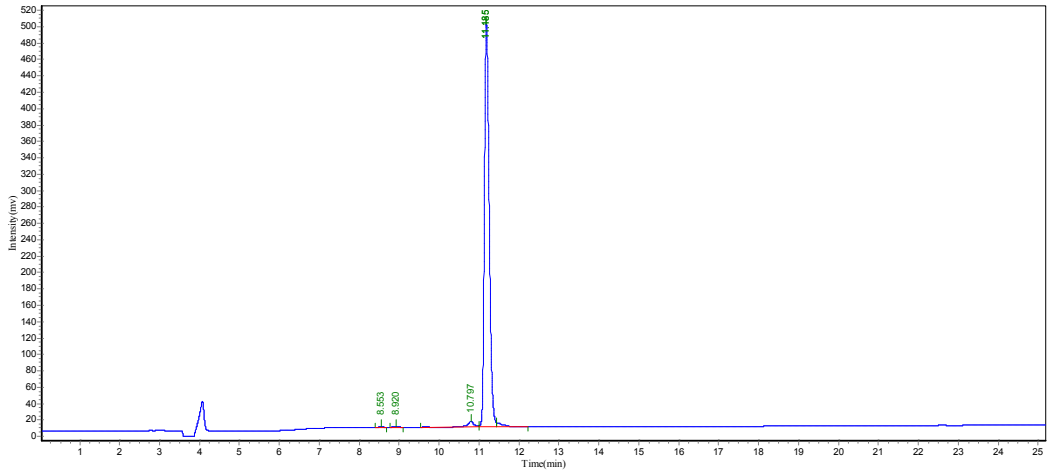
f. FITC-labeled anti-TMD5 scramble

HPLC Report of TMD5

Structure : TMD5
Column : 4.6×250mm,Venusil MP C18-5
Solvent A : 0.1% trifluoroacetic in 100% acetonitrile
Solvent B : 0.1% trifluoroacetic in 100% water
Gradient :

	A	B
0.01min	55%	45%
25min	100%	0%
25.1min	100%	0%
30min	STOP	

Flow rate : 1.0 mL/min
Wavelength : 220nm
Volume : 5ul



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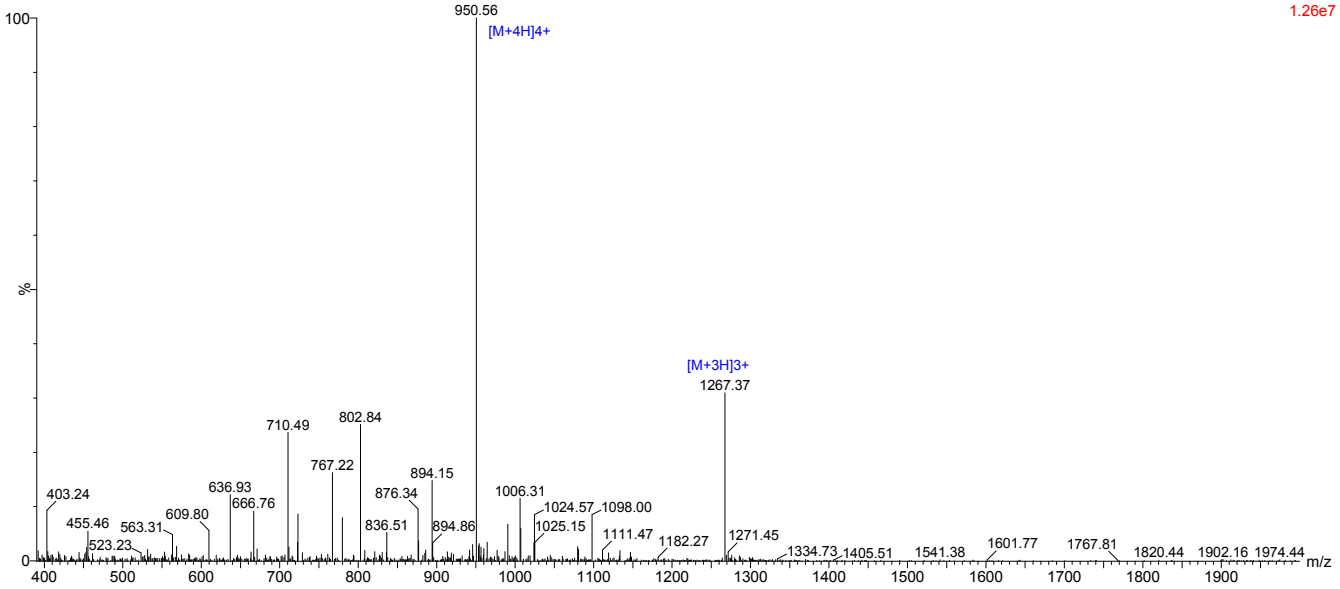
2	8.920	1142.773	8896.888	0.2089
3	10.797	7004.221	75740.398	1.7785
4	11.185	490966.563	4109966.750	96.5070
5	11.185	5274.425	61557.281	1.4454
Total			100.0000	

Mass Spectrometry Report of TMD5



Sample Description		Instrument	Waters ZQ2000		
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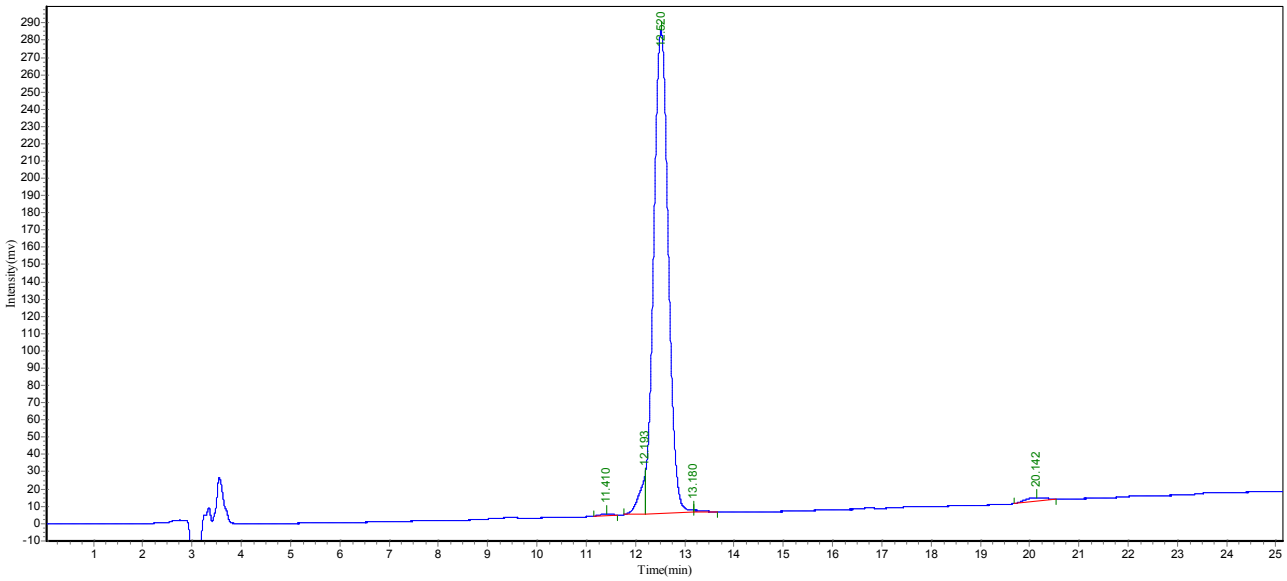
Analyst:	YU	Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
Sample:	TMD5	CDL:	-20.0v	T.Flow:	0.2ml/min
M.W.:	3795.91	CDL Temp.:	250 °C	B. Conc.:	50%H2O/50%ACN
Lot. No.:	P161111-SY545400	Block Temp.:	200 °C		

HPLC Report of Coumarin-tagged TMD5

Structure : coumarin-labeled TMD5
Column : 4.6×250mm,Venusil MP C18-5
Solvent A : 0.1% trifluoroacetic in 100% acetonitrile
Solvent B : 0.1% trifluoroacetic in 100% water
Gradient :

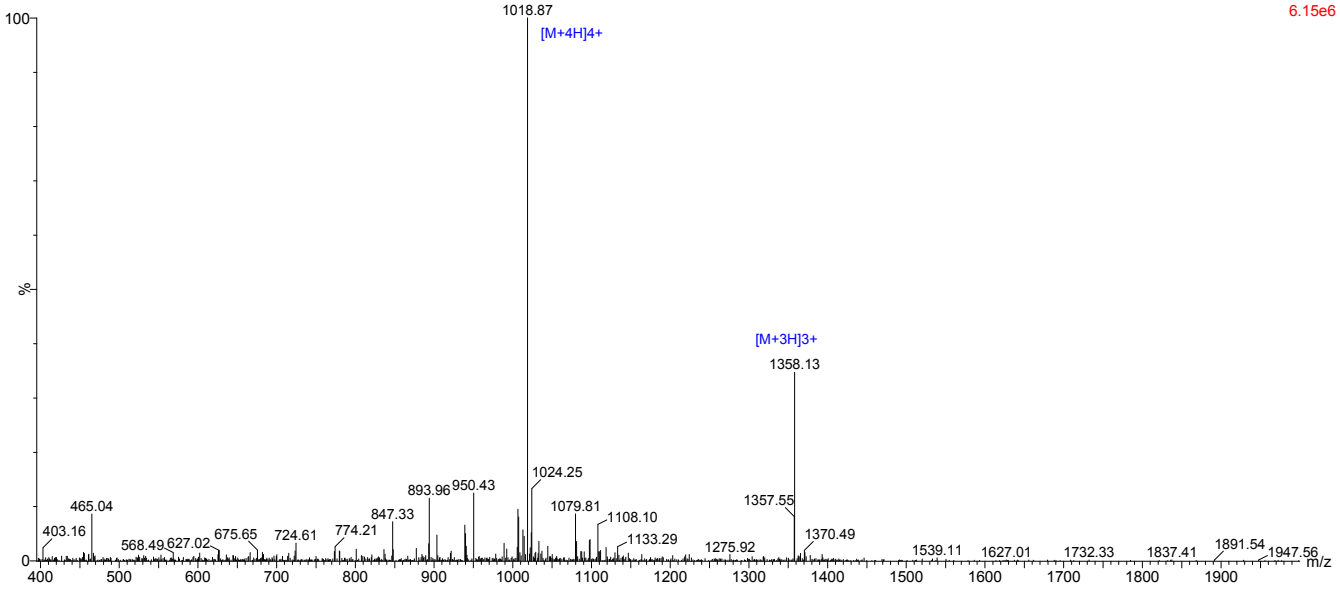
	A	B
0.01min	50%	50%
25min	100%	0%
25.1min	100%	0%
30min	STOP	

Flow rate : 1.0 mL/min
Wavelength : 220nm
Volume : 5ul



Peak No.	Ret Time	Height	Area	Conc..
1	11.410	1141.444	17451.203	0.2892
2	12.193	21842.223	179035.031	2.9671
3	12.520	279863.688	5757074.500	95.4093
4	13.180	1310.283	17379.811	0.2880
5	20.142	2148.968	63140.801	1.0464
Total			100.0000	

Mass Spectrometry Report of Coumarin-tagged TMD5



Sample Description		Instrument	Waters ZQ2000		
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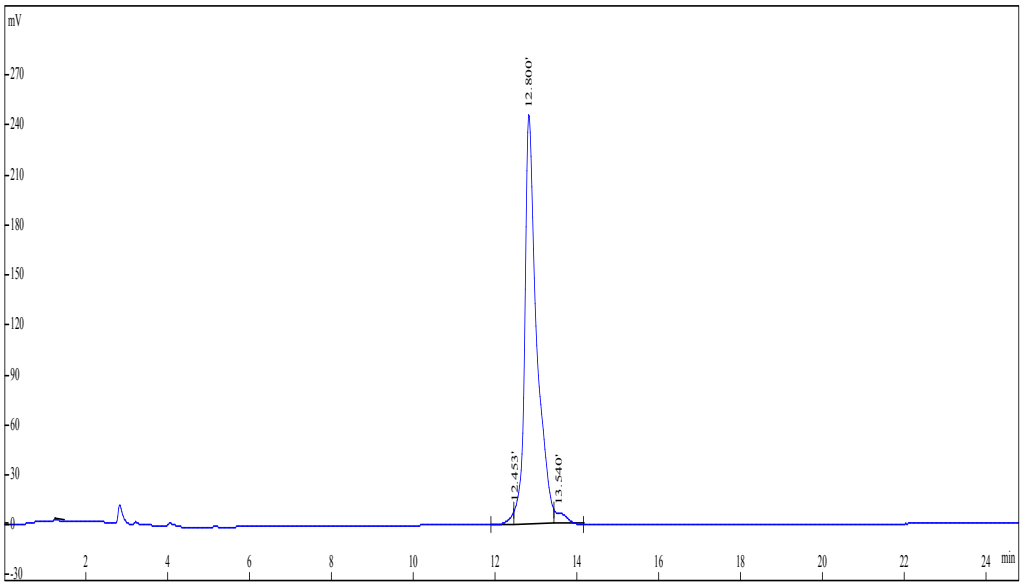
Analyst:	YU	Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
Sample:	coumarin-labeled TMD5	CDL:	-20.0v	T.Flow:	0.2ml/min
M.W.:	4068.96	CDL Temp.:	250 ºC	B. Conc.:	50%H2O/50%ACN
Lot. No.:	P161124-SY545401	Block Temp.:	200 ºC		

HPLC Report of anti-TMD5

Product Name:anti-TMD5
Column :4.6*250mm, 4.6*250mm,SP-300-10-UniPS
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water
Gradient :

	A	B
0.00min	50%	50%
25min	100%	0%
25.1min	100%	0%
30min	Stop	

Flow rate :1.0ml/min
Wavelength :220nm
Volume :10ul



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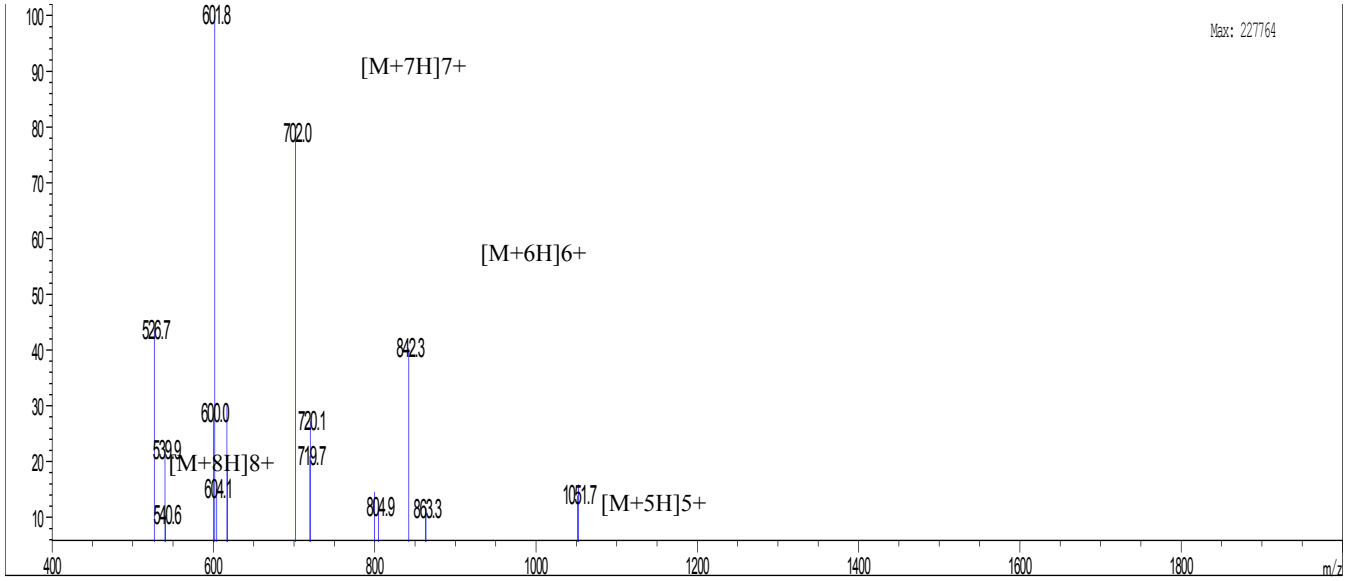
Mass Spectrometry Report of anti-TMD5



Sample Description		Instrument	Agilent-6125B		
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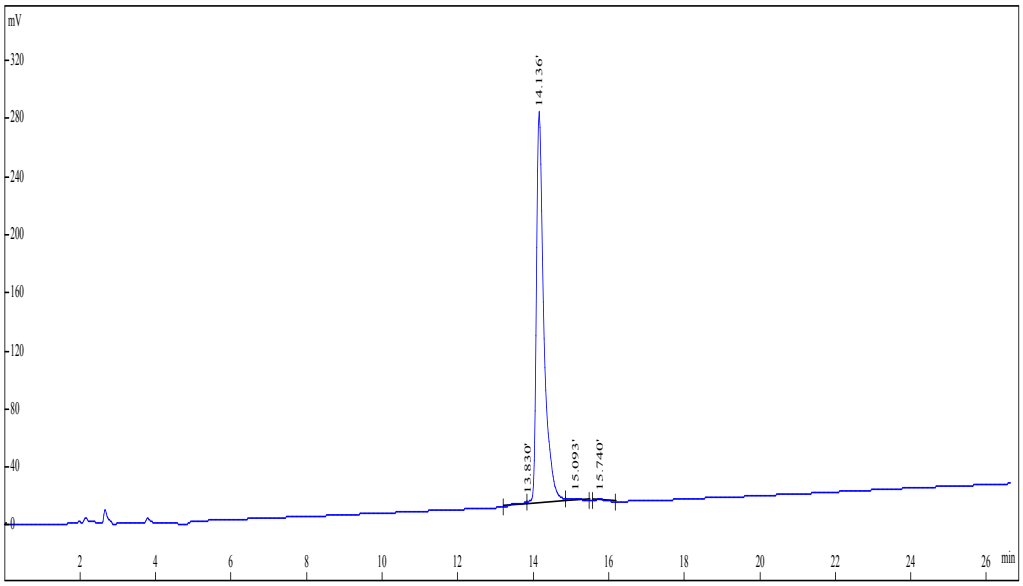
Analyst:	YU	Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
Sample:	anti-TMD5	CDL:	-20.0v	T.Flow:	0.2ml/min
M.W.:	4205.41	CDL Temp.:	250 ^{o}C	B. Conc.:	50%H2O/50%ACN
Lot. No.:	P190220-SL709219	Block Temp.:	200 ^{o}C		

HPLC Report of FITC-tagged anti-TMD5

Product Name:FITC-labeled anti TMD5
Column :4.6*250mm, 4.6*250mm,SP-300-10-UniPS
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water
Gradient : A B
0.00min 50% 50%
25min 100% 0%
25.1min 100% 0%
30min Stop
Flow rate :1.0ml/min
Wavelength :220nm
Volume :10ul



Rank	Time	Conc.	Area	Height
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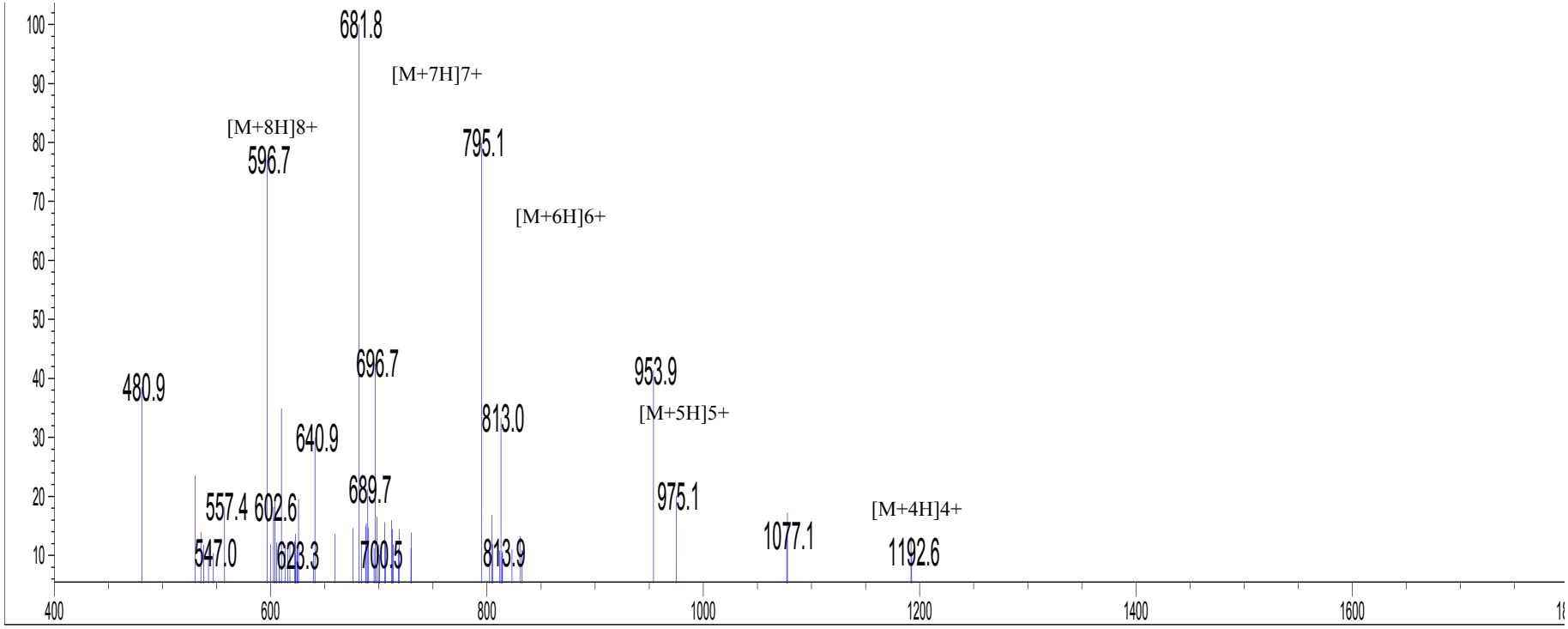
3	15.093	1.2812	50071	2288
4	15.740	0.3957	15464	1542
Total		100	3908190	277781

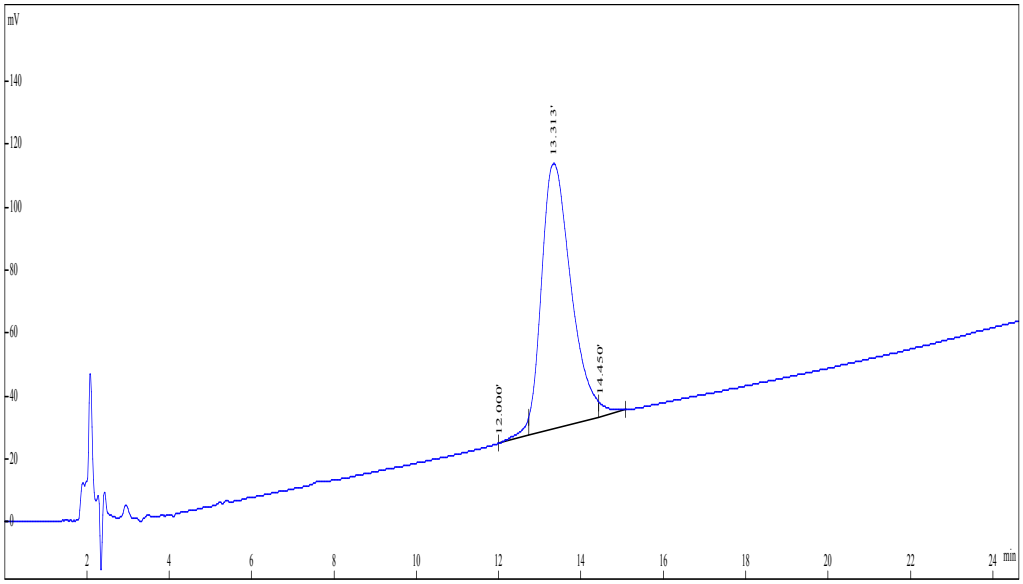
Mass Spectrometry Report of FITC-tagged anti-TMD5



Sample Description		Instrument	Agilent-6125B		
Analyzed date:	2017-03-07	Probe:	ESI	Probe Bias:	＋4.5kv
Analyst:	YU	Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
Sample:	FITC-labeled anti TMD5	CDL:	-20.0v	T.Flow:	0.2ml/min
M.W.:	4765.01	CDL Temp.:	250 ^{o}C	B. Conc.:	50%H2O/50%ACN
Lot. No.:	P190220-SL709220	Block Temp.:	200 ^{o}C		

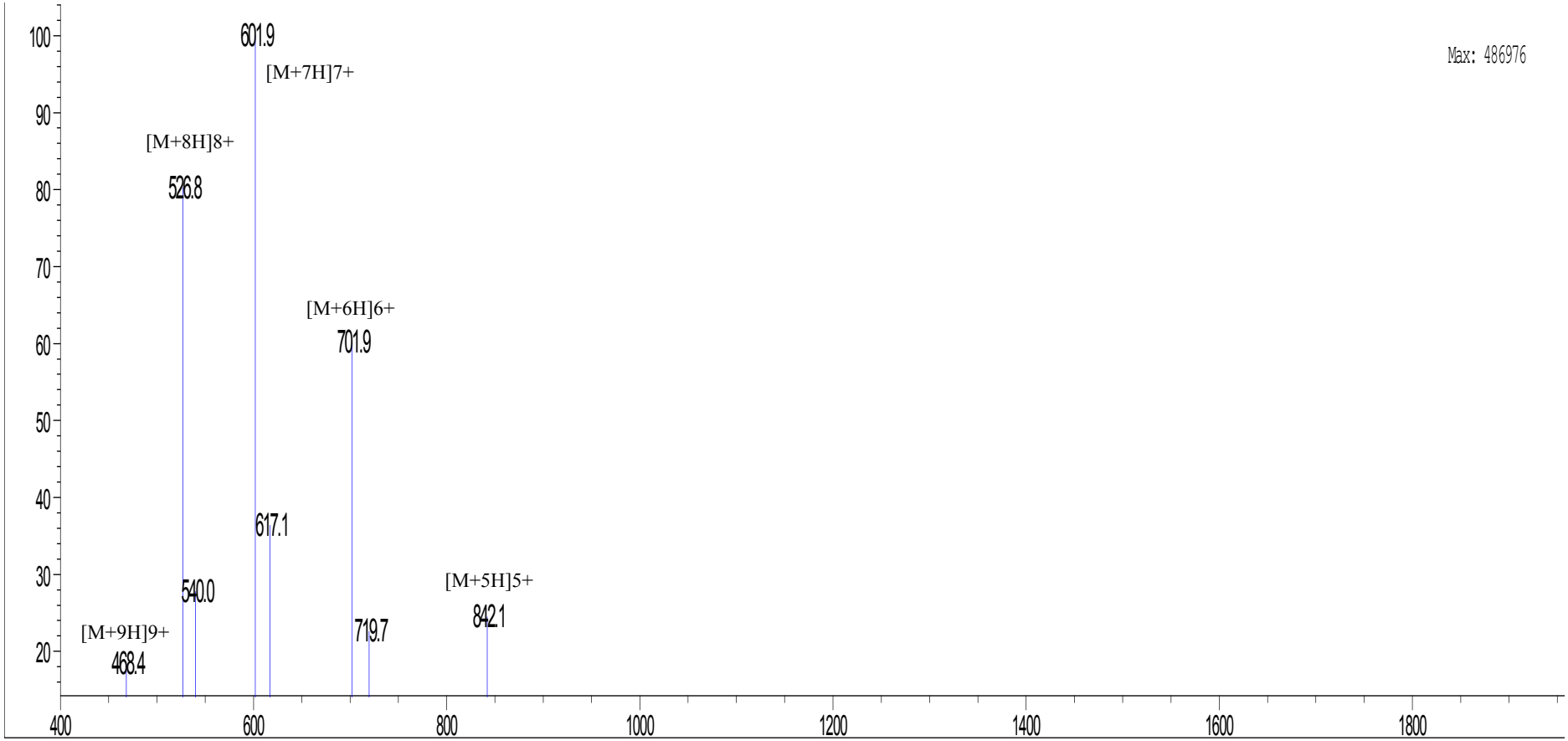
HPLC Report of Anti-TMD5 Scramble

Product Name:anti-TMD5 scramble
Column :4.6*250mm, GS-120-5-C18-BIO
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water
Gradient : A B
0.00min 40% 60%
25min 65% 35%
25.1min 100% 0%
30min Stop
Flow rate :1.0ml/min
Wavelength :220nm
Volume :10ul



Rank	Time	Conc.	Area	Height
1	12.000	1.5470	65093	0
2	13.313	97.0118	4081893	84156
3	14.450	1.4412	60642	4277
Total		100	4207628	88433

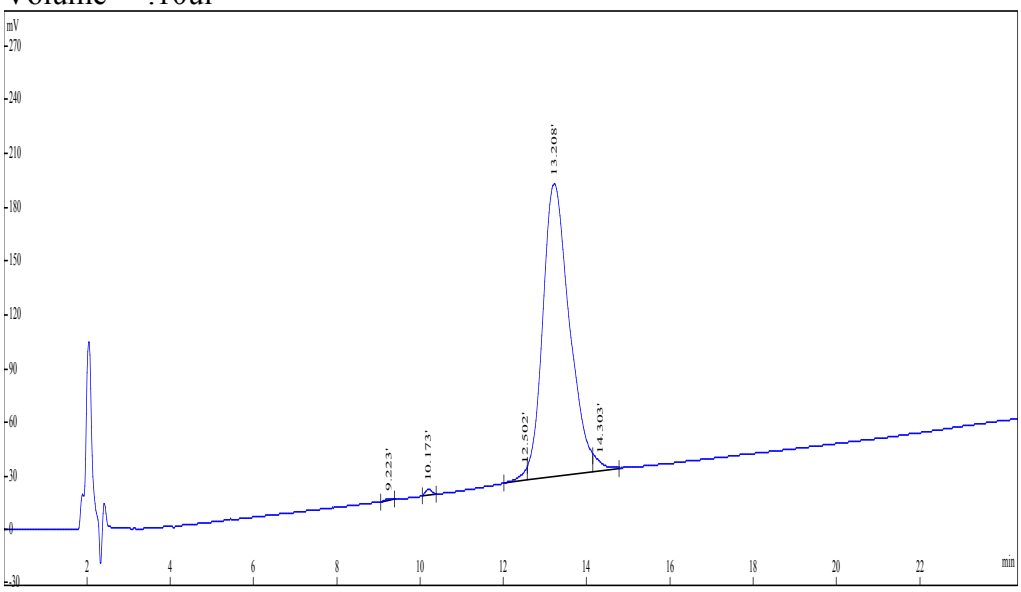
Mass Spectrometry Report of Anti-TMD5 Scramble



Sample Description		Instrument	Agilent-6125B		
Analyzed date:	2017-03-22	Probe:	ESI	Probe Bias:	＋4.5kv
Analyst:	YU	Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
Sample:	anti-TMD5 scramble	CDL:	-20.0v	T.Flow:	0.2ml/min
M.W.:	4205.41	CDL Temp.:	250 ^{o}C	B. Conc.:	50%H2O/50%ACN
Lot. No.:	P190313-SL714398	Block Temp.:	200 ^{o}C		

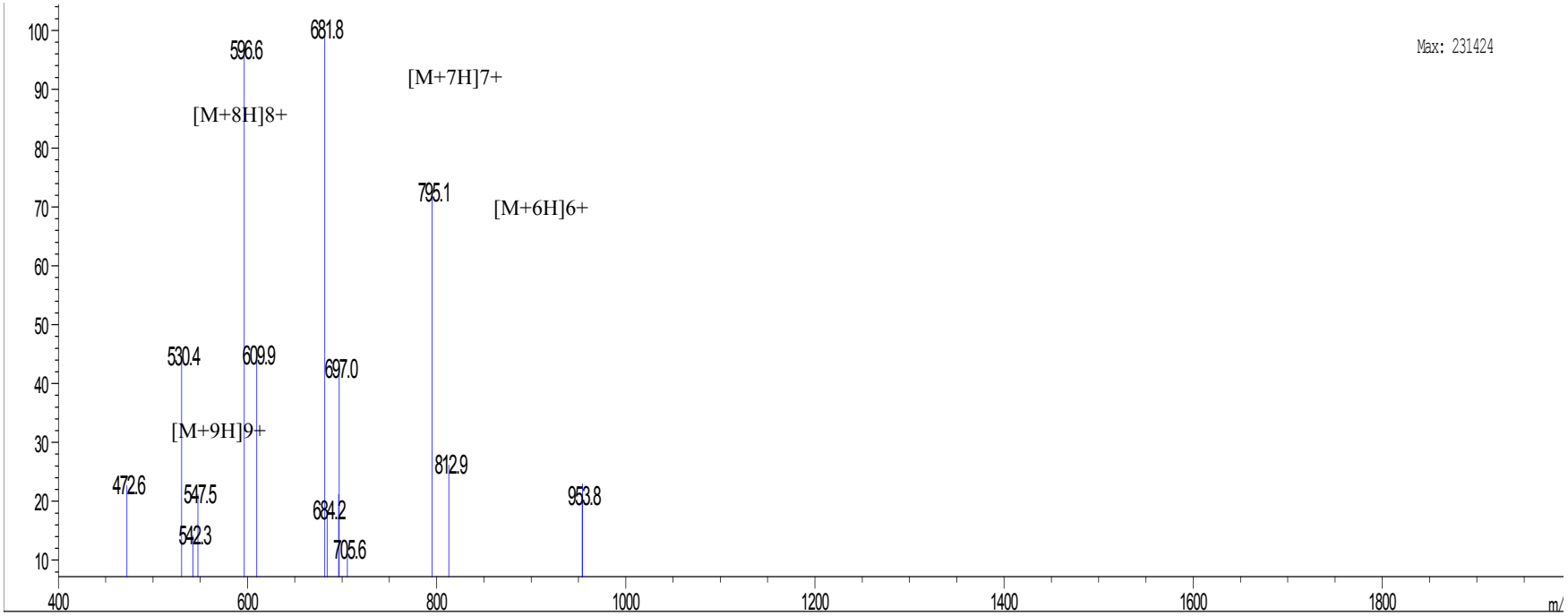
HPLC Report of FITC-tagged anti-TMD5 scramble

Product Name:FITC-anti-TMD5 scramble
Column :4.6*250mm, GS-120-5-C18-BIO
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water
Gradient : A B
0.00min 40% 60%
25min 65% 35%
25.1min 100% 0%
30min Stop
Flow rate :1.0ml/min
Wavelength :220nm
Volume :10ul



Rank	Time	Conc.	Area	Height
1	9.223	0.1294	9381	1016
2	10.173	0.4198	30441	3126
3	12.502	1.0548	76492	5018
4	13.208	96.9865	7033302	162959
5	14.303	1.4095	102217	4763
Total		100	7251833	176882

Mass Spectrometry Report of FITC-tagged anti-TMD5 scramble



Sample Description		Instrument	Agilent-6125B		
Analyzed date:	2017-03-25	Probe:	ESI	Probe Bias:	＋4.5kv
Analyst:	YU	Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
Sample:	FITC-anti-TMD5 scramble	CDL:	-20.0v	T.Flow:	0.2ml/min
M.W.:	4765.01	CDL Temp.:	250 °C	B. Conc.:	50%H2O/50%ACN
Lot. No.:	P190313-SL714400	Block Temp.:	200 °C		