Multiplexed probing of proteolytic enzymes using mass cytometry-compatible activity-based probes.

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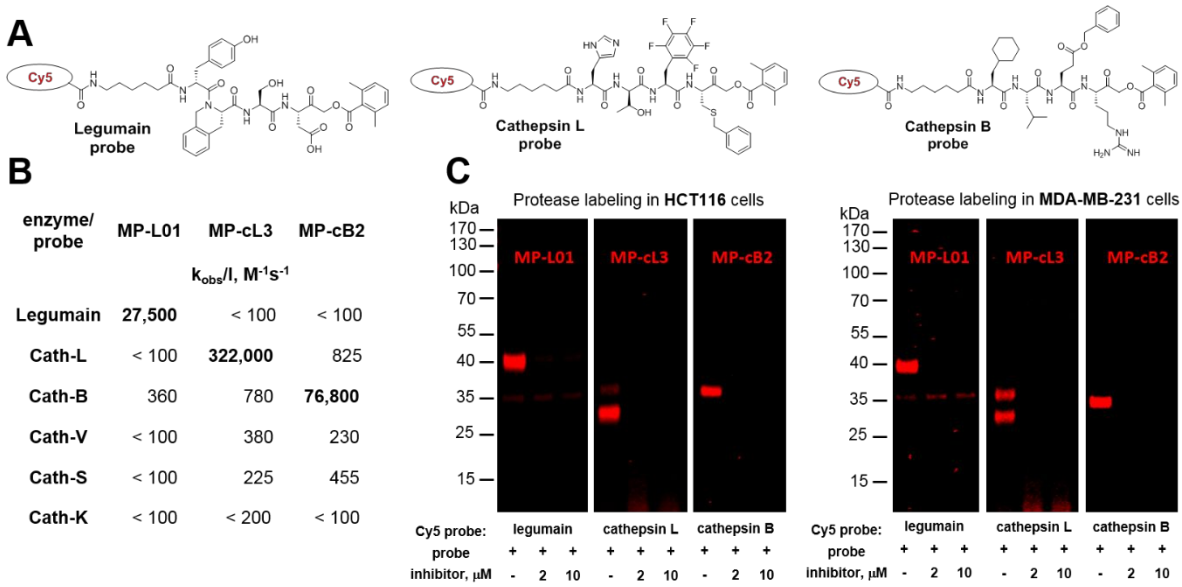
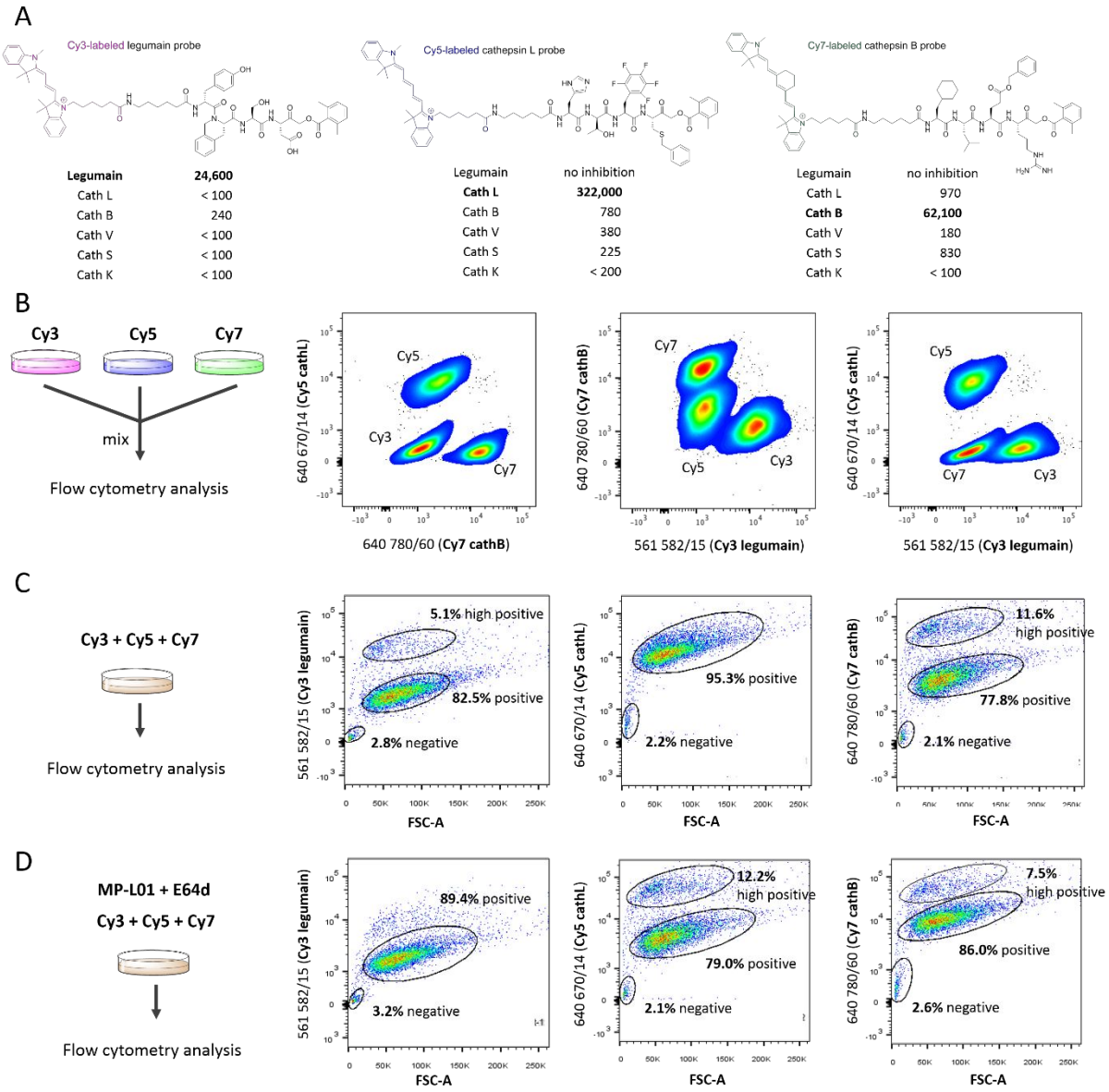
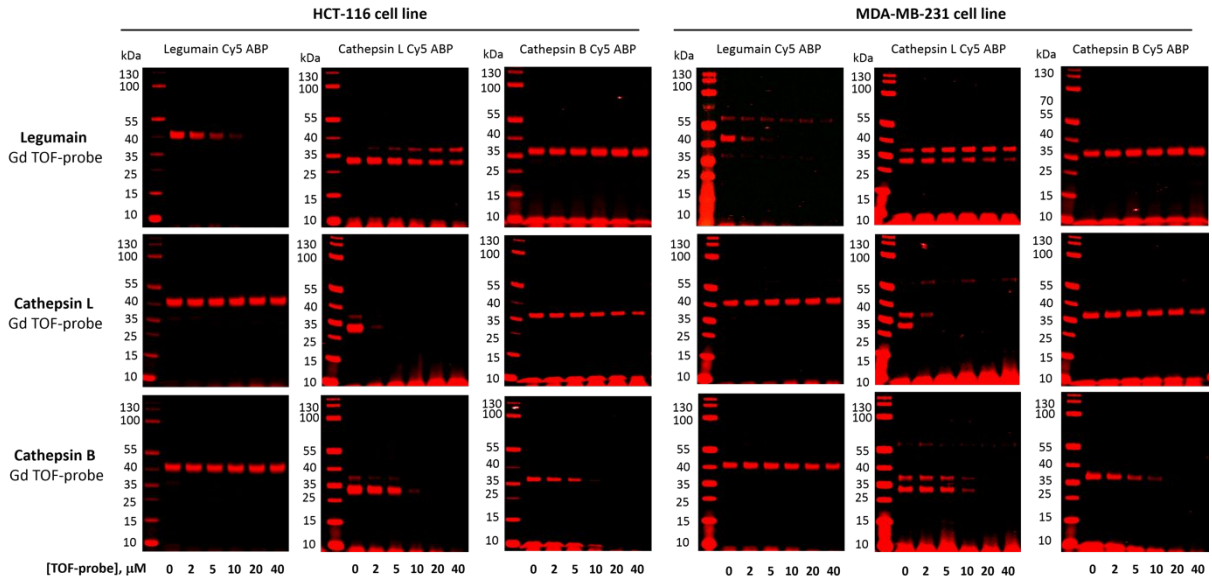


enzyme/ probe	MP-L01	MP-cL3	MP-cB2
		k_{obs}/I, $M^{-1}s^{-1}$	
Legumain	**27,500**	< 100	< 100
Cath-L	< 100	**322,000**	825
Cath-B	360	780	**76,800**
Cath-V	< 100	380	230
Cath-S	< 100	225	455
Cath-K	< 100	< 200	< 100

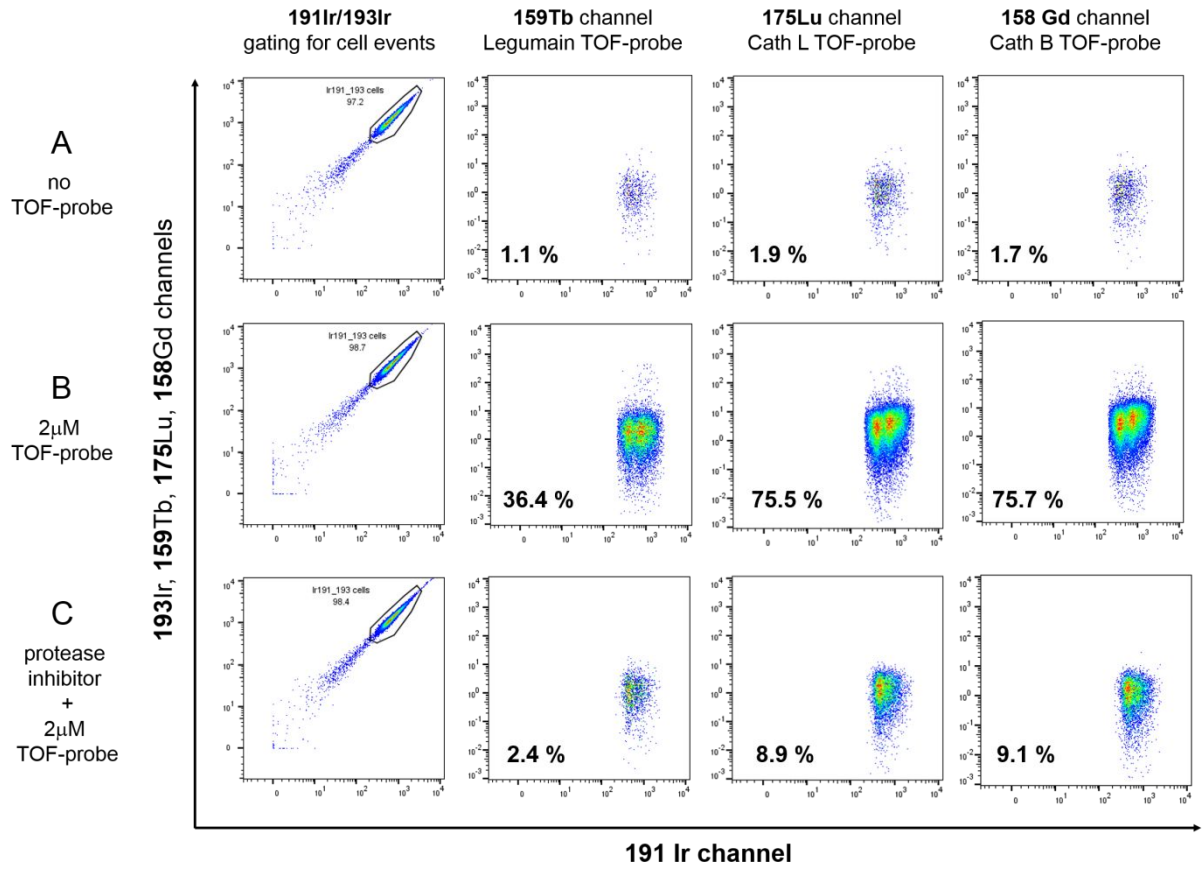
Supplemental Figure 1 Selective Cy5-labeled ABPs for the detection of lysosomal proteases in cancer cells. **A** Structures of three selective Cy5-labeled ABPs for legumain, cathepsin L and cathepsin B. **B** Kinetic parameters (second order inhibition constant, $k_{obs}/[I]$) of ABPs. **C** Selective labeling of active legumain, cathepsin L and cathepsin B in cancer cells lines (HCT-116 and MDA-MB-231) with Cy5-ABPs. Probes (1 μM) were incubated with cells for 4 hours. For enzyme inhibition MP-L01 (for legumain) and E64d (for cathepsins) were incubated for 2 hours prior to enzyme labeling with ABPs.



Supplemental Figure 2 Detection of legumain and cathepsins in HCT116 cancer cells with fluorescent probes and flow cytometry. **A** Structures and inhibition constants (k_{obs}/[I], $M^{-1}s^{-1}$) of three selective ABPs for legumain (Cy3-labeled), cathepsin L (Cy5-labeled) and cathepsin B (Cy7-labeeld). **B** HCT-116 cells were incubated with three ABPs separately, then combined and subjected for flow cytometry. **C** HCT-116 cells were incubated with a mixture of three ABPs followed by flow cytometry analysis. Data from both experiments are presented on separate two-dimensional plots. **D** HCT-116 cells were pre-incubated with legumain (MP-L01) and pan-cathepsin (E64d) inhibitors followed by incubation with a mixture of three ABPs. Data are presented on separate plots where the x-axis show forward scatter area (FSC-A), and y-axis shows the intensity of ABP`s fluorescent signal.

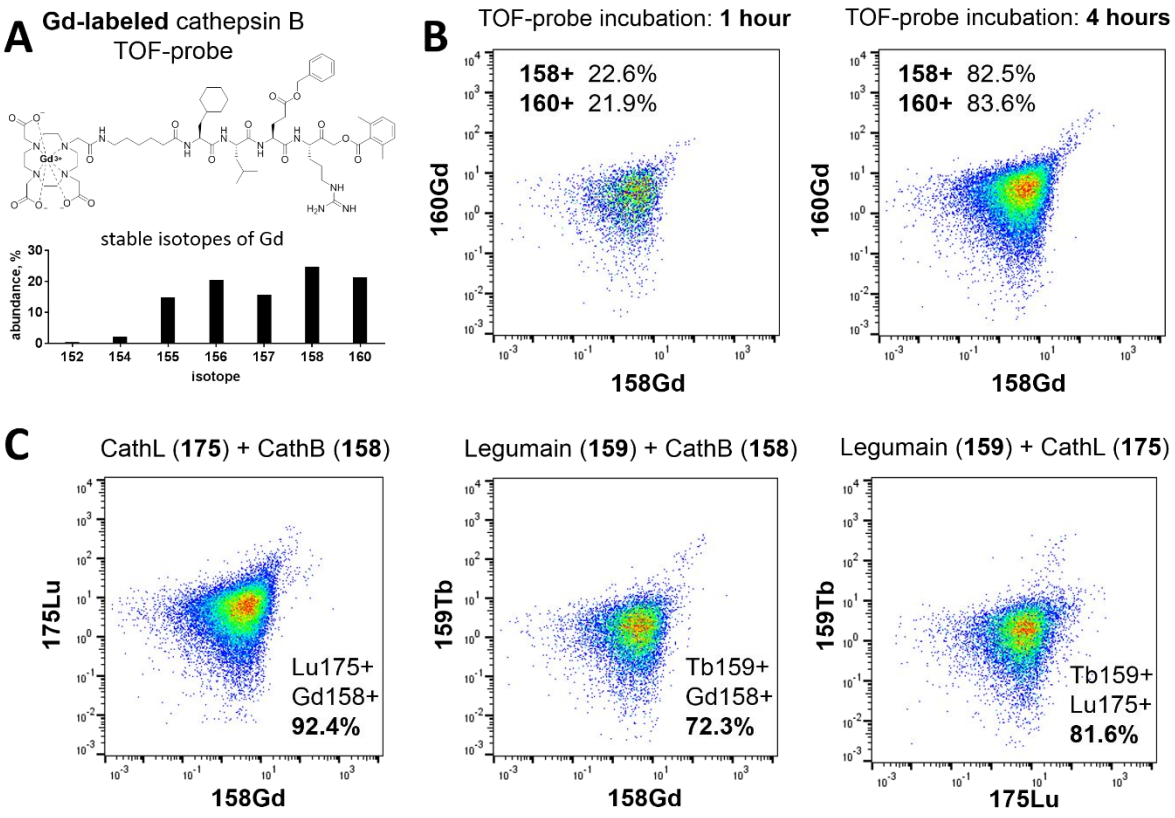


Supplemental Figure 3 Whole gels demonstrating the potency and selectivity of TOF-probes for the labeling/inhibition of legumain, cathepsin L and cathepsin B in human cancer cell lines, HCT-116 and MDA-MB-231. To assess the selectivity of TOF-probes, they were incubated at various concentration range with cancer cells, and next the residual protease activity was detected with selective, Cy5-labelled probes (Cy5-MP-L01 for legumain, Cy5-MP-cL3 for cathepsin L and Cy5-cB2 for cathepsin B). In HCT-116 and MDA-MB-231 cells legumain truncates cathepsin L, therefore one dominant cathepsin L band in visible. The inhibition of legumain with its TOF-probe results in attenuation of cathepsin L truncation, thus two bands are detected. The ratio between truncated and non-truncated forms of cathepsin L depends on legumain activity and differs between cells lines. Cathepsin B Cy5 probe labels single chain cathepsin B form (29 kDa) and also the light chain (6 kDa), which dissociated from the heavy chain after sample boiling and therefore two bands are visible.



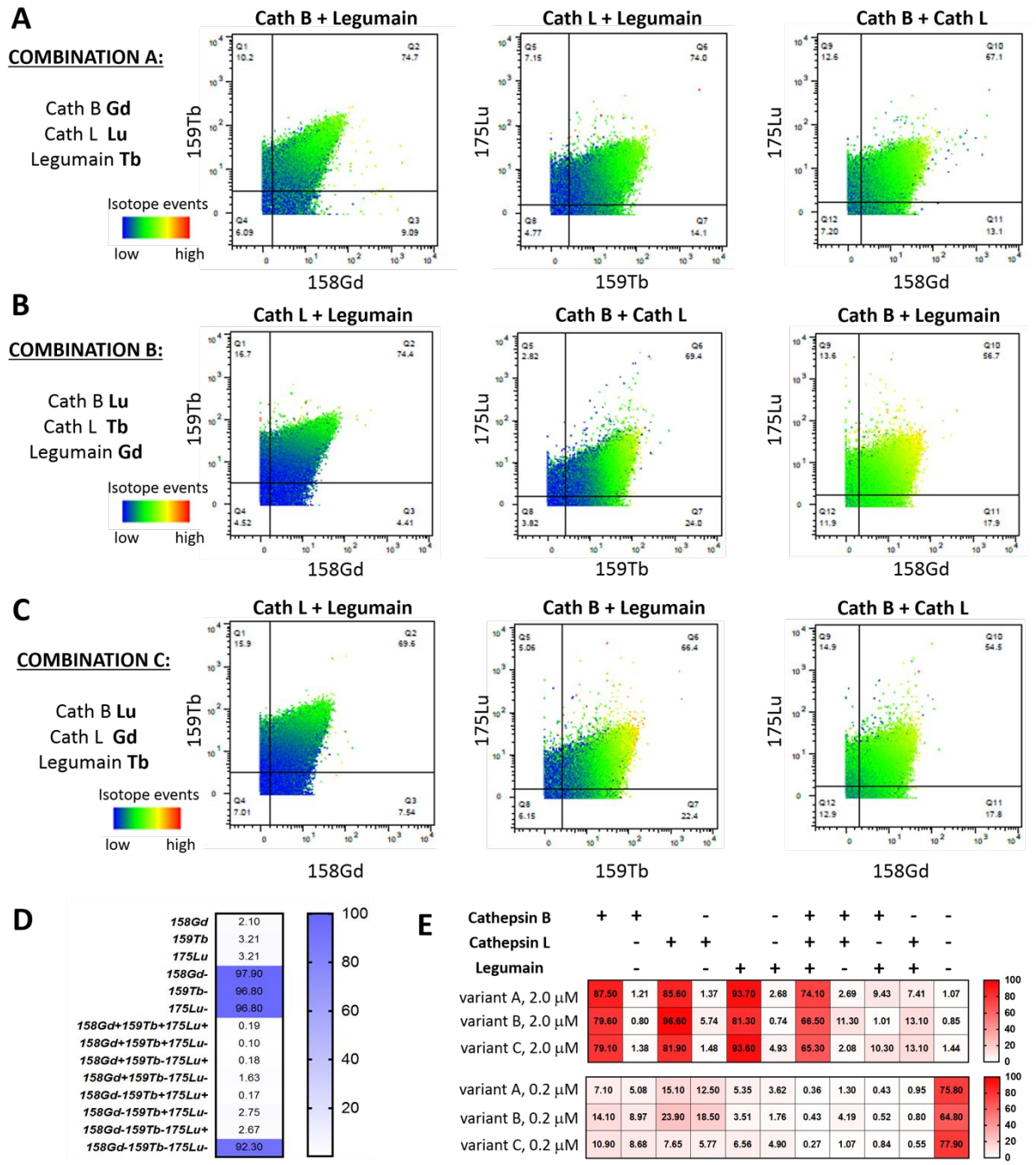
Supplemental Figure 4 Detection of proteases by TOF-probes in HCT-116 cells. TOF-probes that were incubated with cells are taken up and react covalently with proteases. After fixing and permeabilization, unbound probes are washed out. For each measurement around 100,000 cell events were collected and analyzed. Cells were (A) left untreated to measure background signal, (B) incubated with TOF-probes (2 μM, 6 hours), or (C) pre-treated with inhibitors (25 μM, 2 hours), followed by incubation with TOF-probes (2 μM, 6 hours). Cells were stained with 191Ir/193Ir (DNA intercalator to reveal the nucleus) and subjected for mass cytometry analysis. Cells that were left untreated have a low background signal, whereas cells with TOF-probes demonstrate labeling of proteases: legumain (159Tb chan-nel), cathepsin L (175Lu channel) and cathepsin B (158Gd channel). When the cells were pre-treated with inhibitors (MP-L01 for legu-main and E64d for cathepsins) signals from enzymes were substantially reduced. For each sample the percentage of cell positive events was calculated. As cells differed in DNA content, probably due to being at different stage of the cell cycle, we observed two populations. This in

turn influenced overall signal intensities in case of inhibitor pre-treated cells. The percent of probe labeled cells remained the same across these populations.



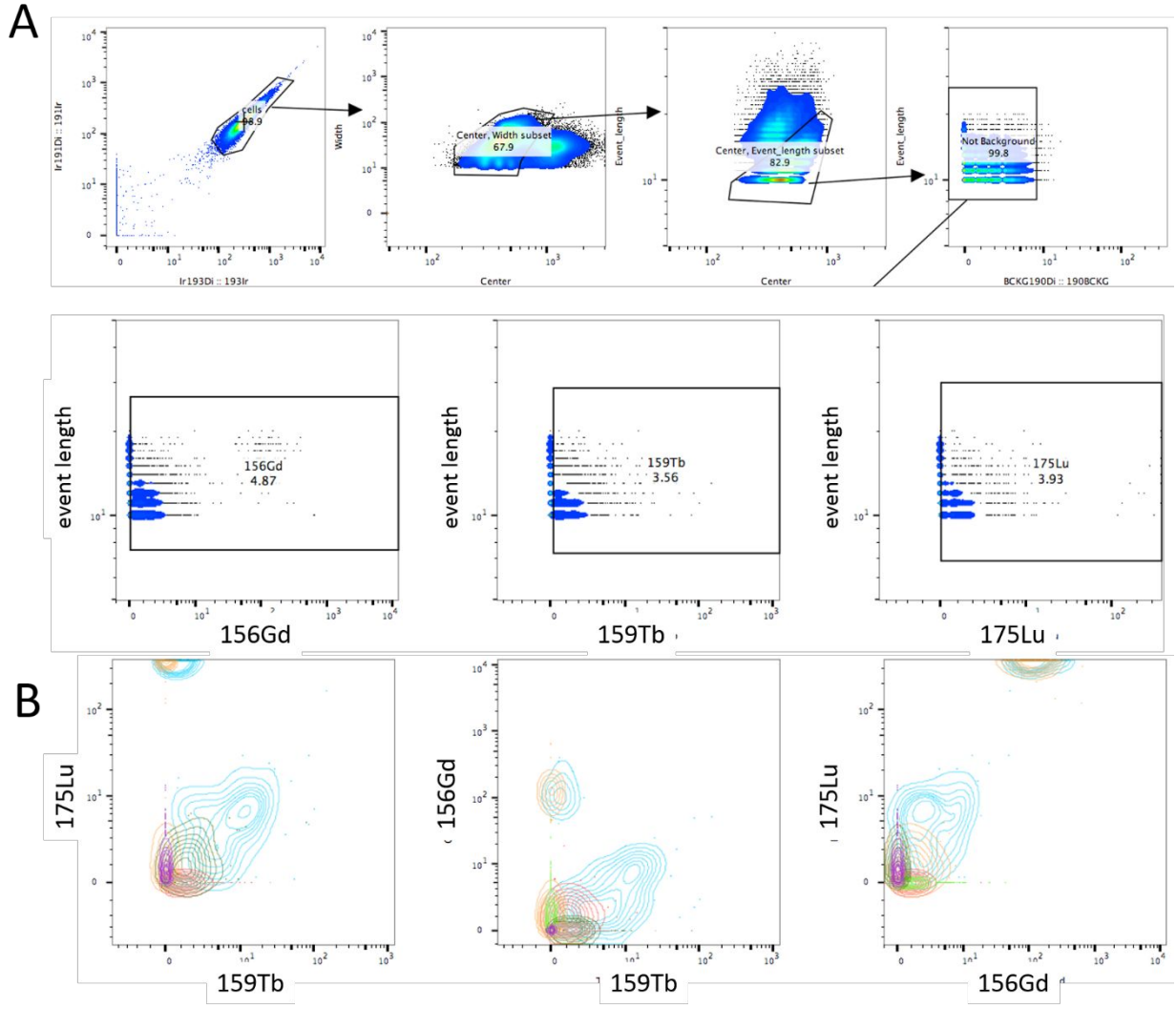
Supplemental Figure 5 Selective detection of legumain, cathepsin L, and cathepsin B in HCT-116 cell line. **A** The structure of cathepsin B TOF-probe labeled with Gd metal. By chelating MP-cB2 probe with non-isotopically pure Gadolinium metal we detected cathepsin B activity on two channels (158 and 160) and demonstrated the homogenous distribution of TOF-probe within single cells. **B** Cathepsin B Gd TOF-probe uptake kinetic in HCT-116 cells. After 1 hour of incubation only around 20% of cells were ^{158}Gd and ^{160}Gd positive, whereas the prolonged incubation (4 hours) resulted in the labeling of cathepsin B in over 80% of cells population. Symmetrical graphs demonstrated that TOF-probe was homogenously distributed. **C** Parallel detection of three proteases in HCT-116 cells. Three TOF-probe

were incubated with cells for 4 hours and subjected for mass cytometry analysis. Results demonstrate that all three probes were taken up by cells and efficiently labeled proteases.



Supplemental Figure 6 Selective labeling of proteases in HCT-116 cells with TOF-probes. **A-C** Cells were incubated with various sets of TOF-probes (in three combinations: A-C). Graphs were divided into four quadrants based on metal (TOF-probe) events. **D** Non-TOF-probe treated cells were subjected to mass cytometry analysis and the data was used for TOF-probe treated cell gating. **E** TOF-probes uptake and protease labeling in HCT-116 cells presented as heat maps. Data demonstrate that regardless of

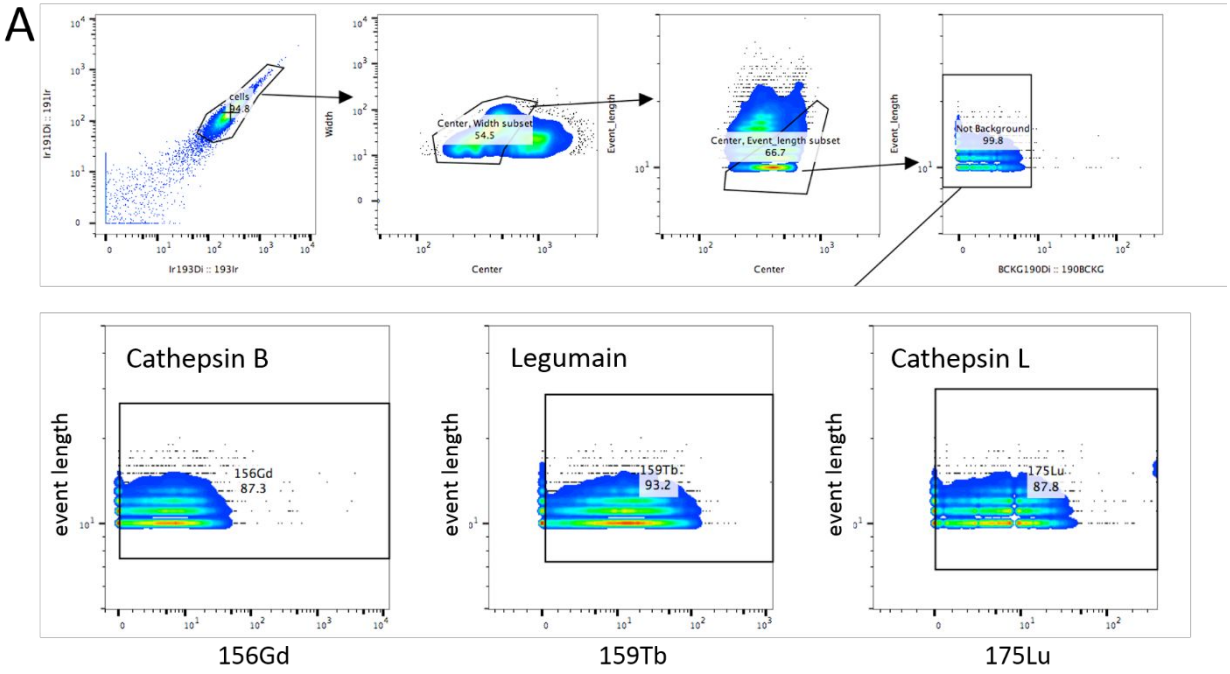
which TOF-probes set was used, the pattern of protease labeling is similar, demonstrating the wide applicability of this technology.



	Sample Name	Subset Name	Count	Freq. of Parent
	HCT116_control_01_01.FCS	156Gd-159Tb-175Lu-	49347	88.9
	HCT116_control_01_01.FCS	156Gd-159Tb-175Lu+	1758	3.17
	HCT116_control_01_01.FCS	156Gd-159Tb+175Lu-	1603	2.89
	HCT116_control_01_01.FCS	156Gd-159Tb+175Lu+	106	0.19
	HCT116_control_01_01.FCS	156Gd+159Tb-175Lu-	2268	4.09
	HCT116_control_01_01.FCS	156Gd+159Tb-175Lu+	164	0.30
	HCT116_control_01_01.FCS	156Gd+159Tb+175Lu-	118	0.21
	HCT116_control_01_01.FCS	156Gd+159Tb+175Lu+	152	0.27

Supplemental Figure 7 Detailed gating strategy for non-TOF-probes treated HCT-116 cells. **A** DNA (^{191}Ir/^{193}Ir) positive events were gated based on their width and length. The signal background as well as doublets were removed form analysis. For each channel analyzed (^{156}Gd, ^{159}Tb and ^{175}Lu) graph plots were gated according to signal intensity. **B** Metal signals were paired (Lu/Tb, Gd/Tb, Lu/Gd) and

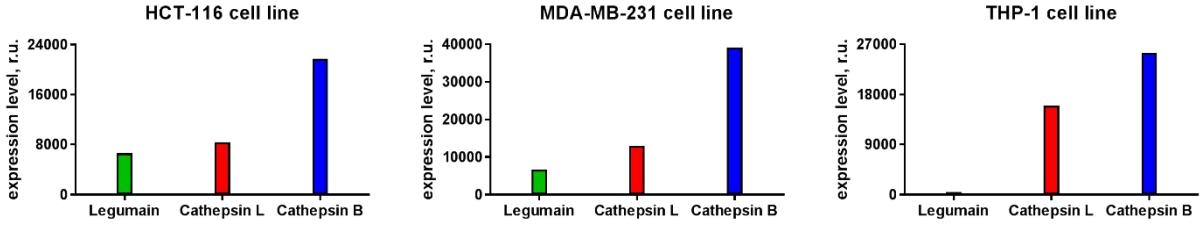
presented on two dimensional graphs. Based on signal counts, positive/negative events were calculated and summarized in table.



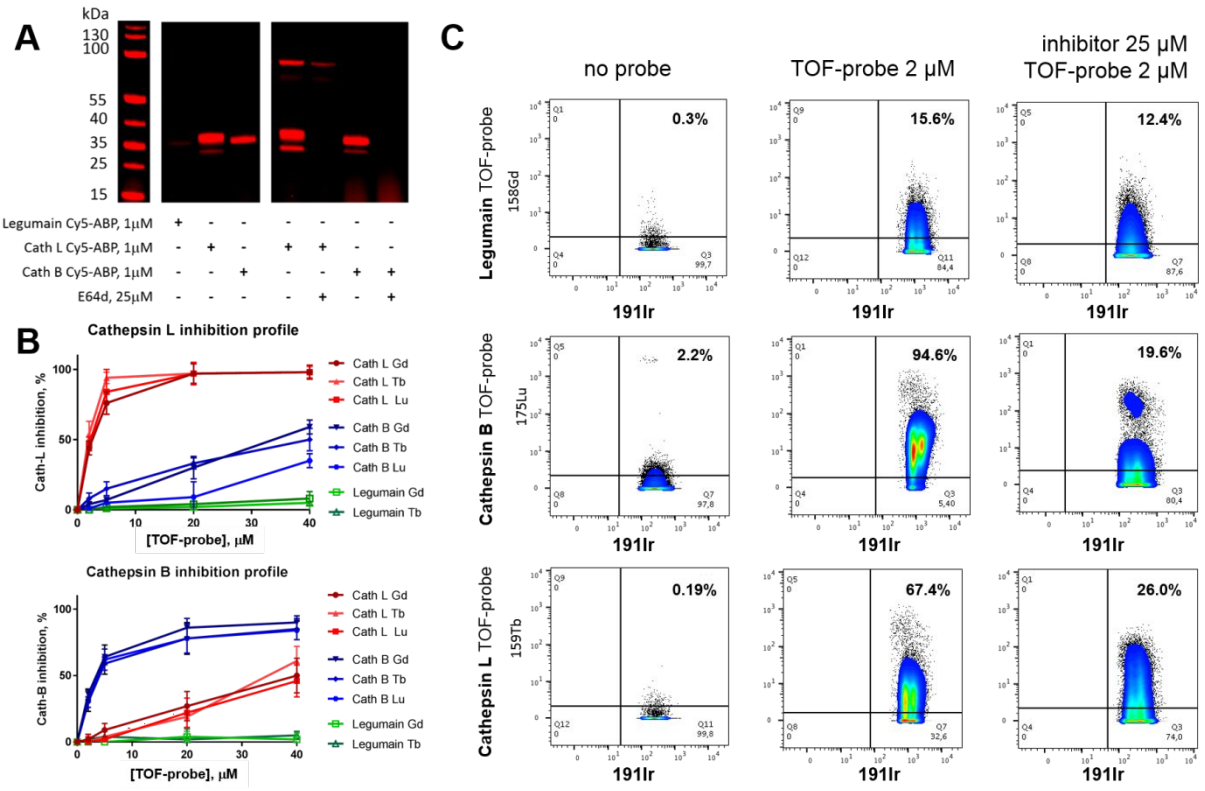
	Sample Name	Subset Name	Count	Freq. of Parent
	HCT_A_2uM_06.FCS	156Gd-159Tb-175Lu-	220	1.16
	HCT_A_2uM_06.FCS	156Gd-159Tb-175Lu+	255	1.34
	HCT_A_2uM_06.FCS	156Gd-159Tb+175Lu-	438	2.30
	HCT_A_2uM_06.FCS	156Gd-159Tb+175Lu+	1504	7.91
	HCT_A_2uM_06.FCS	156Gd+159Tb-175Lu-	236	1.24
	HCT_A_2uM_06.FCS	156Gd+159Tb-175Lu+	588	3.09
	HCT_A_2uM_06.FCS	156Gd+159Tb+175Lu-	1422	7.48
	HCT_A_2uM_06.FCS	156Gd+159Tb+175Lu+	14356	75.5

Supplemental Figure 8 Detailed gating strategy for TOF-probes treated HCT-116 cells exemplified in variant A (Tb probe for legumain, Gd probe for cathepsin B and Lu probe for cathepsin L). **A** DNA (^{191}Ir/^{193}Ir) positive events were gated based on their width and length, and next the signal background was removed. For each channel analyzed (^{156}Gd cathepsin B probe, ^{159}Tb legumain probe and ^{175}Lu cathepsin L probe) channels were gated according to signal intensity. **B** Metal signals were paired

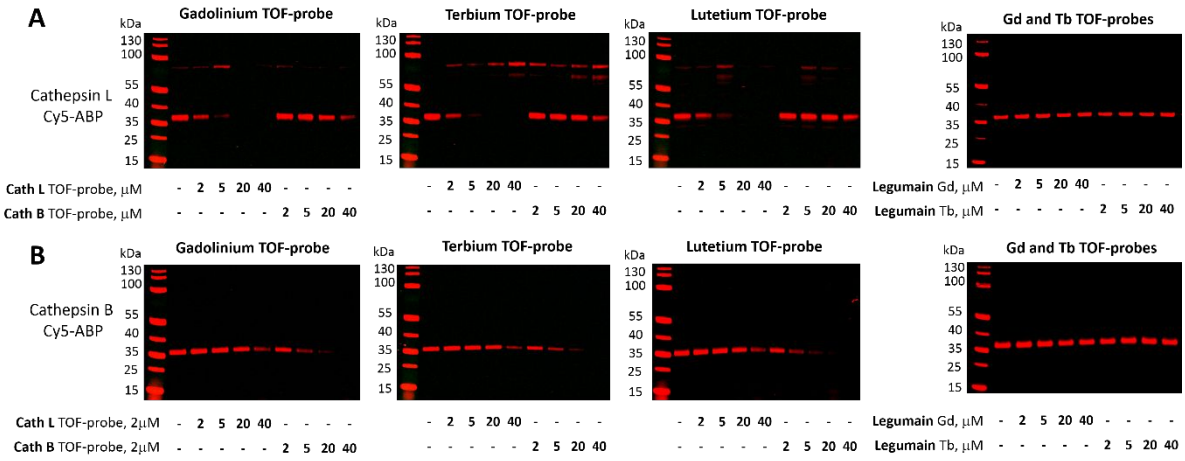
(Lu/Tb, Gd/Tb, Lu/Gd) and presented on two dimensional graphs. Based on signal counts metal positive/negative events were calculated and summarized in table.



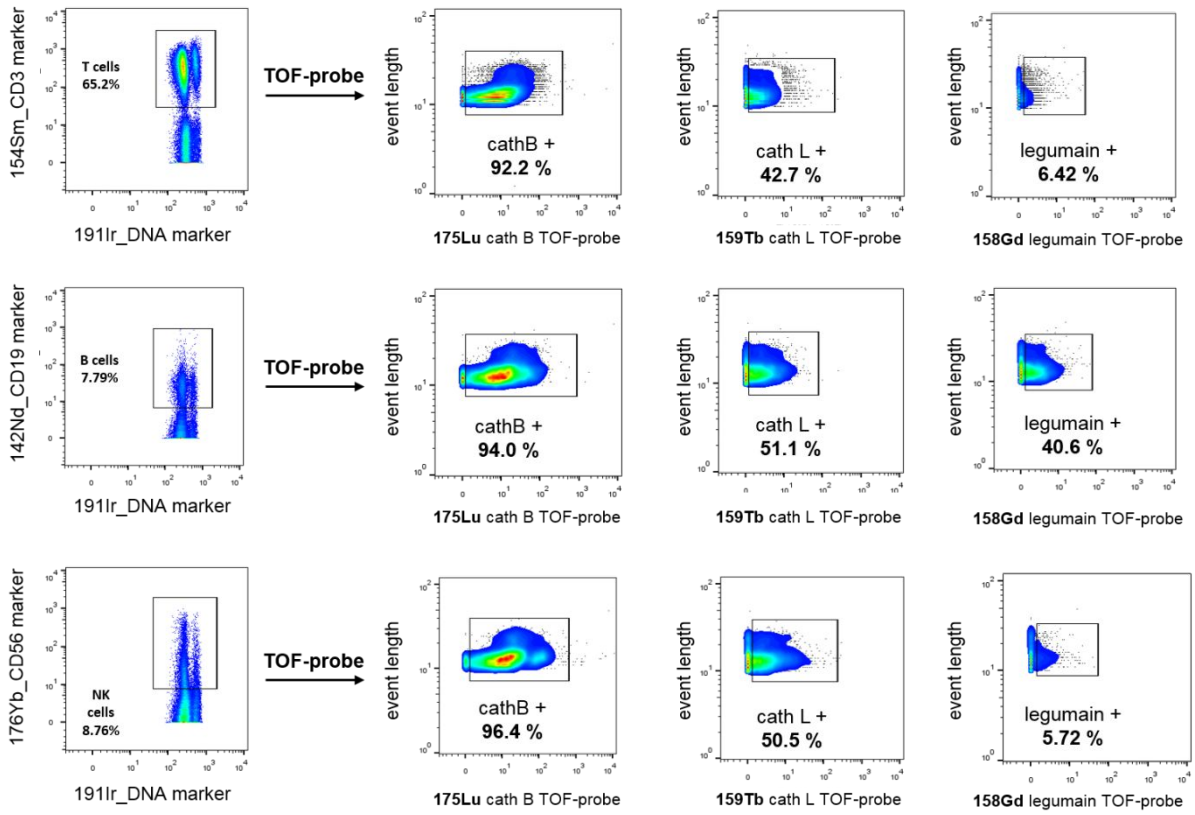
Supplemental Figure 9 Expression level of legumain, cathepsin L and cathepsin B in three human cell lines: HCT-116, MDA-MB-231 and THP-1. Data was extracted from Geneinvestigator database. Only non-stimulated cells were taken for the analysis, and data presents average expression levels in relative units.



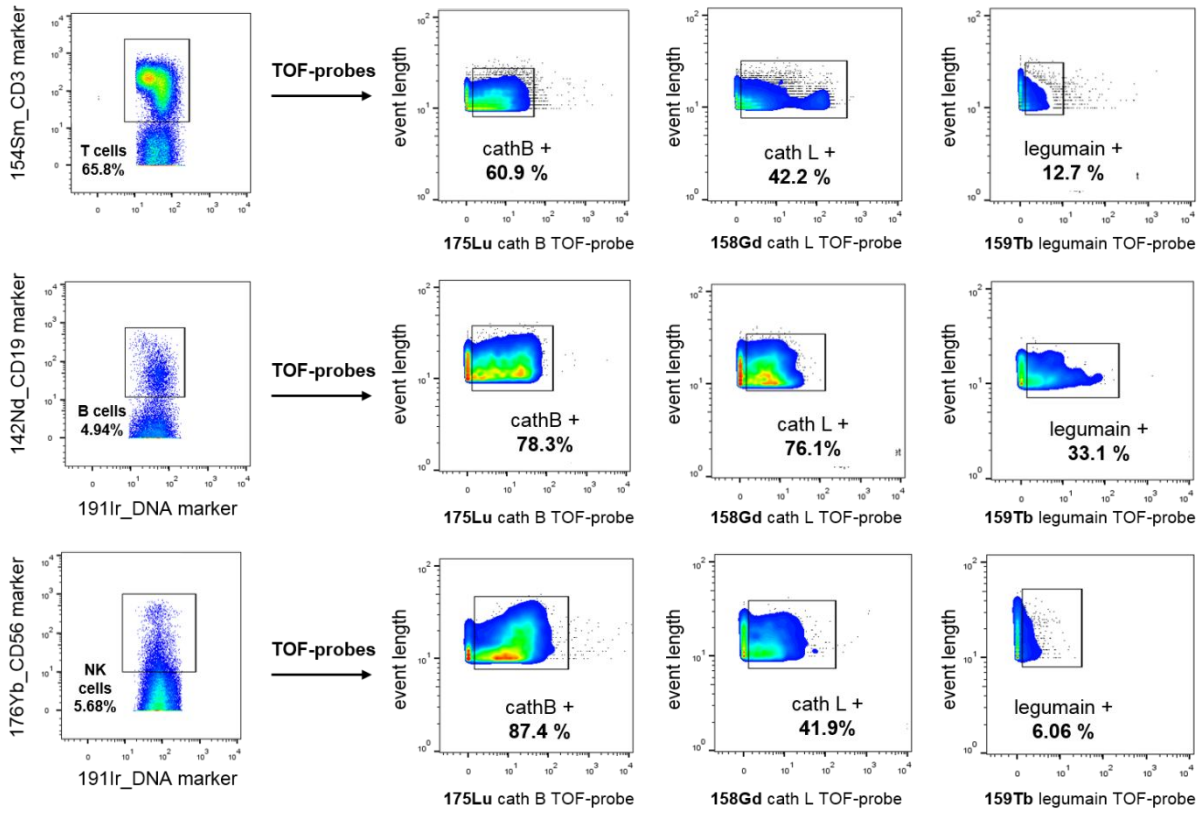
Supplemental Figure 10 Detection of protease activity in THP-1 cells using TOF-probes. **A** Visualization of cathepsin L and cathepsin B activity in THP-1 with Cy5-labeled selective probes. Legumain was not labeled with Cy5-MP-L01 probe, confirming the low expression level of this enzyme in THP-1 cells. **B** Inhibition profiles of cathepsin L and cathepsin B in THP-1 cells. Data demonstrate high selectivity of our TOF-probes. **C** Metal-labeled TOF-probes show high activity of cathepsin L and cathepsin B, but not legumain in THP-1 cells. Left panel: background signal from investigated channels; middle panel: the percentage of cells containing active proteases; right panel: residual protease activity after inhibitor pre-treatment.



Supplemental Figure 11 Determination of TOF-probes selectivity toward cathepsin L (A) and cathepsin B (B) in THP-1 cells. To assess the selectivity of TOF-probes, they were incubated at various concentration range with THP-1 cells, and next the residual protease activity was detected with selective, Cy5-labelled probe (Cy5-MP-cL3 for cathepsin L and Cy5-cB2 for cathepsin B).



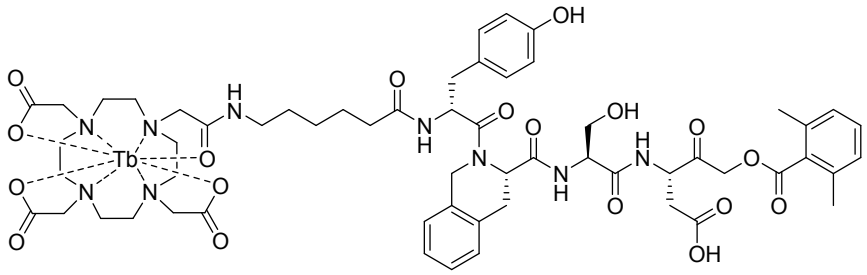
Supplemental Figure 12 Proteases detection in Peripheral Blood Mononuclear Cells. Different PBMCs` populations were detected with the use of metal-conjugated antibodies (^{154}Sm-CD3 for T cells, ^{142}Nd-CD19 for B cells, and ^{176}Yb-CD56 for NK cells). Proteases activity was detected with TOF-probes in the following combination: ^{175}Lu-tagged cat B probe, ^{159}Tb-tagged cat L probe, and Gd-tagged legumain probe (detected at 158 channel). Data present that cathepsin B is the most active enzyme in all type of cells, cathepsin L is also present in all cell populations, but less active than cathepsin B, and legumain activity is B cells specific.

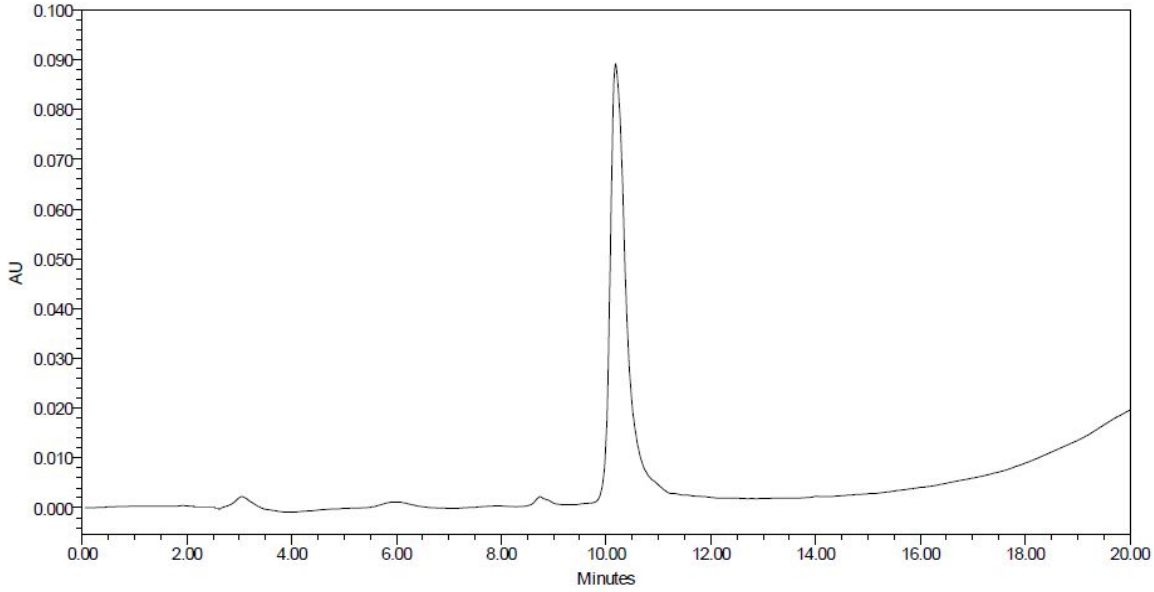


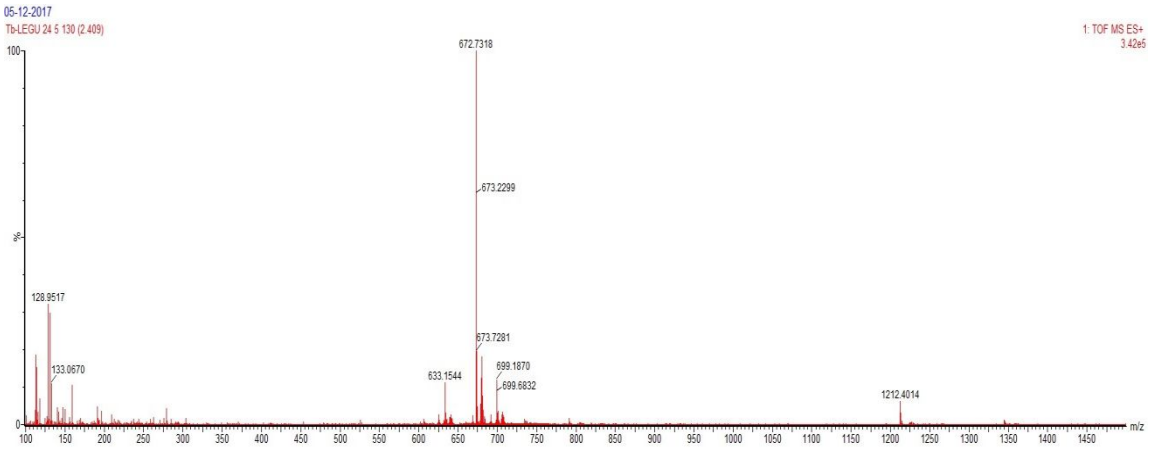
Supplemental Figure 13 Proteases detection in Peripheral Blood Mononuclear Cells. Different PBMCs` populations were detected with the use of metal-conjugated antibodies (^{154}Sm-CD3 for T cells, ^{142}Nd-CD19 for B cells, and ^{176}Yb-CD56 for NK cells). Proteases activity was detected with TOF-probes in the following combination: ^{175}Lu-tagged cat B probe, Gd-tagged cat L probe (detected at 158 channel), and ^{159}Tb-tagged legumain probe. Data present that cathepsin B is the most active enzyme in all type of cells, cathepsin L is also present in all cell populations, but less active than cathepsin B, and legumain activity is B cells specific.

DOTA(^{159}Tb)-Ahx-*D*Tyr-Tic-Ser-Asp-AOMK

HRMS (m/z): $[M+H]^+$ calcd for $C_{58}H_{74}N_9O_{18}Tb$, 672.7202, found 672.7318







DOTA(Gd)-Ahx-*D*Tyr-Tic-Ser-Asp-AOMK

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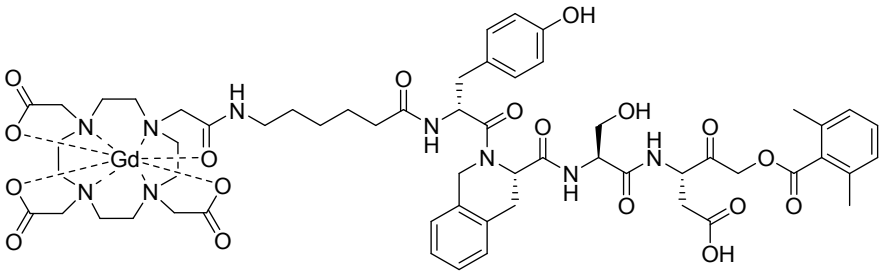

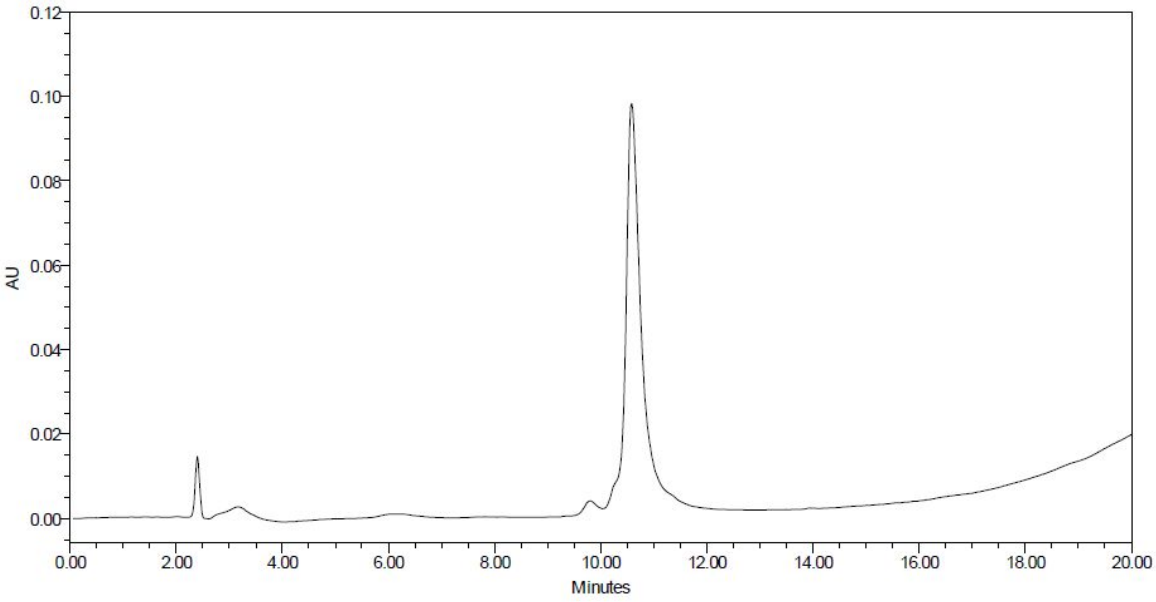

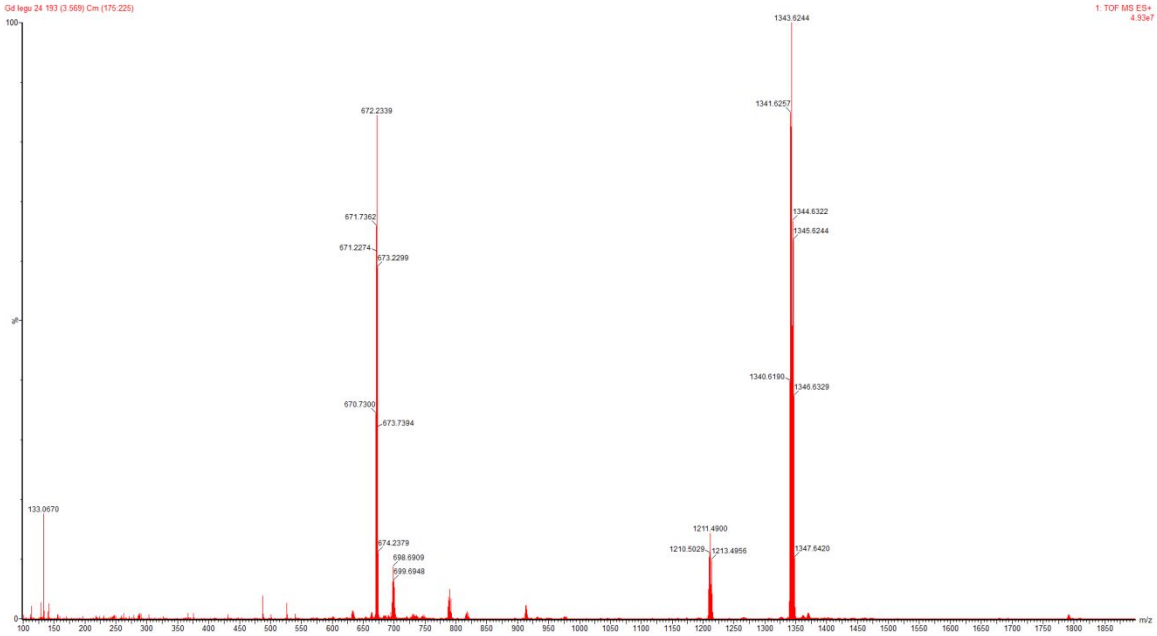

DOTA(^{159}Tb)-Ahx-His-*D*Thr-Phe(F5)-Cys(Bzl)-AOMK

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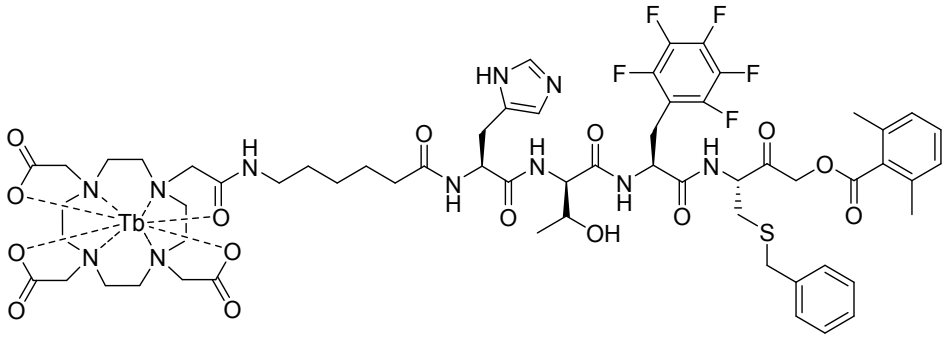

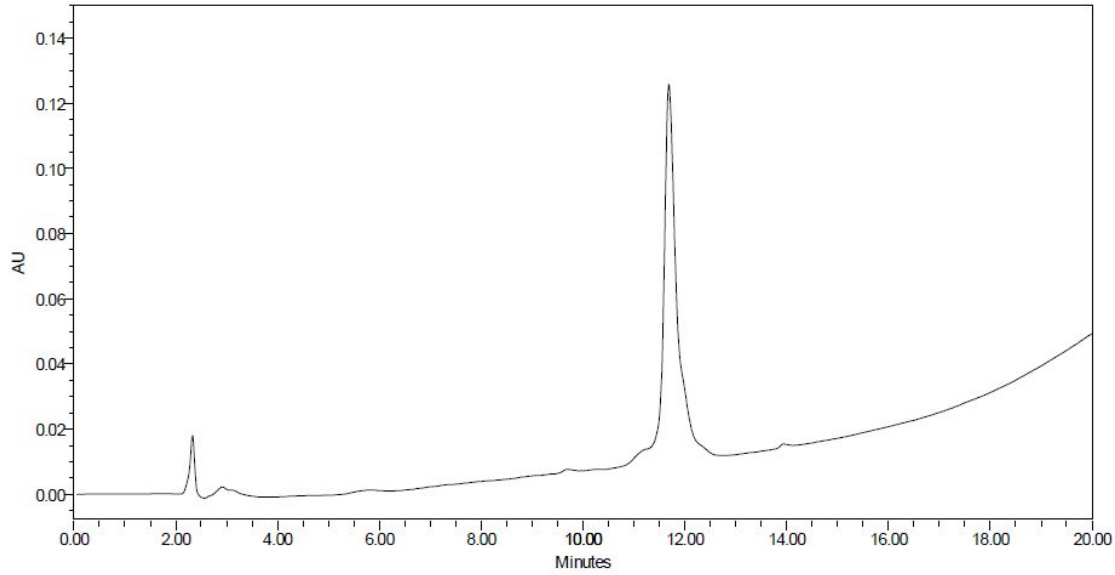

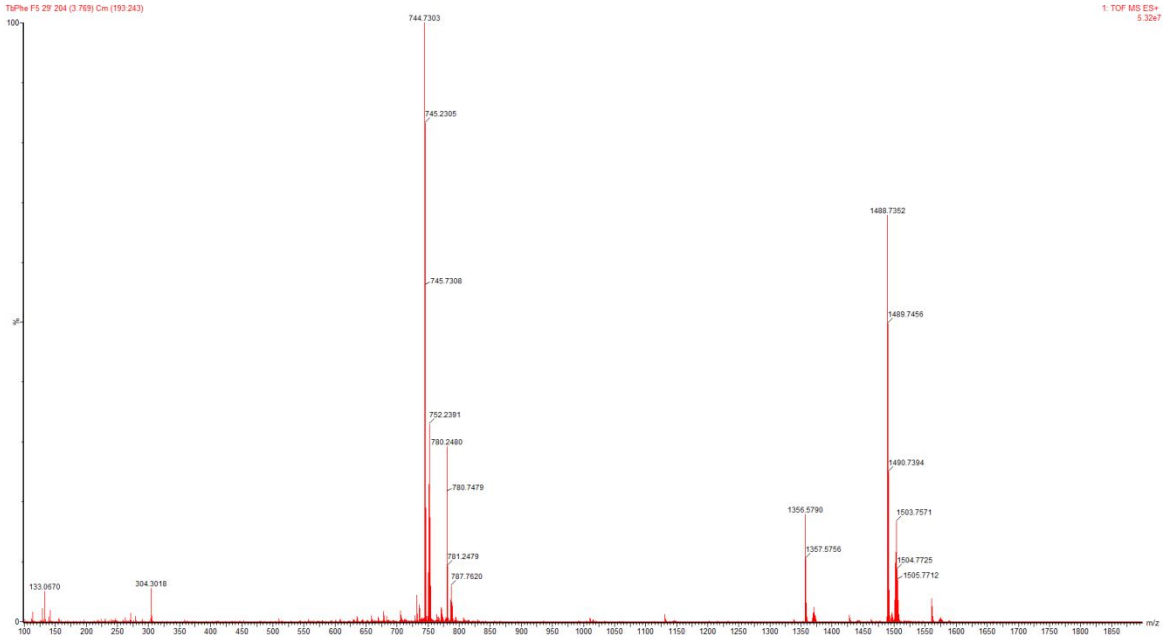

DOTA(^{175}Lu)-Ahx-His-*D*Thr-Phe(F5)-Cys(Bzl)-AOMK

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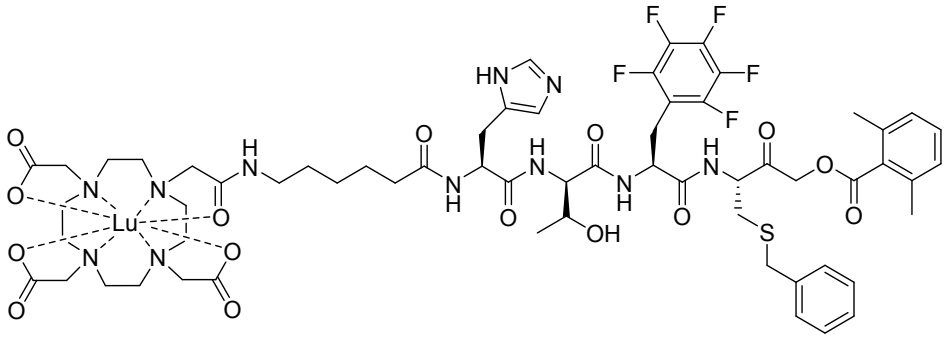

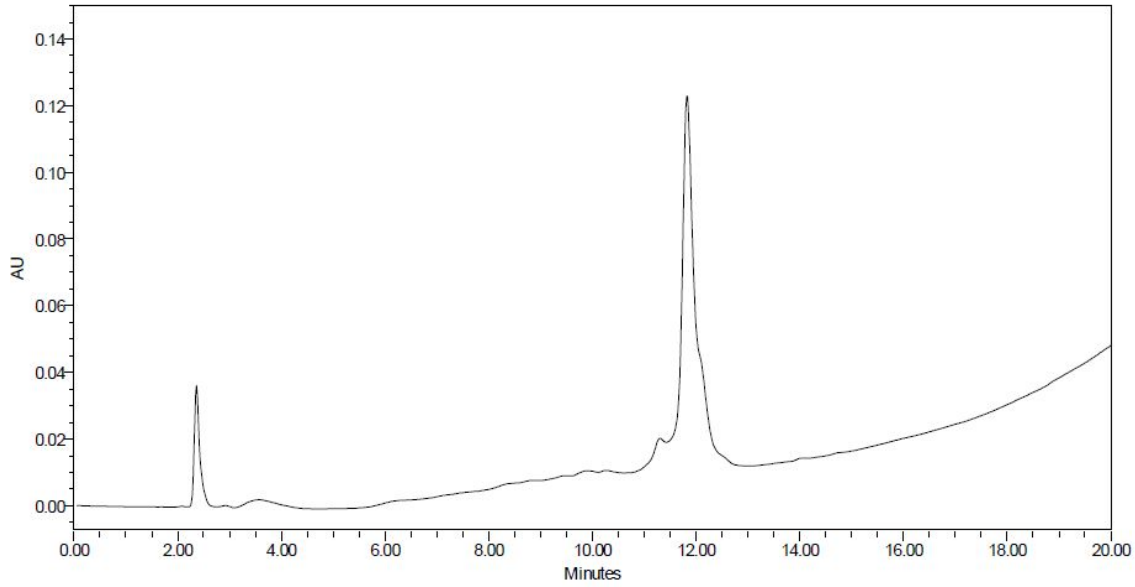

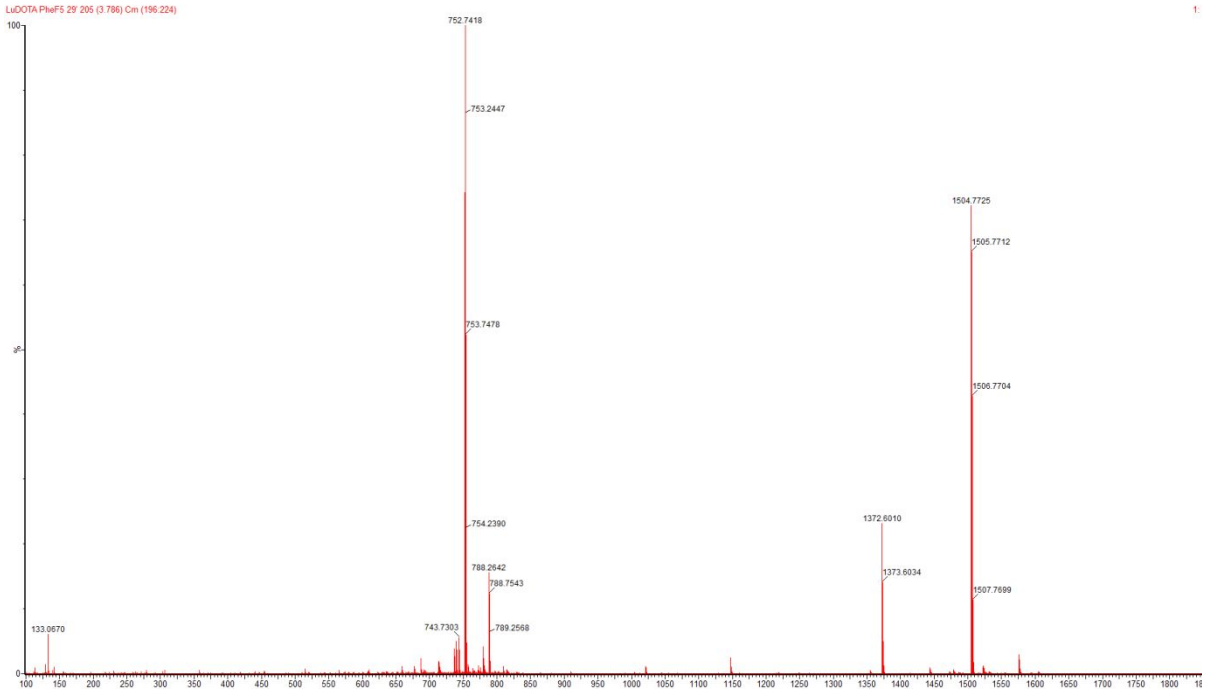

DOTA(Gd)-Ahx-His-DThr-Phe(F5)-Cys(Bzl)-AOMK

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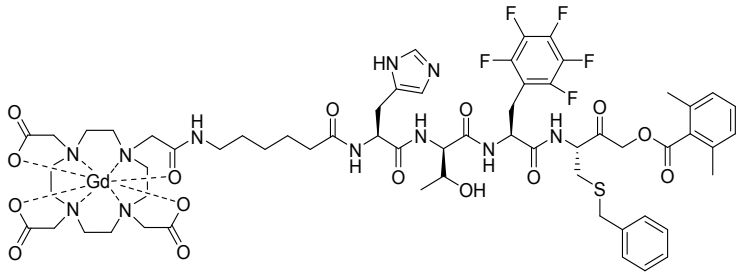

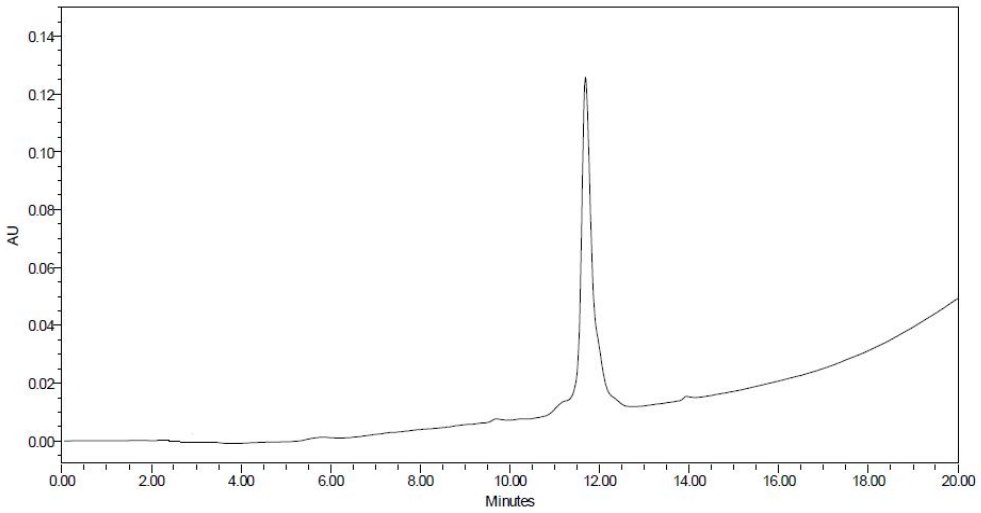

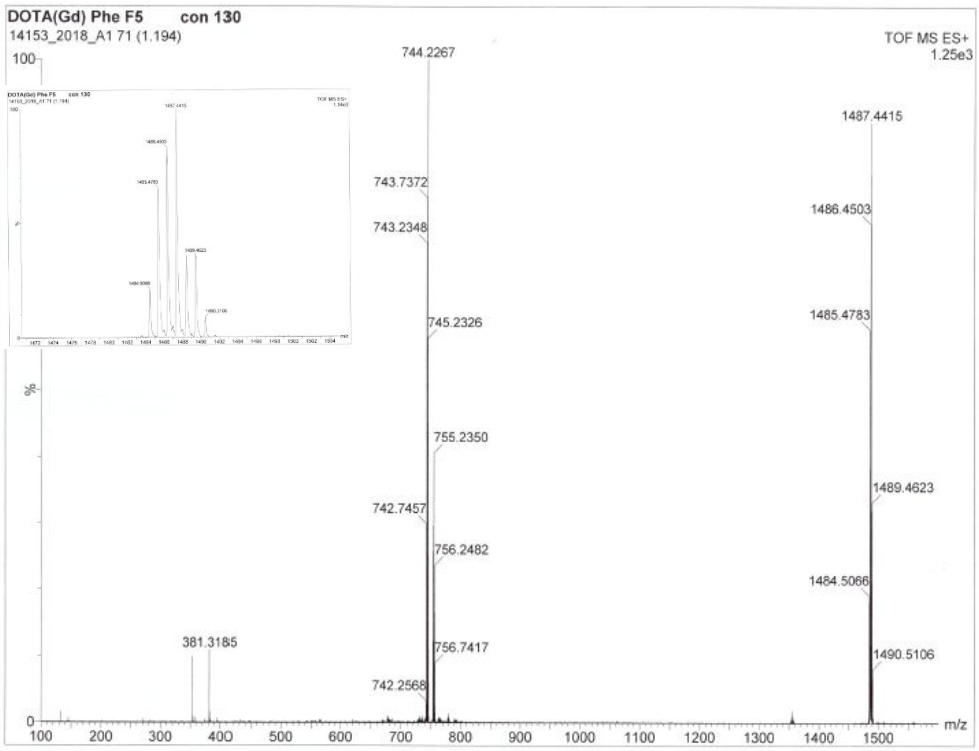

DOTA(^{159}Tb)-Ahx-Phe-Cys(Bzl)-AOMK

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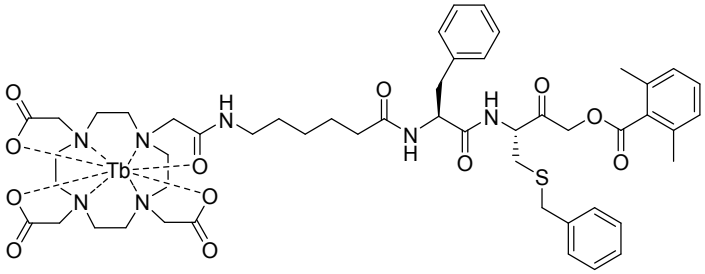
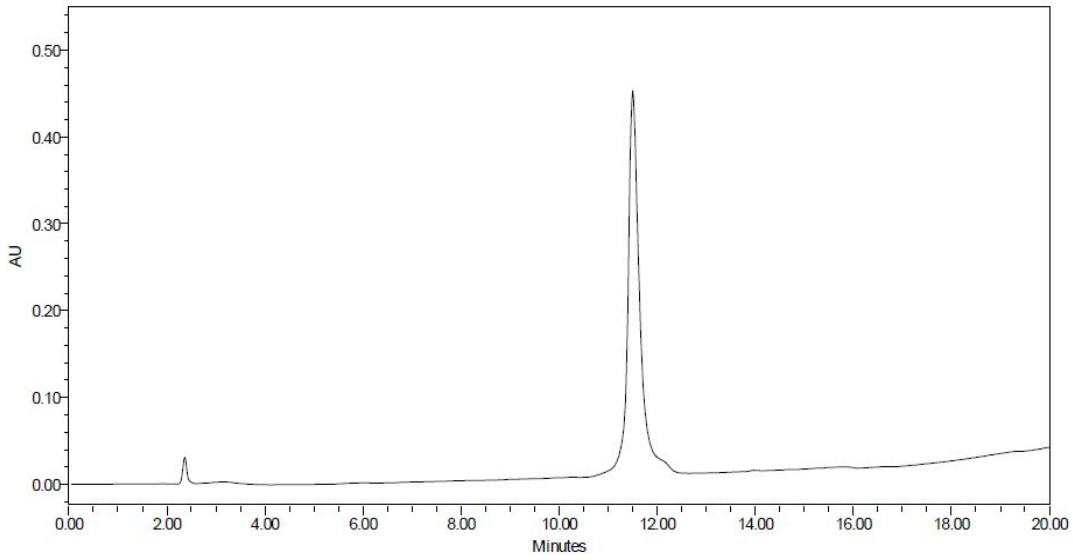
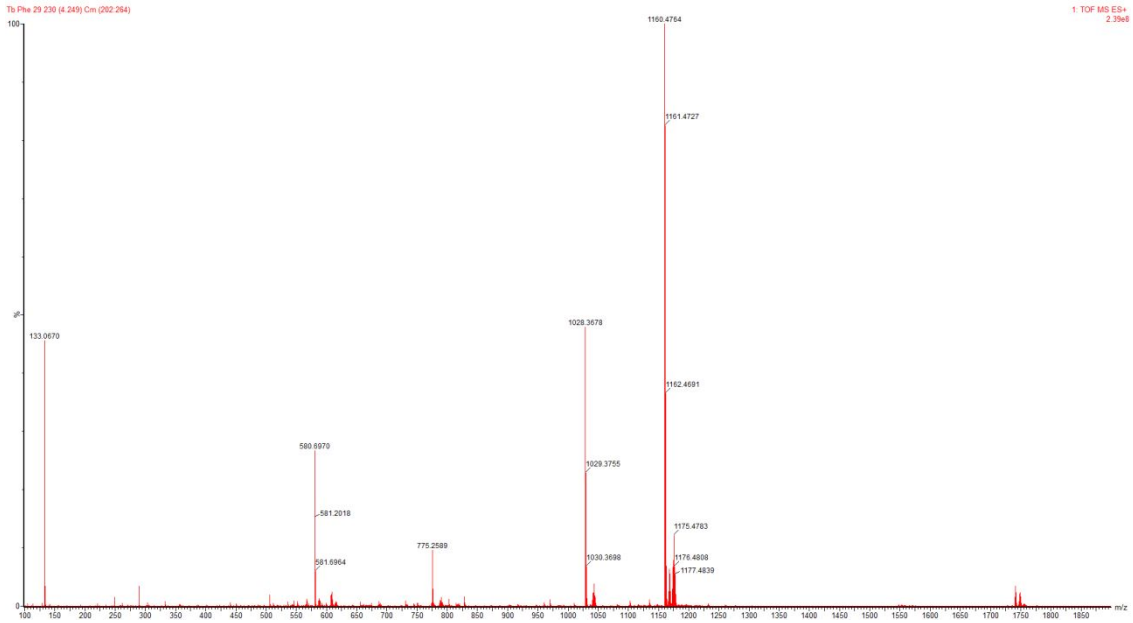
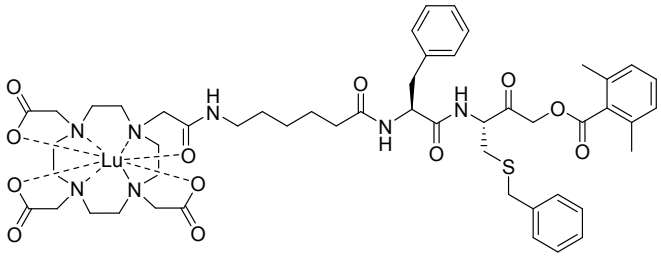


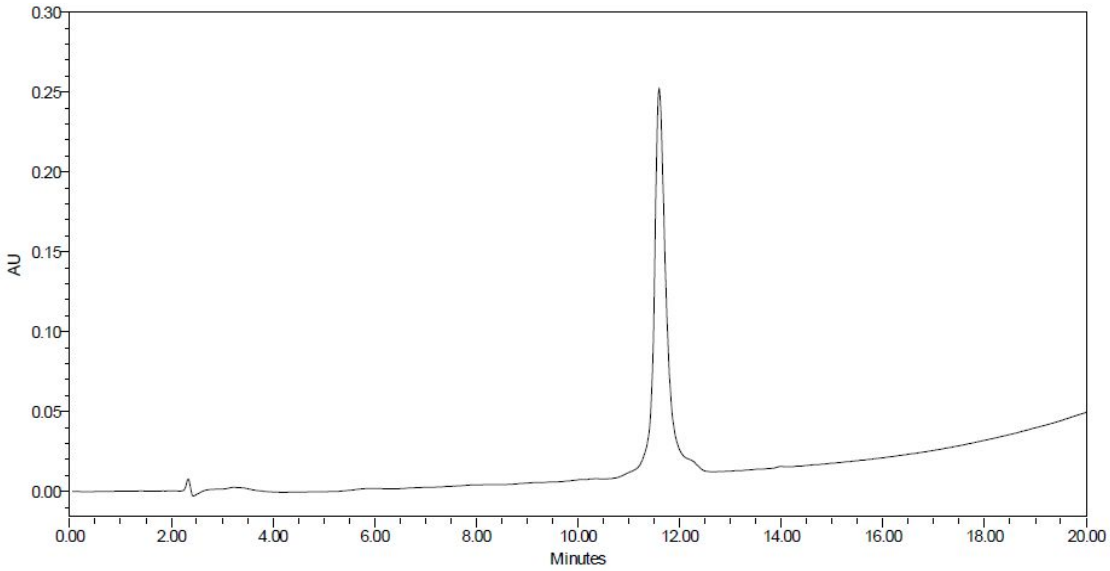


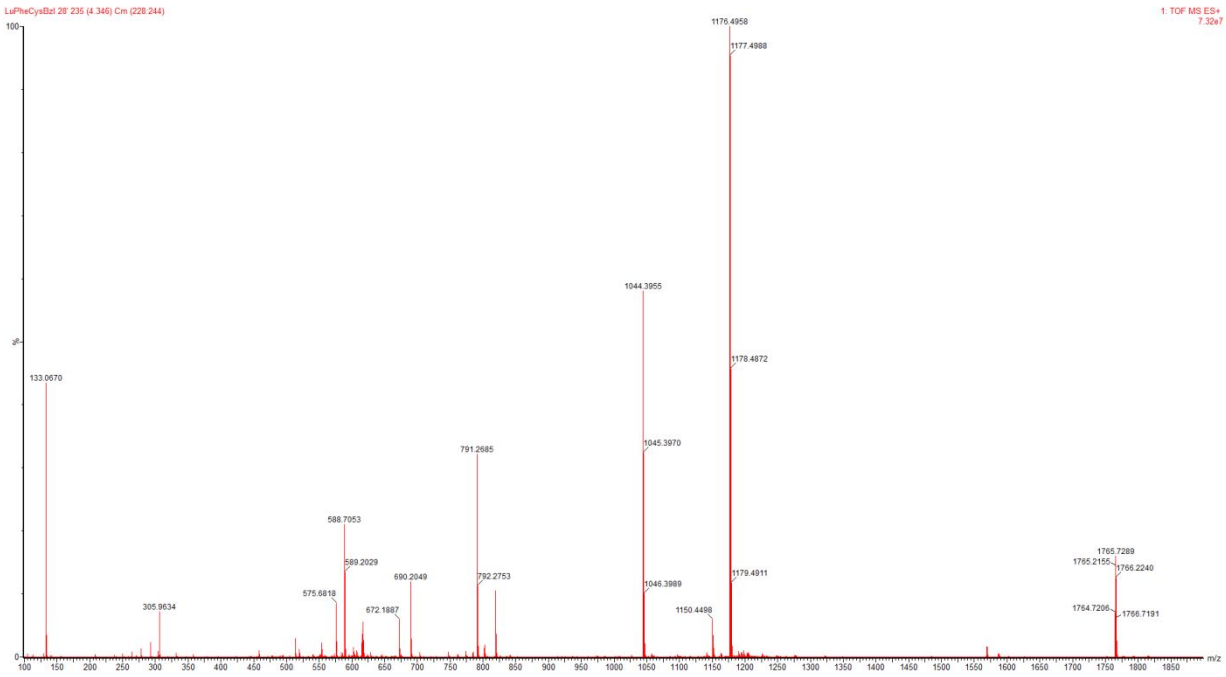


DOTA(^{175}Lu)-Ahx-Phe-Cys(Bzl)-AOMK

HRMS (m/z): $[M+H]^+$ calcd for $C_{51}H_{66}LuN_7O_{12}S$, 1176.3971, found 1176.4958

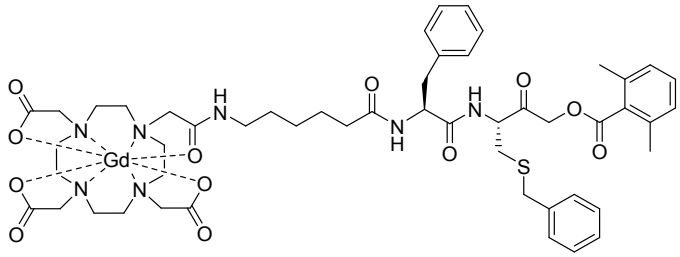


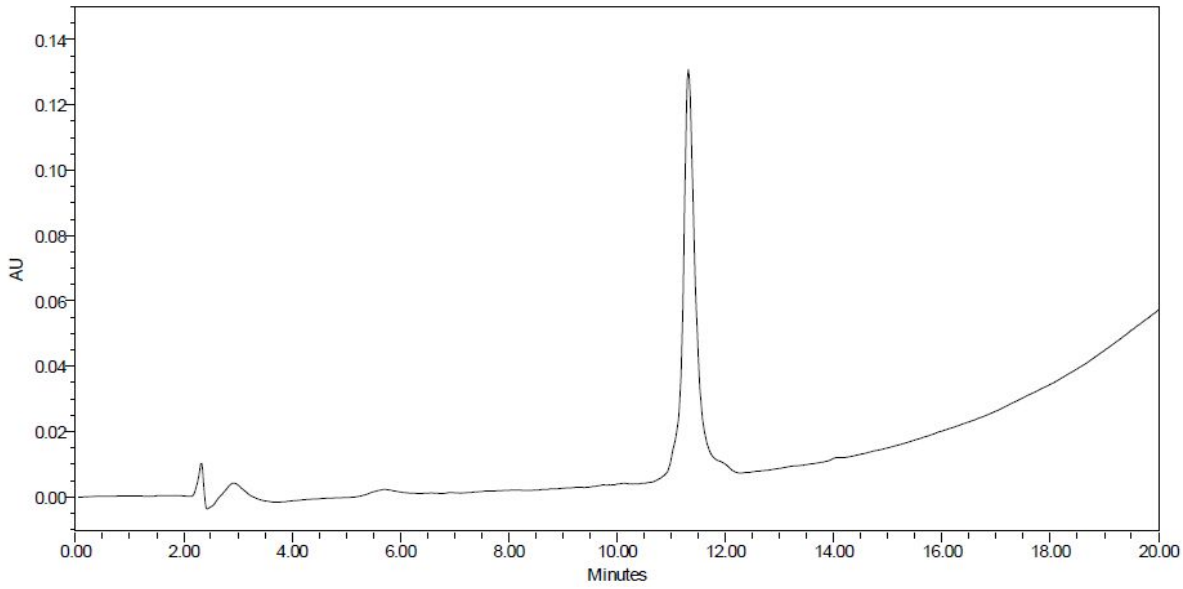


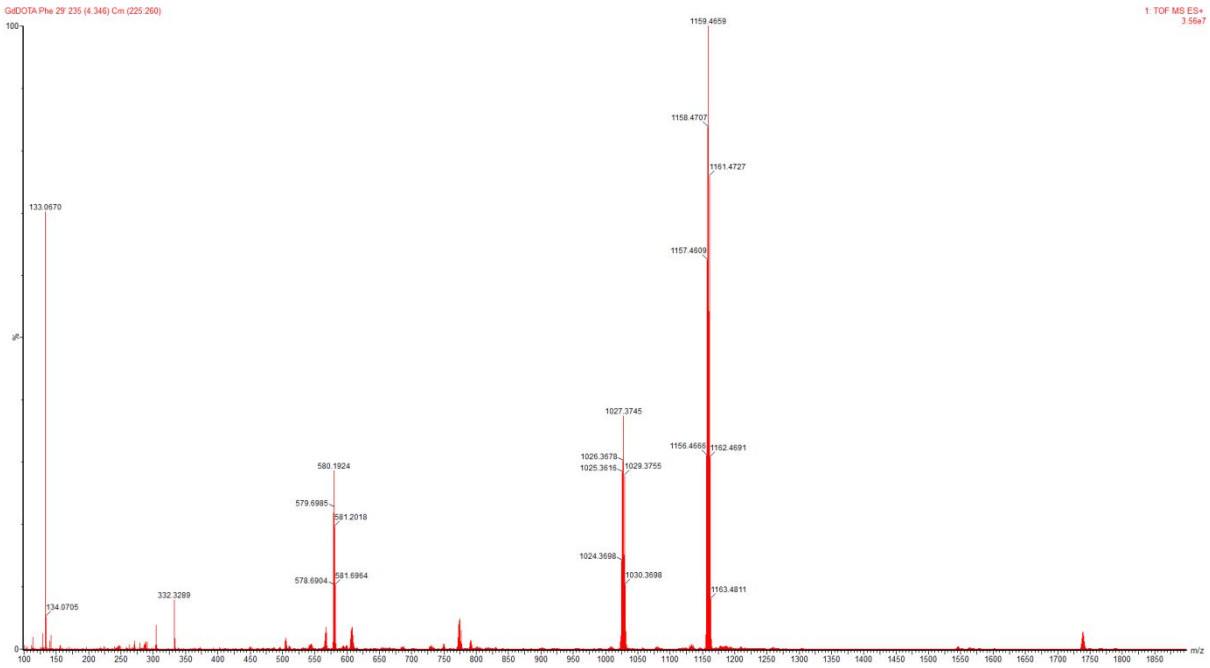


DOTA(Gd)-Ahx-Phe-Cys(Bzl)-AOMK

HRMS (m/z): $[M+H]^+$ calcd for $C_{51}H_{66}GdN_7O_{12}S$, 1159.3804, found 1159.4659

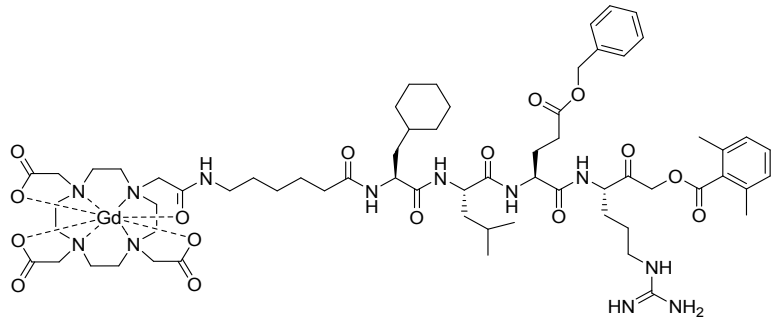


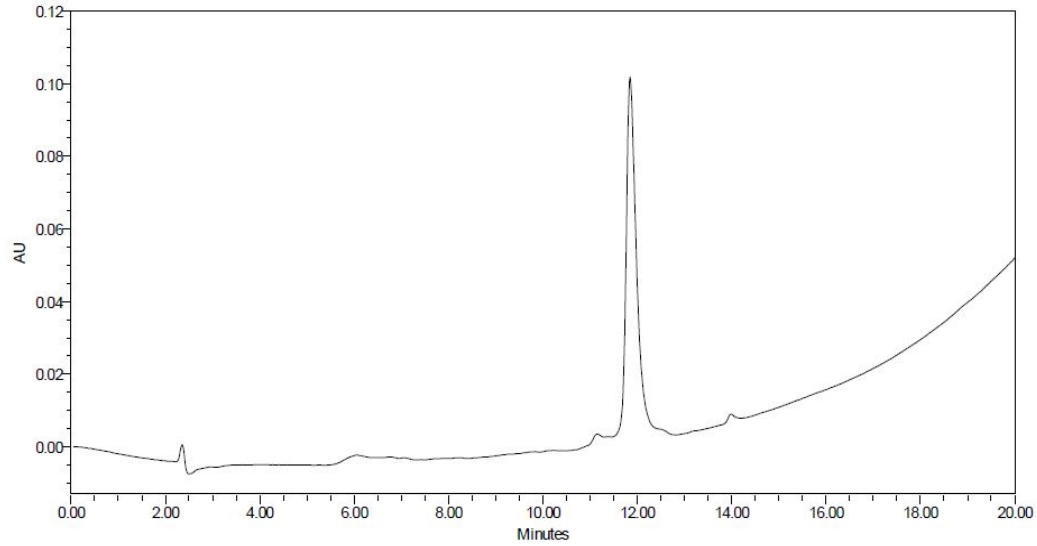


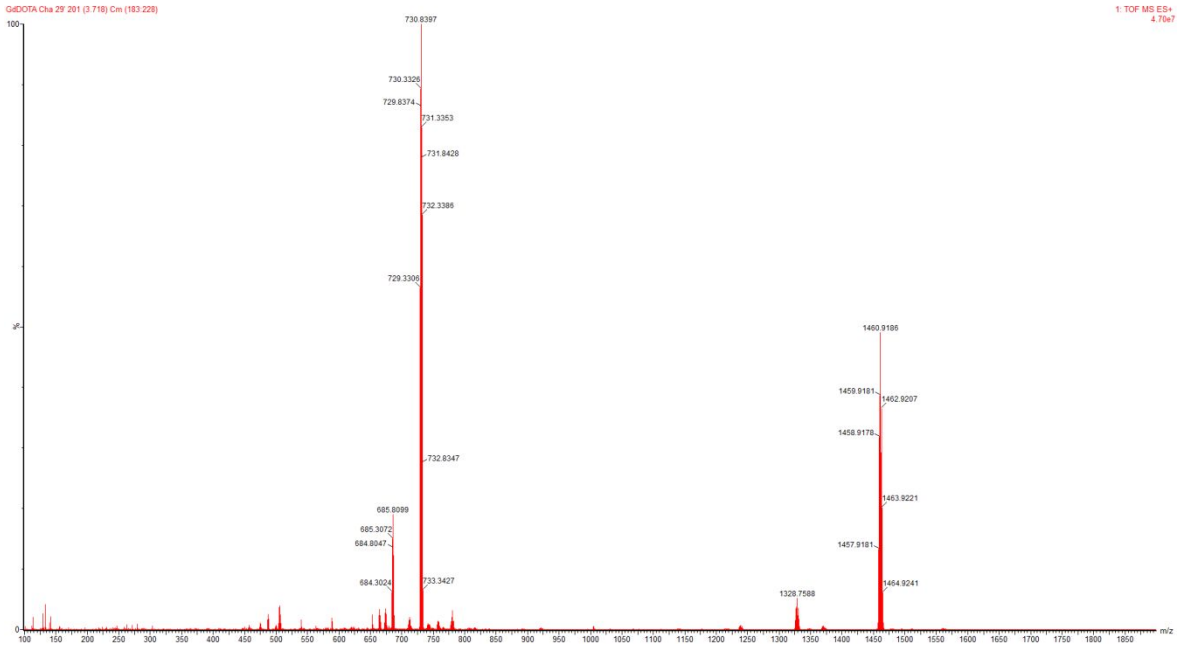


DOTA(Gd)-Ahx-Cha-Leu-Glu(Bzl)-Arg-AOMK

HRMS (m/z): $[M+H]^+$ calcd for $C_{65}H_{97}GdN_{12}O_{16}$, 1460.6460, found 1460.9186

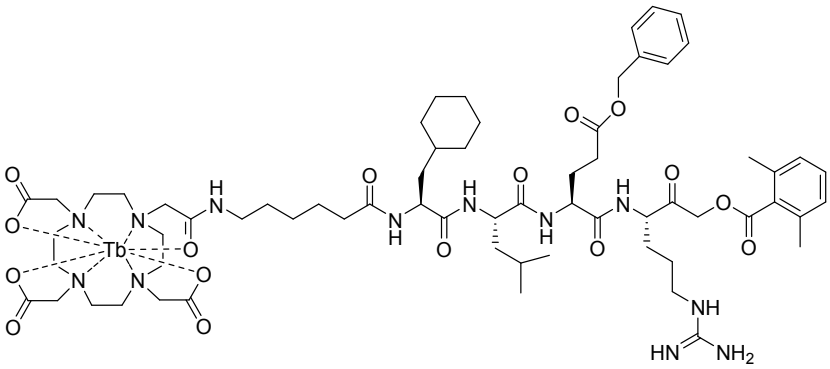


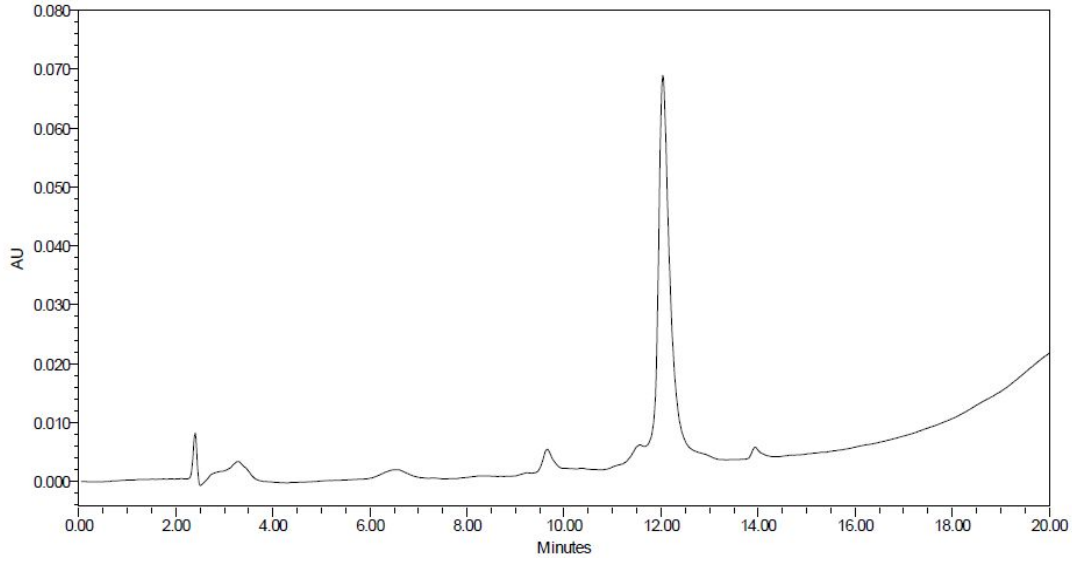


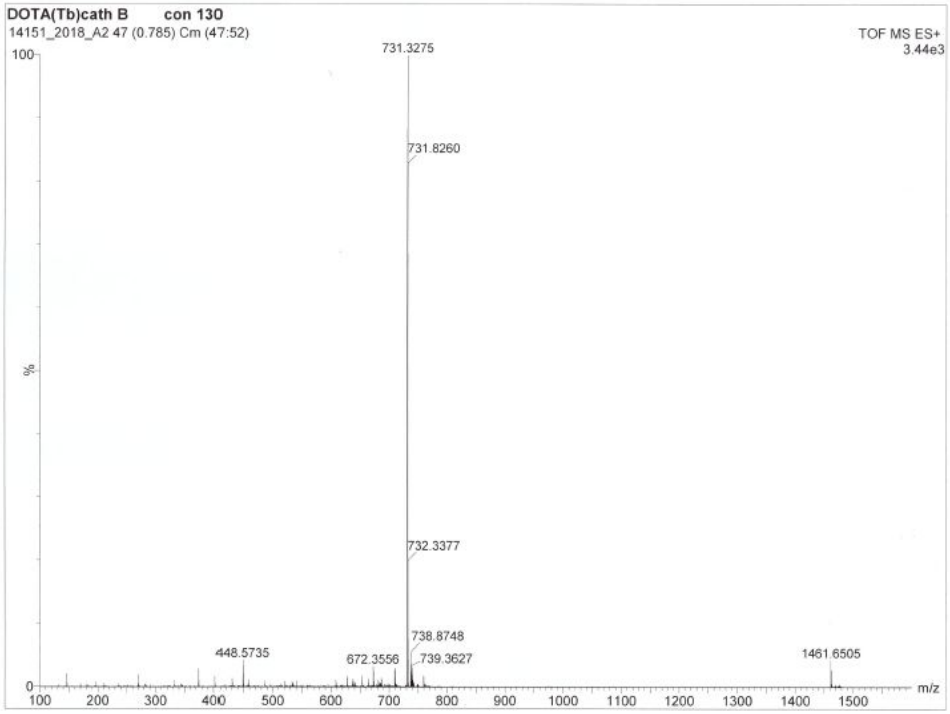


DOTA(^{159}Tb)-Ahx-Cha-Leu-Glu(Bzl)-Arg-AOMK

HRMS (m/z): $[M/2+H]^+$ calcd for $C_{65}H_{97}N_{12}O_{16}Tb$, 731.3273, found 731.3275

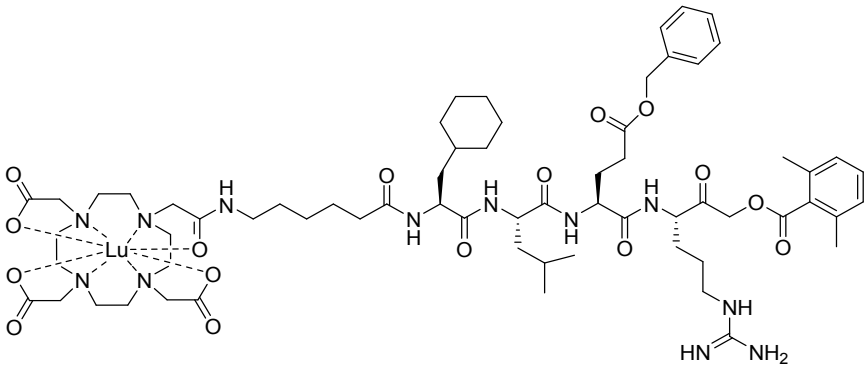


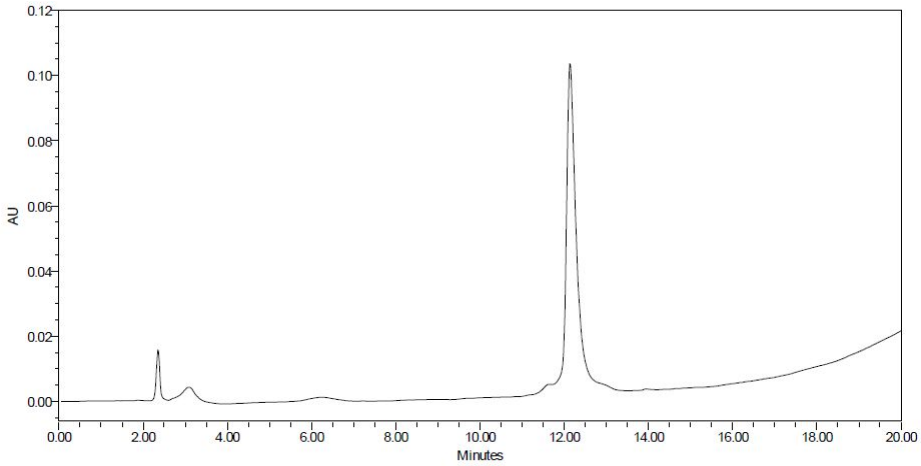


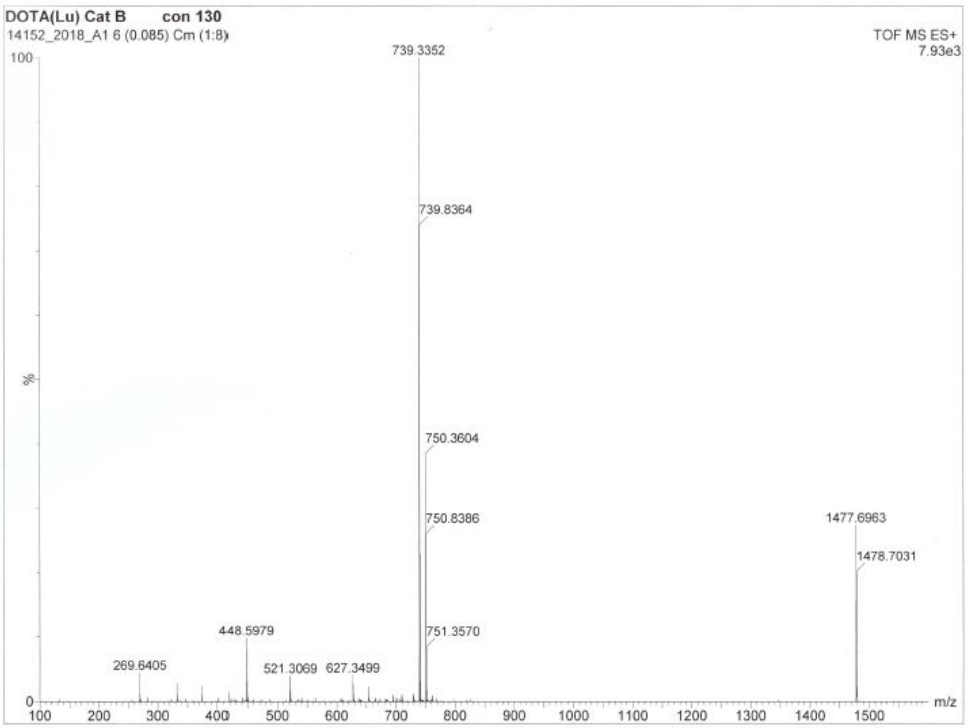


DOTA(^{175}Lu)-Ahx-Cha-Leu-Glu(Bzl)-Arg-AOMK

HRMS (m/z): $[M/2+H]^+$ calcd for $C_{65}H_{97}LuN_{12}O_{16}$, 739.3350, found 739.3352

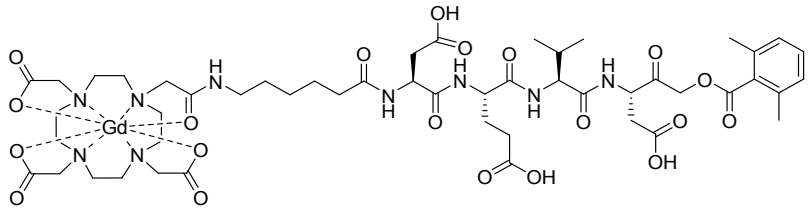


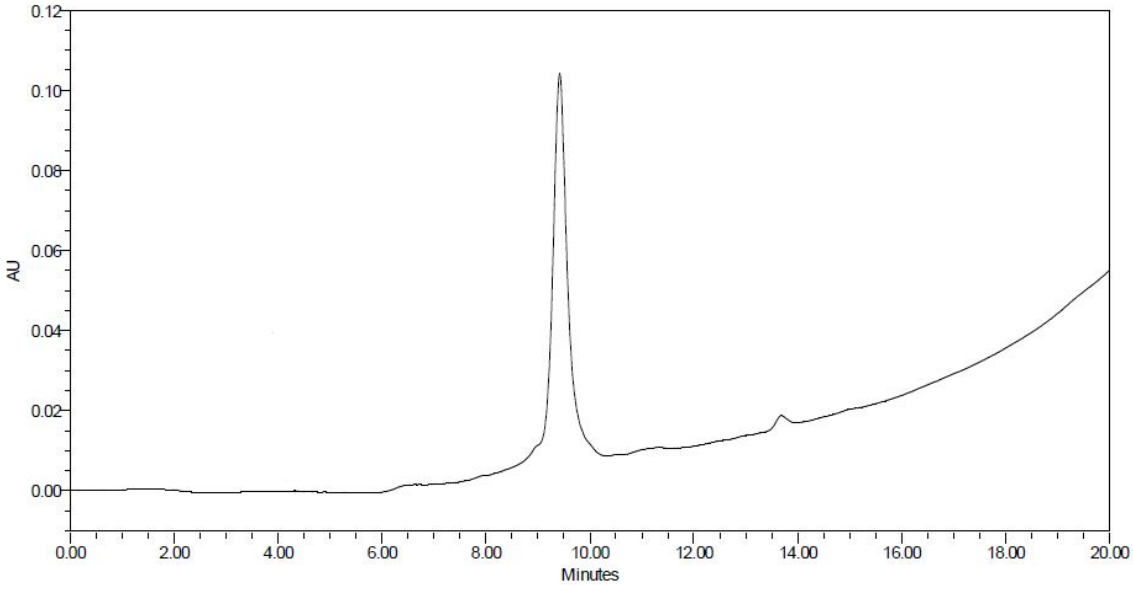


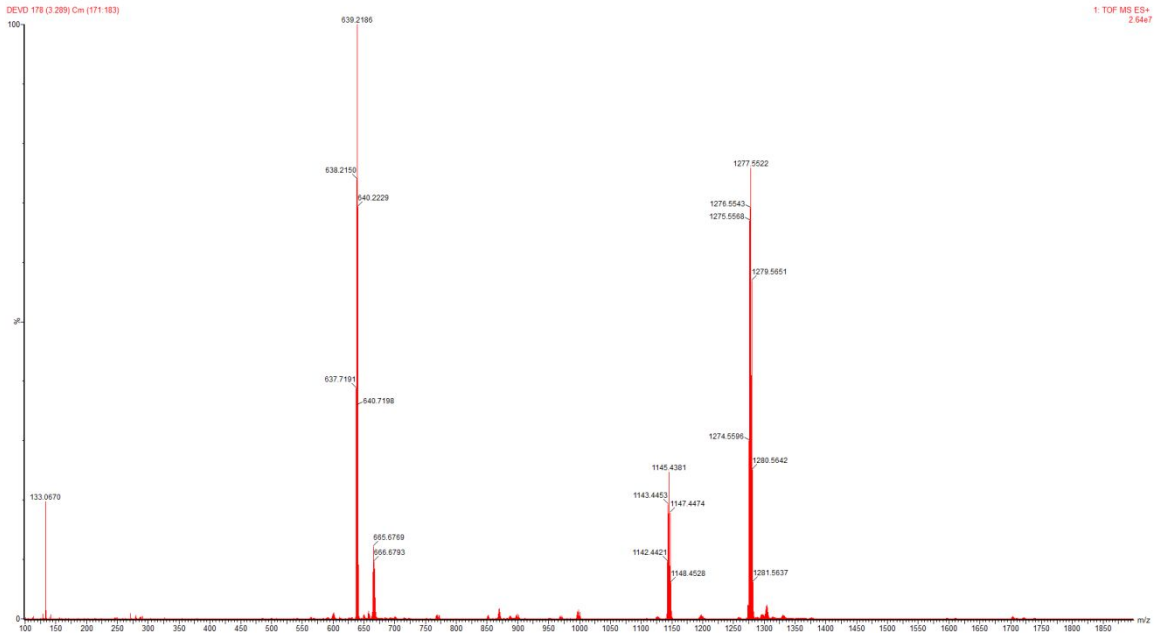


DOTA(Gd)-Ahx-Asp-Glu-Val-Asp-AOMK

HRMS (m/z): $[M+H]^+$ calcd for $C_{50}H_{72}GdN_9O_{20}$, 1277.4208, found 1277.5522

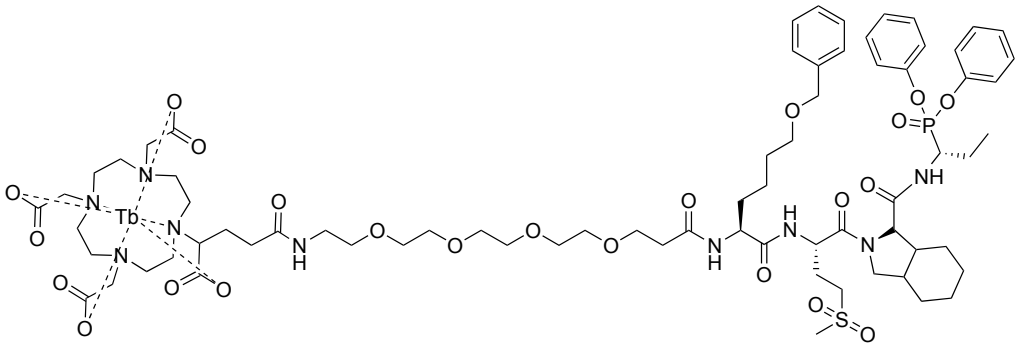


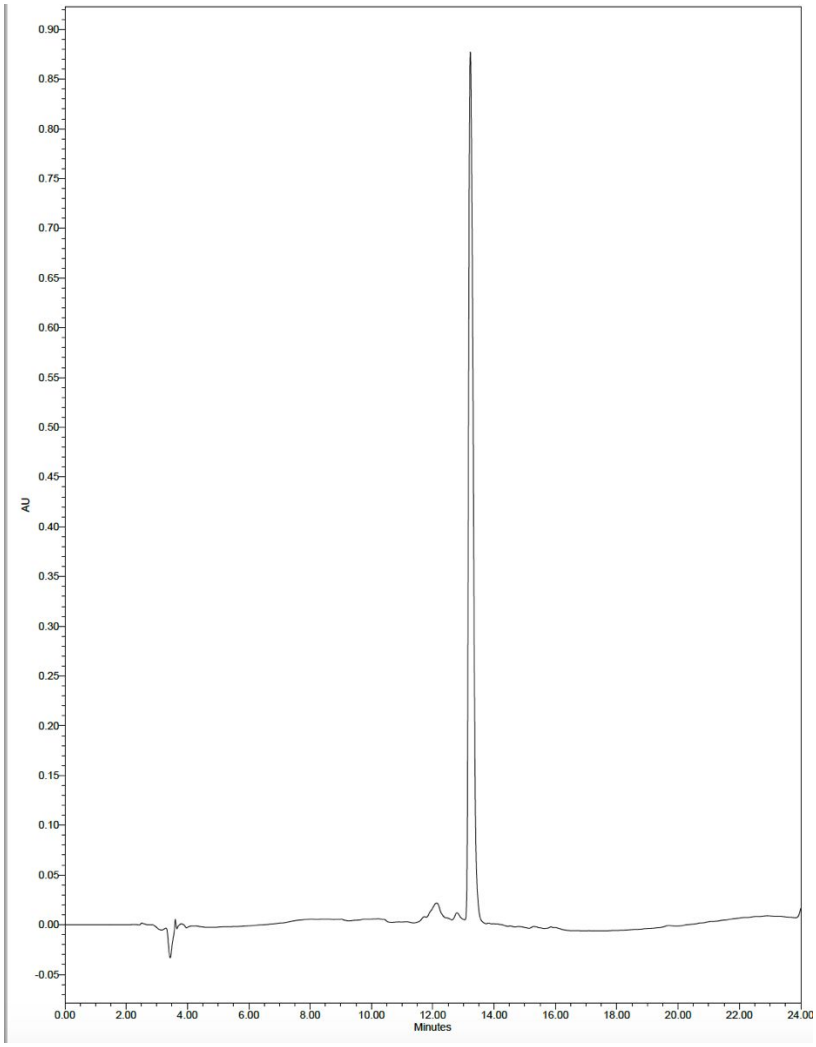




DOTA(^{159}Tb)-PEG-Nle(OBzl)-Met(O_2)-Oic- Abu^{P}($OPh)_2$

HRMS (m/z): $[1/2M+H]^+$ calcd for $C_{72}H_{104}N_9O_{23}PSTb$, 844.3228, found $[1/2M+H]^+$ 844.27




AU
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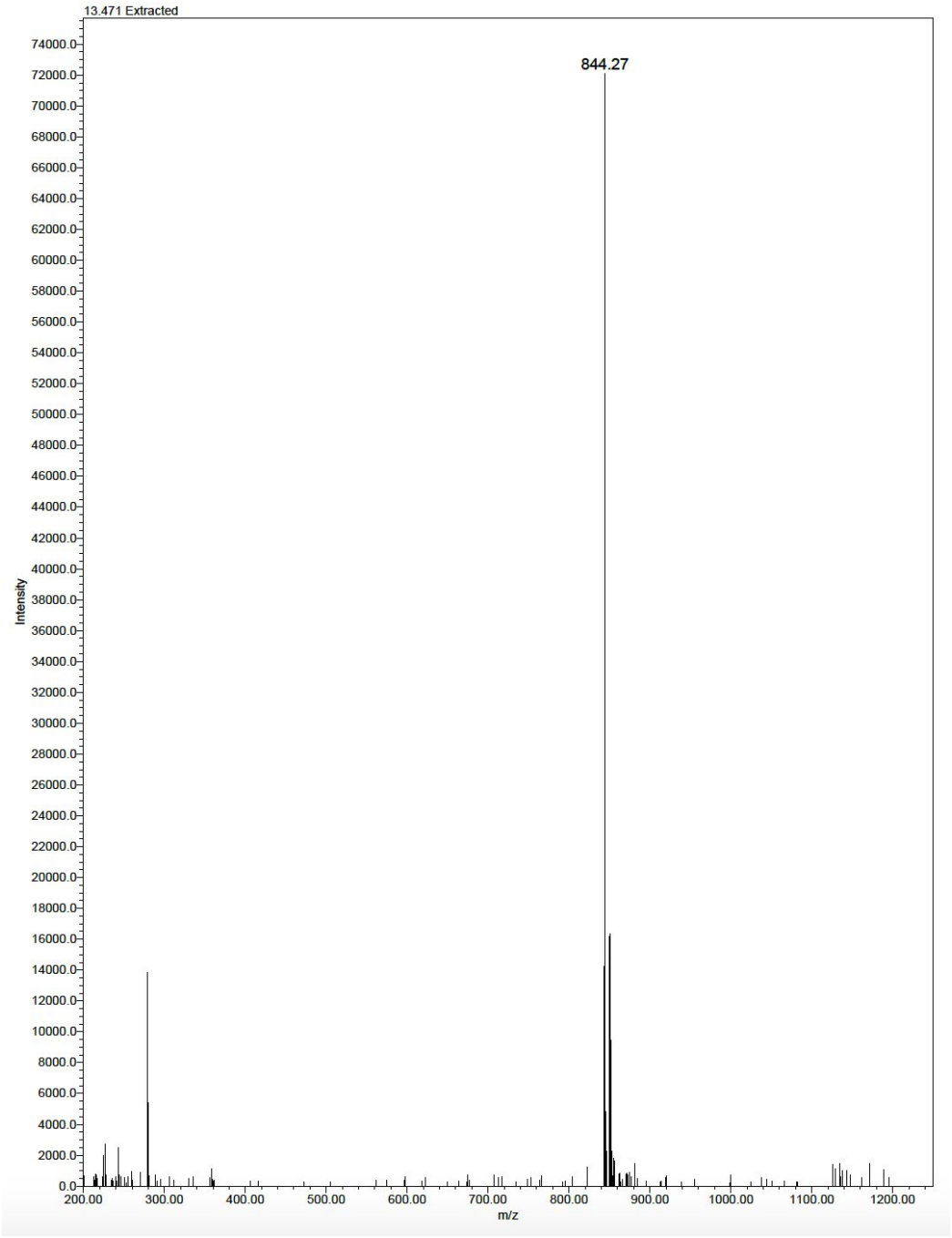
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
Minutes


13.471 Extracted
844.27
Intensity
m/z
74000.0
72000.0
70000.0
68000.0
66000.0
64000.0
62000.0
60000.0
58000.0
56000.0
54000.0
52000.0
50000.0
48000.0
46000.0
44000.0
42000.0
40000.0
38000.0
36000.0
34000.0
32000.0
30000.0
28000.0
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1200.00