Supplementary material for

Design of protease activated optical contrast agents that exploit a latent lysosomotropic effect for use in fluorescence-guided surgery

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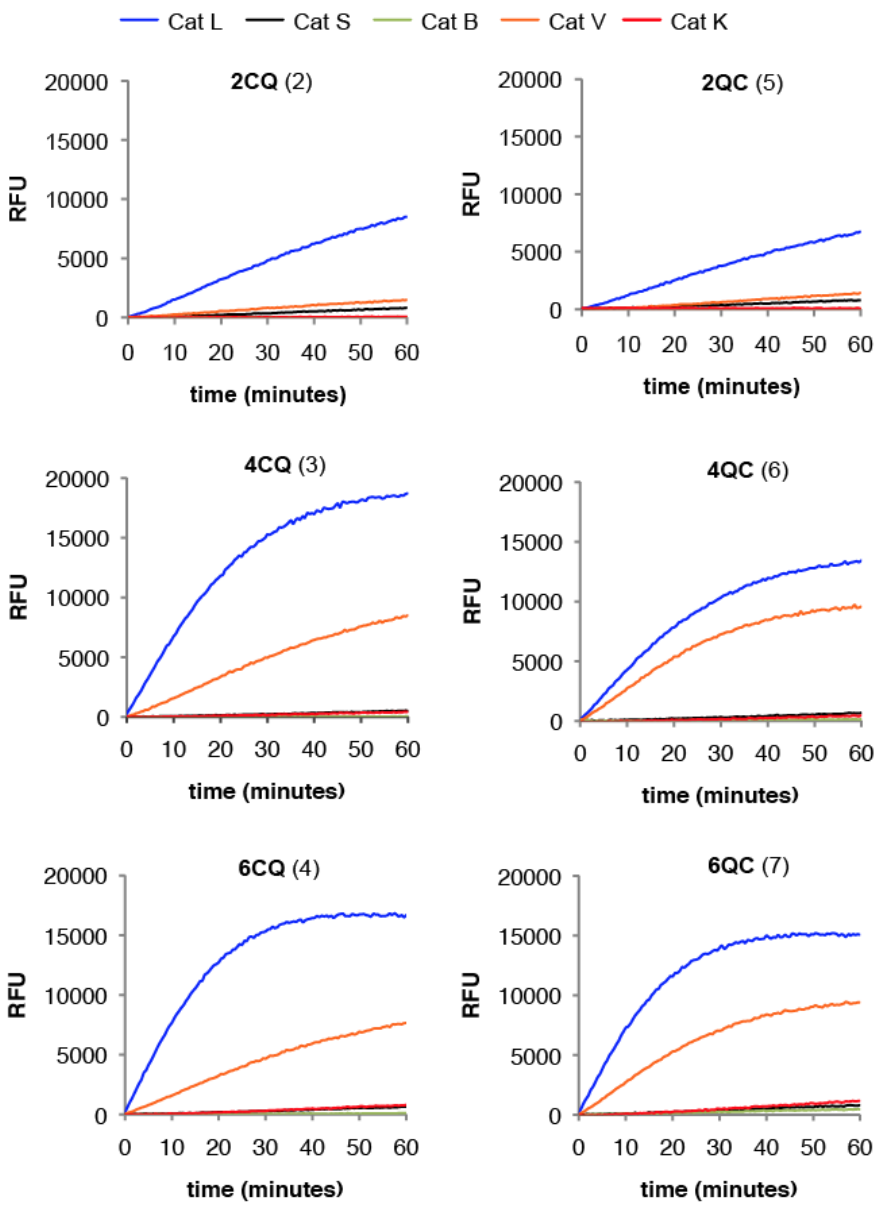
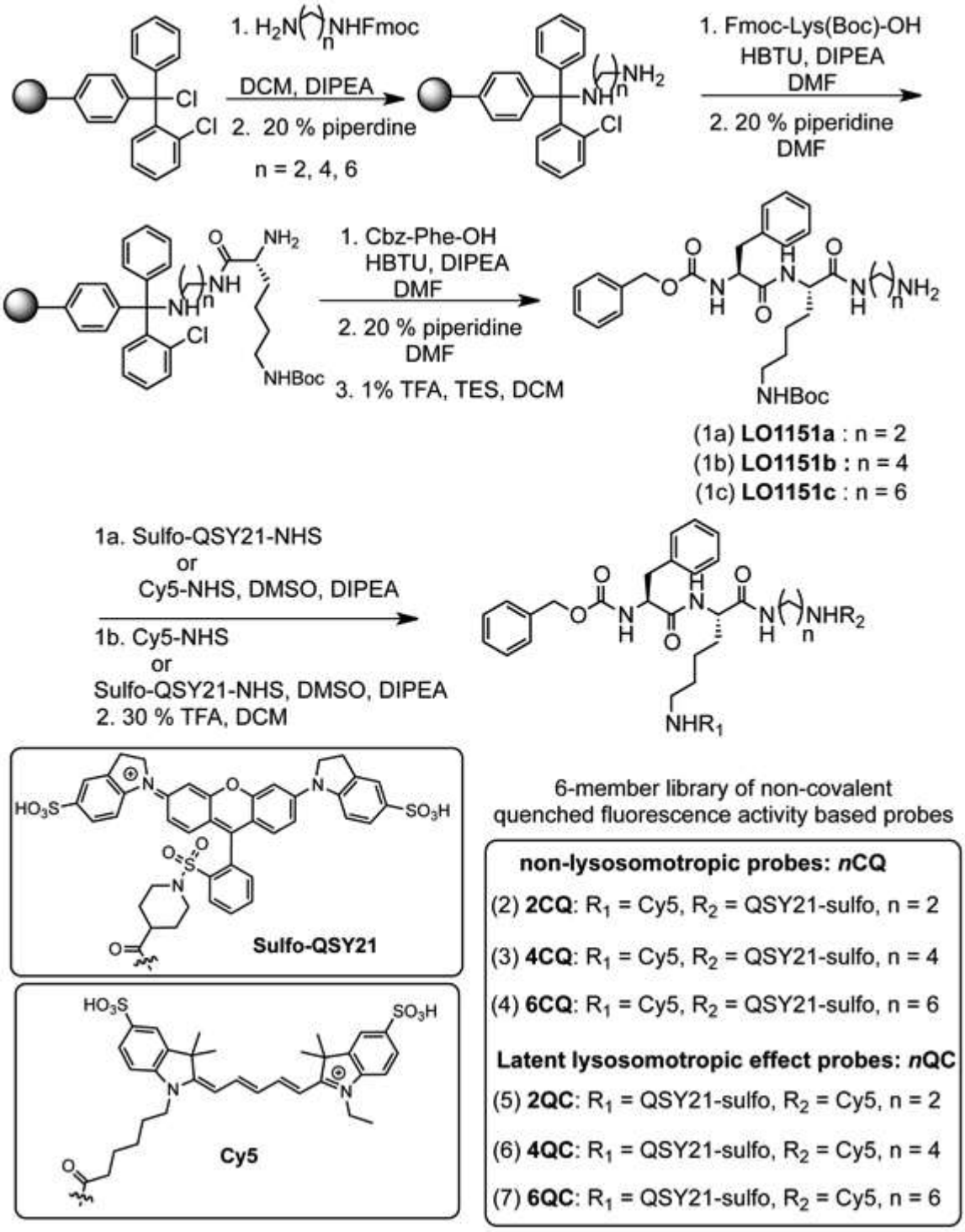


Supplementary Figure 1. Enzymatic turnover of quenched fluorescence ***n*CQ/nQC** substrates using recombinant cysteine cathepsins (10nM each in citric buffer pH 5.5): Cat L (blue), Cat S (black), Cat B (green), Cat V (orange) and K (red), in a total volume of 100 µl . The increase in Cy5 fluorescence was detected at the wavelengths 640/679nm at 37 ºC.

Supplementary Materials and Methods

All resins and reagents were purchased from commercial suppliers and used without further purifications. Water used for reactions and aqueous workup was glass-distilled from a deionized water feed. Reagent grade solvents were used for all non-aqueous extractions. All water-sensitive reactions were preformed in anhydrous solvents under positive pressure of argon. Reactions were analyzed by LC-MS using an API 150EX single-quadrupole mass spectrometer (Applied Biosystems). Synthesized compounds were purified via Reverse-phase HPLC with an ÅKTA explorer 100 (Amersham Pharmacia Biotech) using C18 columns. Compounds were eluted with and a gradient of doubly distilled water and acetonitrile containing 1 % trifluoracetic acid as solvents. NMR spectra were recorded on a Varian 400 MHz (400/100), Varian 500 MHz (500/125) equipped with a pulsed field gradient accessory and processed using MestReNova NMR processing software. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and referenced to the residual protium signal in the NMR solvents ($CDCl_3$, δ = 7.25). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet and q = quartet), coupling constant (*J*) in Hertz (Hz) and integration. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM (GIBCO Cat# 11995)), supplemented with 10 % fetal bovine serum (FBS) and and 100 units/ml % penicillin and 100 µg/ml streptomycin.

Compound synthesis



Supplementary Scheme 1. Synthesis of a 6-membered library of non-covalent quenched fluorescent activity based probes using a combination of solid and solution phase synthesis

Intermediates 1a, 1b, and 1c. 0.5 g of 2-chlorortrityl chloride resin (0.84 mmol/g loading) was weighed into three separate solid phase reaction vessels. The reaction vessels were labeled from a to c with a permanent marker. Dichloromethane (DCM) was added to suspend the resins and then vessels were agitated for 15 minutes using a laboratory shaker. After washing 2 times with DCM, mono-Fmoc-1,3-ethanediamine (0.35 g, 1.26 mmol), mono-Fmoc-1,4-butanediamine (0.39 g, 1.26 mmol), and mono-Fmoc1,6-hexanediamine (0.43 g, 1.26 mmol) in 3 mL of DCM were added to the resin in vessels a, b, and c respectively. Diisopropylthylamine (DIPEA, 3eq) was then added to each vessel and the reaction was agitated for a period of 30 minutes at room temperature. The resin was washed with DCM and then suspended in methanol for 10 minutes in order to deactivate any unreacted trityl-chloride. Fmoc deprotection was achieved by suspending the resin in a solution of 20 % piperidine in DMF for duration of 1 hour, followed by 3 times wash with DCM and DMF respectively. Next, the first amino acid Fmoc-Lys(Boc)-OH (0.58 g, 1.26 mmol) was coupled to the resin using HBTU (0.48 g, 1.26 mmol) and DIPEA (0.17 g, 1.26 mmol) in 3 mL of DMF and rotating the mixture for 2 h. Following a wash cycle, Fmoc deprotection was accomplished using 5 mL of 20% piperidine in DMF for 1 hour , followed again by the wash cycle. The remaining amino acid Cbz-Phe-OH was similarly coupled to the peptide on the resin. The resin was then treated with a solution 1 % trifluoroactic acid in DCM to selectively cleave the peptide (with a from the resin resulting in the products with ω-N-Boc-protected lysine. The solvent was removed from each cleaved product by co-evaporation with toluene, resulting in the intermediate substrates **1a**, **1b** and **1c** respectively (Scheme 1). Each product was then further purified by Reversed phase preparative HPLC followed by lyophilzation to to afford the final peptides as white powder in greater that 95 % purity.

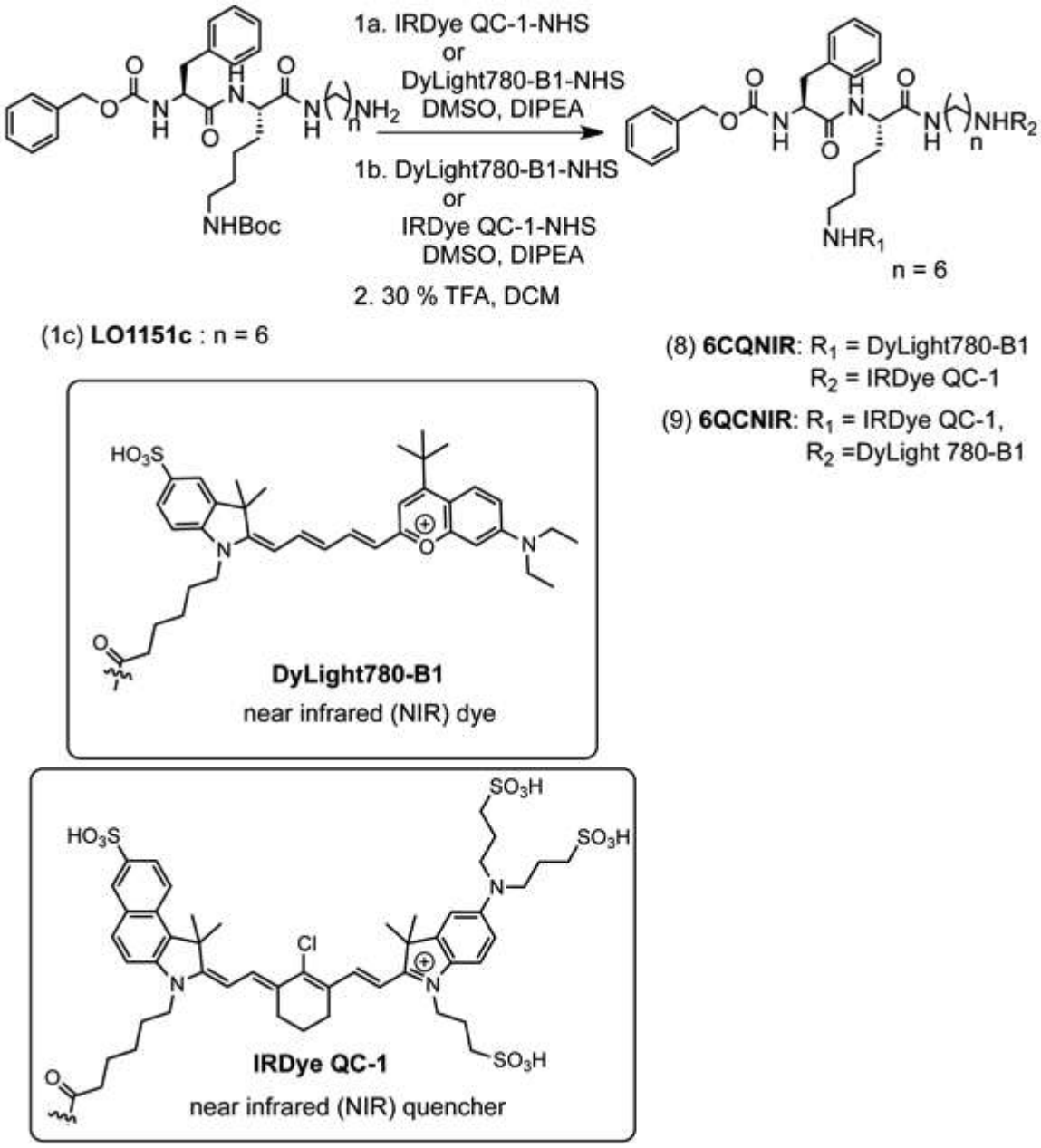
Probes **2CQ** (2), **4CQ** (3), **6CQ** (4). In separate 1.5 mL eppendorf tubes, intermediates **1a** (2.0 mg, 3.51 µmol, 1 equiv), **1b** (2.0 mg, 3.35 µmol, 1 equiv) or **1c** (2.0 mg, 3.2 µmol,

1 equiv) were dissolved in 100 µL of DMSO. DIPEA (5 equiv) was added followed by QSY21-sulfo-NHS (1.1 equiv). The reaction was stirred for 1 hour at room temperature followed by purification of the product by HPLC. The solvent was removed by rotary evaporation after which the Boc-protection on the lysine was removed by treatment with 30 % TFA in DCM for 30 minutes. Following removal of the solvent, the product was lyophilized and then reacted with Cy5-NHS (1.1equiv) in DMSO with 5 equivalent of DIPEA (Supplementary Scheme 1). The final products were each purified by reverse phase HPLC to yield the non-lysosomotropic quenched fluorescence substrates *n*CQ, where n =, 2, 4, and 6 respectively, in greater that 95 % purity. LCMS: 2CQ (2) calculated for $C_{99}H_{108}N_{10}O_{21}S_5^{2+}$: 966.81; found; 966.8, HRMS: calculated for $C_{99}H_{108}N_{10}O_{21}S_5^{2+}$: 966.8159; found: 966.8121. ^{1}H NMR (400 MHz, DMSO): δ 8.40 – 8.29 (m, 2H), 8.18 (d, J = 8.4 Hz, 1H), 8.04 – 7.92 (m, 2H), 7.84 (s, 1H), 7.81 (s, 2H), 7.73 (dd, J = 10.7, 5.6 Hz, 2H), 7.70 – 7.57 (m, 9H), 7.57 – 7.47 (m, 5H), 7.34 – 7.15 (m, 14H), 6.54 (t, J = 10.2 Hz, 7H), 6.29 (dd, J = 13.6, 7.9 Hz, 2H), 4.92 (s, 2H), 4.35 (dd, J = 9.5, 7.2 Hz, 4H), 4.30 – 4.22 (m, 1H), 4.10 (tdd, J = 12.7, 7.4, 5.6 Hz, 4H), 3.28 – 3.20 (m, 9H), 3.09 – 2.90 (m, 8H), 2.70 (d, J = 2.1 Hz, 1H), 2.69 – 2.64 (m, 1H), 2.34 – 2.32 (m, 1H), 2.02 (t, J = 6.6 Hz, 2H), 1.67 (s, 13H), 1.62 – 1.47 (m, 6H), 1.35 – 1.21 (m, 10H). LCMS: 4CQ (3) calculated for $C_{101}H_{112}N_{10}O_{21}S_5^{2+}$: 980.8; found; 980.6, HRMS: calculated for $C_{101}H_{112}N_{10}O_{21}S_5^{2+}$: 980.8315; found: 980.8264. ^{1}H NMR (400 MHz, DMSO): δ 8.33 (t, J = 13.0 Hz, 2H), 8.18 (dd, J = 7.7, 1.1 Hz, 1H), 8.03 – 7.91 (m, 3H), 7.79 (s, 3H), 7.75 – 7.55 (m, 12H), 7.55 – 7.49 (m, 4H), 7.47 (d, J = 8.1 Hz, 1H), 7.35 – 7.11 (m, 15H), 6.54 (t, J = 12.5 Hz, 2H), 6.27 (dd, J = 14.0, 8.0 Hz, 2H), 4.90 (s, 2H), 4.41 – 4.29 (m, 4H), 4.29 – 4.18 (m, 1H), 4.19 – 3.95 (m, 6H), 3.29 – 3.12 (m, 9H), 3.04 – 2.80 (m, 8H), 2.67 (ddd, J = 8.8, 6.3, 5.4 Hz, 2H), 2.31 (dt, J = 3.5, 1.9 Hz, 1H), 2.00 (t, J = 7.0 Hz, 2H), 1.65 (s, 14H), 1.50 (dd, J = 11.2, 6.4 Hz, 6H), 1.36 – 1.17 (m, 16H). LCMS: 6CQ (4) calculated for $C_{103}H_{116}N_{10}O_{21}S_5^{2+}$: 994.8; found; 994.8, HRMS:

calculated for $C_{103}H_{116}N_{10}O_{21}S_5^{2+}$: 994.8472; found: 994.8444. ^{1}H NMR (400 MHz, DMSO) δ 8.34 (t, J = 12.9 Hz, 2H), 8.19 (d, J = 9.0 Hz, 1H), 8.04 – 7.91 (m, 3H), 7.79 (s, 2H), 7.71 (t, J = 5.5 Hz, 1H), 7.63 (ddd, J = 20.6, 10.2, 5.4 Hz, 10H), 7.50 (dd, J = 17.0, 7.9 Hz, 5H), 7.21 (ddt, J = 23.7, 21.2, 7.0 Hz, 14H), 6.54 (t, J = 12.3 Hz, 4H), 6.27 (dd, J = 13.7, 7.8 Hz, 2H), 4.90 (s, 2H), 4.34 (t, J = 9.9 Hz, 4H), 4.25 (dd, J = 10.2, 6.0 Hz, 1H), 4.19 – 3.97 (m, 5H), 3.23 (dt, J = 17.2, 8.7 Hz, 9H), 3.04 – 2.84 (m, 7H), 2.71 (d, J = 12.6 Hz, 1H), 2.67 – 2.63 (m, 1H), 2.31 (dt, J = 3.7, 1.9 Hz, 1H), 2.00 (t, J = 7.1 Hz, 3H), 1.65 (s, 13H), 1.50 (t, J = 11.5 Hz, 6H), 1.36 – 1.05 (m, 20H).

Probes **2QC** (5), **4QC** (6), **6QC** (7). In a separate 1.5 mL eppendorf tubes, intermediates **1a** (2.0 mg, 3.51 μmol, 1 equiv), **1b** (2.0 mg, 3.35 μmol, 1 equiv) and **1c** (2.0 mg, 3.2 μmol, 1 equiv) were dissolved in 100 μL of DMSO. DIPEA (5equiv) was added to each compound followed by Cy5-NHS (1.1 equiv). The reaction was stirred for 1 hour at room temperature followed by purification of the product by HPLC. The solvent was removed by rotary evaporation after which the Boc-protection on the lysine was removed by treatment with 30 % TFA in DCM for 30 minutes. Following removal of the solvent, the products were lyophilized and then reacted with QSY21-sulfo-NHS in DMSO with 5 equivalent of DIPEA for a duration of 1h. LCMS analysis showed completion of the reaction. (Supplementary Scheme 1). The final products were each purified by reverse phase HPLC using a gradient of 5 % to 50% acetonitrile over 30 minutes to yield the latent lysosomotropic effect quenched fluorescence substrates *n*QC, where n =, 2, 4, and 6 respectively in greater that 95 % purity. LCMS: 2CQ (2) calculated for $C_{99}H_{108}N_{10}O_{21}S_5^{2+}$: 966.81; found; 966.8, HRMS: calculated for $C_{99}H_{108}N_{10}O_{21}S_5^{2+}$: 966.8159; found: 966.8122. ^{1}H NMR (400 MHz, DMSO) δ 8.33 (t, J = 12.4 Hz, 2H), 8.18 (d, J = 8.0 Hz, 1H), 8.07 – 7.91 (m, 3H), 7.90 – 7.84 (m, 1H), 7.79 (s, 3H), 7.70 – 7.57 (m, 10H), 7.50 (dd, J = 18.9, 10.0 Hz, 4H), 7.23 (ddt, J = 24.4, 16.0, 8.0 Hz, 14H), 6.60 –

6.50 (m, 2H), 6.27 (dd, J = 13.4, 10.2 Hz, 2H), 4.89 (s, 2H), 4.43 – 4.29 (m, 4H), 4.25 (td, J = 11.1, 6.0 Hz, 1H), 4.17 – 4.00 (m, 5H), 3.28 – 3.17 (m, 8H), 3.09 – 2.94 (m, 6H), 2.87 (dt, J = 12.4, 6.4 Hz, 2H), 2.75 – 2.63 (m, 2H), 2.34 – 2.28 (m, 1H), 2.05 – 1.98 (m, 3H), 1.73 (s, 1H), 1.65 (s, 13H), 1.52 (s, 6H), 1.34 – 1.07 (m, 13H). LCMS: 4QC (6) calculated for $C_{101}H_{112}N_{10}O_{21}S_5^{2+}$: 980.8; found; 980.8, HRMS: calculated for $C_{101}H_{112}N_{10}O_{21}S_5^{2+}$: 980.8315; found: 980.8267. ^{1}H NMR (400 MHz, DMSO) δ 8.33 (t, J = 13.5 Hz, 2H), 8.18 (d, J = 7.0 Hz, 1H), 8.03 – 7.90 (m, 3H), 7.79 (s, 3H), 7.72 (t, J = 5.4 Hz, 1H), 7.68 – 7.56 (m, 10H), 7.52 (d, J = 8.4 Hz, 4H), 7.47 (d, J = 8.7 Hz, 1H), 7.23 (dq, J = 24.2, 8.1 Hz, 14H), 6.55 (t, J = 12.3 Hz, 3H), 6.27 (dd, J = 13.8, 7.1 Hz, 2H), 4.89 (s, 2H), 4.34 (t, J = 9.5 Hz, 4H), 4.22 (dd, J = 16.2, 7.4 Hz, 1H), 4.16 – 4.00 (m, 5H), 3.28 – 3.15 (m, 8H), 3.07 – 2.90 (m, 6H), 2.86 (dd, J = 11.4, 5.5 Hz, 2H), 2.74 – 2.63 (m, 2H), 2.43 (d, J = 11.5 Hz, 2H), 2.31 (dd, J = 3.5, 1.8 Hz, 1H), 1.99 (t, J = 7.2 Hz, 2H), 1.65 (s, 14H), 1.57 – 1.43 (m, 6H), 1.36 – 1.17 (m, 15H). LCMS: 6QC (7) calculated for $C_{103}H_{116}N_{10}O_{21}S_5^{2+}$: 994.8; found; 994.8, HRMS: calculated for $C_{103}H_{116}N_{10}O_{21}S_5^{2+}$: 994.8472; found: 994.8429. 1H NMR (400 MHz, DMSO) δ 8.34 (t, J = 13.1 Hz, 2H), 8.21 – 8.12 (m, 1H), 7.96 (dt, J = 15.3, 8.5 Hz, 3H), 7.79 (s, 3H), 7.73 – 7.55 (m, 11H), 7.50 (dd, J = 19.7, 8.5 Hz, 5H), 7.23 (dq, J = 16.3, 7.7 Hz, 14H), 6.53 (s, 8H), 6.27 (dd, J = 13.8, 5.6 Hz, 2H), 4.89 (s, 2H), 4.28 (ddd, J = 19.8, 16.3, 7.2 Hz, 5H), 4.08 (ddd, J = 16.0, 11.8, 6.5 Hz, 5H), 3.27 – 3.13 (m, 7H), 3.04 – 2.90 (m, 5H), 2.86 (dd, J = 12.5, 5.9 Hz, 2H), 2.74 – 2.63 (m, 2H), 2.31 (dt, J = 3.5, 1.7 Hz, 1H), 2.00 (t, J = 7.0 Hz, 2H), 1.66 (s, 12H), 1.58 – 1.44 (m, 5H), 1.38 – 1.13 (m, 20H).



Supplementary Scheme 2. Synthesis of near infrared non-covalent quenched fluorescent activity based probe that iscompatible with the da Vinci surgical system for FGS.

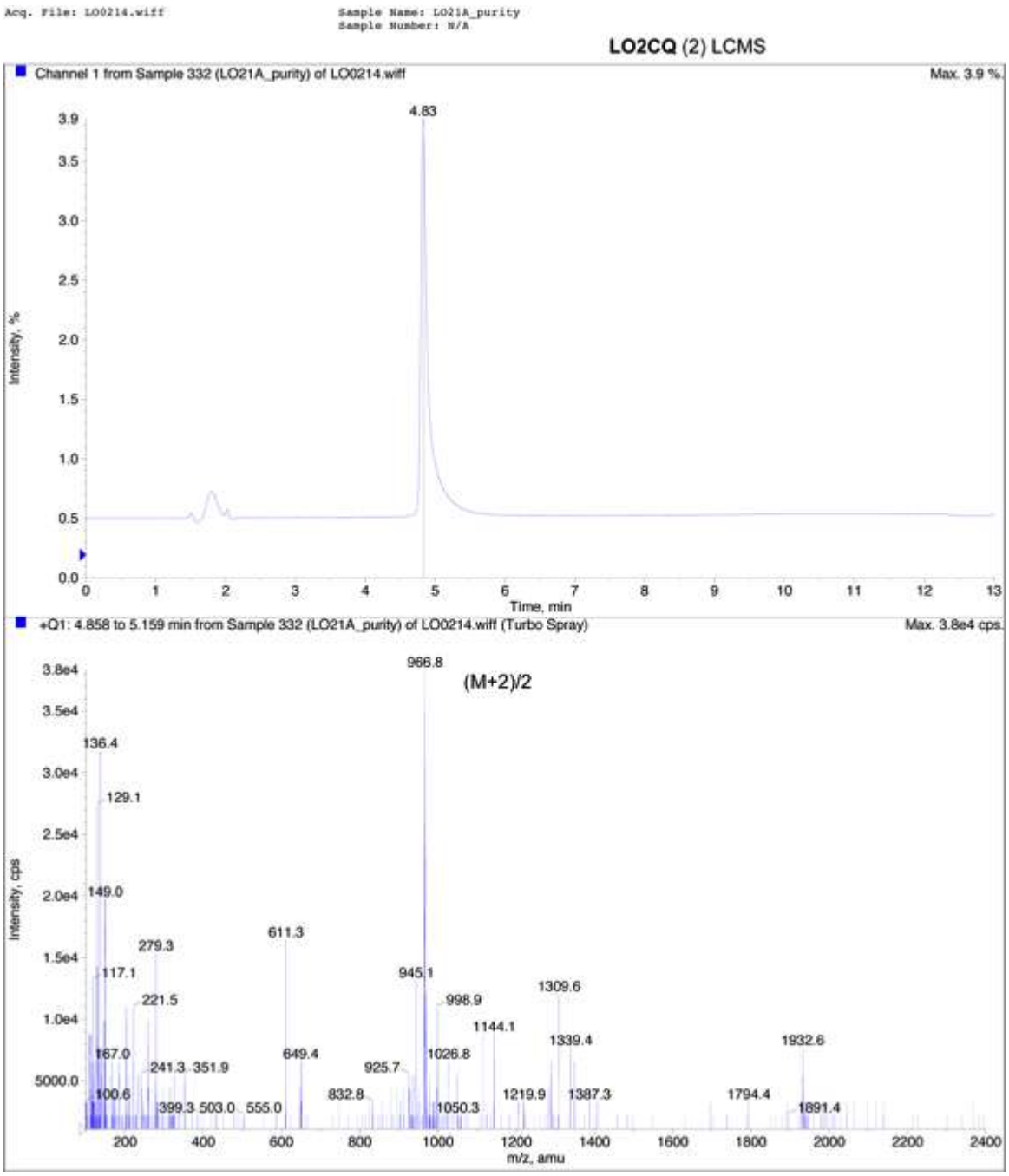
Probe **6CQNIR (8**). Intermediate 1c (1.0 mg, 1.6 µmol, 1 equiv) was dissolved in 100µl of DMSO. DIPEA (1.4 µl, 8 µmol) and IRDye QC-1-NHS (2.1mg, 1.76 µmol, 1.1 equiv) were added and reacted for 1 hour. The product was purified by Reverse phase

preparative HPLC using a gradient stating from 20 % acetonitrile to 60 % over 25 minutes. The solvent was removed by evaporation and then treated with 50 % TFA in DCM for 30 minutes to remove the Boc protection group on the lysine. The product was lyophilized to yield 1.8 mg (1.1 µmol) of the intermediate amine product, representing a yield of 72 % over 2 steps. This product was taken up in 100 µl DMSO, DIPEA (5equiv) was added and then reacted for hour with DyLigth780-B1-NHS (0.94 mg, 1.21 µmol, 1.1equiv). The final product was purified by HPLC using a gradient starting from 10 % to 60 % acetonitrile over 30 minutes at flow rate of 5µl/min. The product was lyophilized to obtain 1.9 mg of a dark green powder, representing a yield of 76%. LCMS (ESI) : m/z calculated for $C_{116}H_{151}ClN_{10}O_{22}S_5^{2+}$: 1115.96; found: 1116.1. HRMS, calculated for $C_{116}H_{151}ClN_{10}O_{22}S_5^{2+}$; 1115.4643; found: 1115.4617, calculated for $C_{116}H_{152}ClN_{10}O_{22}S_5^{3+}$; 744.9786; found: 743.9777

Probe **6QCNIR (9)**. Intermediate 1c (1.0 mg, 1.6 µmol, 1 equiv) was dissolved in 100µl of DMSO. DIPEA (1.4 µl, 8 µmol) and DyLigth780-B1-NHS (1.33 mg, 1.76 µmol, 1.1 equiv) were added and reacted for 1 hour. The product was purified by Reverse phase preparative HPLC using a gradient stating from 20 % acetonitrile to 60 % over 25 minutes. The solvent was removed by evaporation and then treated with 50 % TFA in DCM for 30 minutes to remove the Boc protection group on the lysine. The product was lyophilized to yield 1.3 mg (1.1 µmol) of the intermediate amine product, representing a yield of 69 % over 2 steps. This product was taken up in 100 µl DMSO, DIPEA (5 equiv) was added and then reacted with IRDye QC-1-NHS (1.42 mg, 1.21 µmol, 1.1equiv) for hour. The final product was purified by HPLC using a gradient starting from 10 % to 60 % acetonitrile over 30 minutes at flow rate of 5 µl/min. The product was lyophilized to obtain 1.5 mg of a dark green powder, representing a yield of 62 %. LCMS (ESI): m/z calculated for $C_{116}H_{151}ClN_{10}O_{22}S_5^{2+}$: 1115.96; found: 1116.1. HRMS, calculated for

$C_{116}H_{151}ClN_{10}O_{22}S_5^{2+}$; 1115.4643; found: 1115.4632, calculated for $C_{116}H_{152}ClN_{10}O_{22}S_5^{3+}$; 744.9786; found: 743.9790.

LCMS of **2CQ** (2)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Sample Number: N/A
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Max. 3.9 %.
4.83
Intensity, %
Time, min
+Q1: 4.858 to 5.159 min from Sample 332 (LO21A_purity) of LO0214.wiff (Turbo Spray)
Max. 3.8e4 cps.
966.8
(M+2)/2
136.4
129.1
149.0
279.3
117.1
221.5
611.3
945.1
998.9
1309.6
1144.1
1339.4
1932.6
167.0
241.3
351.9
649.4
925.7
1026.8
100.6
399.3
503.0
555.0
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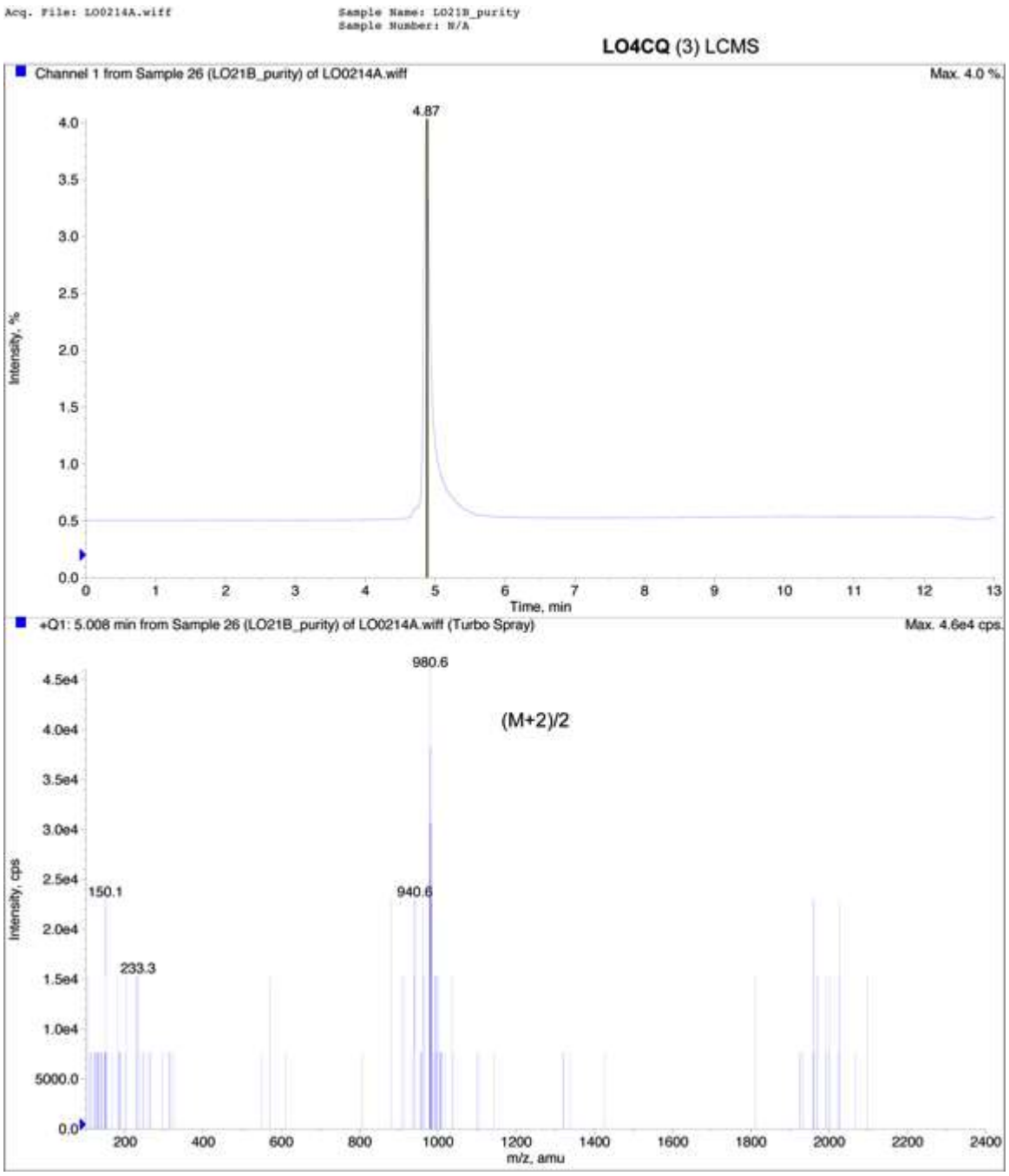
Intensity, cps
m/z, amu

LCMS for **4CQ** (3)



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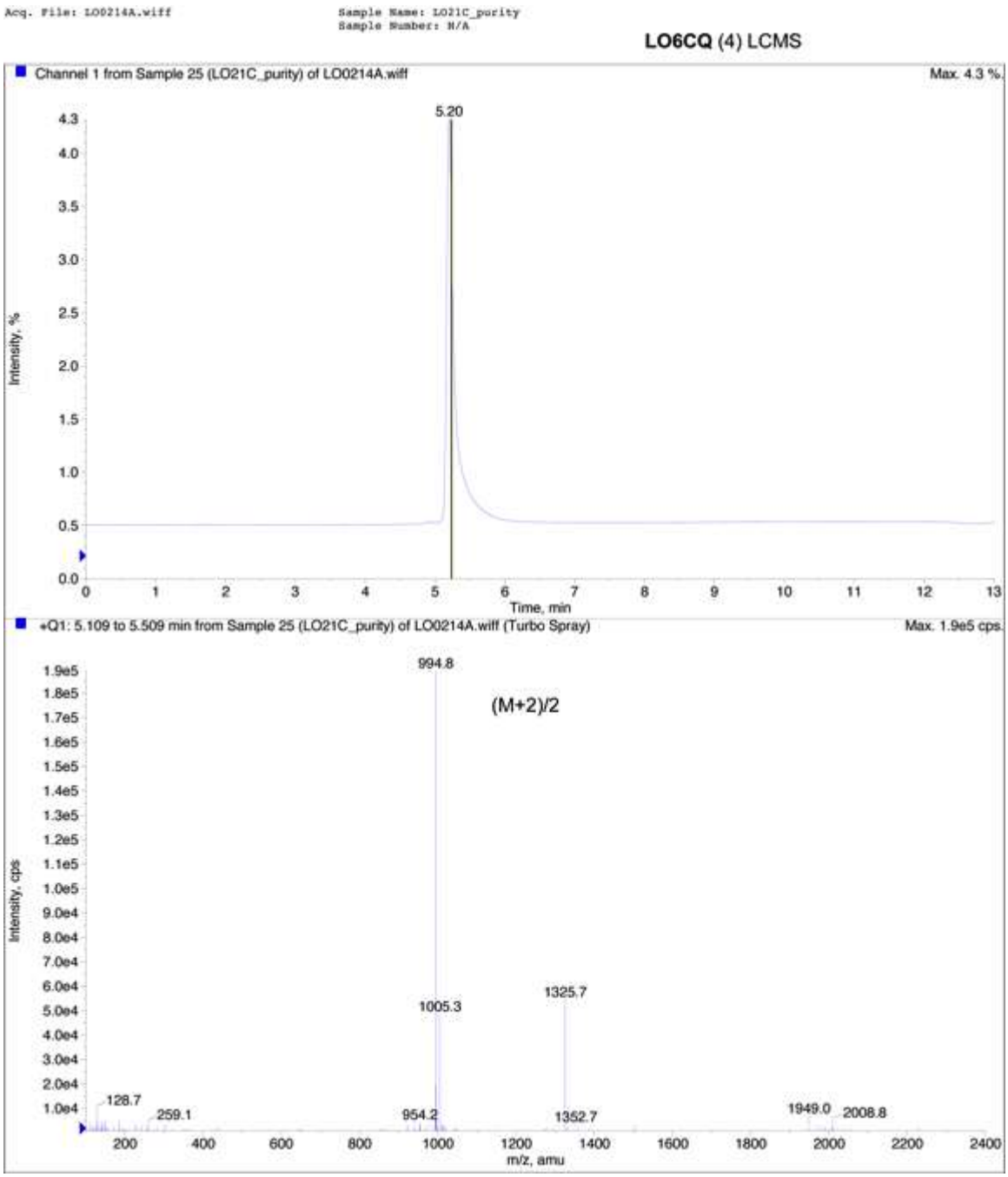

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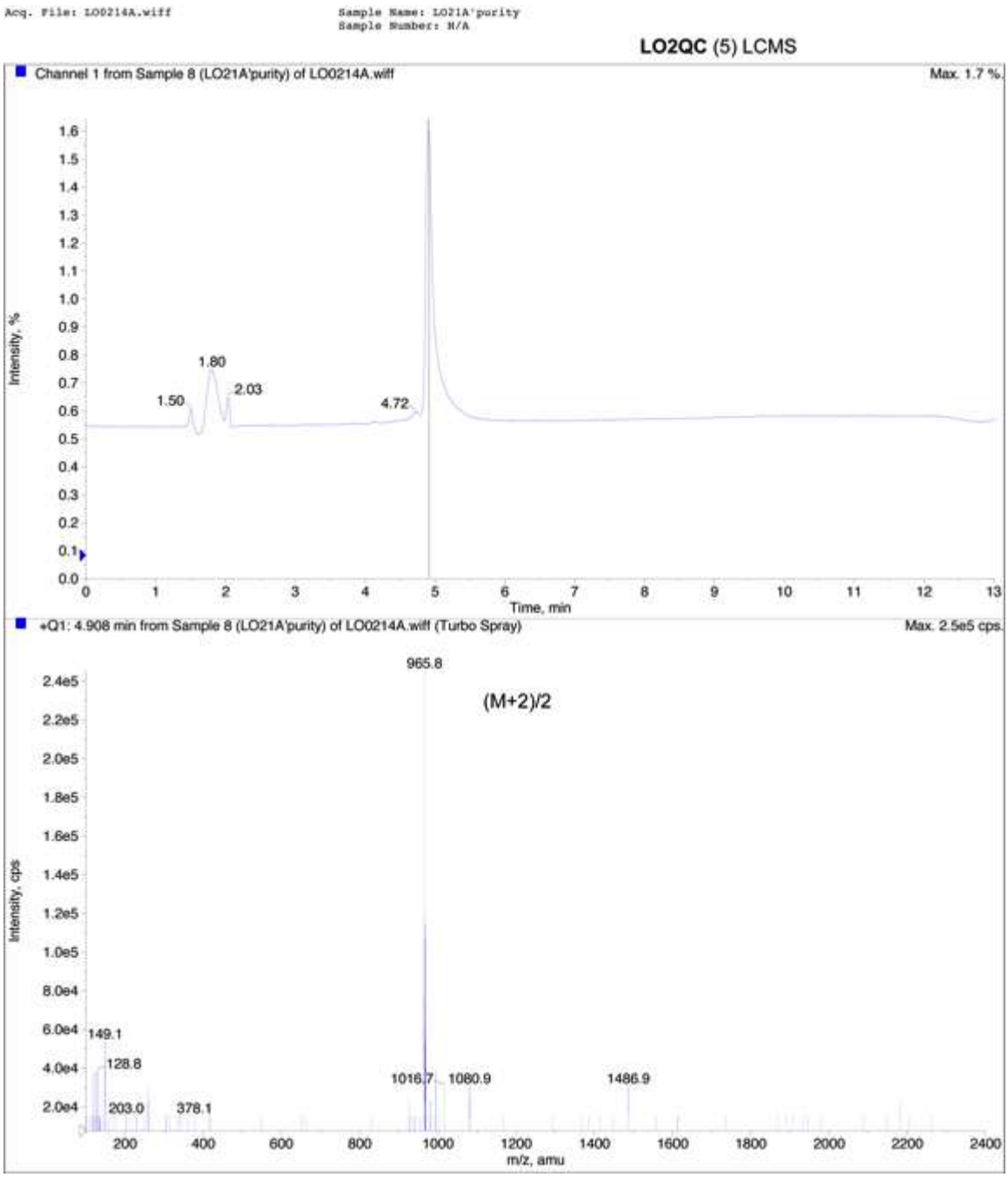

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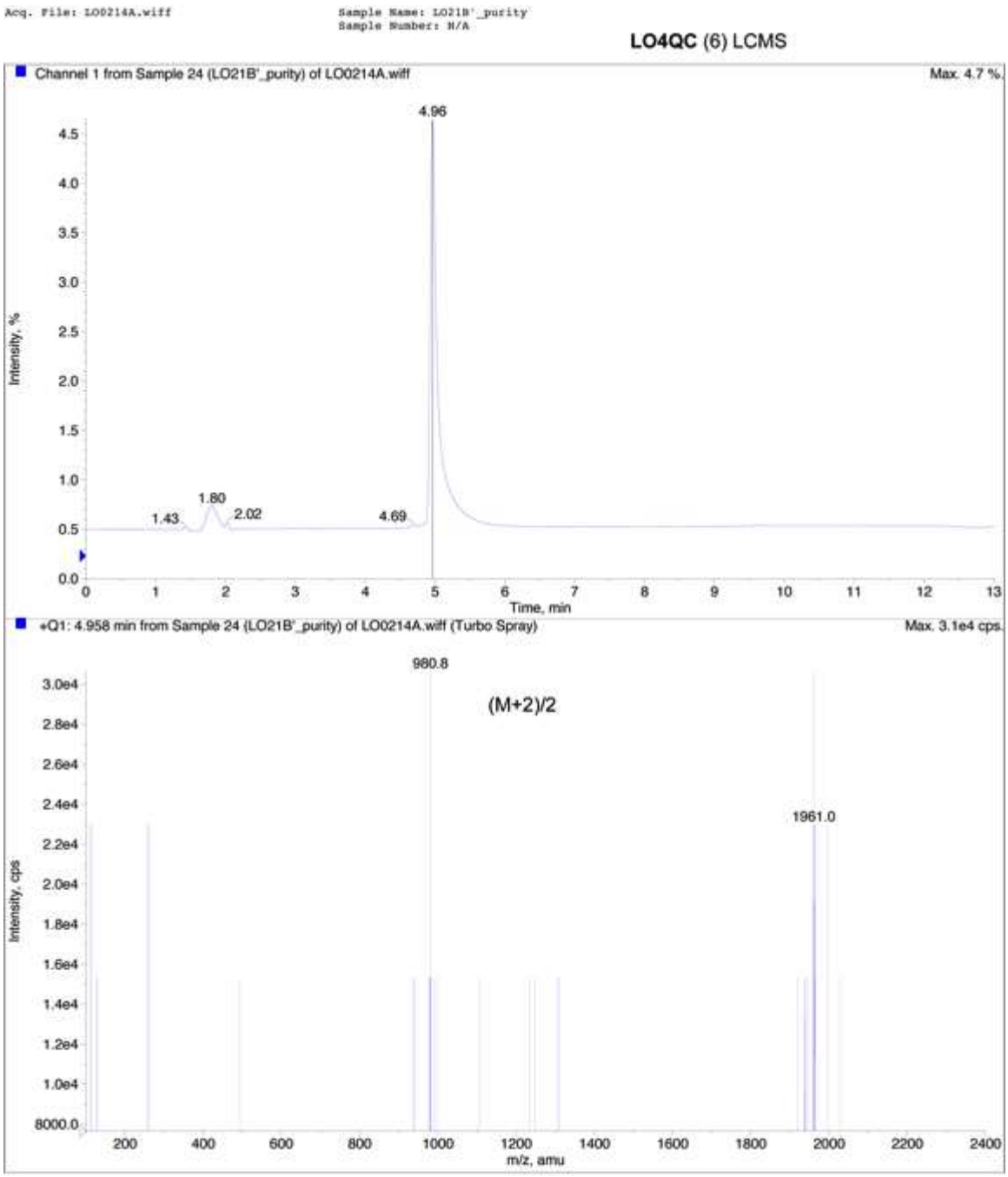
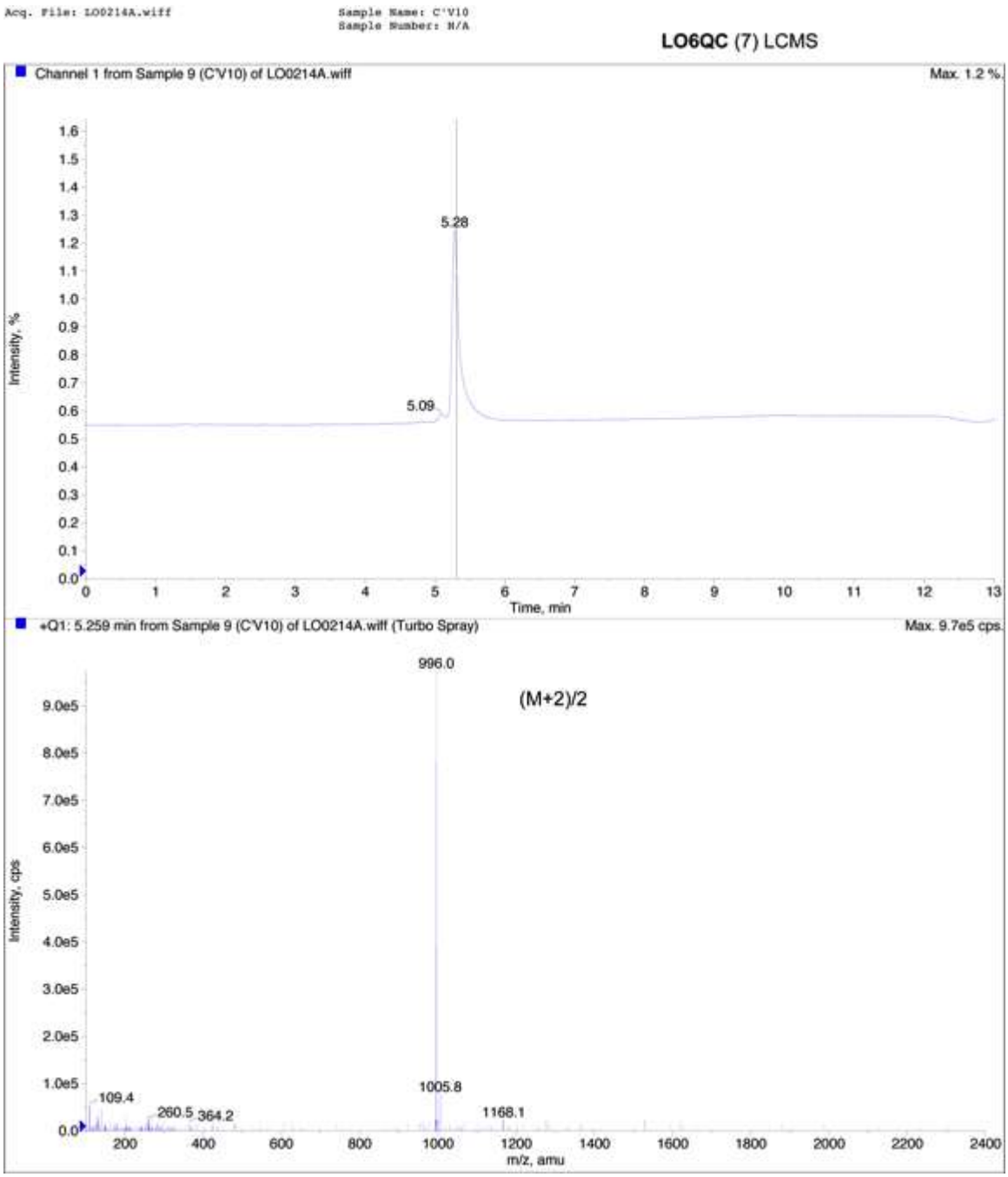


LCMS for **6QC** (7)



LCMS for **6CQNIR** (8)

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Sample Number: N/A

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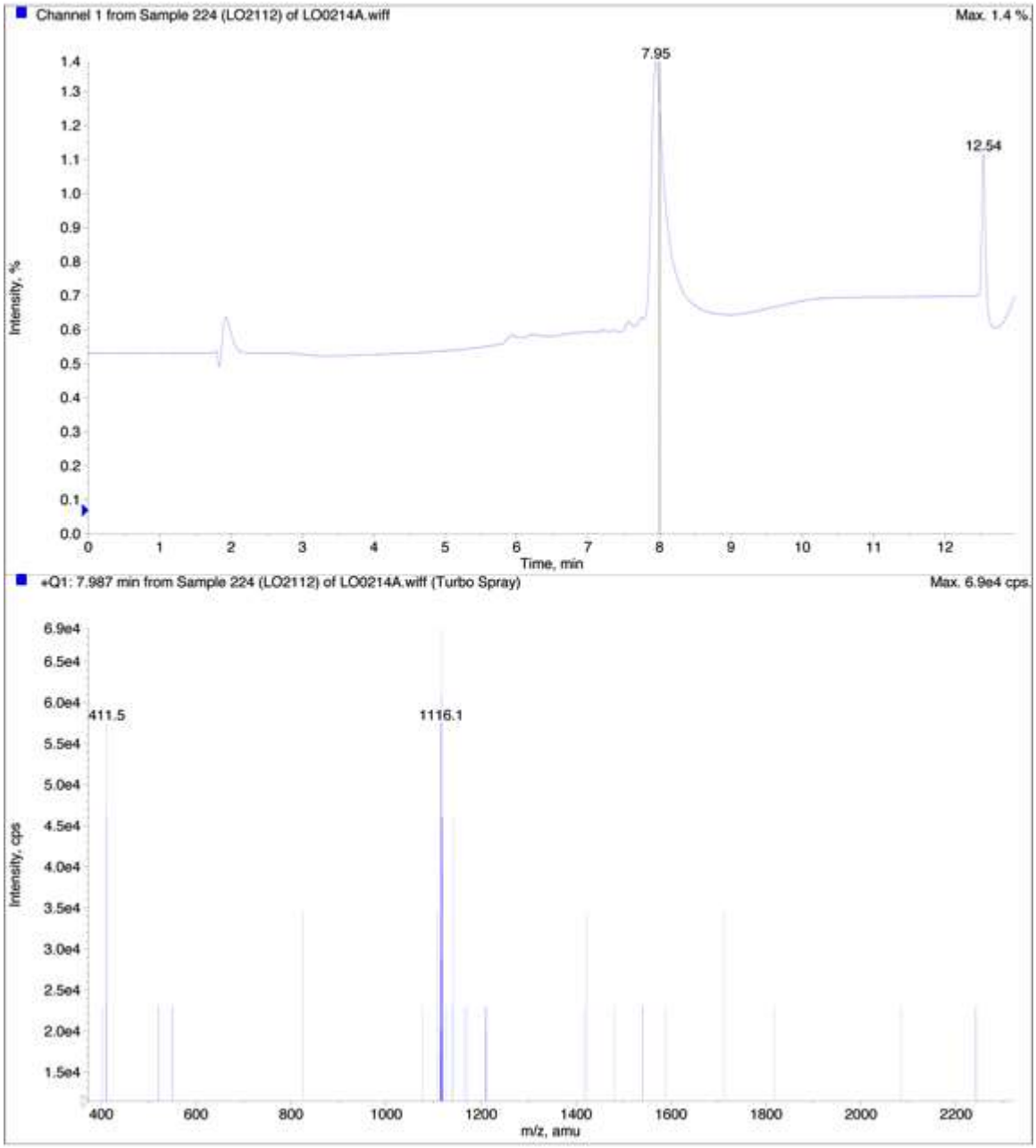

LCMS for **6QCNIR** (9)

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