Electronic Supplementary Information for

A phage display-based strategy for the *de novo* creation disulfide-constrained and isomer-free bicyclic peptide affinity reagents

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Supplementary method

Supplementary data

1.1 Construction of Phage library

The genes encoding random peptides -GCXCX$_5$CX$_5$C- were appended in a PCR reaction. Gel-purified *Sfi I*/*Not I*-digested DNA and pCantab 5E was ligated after the determination of the optimum insert/vector ratio (10/1). Transforming the ligation mix into ER2738 yields 4.52×10^9 colonies on plates, and then colonies were scraped from the plates with the 2YT media and stored at –80℃ with the addition of 20% v/v glycerol.

1.2 Rescue and Amplification of Phage

Inoculated 2YT-G (2YT with 2% w/v Glucose) with the library glycerol stock to reach an OD600 of 0.1, incubated at 37 ℃ until OD600 reach 0.6-0.8. 20 Fold-excess of helper phage M13KO7 was then added to the culture and incubated at 37 ℃ for 1 h to infect the cells. Centrifuged the cells and discarded the supernatant. Resuspended the cell pellet in 2YT-AK (2YT with 100 μg/mL Ampicilin and 50 μg/mL Kanamycin) and incubated at 30 ℃ overnight. Centrifuged the cells and added 20% v/v PEG-NaCl (20% w/v PEG-8000 with 2.5 M NaCl) to the supernatant to precipitate the phages. Centrifuged and discarded the supernatant, and resuspended the phage pellets in PBS (pH 7.4), and then centrifuged to remove any remaining cell debris. Repeated the process of the phage precipitation one more time to get purer phage pellets and resuspended the phage library in PBS (pH 7.4) containing 30% v/v glycerol. Finally, the phage library was filtered through a 0.45 μm filter and stored at –80 ℃.

1.3 Expression and purification of Keap1 kelch domain

The DNA region coding for Keap1 kelch domain (residues 321-609) was constructed and cloned into pET-15b vector, the resulting plasmid was used to transform BL21 (DE3) competent cells. Cells were grown in LB medium at 37 ℃ and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD600 of 0.6. After 8~12 h, Cells were harvested and lysed in Buffer A (100 mM Tris/500 mM NaCl/1 mM PMSF, pH 7.9) by sonication. The supernatant was applied to Ni-sepharose column bound with Ni^{2+} and washed with Buffer A. Then, the Keap1 kelch domain was eluted

with Buffer E (100 mM Tris/500 mM NaCl/200 mM imidazole, pH 7.9) and further purified on a Superdex^{TM} 75 10/300 GL column (GE Healthcare).

1.4 Biotinylation of Keap1 protein

The KEAP1 kelch-domain was biotinylated at a concentration of 5 μM with a 5-fold molar excess of Sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) in PBS (pH 7.4) at RT for 0.5 h. The unreacted free Sulfo-NHS-LC-biotin was removed by size exclusion chromatography with desalt column on an AKTA pure system (GE Healthcare) with PBS (pH 7.4) as the running buffer.

1.5 Target immobilization and panning

The target immobilization and panning experiments were conducted as described by Chen et al[1]. Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen, 100 μL; or neutravidin-coated beads for the second round) was washed twice with Binding buffer (10 mM Tris-Cl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, pH7.4) before distributing into two tubes equally. Biotinylated target (1~5 μg) was added to one of the two tubes, and the same volume of buffer to the second tube, incubate the tubes on a rotator at room temperature for 10 min. Put the tubes on a magnet for 2 min and discarded the supernatants. The beads were washed with binding buffer for three times prior to blocking of the beads with 150 μL binding buffer and 300μL Blocking buffer (Binding buffer with 0.3% v/v Tween-20 and 3% w/v BSA) on a rotator for 30min at room temperature, in parallel, the phage library (10^{12}~10^{13} t.u.) dissolved in 3ml binding buffer were blocked by addition of 1.5 mL Blocking buffer. The blocked beads with and without immobilized target proteins were mixed with blocked phage library and co-incubated on a rotator for 30 min at room temperature. After the incubation, discarded the supernatant and wash the beads for eight times with Washing buffer (Binding buffer with 0.1% v/v Tween-20) and twice with Binding buffer. During the period, the tubes were replaced at least three times. After all the washing steps, the buffer was removed, and the phages were

eluated by resuspending the beads in 200 μL Elution buffer (50 mM glycine, pH 2.2) and incubate for exactly 5 min, then the supernatant is transferred to a tube containing 50 μL Neutralization buffer (1 M Tris-Cl, pH 8.0).

1.6 Phage Titration

Serially diluted the phages by ten folds with the 2YT media. 20 μL of each dilution was added to 180 μL ER2738 log-phase culture and incubated for 30min at 37 ℃ (No Shaking). Spread 10 μL of each infected cell onto LB-A plates (LB plates containing 100μg/mL Ampicilin) and incubated overnight at 37 ℃. Counted the colonies on the plate, and calculated the titer of the phage preparation (Colony Forming Unit per ml).

1.7 Amplification of Monoclonal Phage

Inoculated 1 mL of 2YT-AG with individual colonies from the plates generated by titration of eluted phages and incubated overnight with shaking at 37 ℃. Transfer 600 μL culture of each colony to 6 mL 2YT-AG and incubate with shaking at 37 ℃ for 2 h (add 15% v/v glycerol to the remaining 400 μL culture and store at –80℃), added 20 μL PBS containing 6×10^{10} helper phage M13KO7 to each culture and incubated for 30 min at 37 ℃ without shaking and followed by 30min with vigorous shaking. Centrifuged at 4500g for 10 min, and resuspended the pellets in 6 mL 2YT-AK media and incubated at 30 ℃ overnight with shaking. Centrifuged at 4500 g for 10 min to discard the cell pellet, and added 20% v/v PEG-NaCl to the supernatant and incubated on ice for at least 1 h. Centrifuged at 9000 g for 10 min and resuspended the phages in 1 mL blocking buffer.

1.8 Monoclonal Phage ELISA

Coated Maxisorp 96-plate with 0.1 μg/well Keap1 kelch domain (0.1 μg/100 μL in Binding buffer) and incubated for 1 h at room temperature. Wash the wells with 3×200 μL Washing buffer. Block the wells with 200 μL/well Blocking buffer and incubate for 1.5 h at room temperature. Washed the wells with 3×200 μL Washing buffer. Added 100 μL/well phage stock of each colony to plate and incubate for 1.5 h

at room temperature. Add 100 μL/well anti-M13-Mab-HRP antibody dissolved in Blocking buffer to the plate and incubate for 30min at room temperature. Washed the wells with 5×200 μL Washing buffer. Developed ELISA with TMB substrate (Sangon. China) and measured the optical density at 450 nm.

1.9 Peptides synthesis and purification

Acetonitrile (ACN), trifluoroacetic acid (TFA), N,N'-diisopropylcarbodiimide (DIC) and thioanisole were purchased from Tokyo Chemical Industry (Shanghai). 1,2-Ethanedithiol (EDT) was purchased from Macklin (Shanghai). MBHA resin and Fmoc-protected amino acids were purchased from GL Biochem (Shanghai). 1-Hydroxybenzotriazole (HOBt) was purchased from Adamas-beta (Shanghai). N-dimethyl formamide (DMF), diethyl ether, and phenol were purchased from Sinopharm (Shanghai). All linear peptides were synthesized on a CEM Discover Liberty BLUE microwave-assisted peptide synthesizer by standard solid phase peptide synthesis approach on MBHA resin. Fmoc groups were removed by 20% vol piperidine in DMF, peptides were cleaved from the resin and side-chains were deprotected using TFA /thioanisole/H2O/phenol/EDT (90 /2.5/ 2.5/2.5 /2.5 v/v, 4 ml). The ether-precipitated crude peptides were purified by semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC), and the molecular masses of the purified peptides were confirmed with MALDI-TOF mass spectroscopy.

1.10 Oxidative folding of peptides

All the peptides screened from the library were oxidized in 50% v/v DMSO /100 mM phosphate buffer (pH 7.4) in room temperature for 12 h at a concentration of 100 μM unless otherwise stated. And all Pen-substituted peptides were oxidized in 50% v/v DMSO/100 mM phosphate buffer containing 0.5 mM oxidized glutathione (pH 7.4) in room temperature for 6 h at a concentration of 100 μM.

1.11 Fluorescence polarization (FP) competition assay

Fluorescence polarization (FP) competition assays were conducted as described

before[2]. All the FP assays were performed in 10 mM PBS on 96-well flat-bottom OptiPlate black plate using an Infinite 200 PRO microplate reader. For the FP binding assay, a 8-point dilution was added to the Lobind tubes (Eppendorf, Germany) , followed by FITC-ETGE (FITC-[β-ala]-DEETGEF-OH) for a total volume to 300μL with a final peptide concentration of 20 nM . Samples were incubated at room temperature for 10 min before read on plate by 125 μL/well. The binding curves were fitted by equation below.

$$y = A_0 + (A_{max} - A_0) \times \frac{(x + c + K_d) - \sqrt{(x + c + K_d)^2 - 4 \times x \times c}}{2c}$$

x is the concentration of protein, y is the recorded fluorescence anisotropy, c is the concentration of FITE-ETGE, A_0 and A_{max} is the minimum and maximum value on the curve, respectively.

For the FP competition assay, 9-point dilutions of peptides were added to the 10 mM PBS containing 20 nM FITC-ETGE and 300 nM Keap1 kelch domain. Samples were incubated at room temperature for 10 min (125 μL/well) before read on plate. The competition curves were fitted by the one-site competition model in Graphpad and the K_i values were calculated. All FP assays were performed in triplicate.

1.12 NMR experiments

All NMR experiments were conducted at 298 K on a Bruker 850 MHz spectrometer. 2D ^{13}C-^{1}H HSQC and ^{1}H-^{1}H TOCSY (τ_m=80 ms) spectra were measured to obtain backbone and side chain resonance assignments. 2D ^{1}H-^{1}H NOESY spectra with a mixing time of 300 ms were measured to analyze the disulfide connectivity. All NMR spectra were processed using NMRPipe[3] and analyzed using NMRFAM-SPARKY[4]. ^{1}H chemical shifts were referenced to internal 3-(Trimethylsilyl) propionic acid-*d*4 (TSP), and ^{13}C chemical shifts were indirectly referenced to TSP[5].

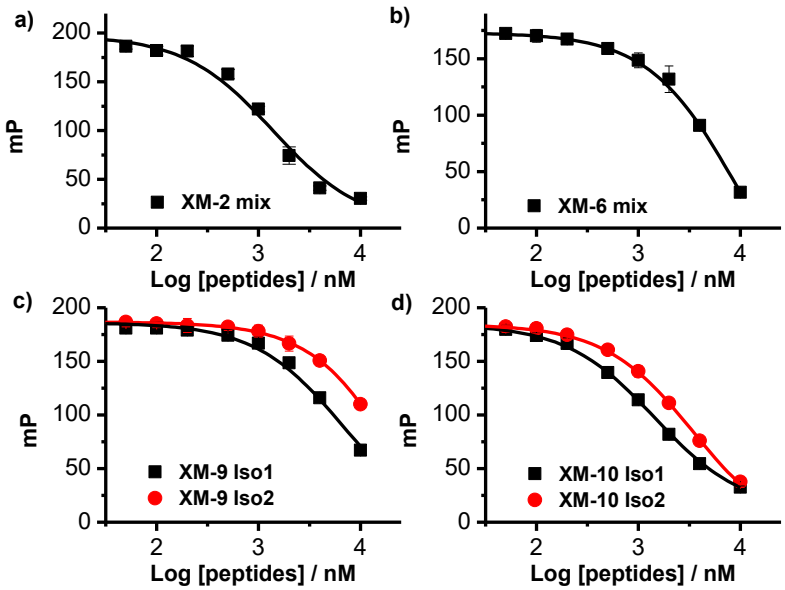

Figure S1. FP competition assays determine the binding interaction of bicyclic peptides with the Keap1 protein. a) XM-2 mixtures formed in 50% DMSO/100mM phosphate buffer (pH 7.4) (K_i = 1167 ± 172 nM). b) XM-6 mixtures formed in 50% DMSO/100mM phosphate buffer (pH 7.4) (K_i = 8684 ± 2530 nM). c) XM-9 bicyclic isomer-I (with the longer retention time on the HPLC chromatogram, K_i = 9522 ± 2024 nM) and isomer-II (K_i = 26150 ± 6709 nM). d) XM-10 bicyclic isomer-I (with the longer retention time on HPLC chromatogram, K_i = 1030 ± 74 nM) and isomer-II (K_i = 2407 ± 115 nM).

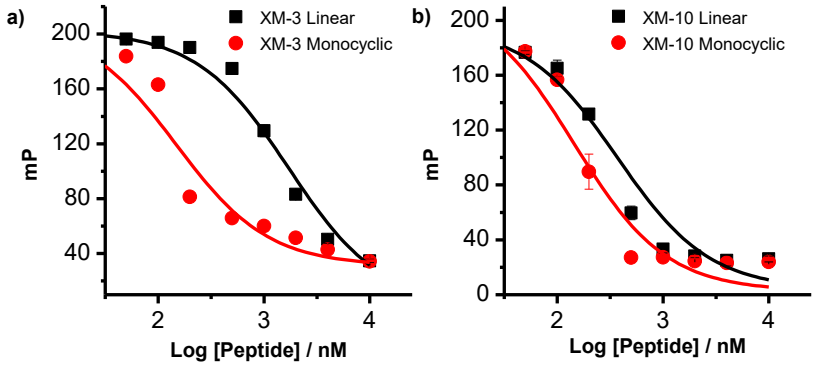

Figure S2. FP competition assays determine the interaction of peptides with the Keap1 kelch domain; a) XM-3 Linear (K_i = 120.8 ±5.5 nM) and XM-3 Monocyclic (K_i = 1255.3 ±76.0 nM); b) XM-10 Linear (K_i = 129.6 ±9.8 nM) and XM-10 Monocyclic (K_i = 205.9 ±6.8 nM).

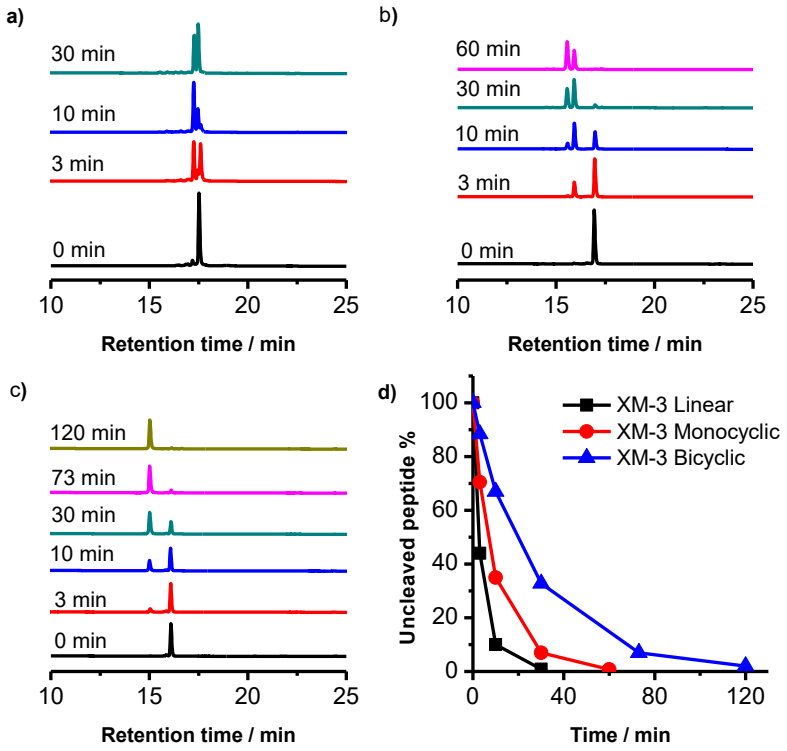

Figure S3. HPLC chromatograms of 40 μM peptides digested by 20 nM Trypsin; a) XM-3 Linear: reduced form of XM-3 Mono (GCGAAGWRDAESGERC); b) XM-3 Monocyclic: monocyclic peptide formed by oxidative folding of reduced XM-3 Mono in 100 mM phosphate buffer containing 50% DMSO; c) XM-3 Bicyclic: XM-3 isomer-I. d) Digestion kinetics of the three peptides mentioned above by trypsin.

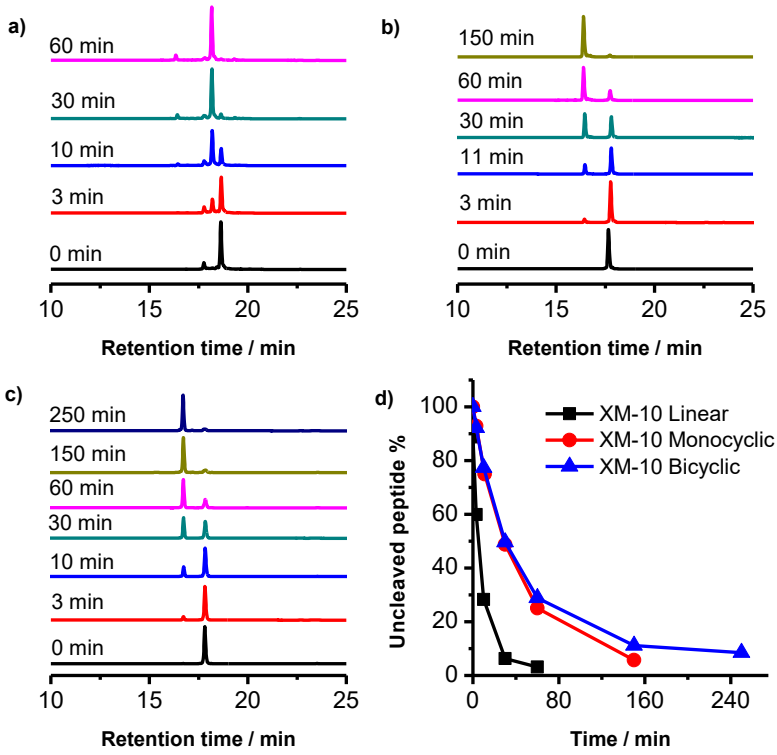

Figure S4. HPLC chromatograms of 40 μM peptides digested by 20 nM Trypsin; a) XM-10 Linear: reduced form of XM-10 Mono (GCGAWEEEDAETGERC); b) XM-10 Monocyclic: monocyclic peptide formed by oxidative folding of reduced XM-10 Mono in 100 mM phosphate buffer containing 50% DMSO; c) XM-10 Bicyclic: XM-10 isomer-I. d) Digestion kinetics of the three peptides mentioned above by trypsin.

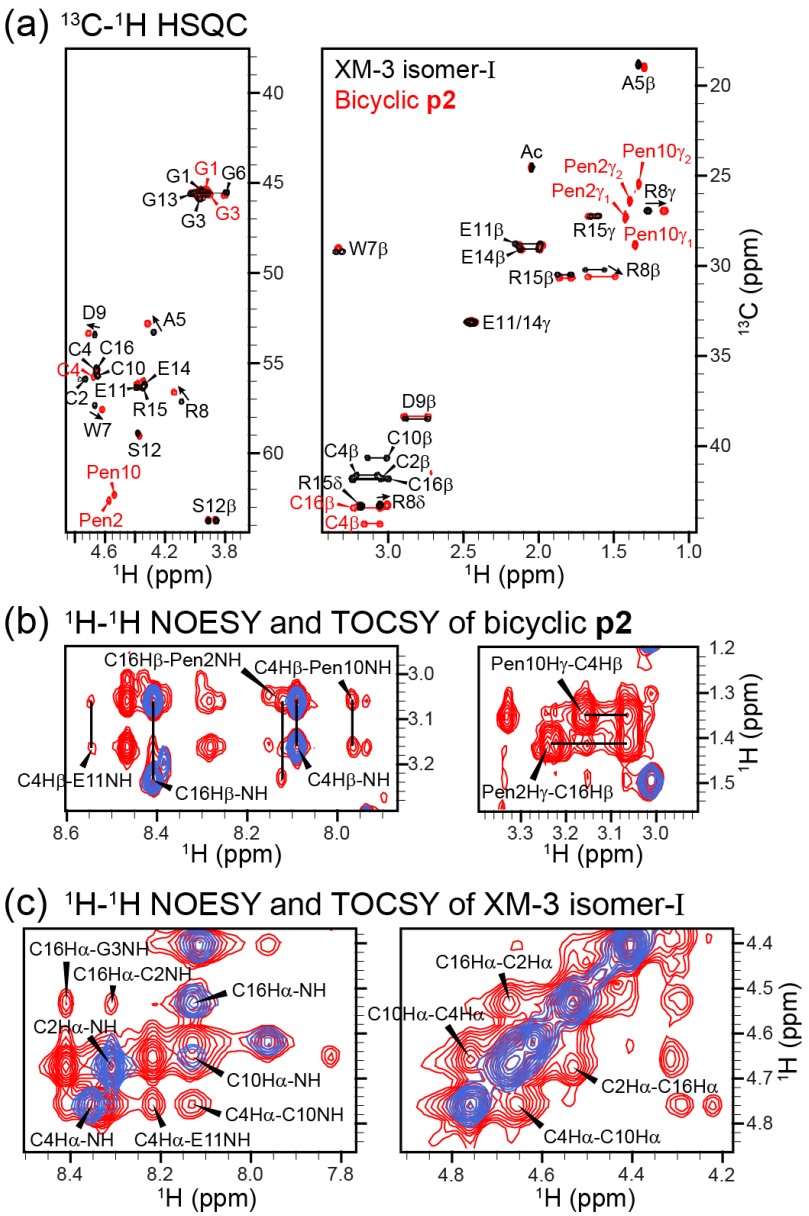

Figure S5. NMR characterization of XM-3 isomer-I and **p2**. a) 2D ^{13}C-^{1}H and ^{1}H-^{1}H correlation spectra of XM-3 isomer-I (black) and **p2** (red) in H_2O/D_2O (90%/10%, v/v); left: backbones, right: side chains. b) 2D ^{1}H-^{1}H NOESY (red) and TOCSY (blue) spectra of **p2** in H_2O/D_2O (90%/10%, v/v). c) 2D ^{1}H-^{1}H NOESY (red) and TOCSY (blue) spectra of XM-3 isomer-I in DMSO-d_6. For clarity, only two local regions were shown and cysteine related assignments were labeled.

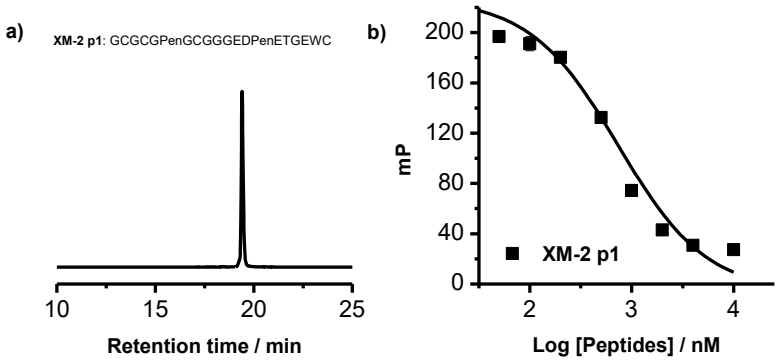

Figure S6. a) Amino acid sequence of XM-2 **p1** and HPLC chromatogram showing the formation of bicyclic peptides from oxidative folding of the reduced peptide in 100 mM PB buffer containing 50% DMSO and 0.5mM GSSG (pH 7.4). b) FP competition assays determine the interaction of peptides XM-2 **p1** (K_i = 502.4±29.2 nM) with the Keap1 kelch domain.

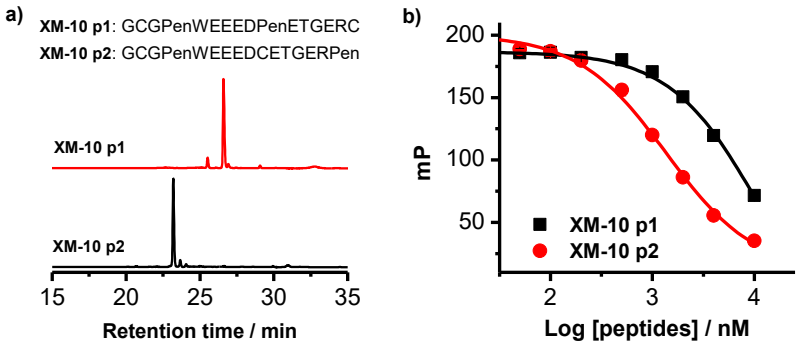

Figure S7. a) Amino acid sequence of XM-10 **p1** and **p2** and HPLC chromatograms showing the formation of bicyclic peptides from oxidative folding of the reduced peptides in 100 mM PB buffer containing 50% DMSO and 0.5mM GSSG (pH 7.4). b) FP competition assays determine the interaction of peptides XM-10 **p1** (K_i = 7524 ± 728 nM) and **p2** (K_i = 1139 ± 51 nM) with the Keap1 kelch domain.

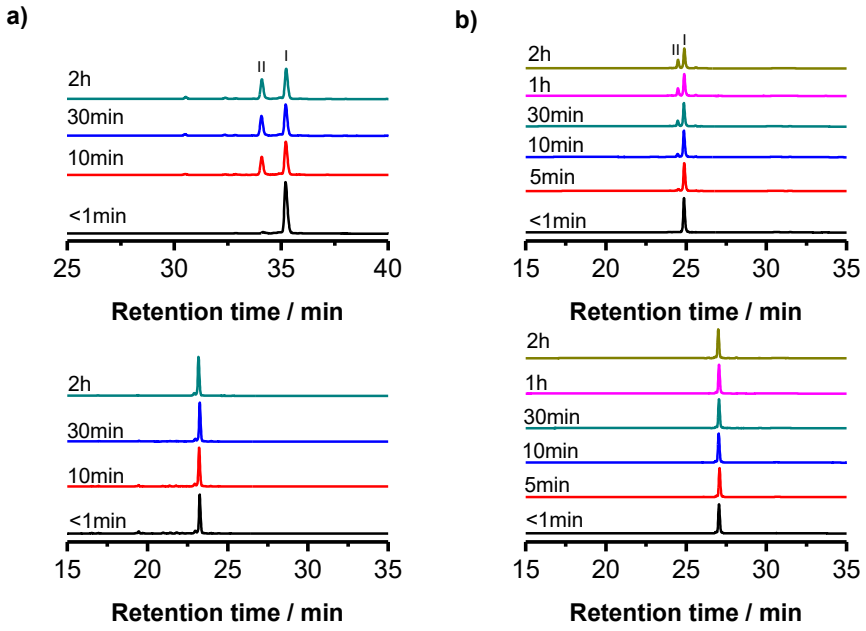

Figure S8. HPLC chromatograms showing the disulfide isomerization of bicyclic peptides in GSH/GSSG buffer; a) Top: 40 μM XM-3 isomer-I in 0.5 mM GSSG/0.5 mM GSH/100 mM phosphate buffer (pH 7.4). Bottom: 40 μM XM-3 **p2** under the same condition; b) Top: 40 μM XM-10 isomer-I in 0.5 mM GSSG/0.5 mM GSH/100 mM phosphate buffer (pH 7.4). Bottom: 40 μM XM-10 **p2** under the same condition.

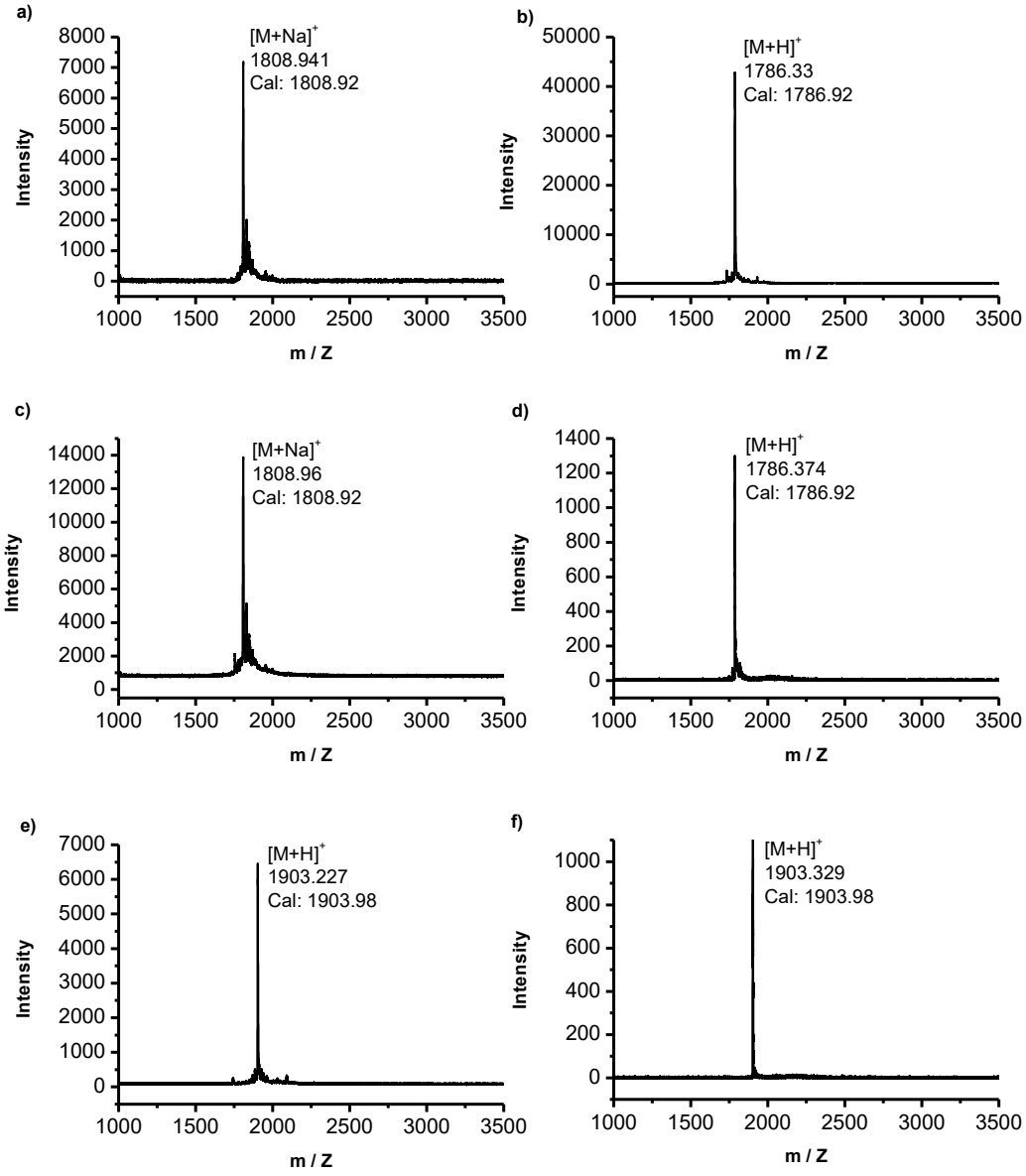

Figure S9. Mass spectra of the Pen-substituted peptides; a) XM-3 **p1**, b) XM-3 **p2**, c) XM-3 **p3**, d) XM-3 **p4**, e) XM-10 **p1**, and f) XM-10 **p2**.

References

[1] S. Chen, C. Heinis, *Methods Mol. Biol.*, **2015**, 1248, 119-137.

[2] J. Wang, M. Zha, Q. Fei, W. Liu, Y. Zhao, C. Wu, *Chem. Eur. J.*, **2017**, 23, 15150-15155.

[3] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J. Biomol. NMR.* **1995**, 6, 277-293.

[4] W. Lee, M. Tonelli, J.L. Markley, *Bioinformatics*. **2015**, 31, 1325-1327.

[5] J.L. Markley, A. Bax, Y. Arata, C.W. Hilbers, R. Kaptein, B.D. Sykes, P.E. Wright, K. Wuthrich, *Pure Appl. Chem.* **1998**, 70, 117-142.

Chromatograms of the Sanger sequencing of the 23 peptide sequences

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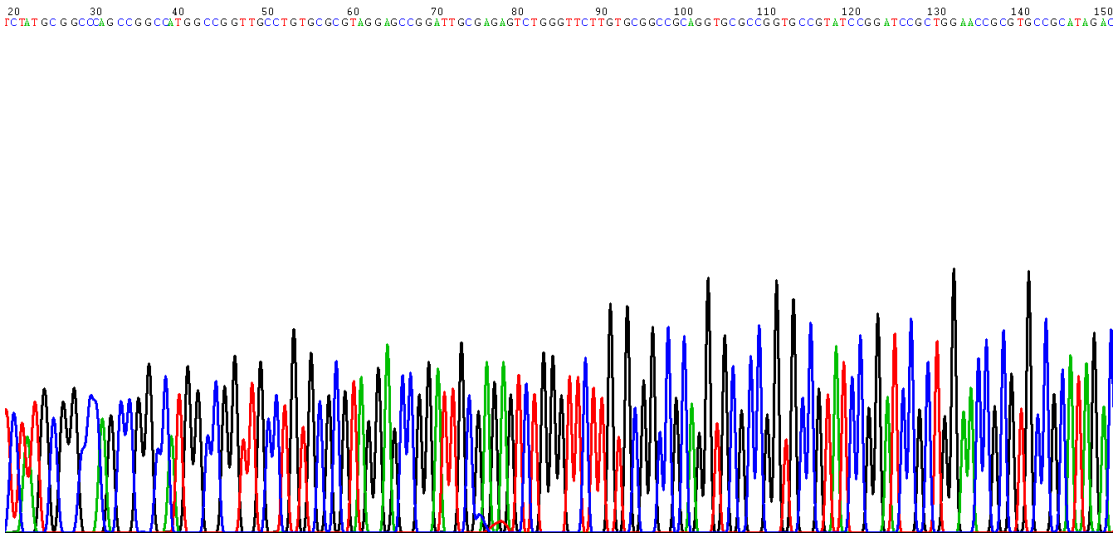

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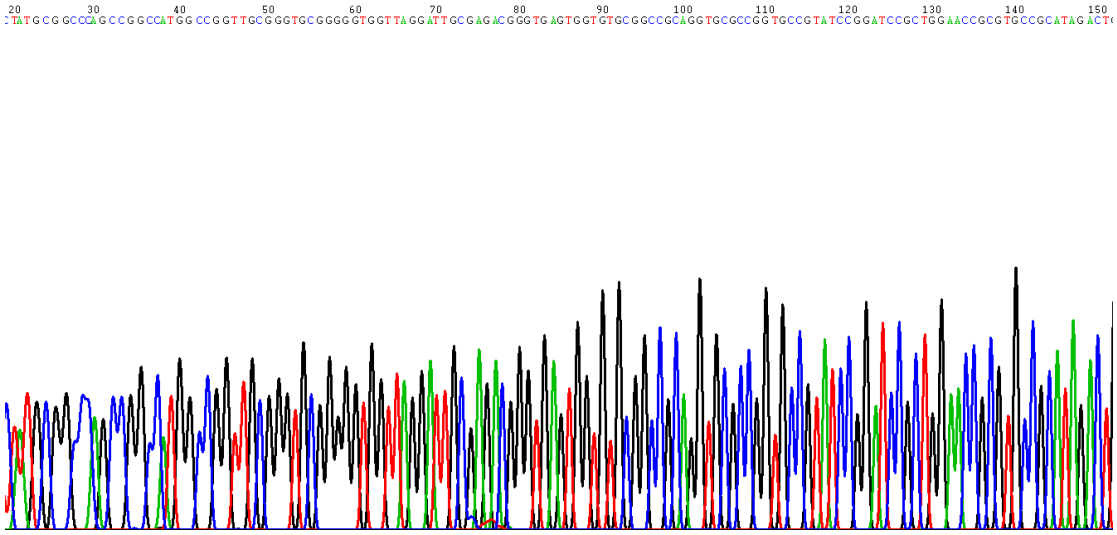

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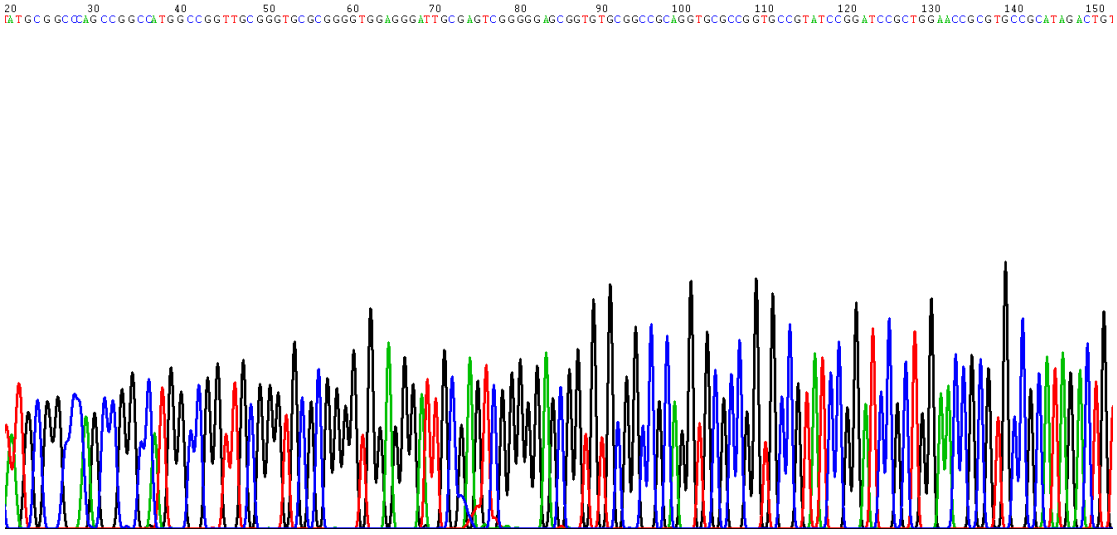
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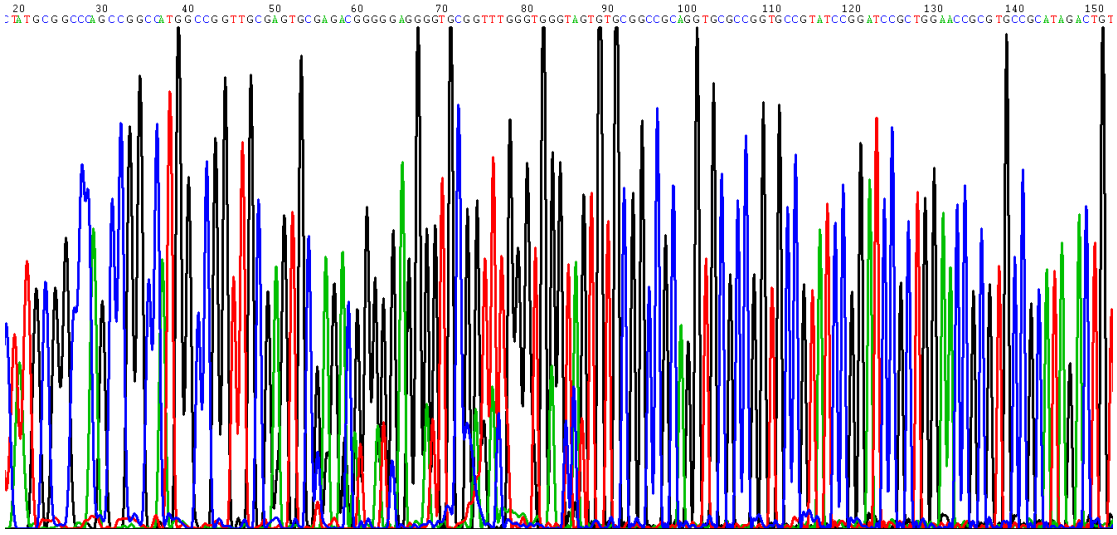
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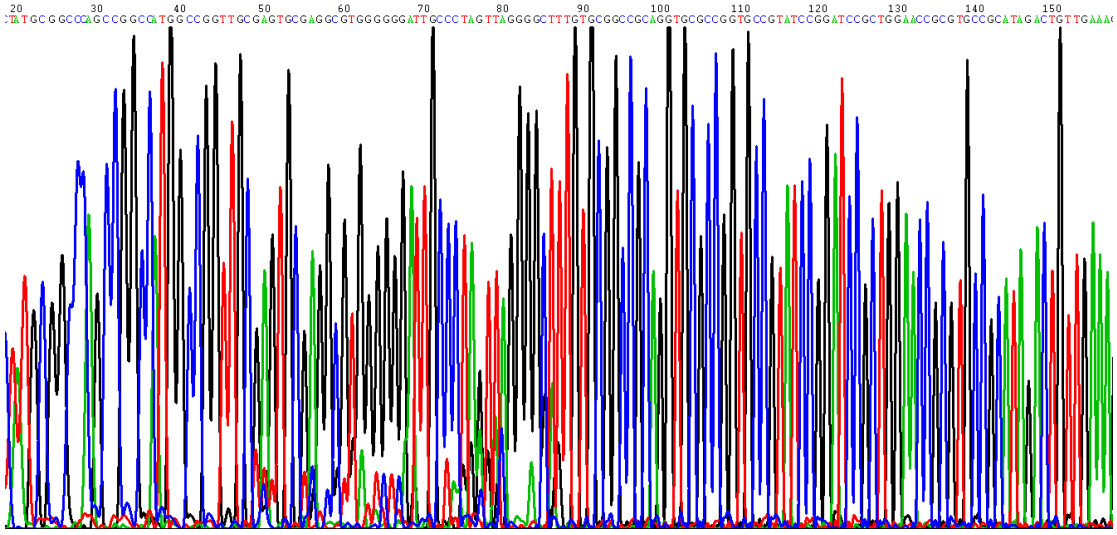

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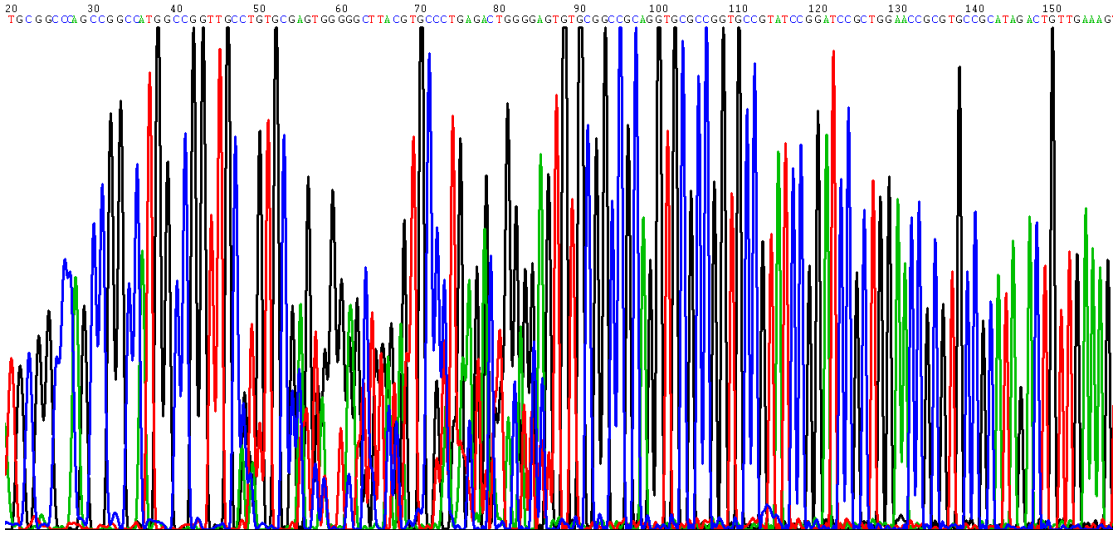

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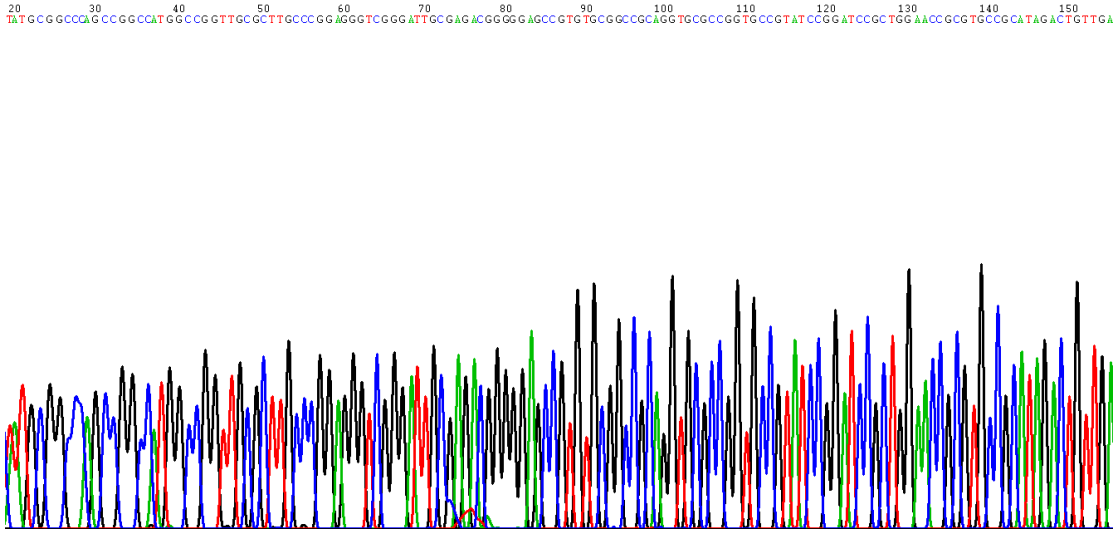
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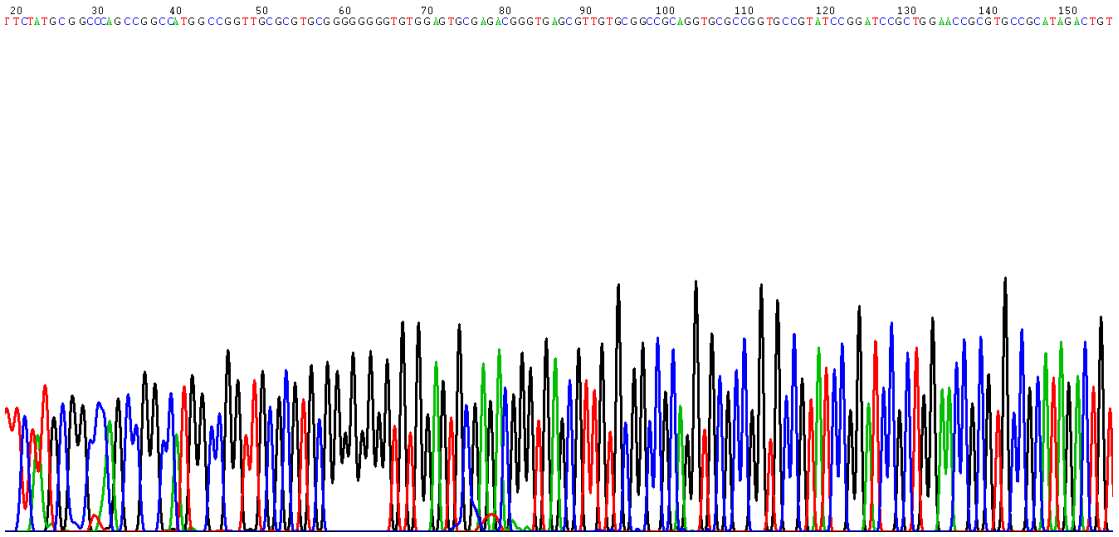
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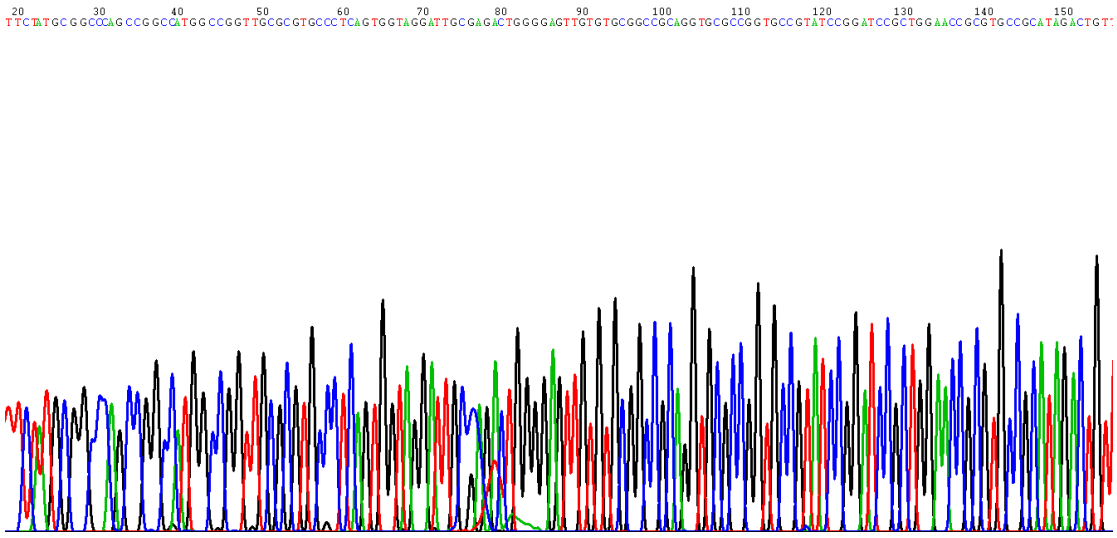

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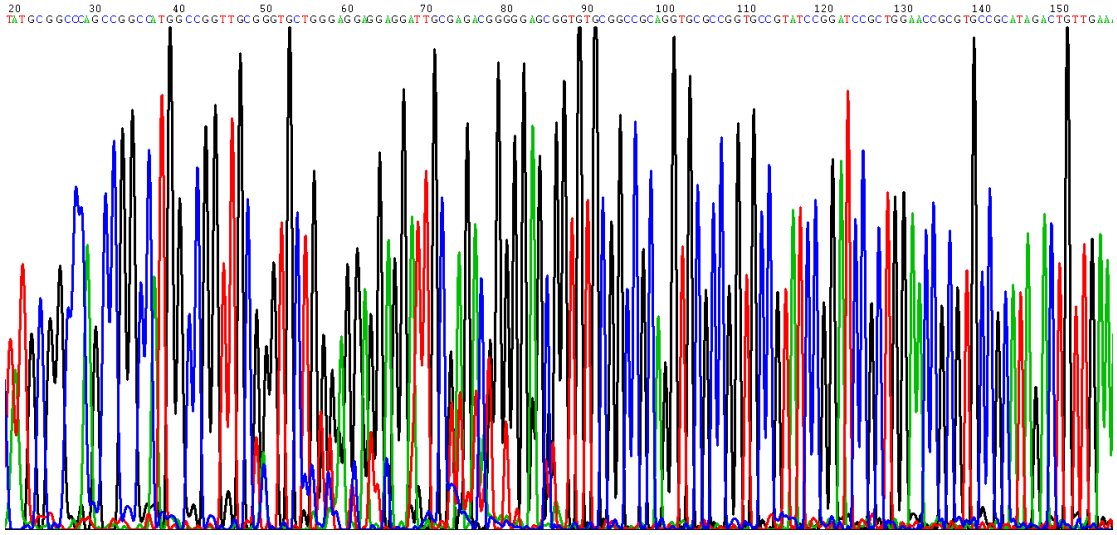

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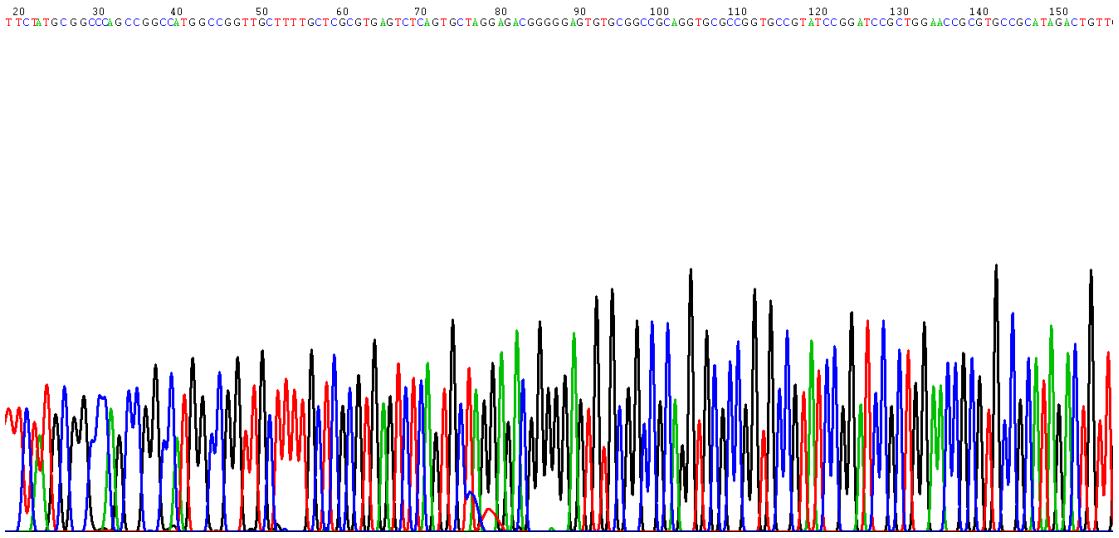

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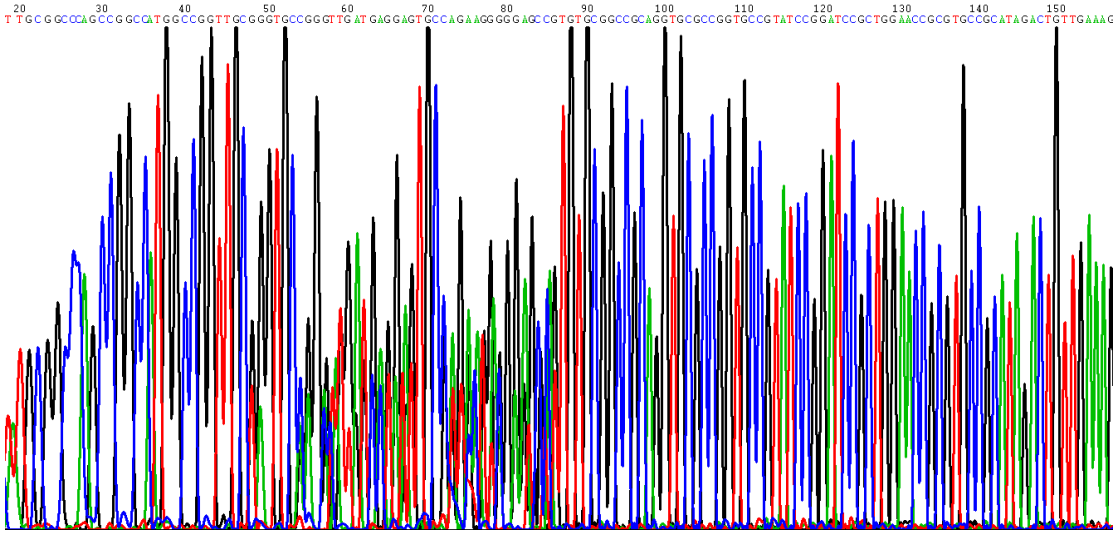

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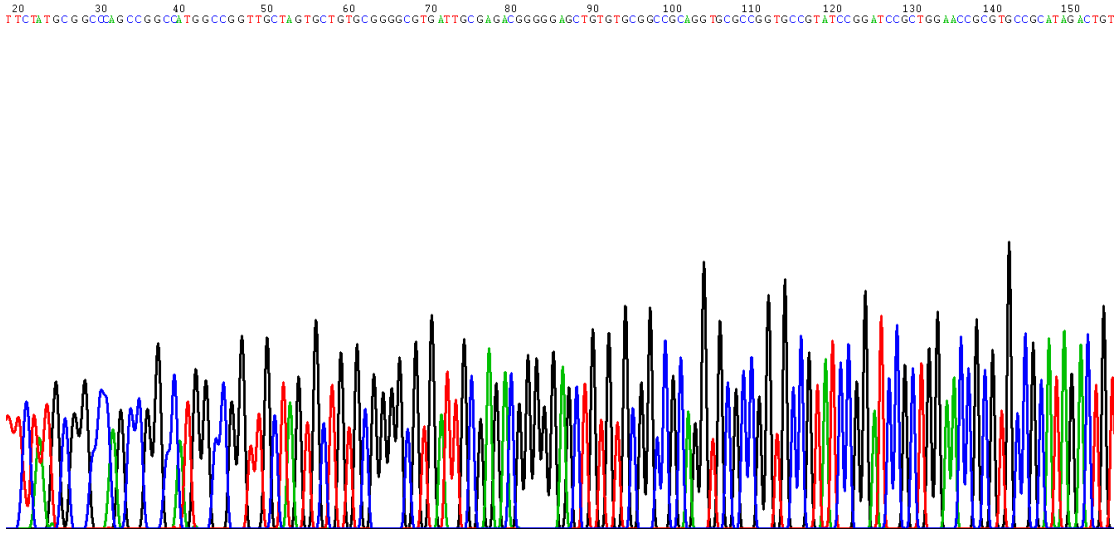

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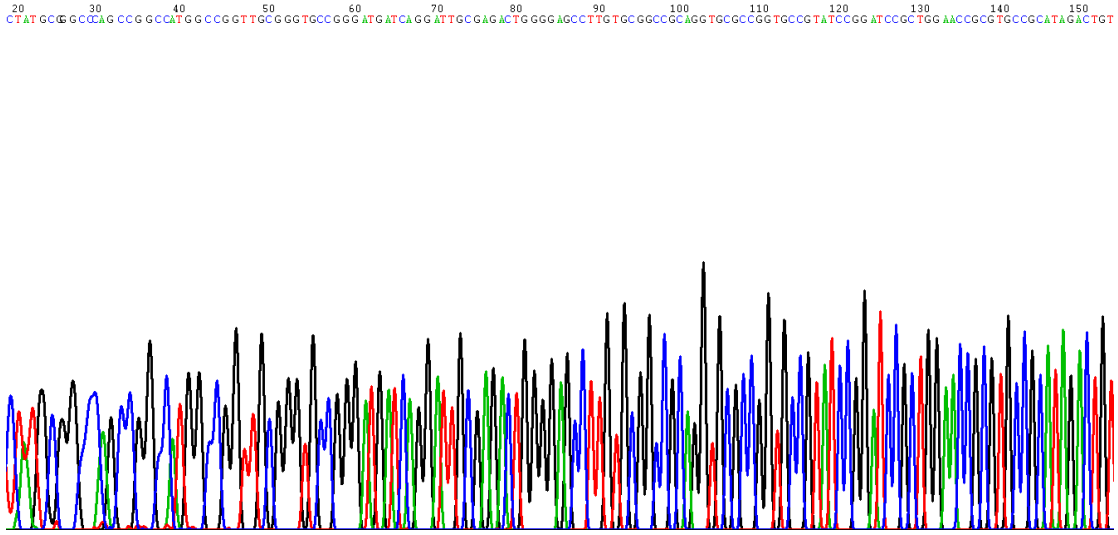

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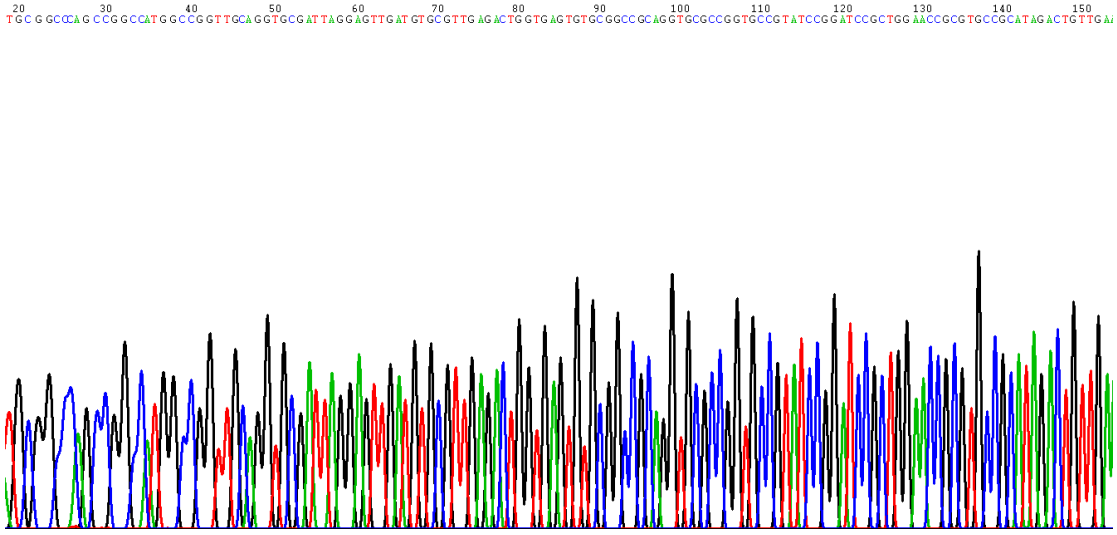

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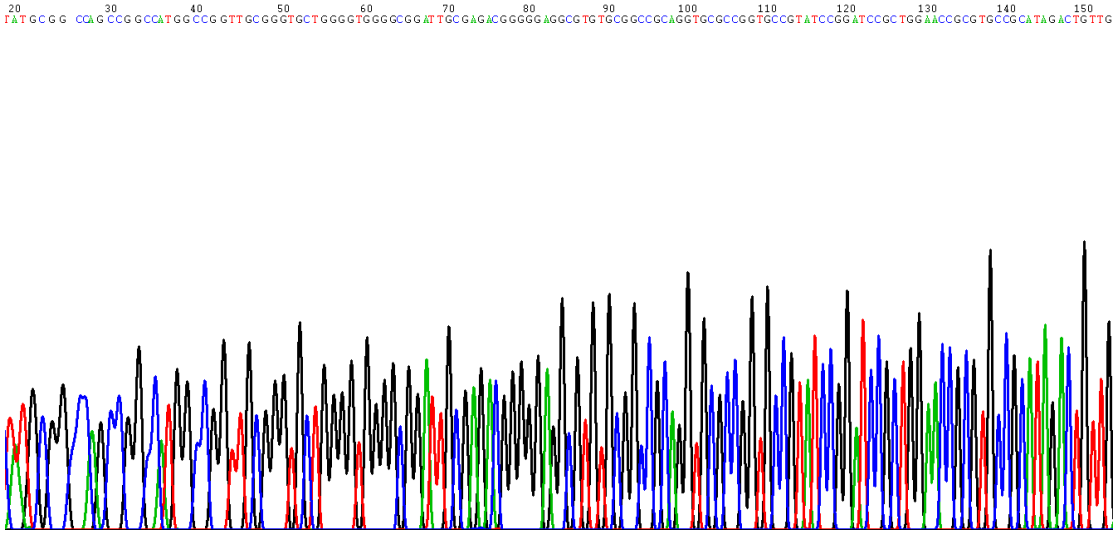

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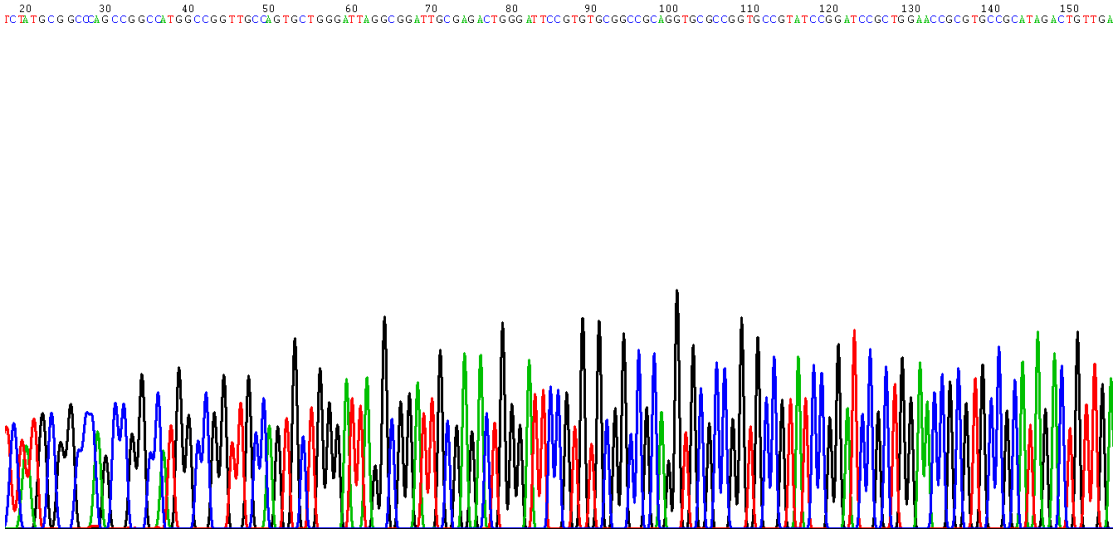
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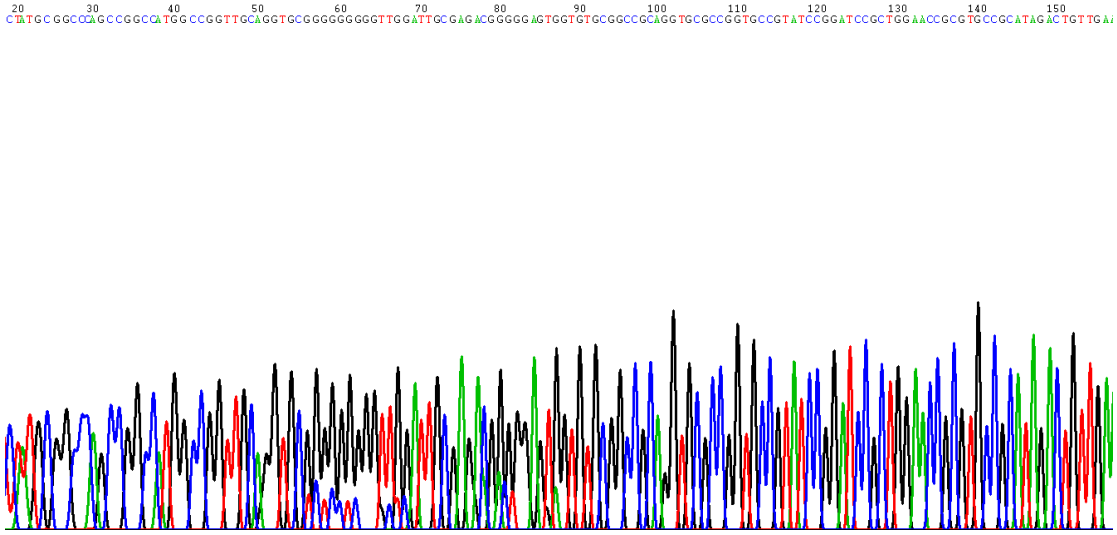
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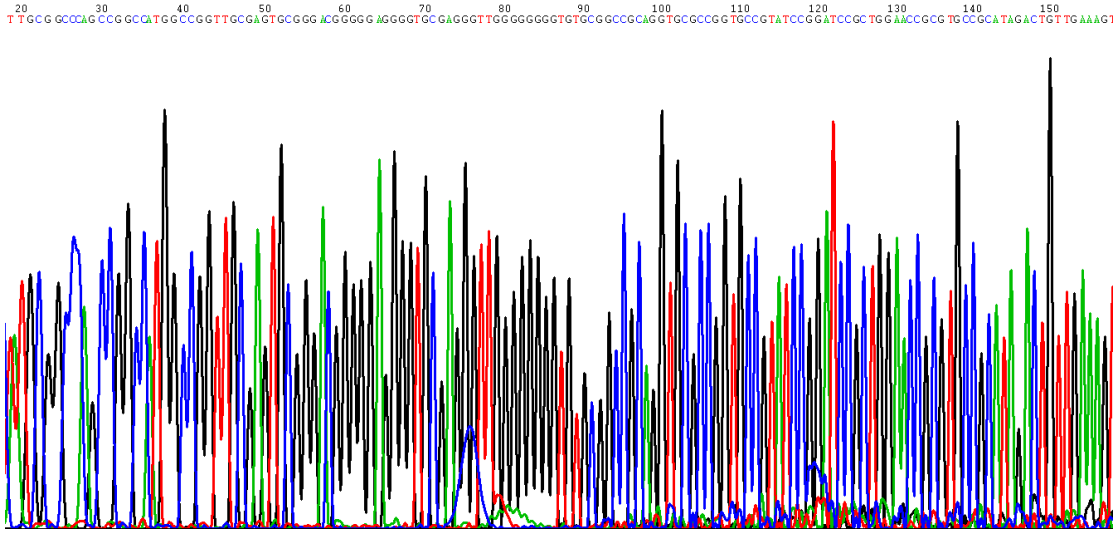
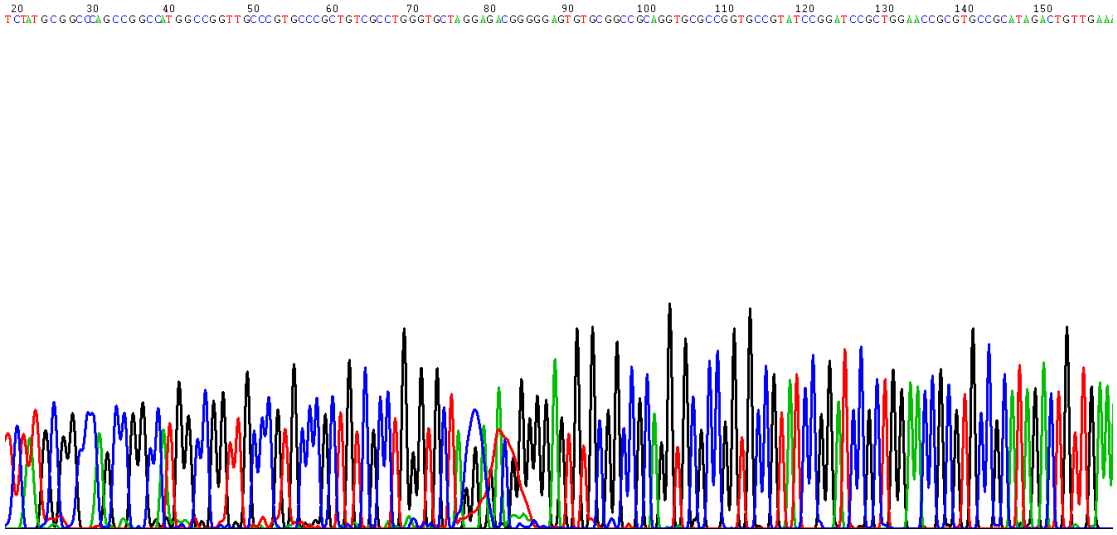
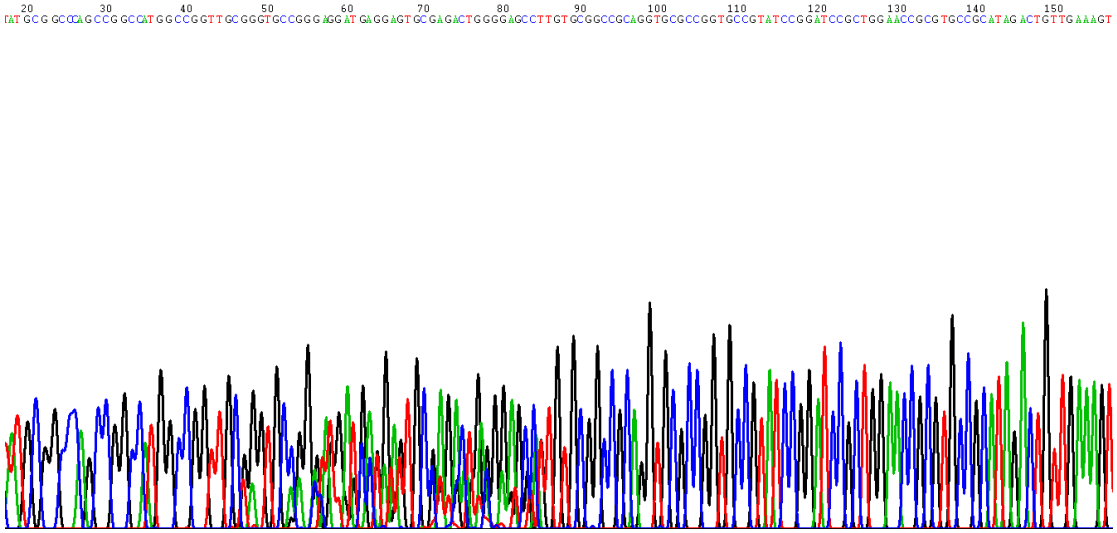
XM-19:



XM-20:



XM-21:



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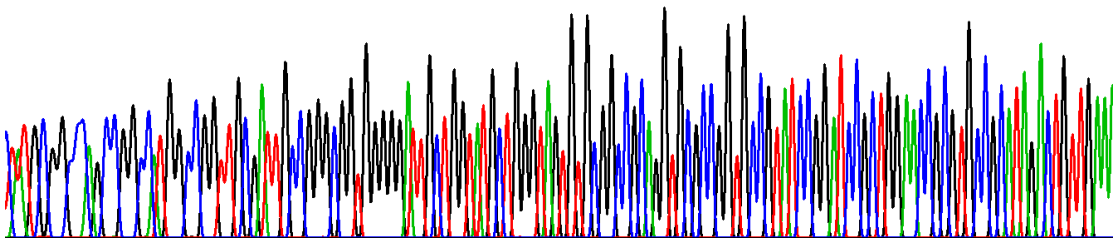
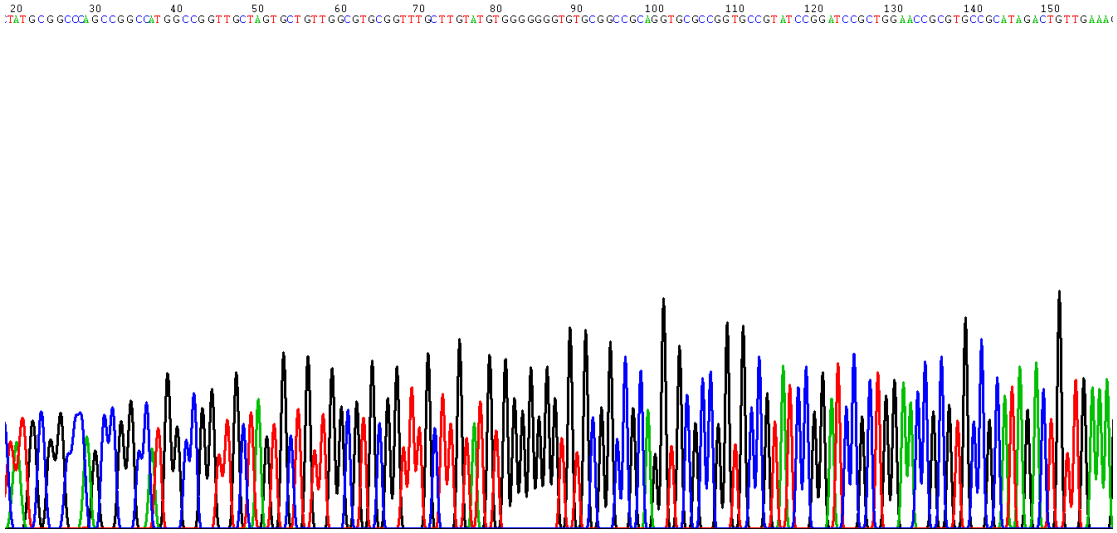


XM-23: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