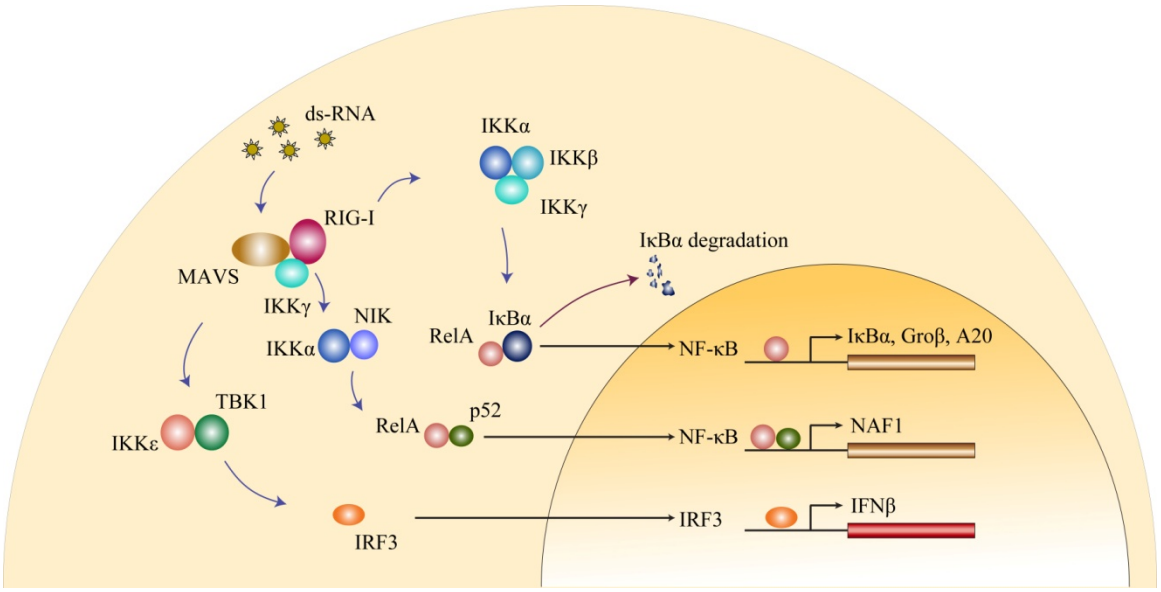
Supplemental table 1. The completeness of trypsin digestion of the signature peptides with native flanking sequence. The signature peptides with native flanking sequence were digested with trypsin as described in the Experimental Section. The amount of each peptide before and after trypsin digestion was analyzed by SRM-MS. The trypsin digestion completeness was calculated as the remaining percentage of the peptides after trypsin digestion. ND: not detectable.

Protein	Sequence[a]	Peak intensity						Trypsin digestion completeness
		Before trypsin digestion			After digestion with trypsin for 24 h			
		Mean	S.D.	CV%	Mean	S.D.	CV%	
MAVS	LTK-**VSASTVPTDGSSR-**NEE	1.84E+08	252384.1	0.1%	107340	5444.722	5.1%	99.94%
RIG-I	KGK-**VVFFANQIPVYEQQK-**SVFS	781284	49879.31	6.4%	ND[b]	-	-	100.00%
IKKA	DSK-**IQLPIIQLRK**-VW	95177359	1690270	1.8%	29552.5	6087.482	20.6%	99.97%
NIK	CLK-**IASEPPPVR**-EIP	9449289	599432.1	6.3%	3633	565.6854	15.6%	99.96%
p52/p100	PSR-**DSGEEAAEPSAPSR**-TPQ	67818182	1544634	2.3%	243898.5	5189.457	2.1%	99.64%
p100	SAR-**ALLDYGVTADAR**-ALL	43114176	1449659	3.4%	148691	30091.64	20.2%	99.66%
IKKB	FFK-**TSIQIDLEK**-YSE	691759.5	40847.44	5.9%	2002	33.94113	1.7%	99.71%
IKKE	TER-**FSSVAGTPEIQELK**-AAA	1600638	50114.07	3.1%	ND[c]	-	-	100.00%
IRF3	GLR-**LVGSEVGDR**-TLP	41209166	262283.6	0.6%	29752.5	3246.327	10.9%	99.93%
TBK1	GLR-**LVGSEVGDR**-TLP	13007425	284291.6	2.2%	ND[c]	-	-	100.00%
RelA	VFR-**TPPYADPSLQAPVR**-VSM	2.84E+09	39120017	1.4%	6608589	132852.6	2.0%	99.77%
IKBa	QEIR-**LEPQEVPR**-GSE	9896976	1413103	14.3%	1142.245	218.8566	19.2%	99.99%

a. The peptide sequences in bold are signature peptides.
b. Noise level is about 100 counts.
c. Noise level is about 20 counts.

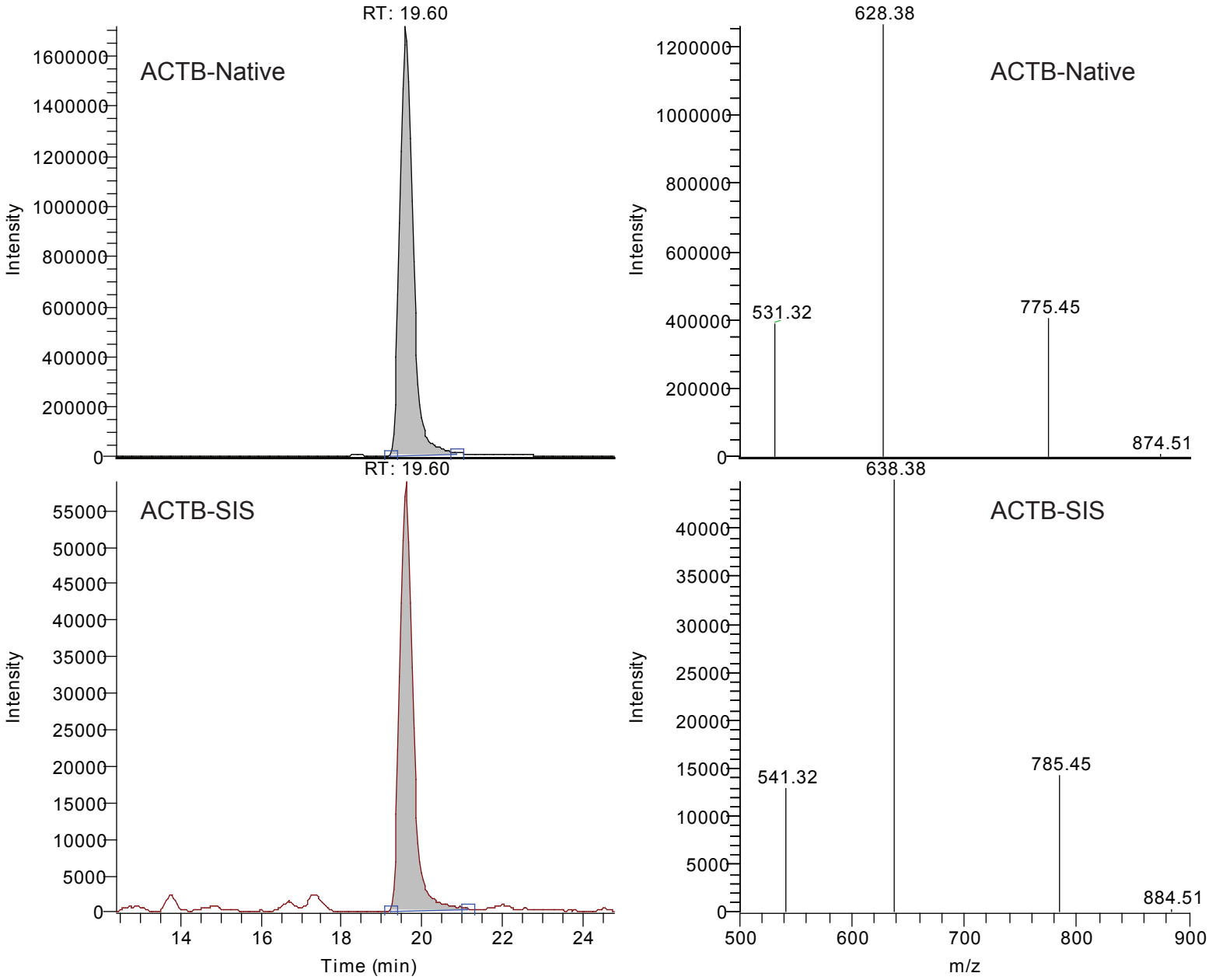
Supplemental Table 2. Activation of noncanonical NF-κB pathway by the stimulation of ds-RNA. A549 cells were treated with ds-RNA for 0, 1 and 4 h, or treated with TNF for 0, 0.5 and 1 h, respectively. The nuclear proteins were extracted and subjected to SID-SRM-MS analysis. Each sample was analyzed by LC-SRM-MS twice. All data were manually inspected to ensure peak detection and accurate integration as described in Experimental Procedure section. The peak area of extract ion chromatogram of native peptides and SIS-peptides of p52 and p100 were integrated using Xcalibur® 2.1. The default values for noise percentage and base-line subtraction window were used. The ratio between the peak area of native and SIS version of each peptide were calculated. Beta-actin was used as loading control. All of the measured native peptide versus SIS-peptide ratios were normalized by beta-actin. The native verse SIS-peptide ratios measured in the two replicate LC-SRM-MS analysis were averaged, and a mean and standard deviation (SD) were calculated.

Stimuli	Time (h)	p52		p100	
		Mean ratio (native/SIS-peptide)	SD	Mean ratio (native/SIS-peptide)	SD
ds-RNA	0	0.0384	0.0079	0.0038	0.0006
	1	0.045	0.0031	0.0035	0.0002
	4	0.067	0.0064	0.0034	0.0011
TNF	0	0.0656	0.0077	0.0032	0.0004
	0.5	0.0407	0.0012	0.0027	0.0008
	1	0.0310	0.0023	0.0023	0.0004



Supplemental Figure 1. Overview of innate immune response highlighting the NF-κB and IRF3 pathways. Shown is a schematic view of the IIR displaying the canonical NF-κB, noncanonical NF-κB and IRF3 signaling pathways of the IIR. The proteins listed are targeted for SID-SRM assay development. Abbreviations: IKK, IκB kinase, IκBα, inhibitor of IκBα isoform; RIG-I, retinoic acid inducible gene-I; MAVS, mitochondrial antivirals signaling; TBK1, Tank binding kinase 1.

Supplemental Figure 2. Evaluation of the specificity of SRM assays. The SIS peptides were spiked in the trypsin digests of nuclear fraction. The native and SIS peptide were analyzed by SRM. In each figure, the left panels are the extract ion chromatogram, and the right panels are the SRM spectra.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Intensity
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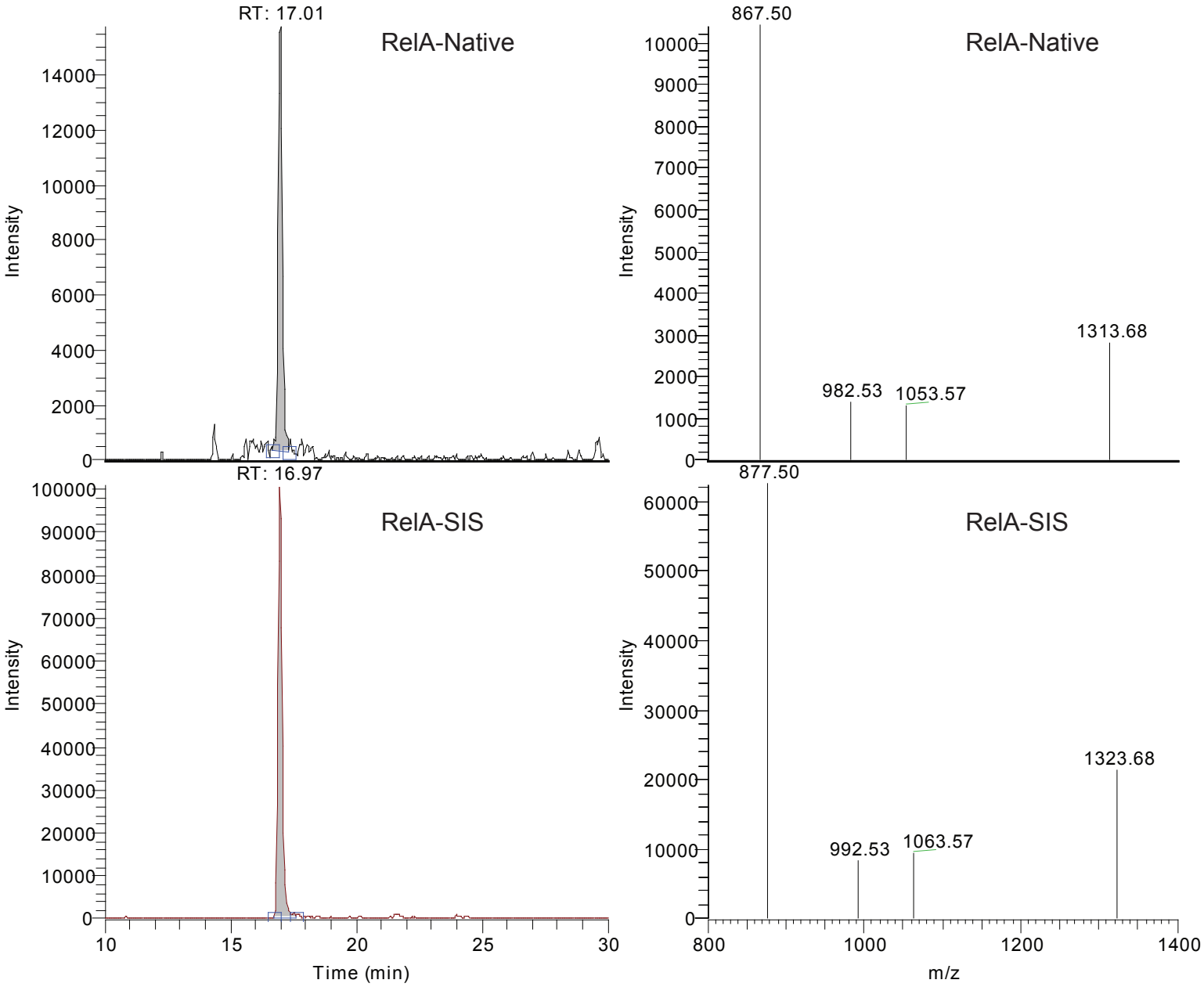
1200000
1000000
800000
600000
400000
200000
0
628.38
ACTB-Native
1200000
1000000
800000
600000
400000
200000
0
531.32
775.45
874.51
RT: 19.60
ACTB-SIS
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
14
16
18
20
22
24
Time (min)
638.38
ACTB-SIS
40000
35000
30000
25000
20000
15000
10000
5000
0
541.32
785.45
884.51
500
600
700
800
900
m/z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Intensity
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RT: 16.97
RelA-SIS
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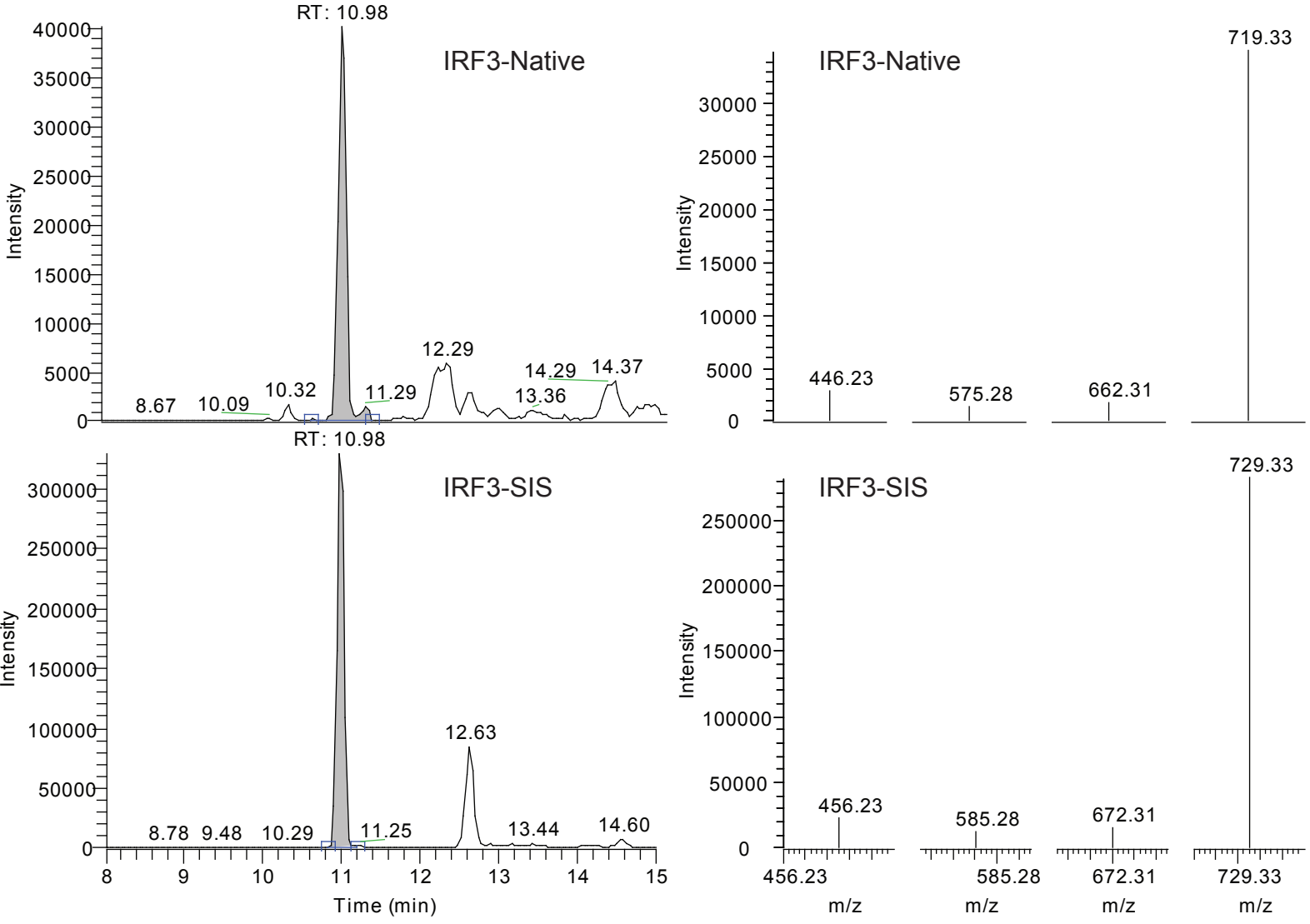
1323.68
992.53
1063.57
Time (min)
m/z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Intensity
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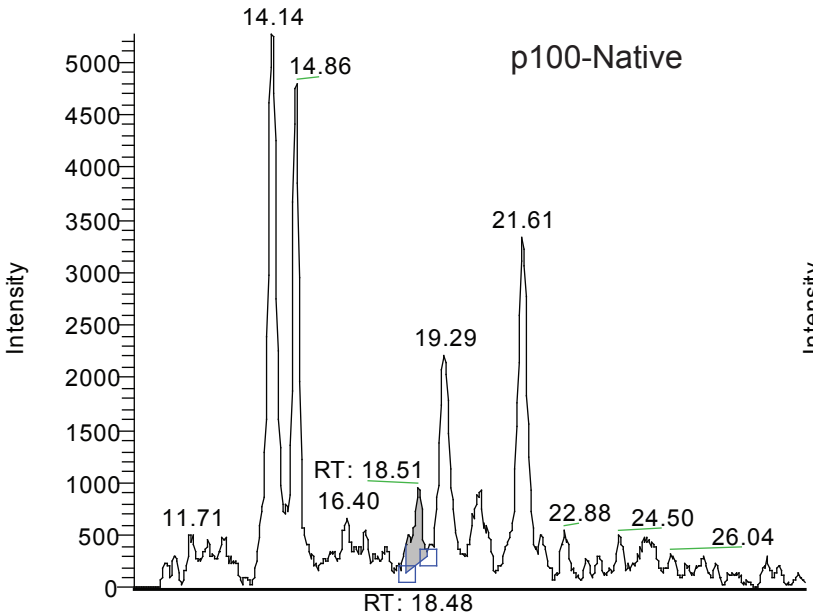
10.32
11.29
12.29
13.36
14.29
14.37
IRF3-Native
719.33
446.23
575.28
662.31
RT: 10.98
IRF3-SIS
8.78
9.48
10.29
11.25
12.63
13.44
14.60
Time (min)
IRF3-SIS
729.33
456.23
585.28
672.31
m/z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19.29
RT: 18.51
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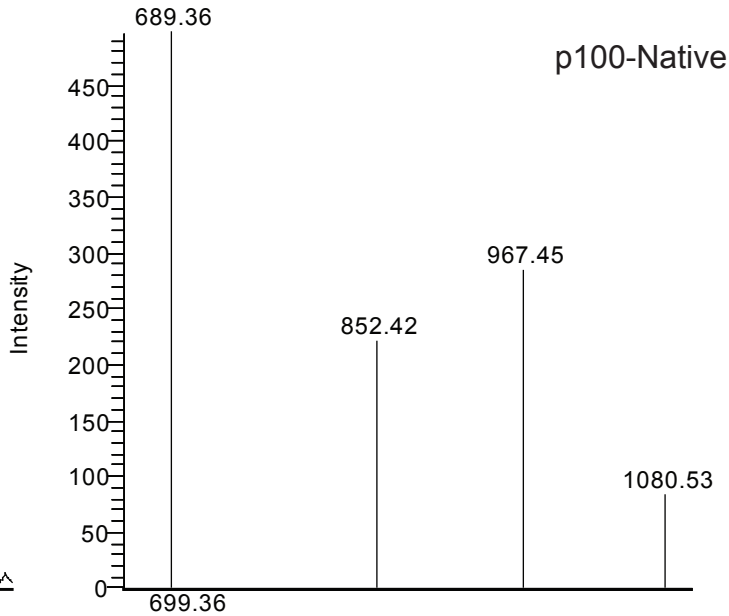
22.88
24.50
26.04
RT: 18.48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Intensity
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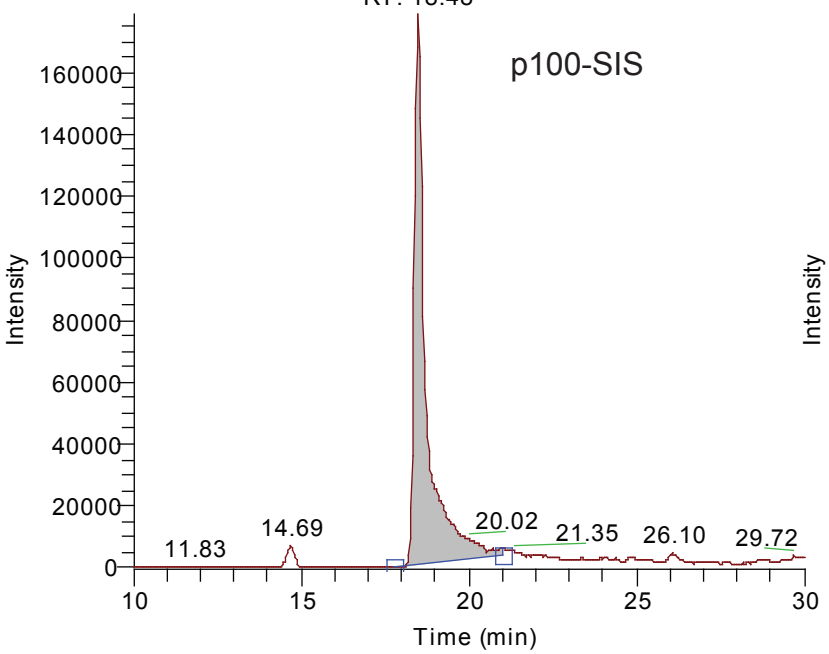
967.45
852.42
1080.53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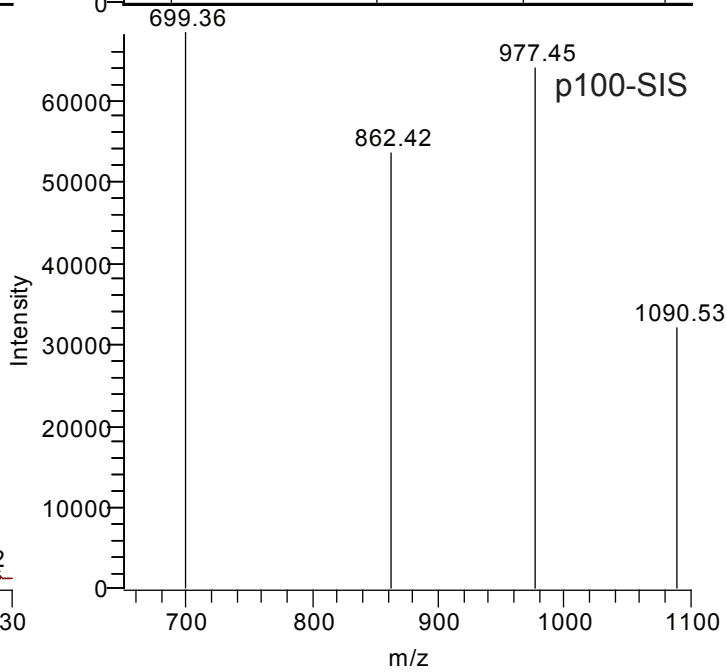
26.10
29.72
Time (min)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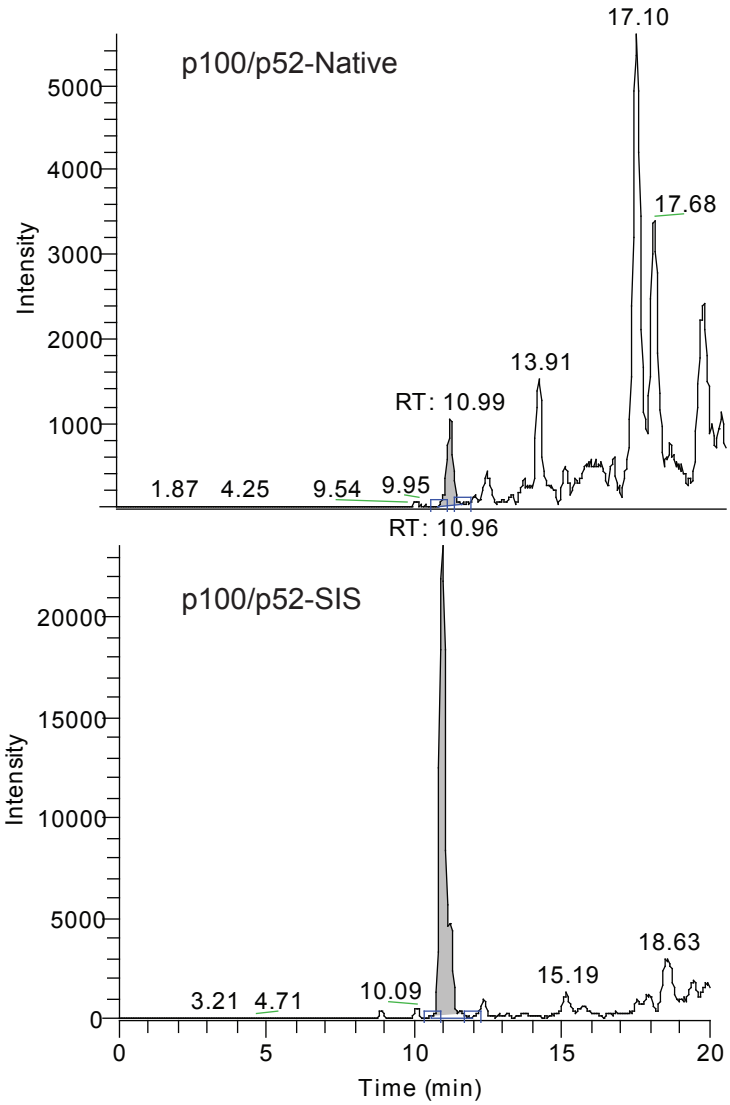
862.42
1090.53
m/z


p100/p52-Native
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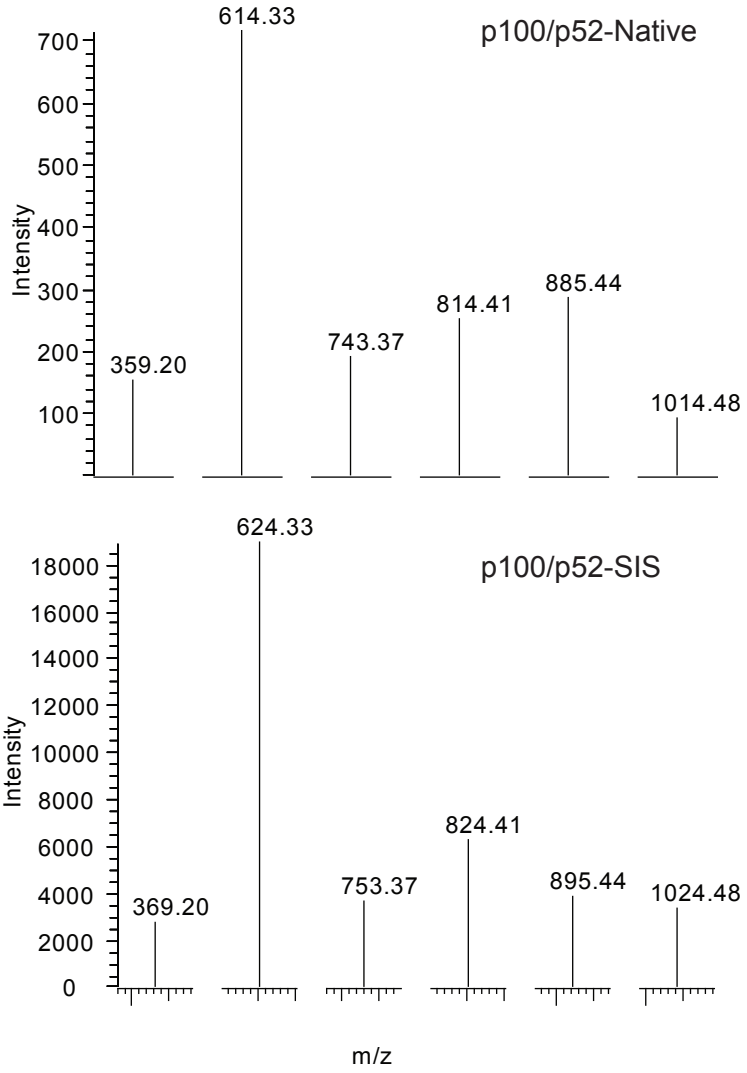
17.10
17.68
13.91
RT: 10.99
1.87
4.25
9.54
9.95
p100/p52-SIS
RT: 10.96
Intensity
18.63
15.19
3.21
4.71
10.09
Time (min)


p100/p52-Native
Intensity
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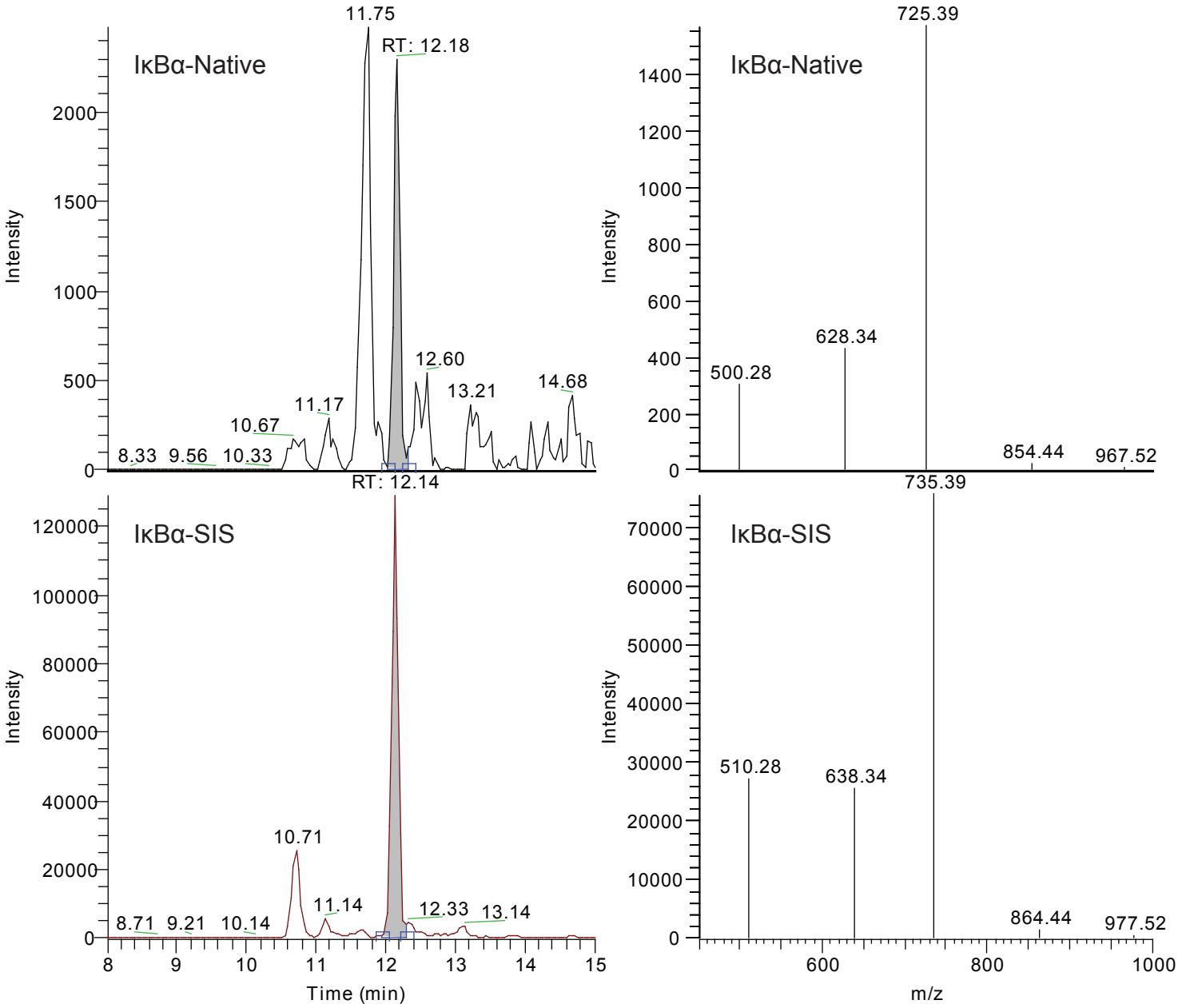
359.20
743.37
814.41
885.44
1014.48
p100/p52-SIS
624.33
369.20
753.37
824.41
895.44
1024.48
m/z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12.60
13.21
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11.17
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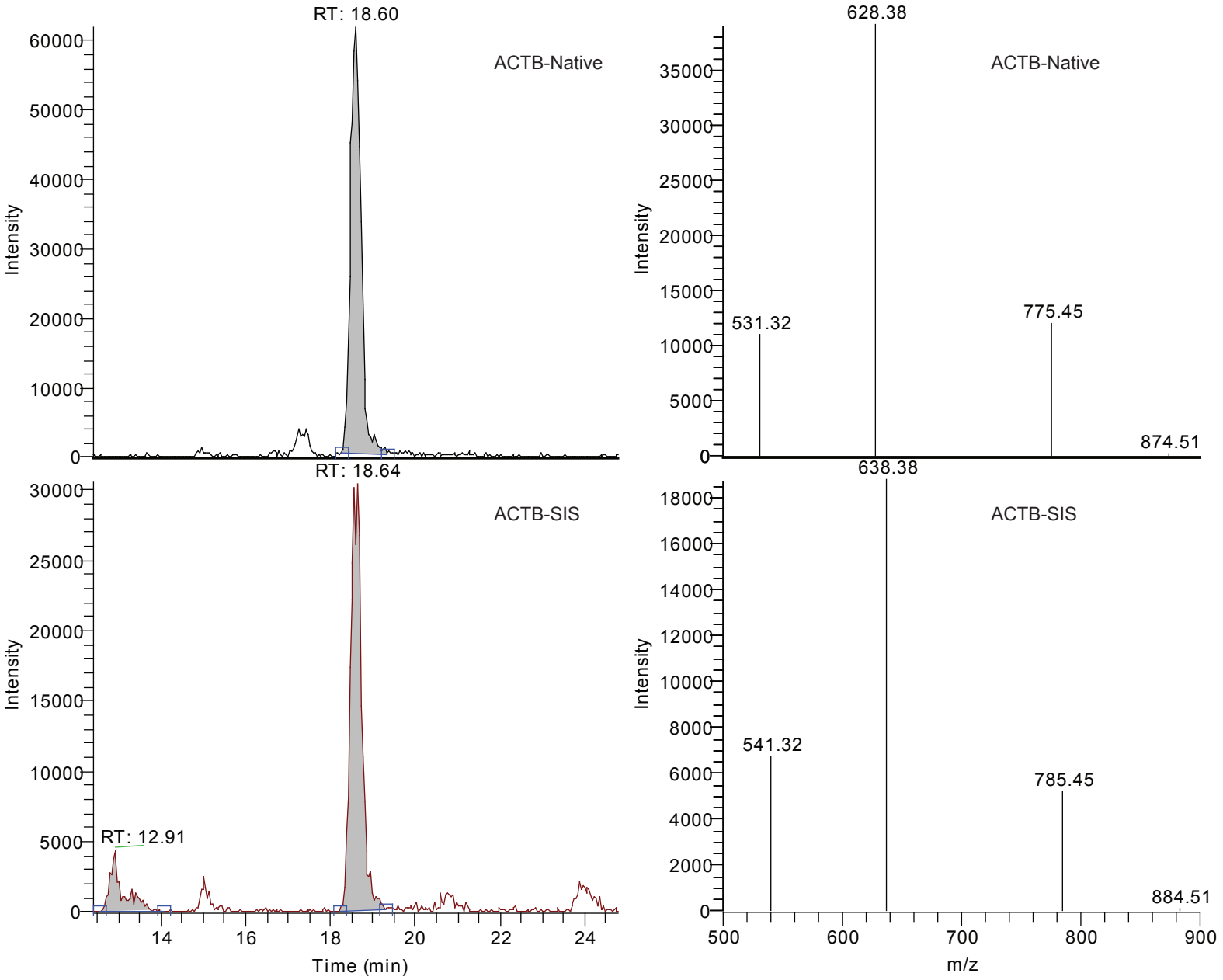
8.33
9.56
10.33
Intensity
IκBα-Native
725.39
628.34
500.28
854.44
967.52
IκBα-SIS
RT: 12.14
10.71
11.14
12.33
13.14
8.71
9.21
10.14
Time (min)
IκBα-SIS
735.39
510.28
638.34
864.44
977.52
m/z

Supplemental Figure 3. Evaluation of the specificity of SRM assays. The SIS peptides were spiked in the trypsin digests of cytoplasma fraction. The native and SIS peptide were analyzed by SRM. In each figure, the left panels are the extract ion chromatogram, and the right panels are the SRM spectra.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ACTB-Native
Intensity
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RT: 18.64
ACTB-SIS
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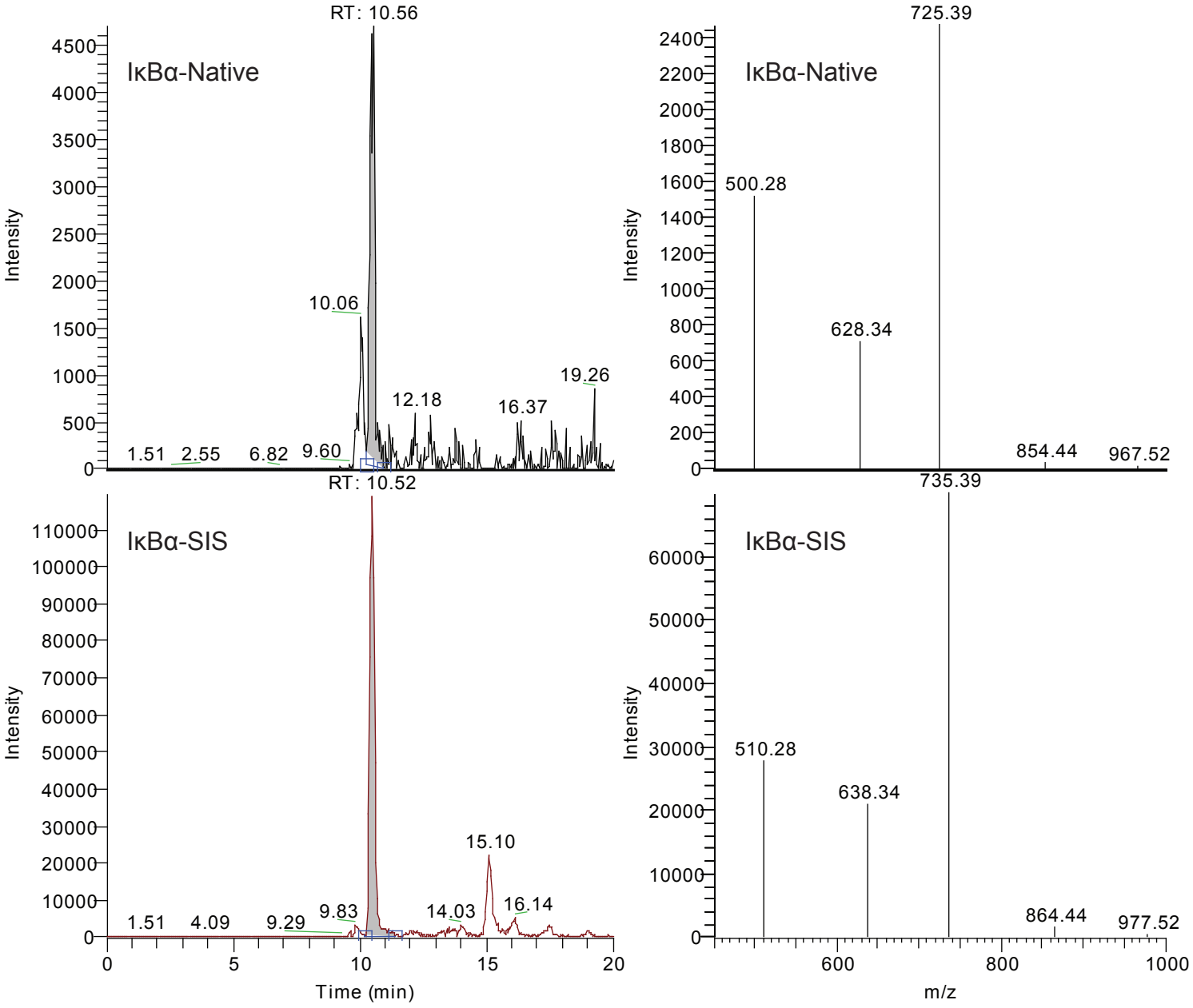
638.38
541.32
785.45
884.51
Time (min)
m/z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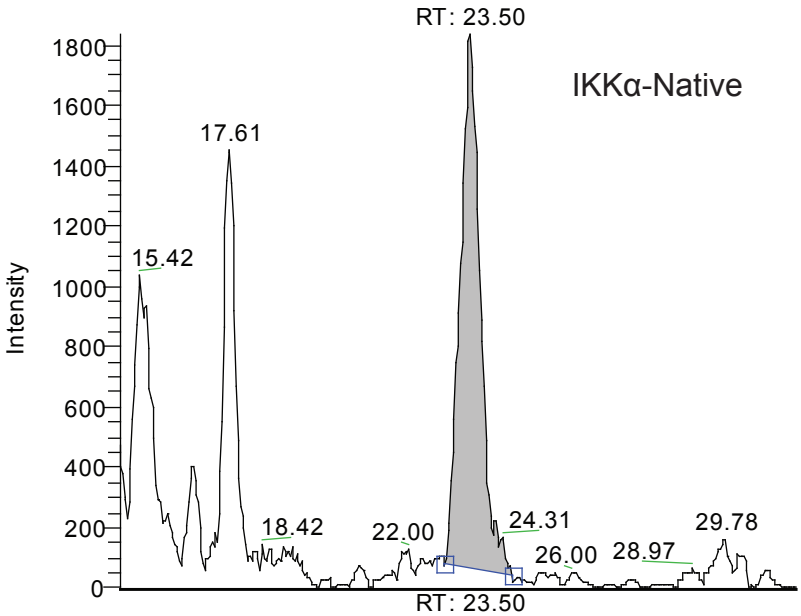
725.39
500.28
628.34
854.44
967.52
RT: 10.52
IκBα-SIS
15.10
16.14
1.51
4.09
9.29
9.83
14.03
735.39
510.28
638.34
864.44
977.52
Time (min)
m/z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Intensity
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18.42
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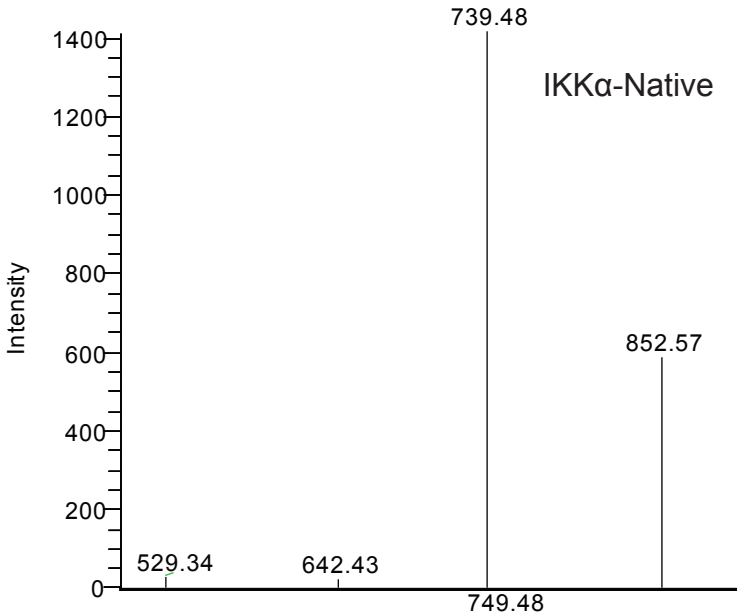
24.31
26.00
28.97
29.78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Intensity
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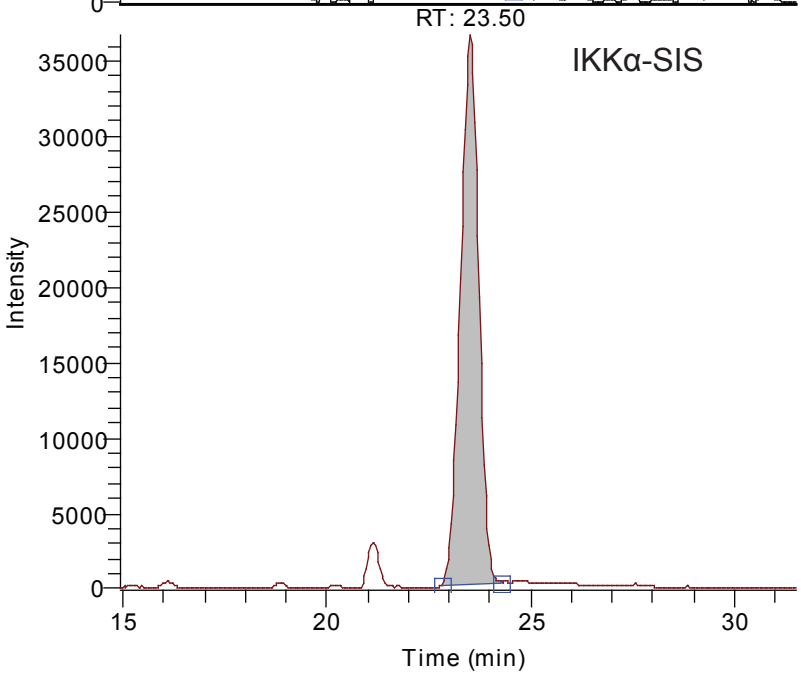
642.43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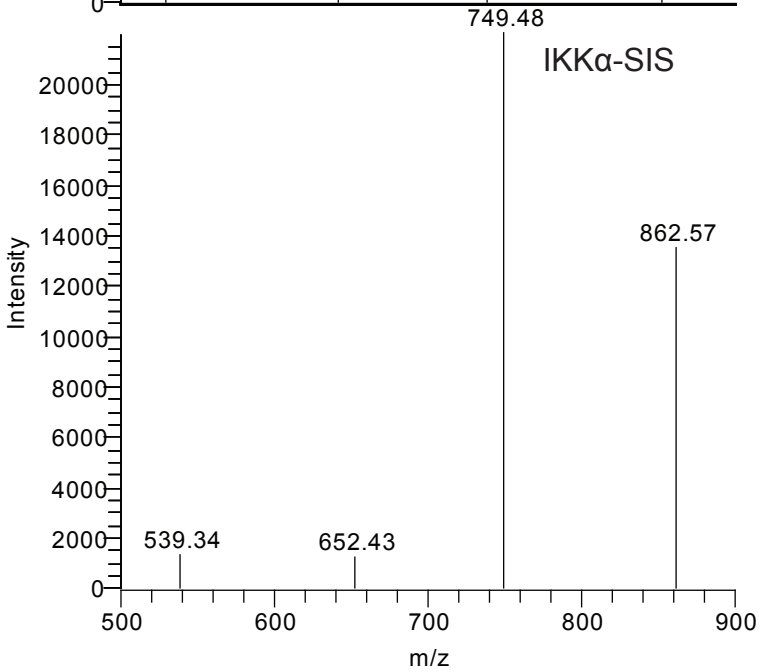
IKKα-SIS
Intensity
Time (min)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Intensity
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539.34
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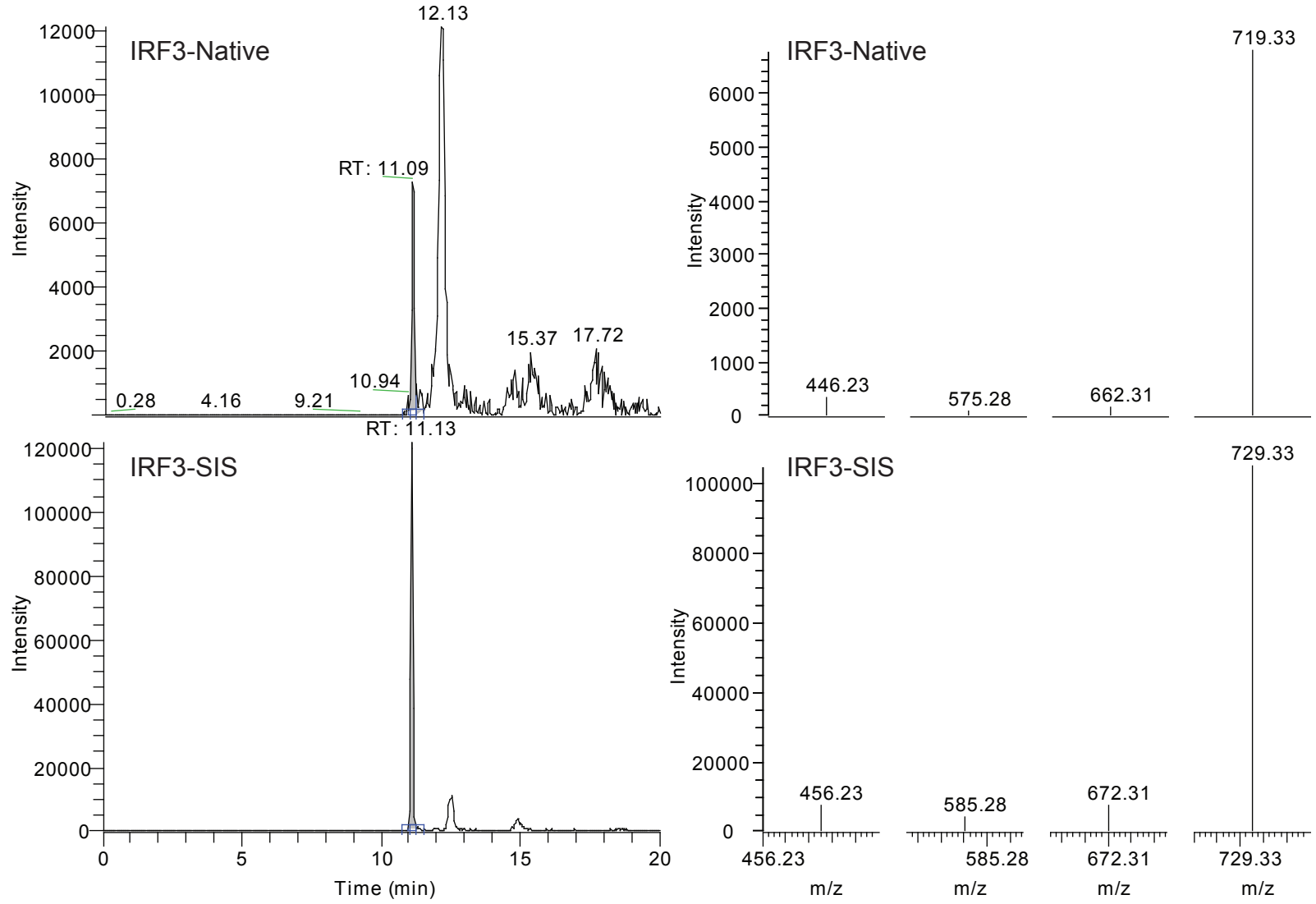
m/z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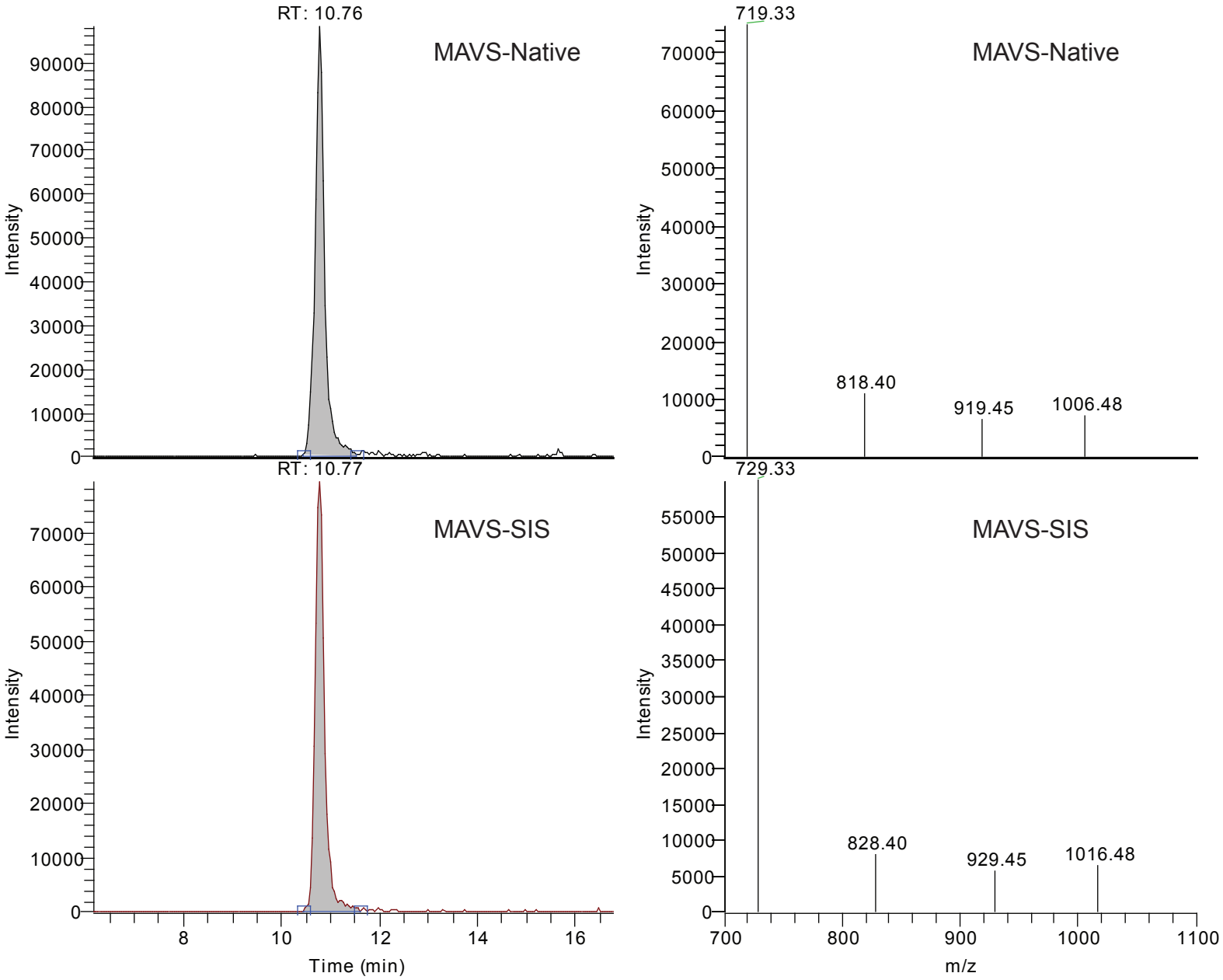
Intensity
IRF3-Native
719.33
446.23
575.28
662.31
IRF3-SIS
RT: 11.13
Intensity
Time (min)
IRF3-SIS
729.33
456.23
585.28
672.31
m/z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MAVS-SIS
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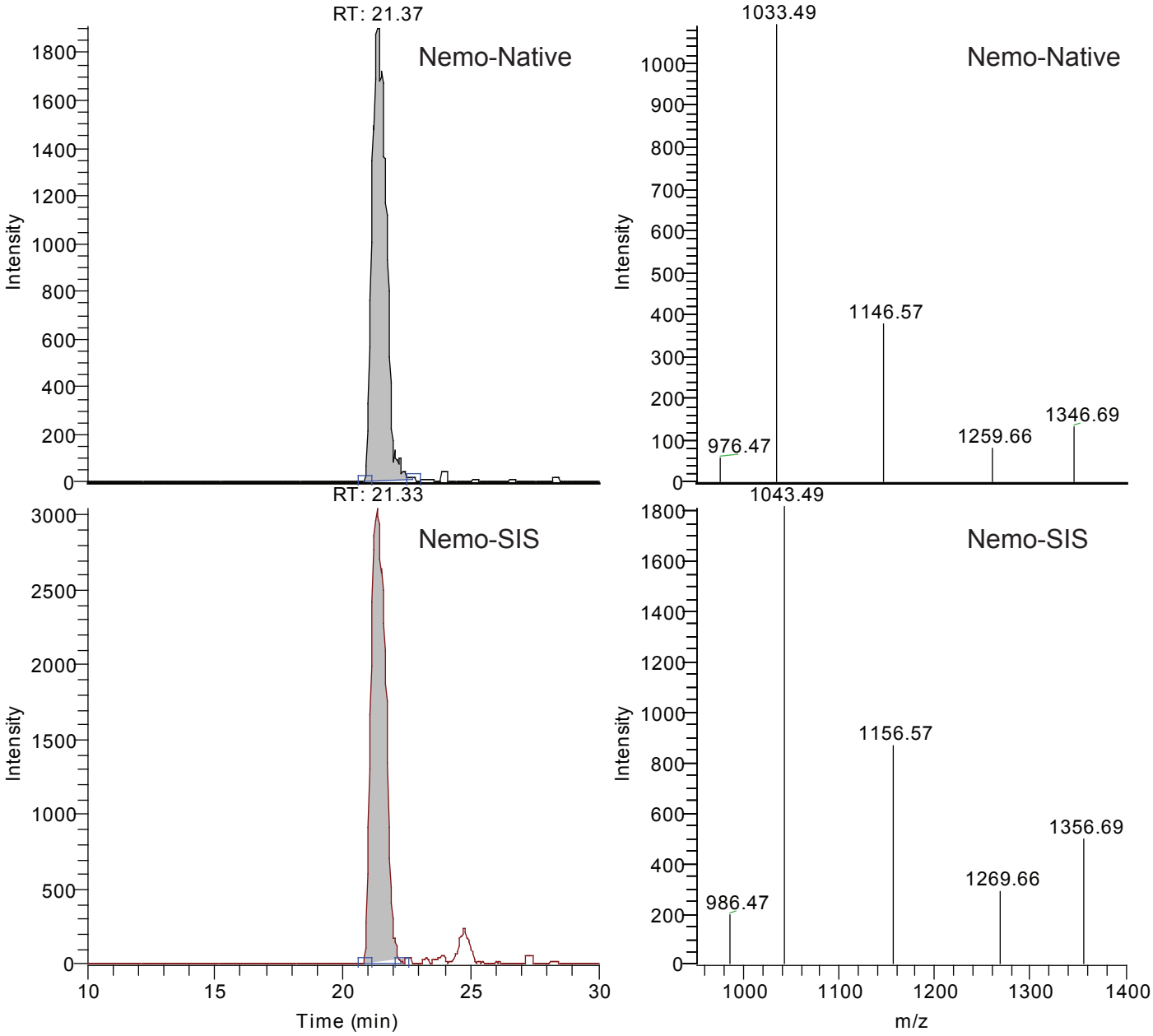
1016.48
Time (min)
m/z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Intensity
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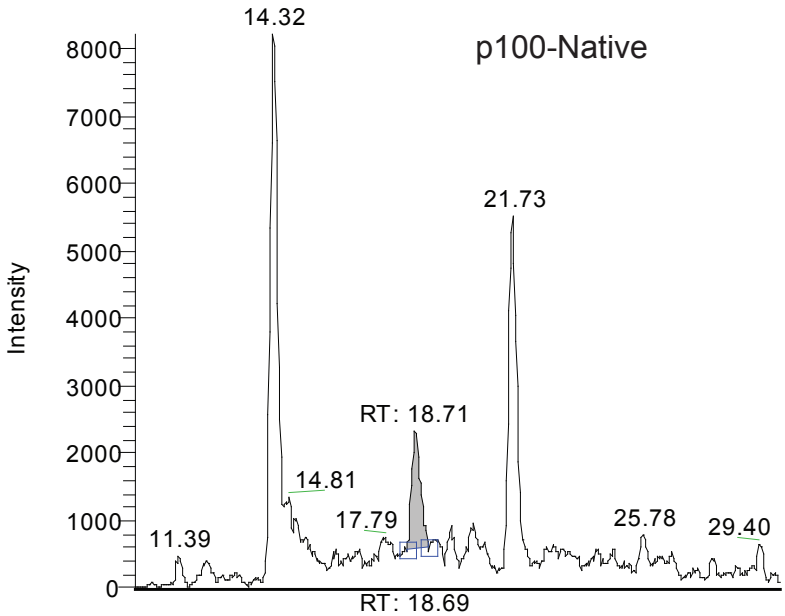
Time (min)
m/z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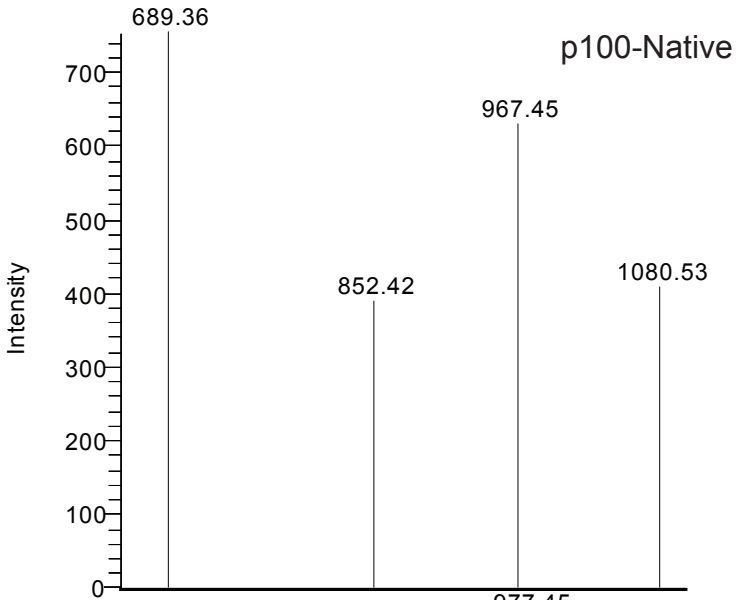
Intensity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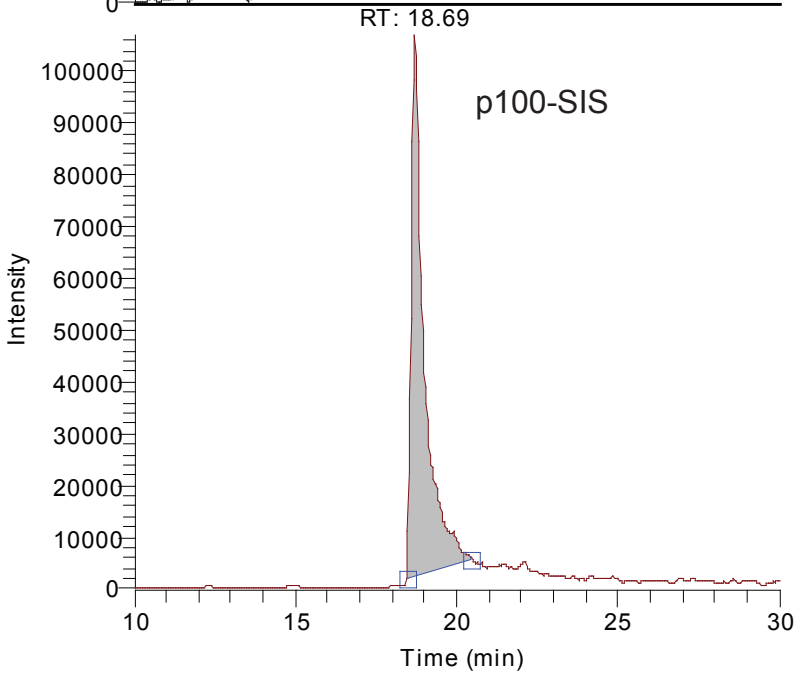
967.45
852.42
1080.53
Intensity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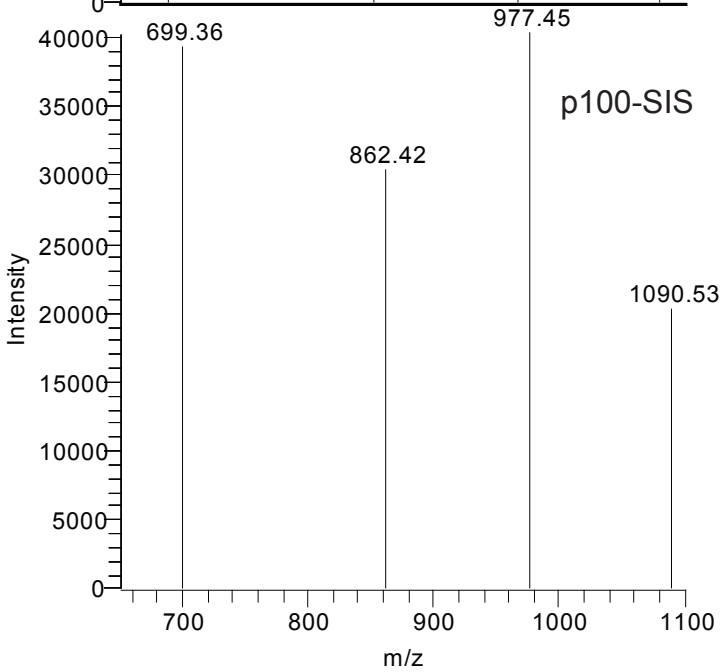
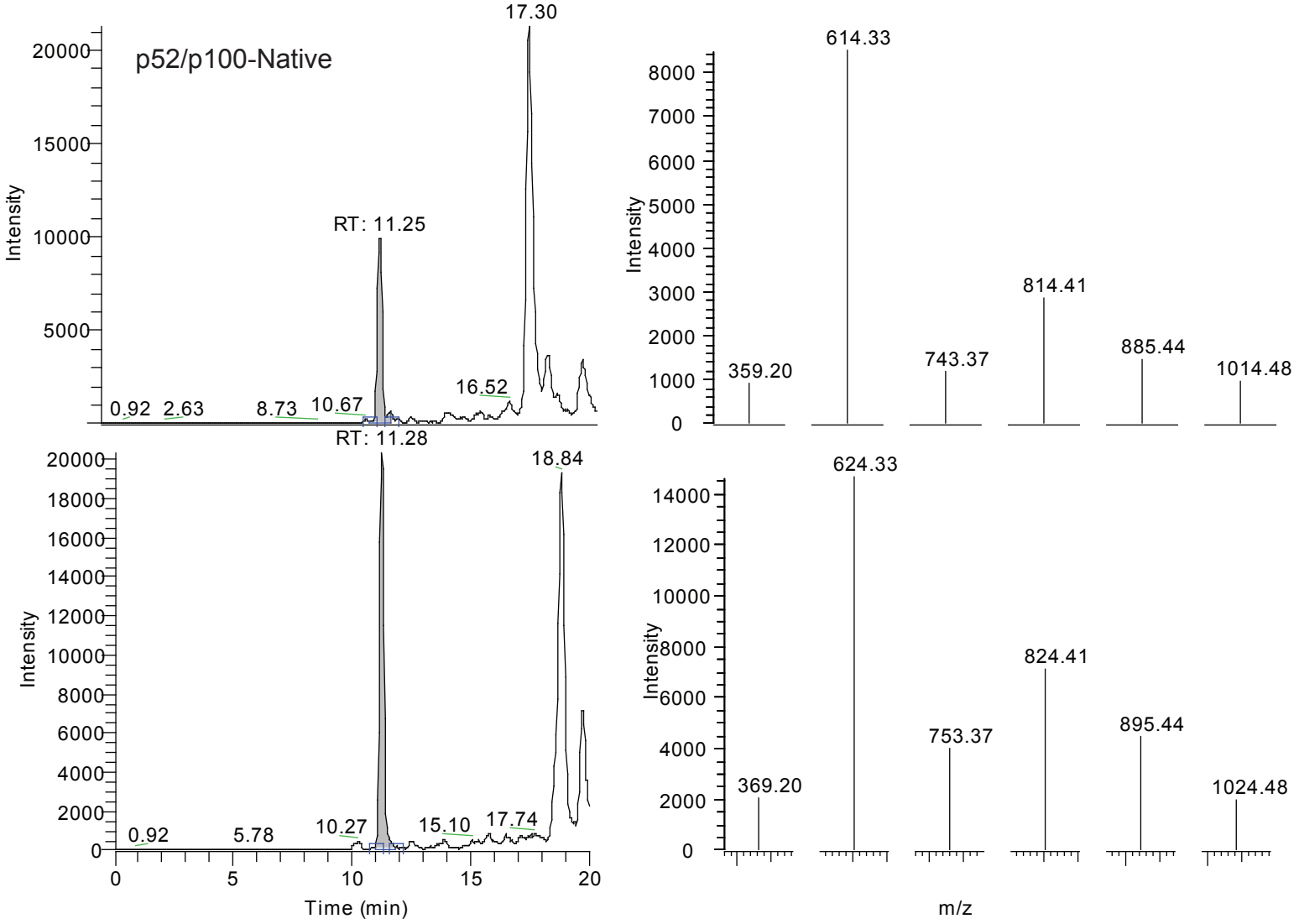
p100-SIS
Intensity
Time (min)


699.36
977.45
p100-SIS
862.42
1090.53
Intensity
m/z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Intensity
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2.63
8.73
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359.20
743.37
814.41
885.44
1014.48
RT: 11.28
18.84
0.92
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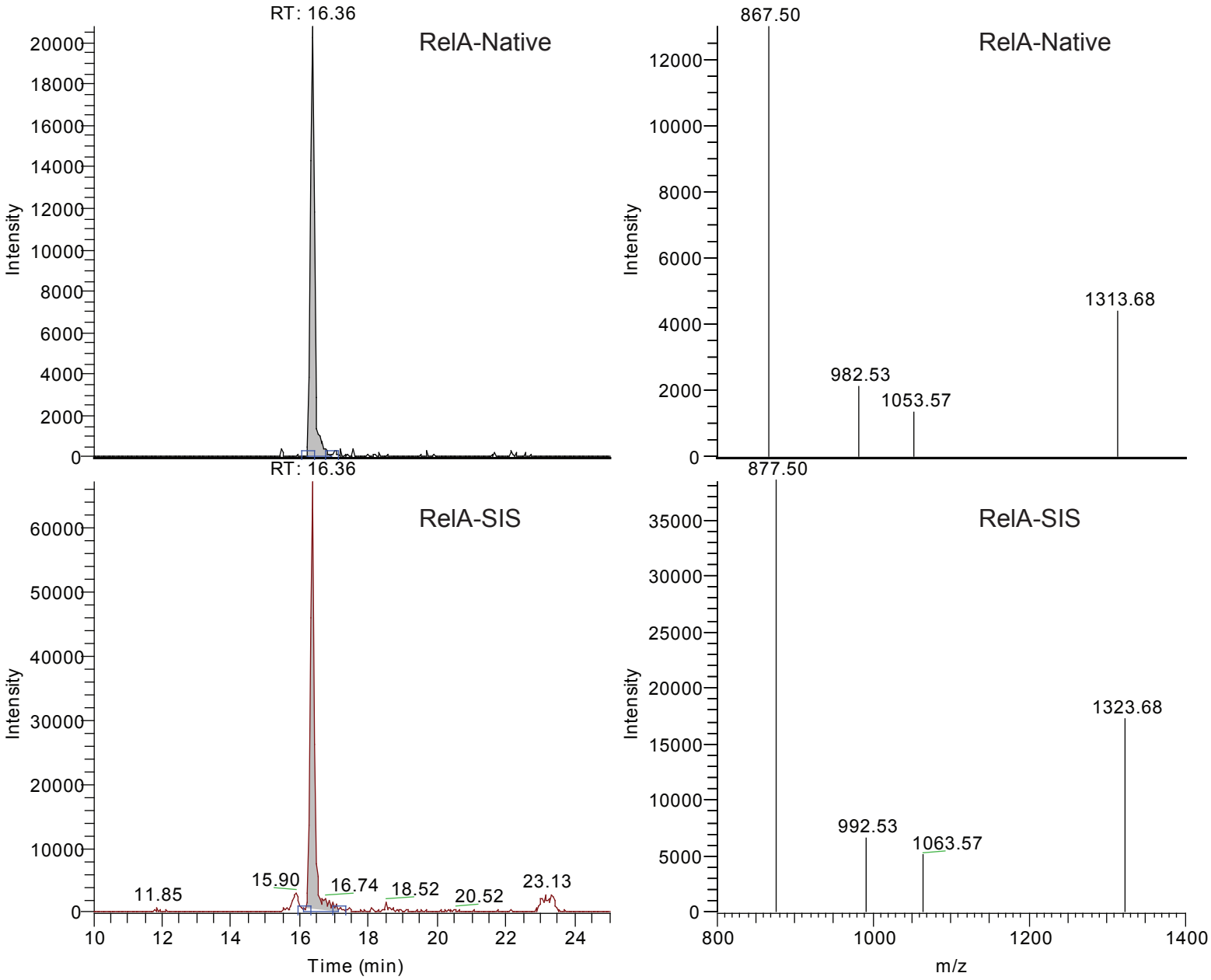
17.74
624.33
369.20
753.37
824.41
895.44
1024.48
Time (min)
m/z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Intensity
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1053.57
RT: 16.36
RelA-SIS
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1063.57
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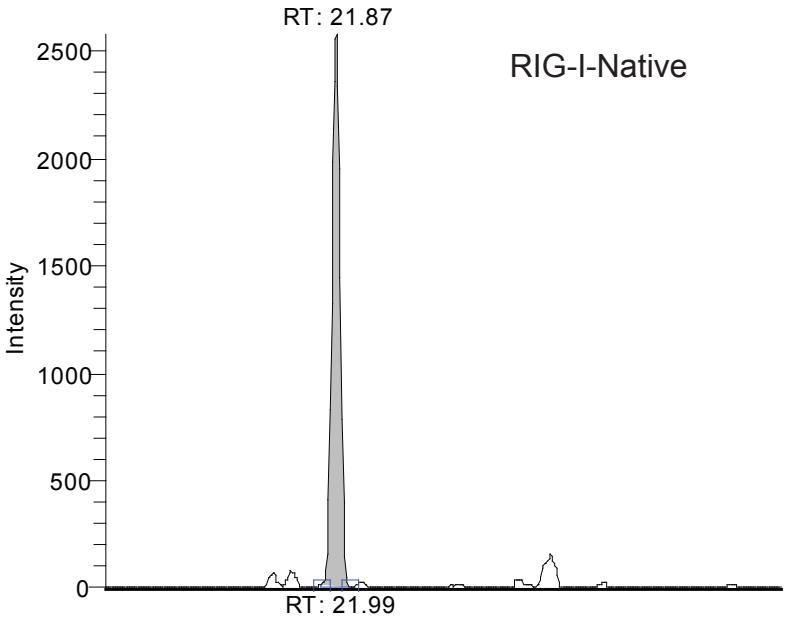
16.74
18.52
20.52
23.13
Time (min)
m/z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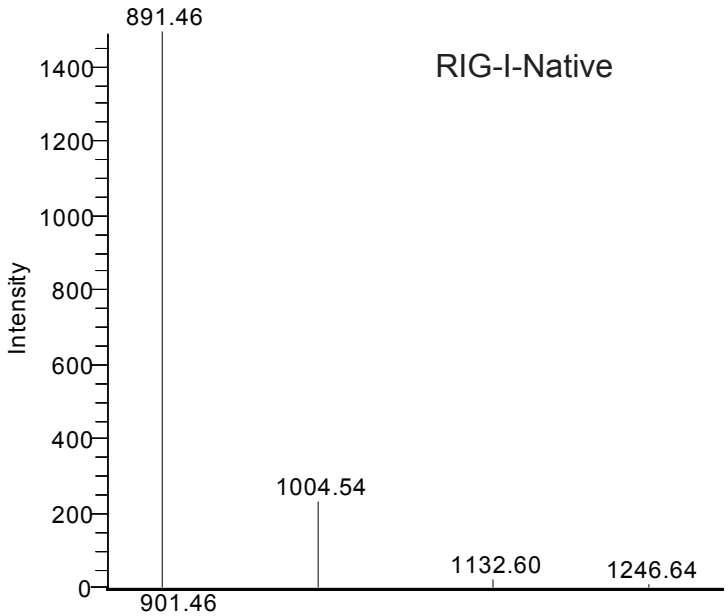
1500
1000
500
0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Intensity
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200
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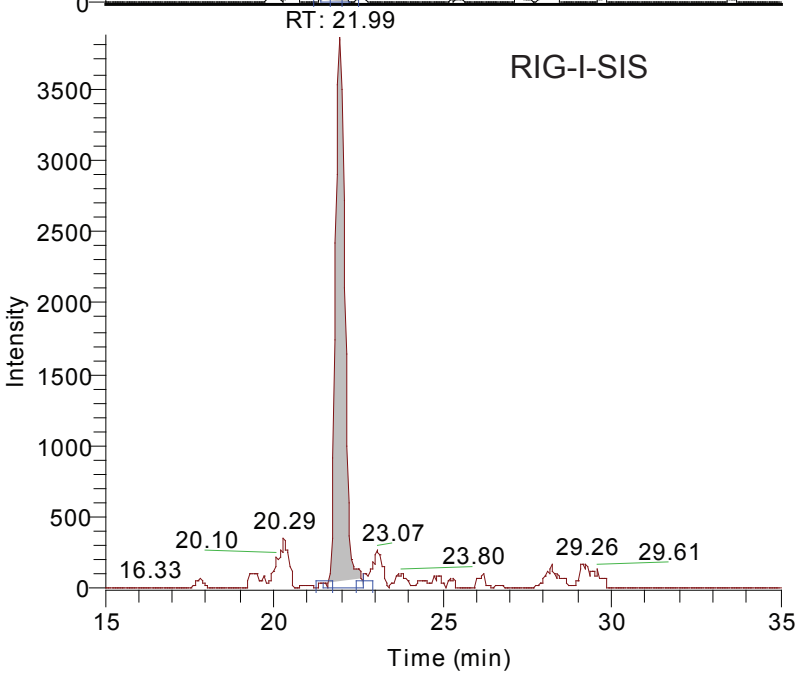
1004.54
1132.60
1246.64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Intensity
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2500
2000
1500
1000
500
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16.33
20.10
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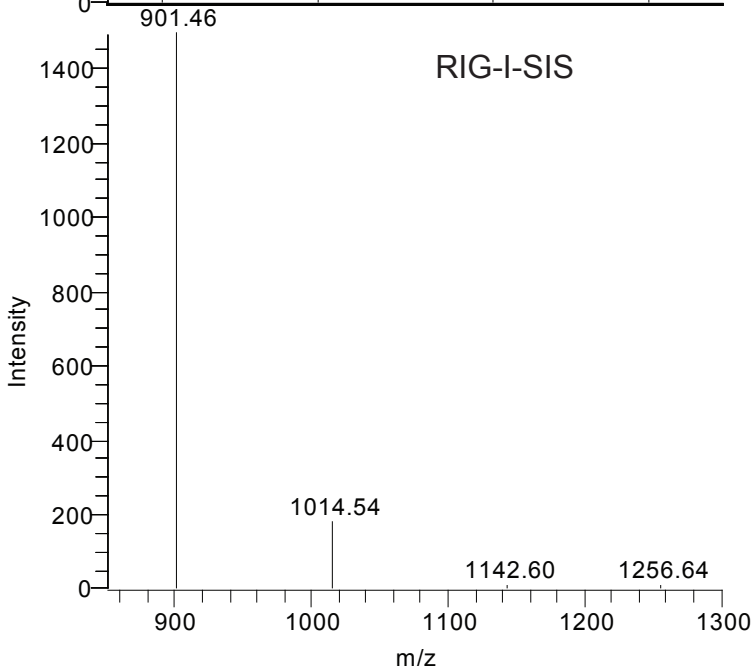
23.07
23.80
29.26
29.61
15
20
25
30
35
Time (min)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RIG-I-SIS
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1000
800
600
400
200
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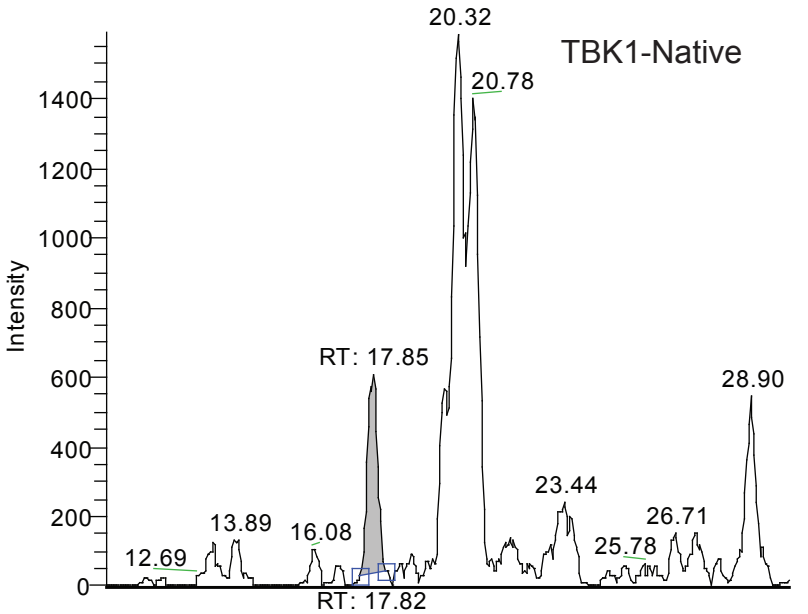
1256.64
900
1000
1100
1200
1300
m/z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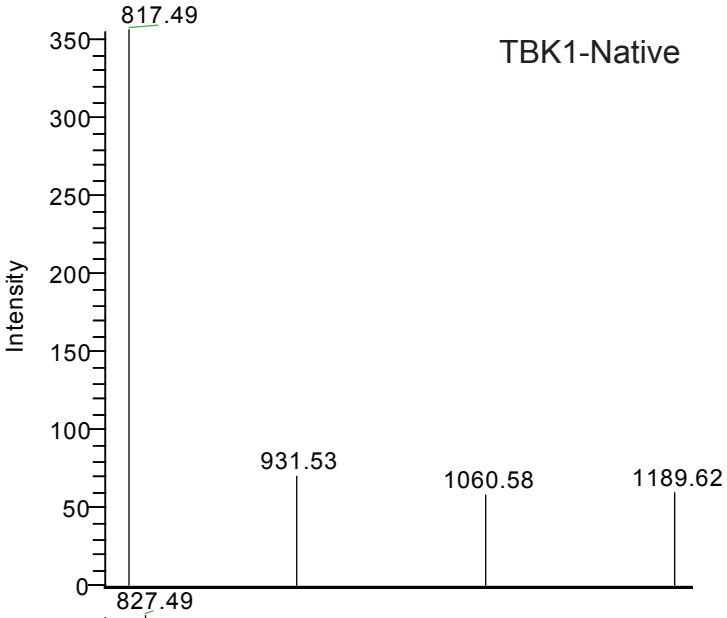
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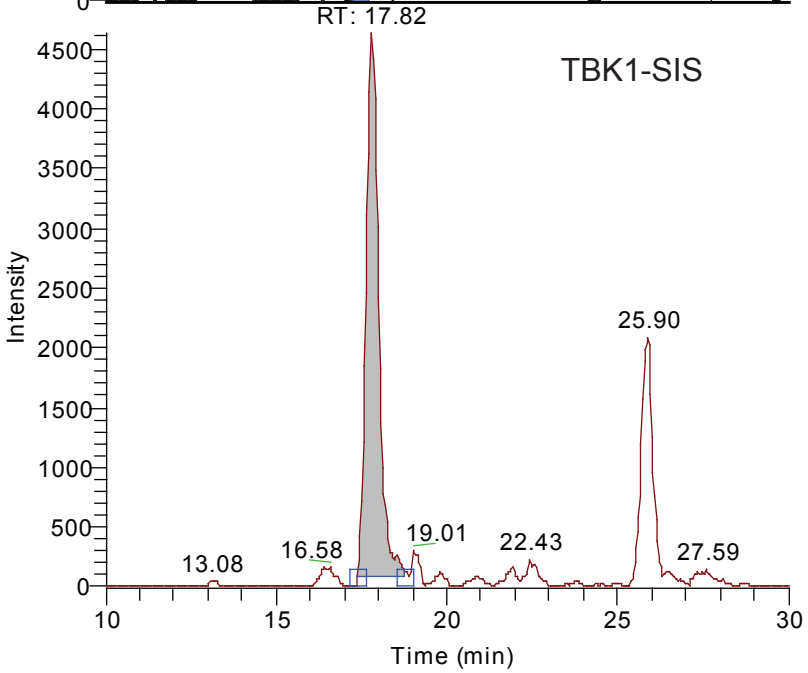
300
350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Intensity
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16.58
19.01
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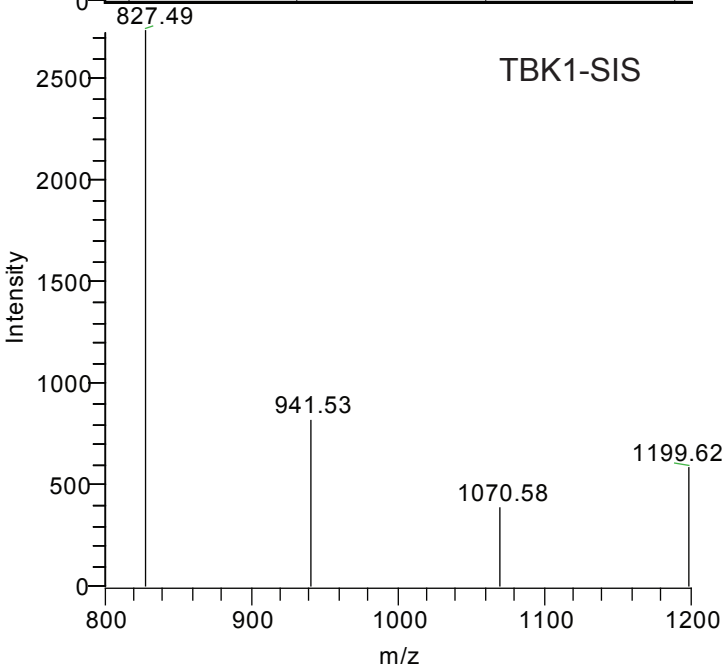
4500
10
15
20
25
30
Time (min)


TBK1-SIS
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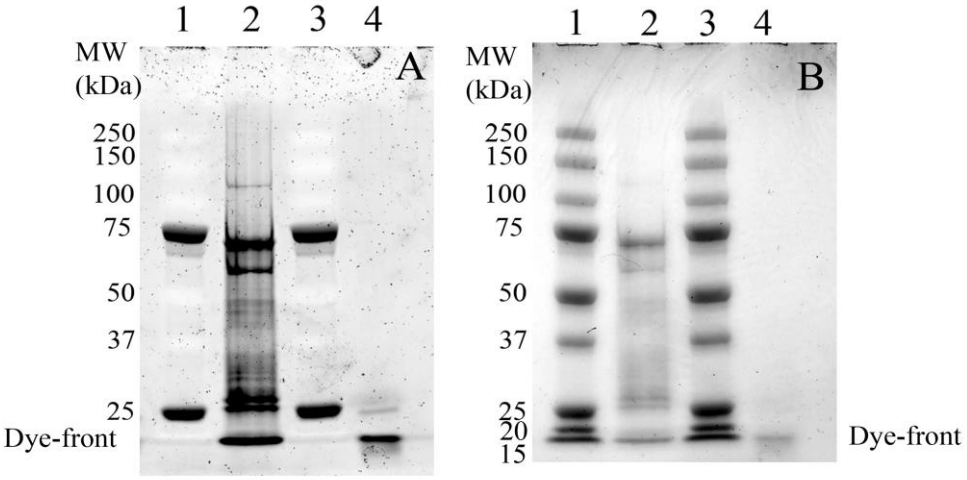
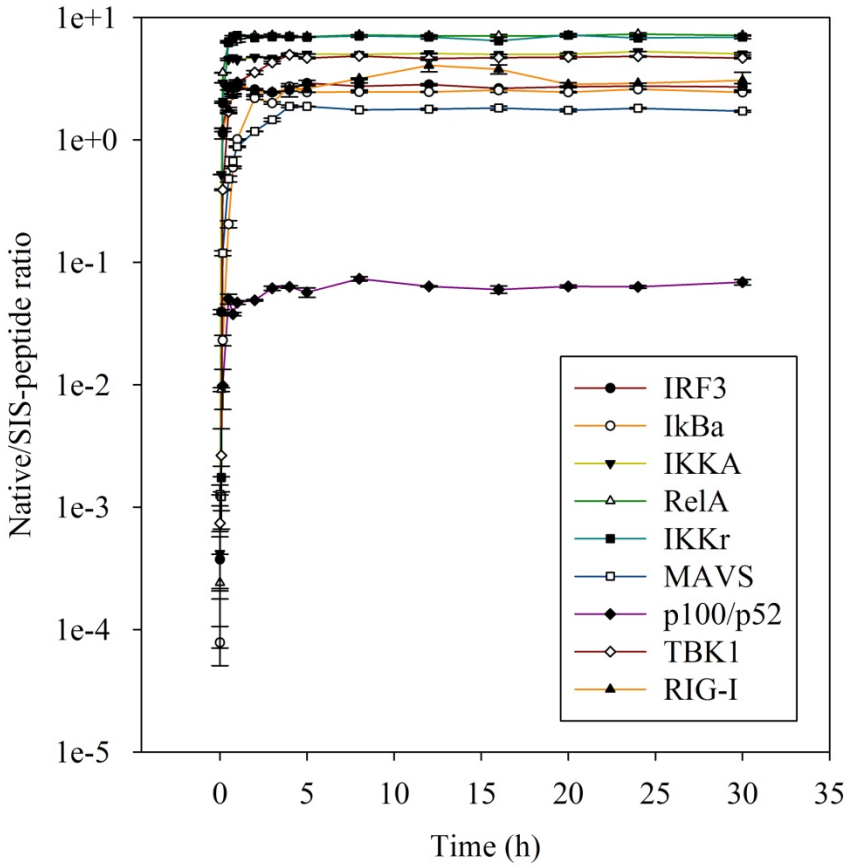
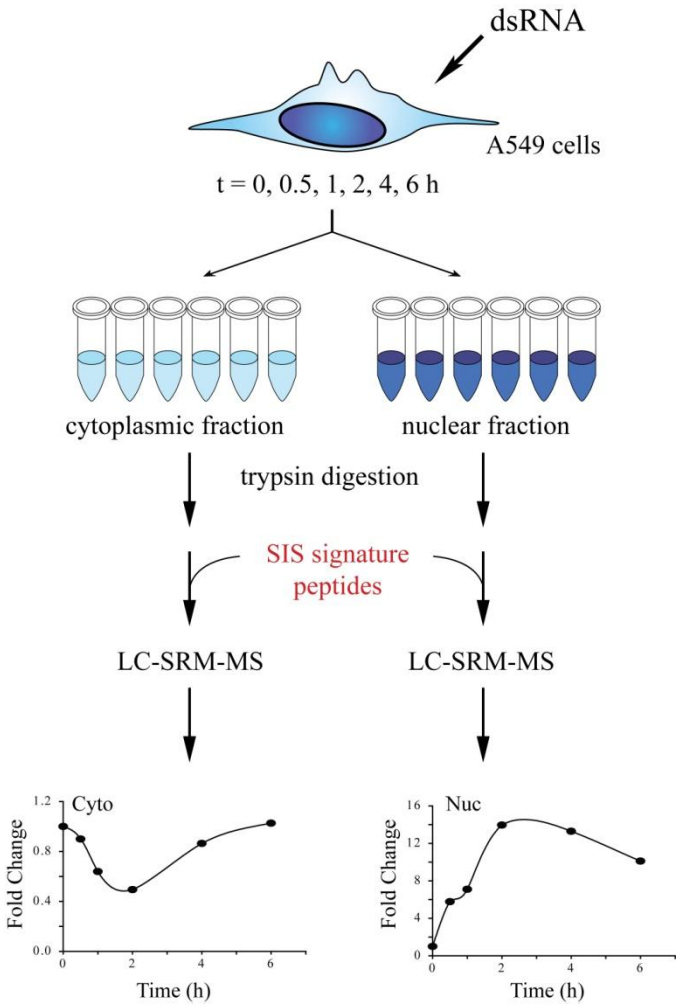
1000
1100
1200
m/z



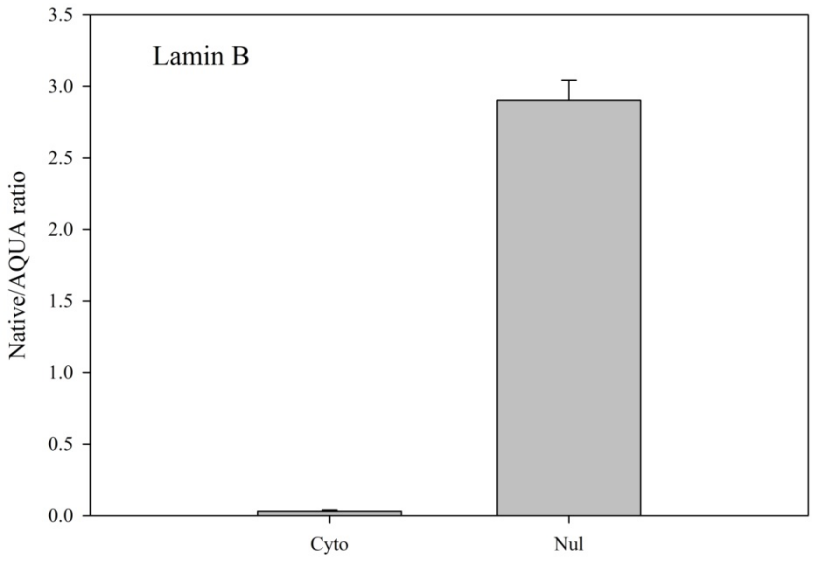
Supplemental Figure 4. SDS-PAGE analysis of recombinant IIR target proteins before and after trypsin digestion. The mixture of recombinant IIR target proteins was digested with trypsin for 24 h. The recombinant protein mix and tryptic digest were loaded on the 10% SDS-PAGE gel. After electrophoresis, the gel was stained with SYPRO® Ruby staining (A). Line 2 is the recombinant protein mix before trypsin digestion; Line 4 is the tryptic digest of the recombinant protein mix. Line 1 and 3 are Precision Plus Protein™ Duel Color standard. Because the MW maker was prestained, some MW maker protein bands cannot be visualized by SYPRO® Ruby. The same gel was later stained with Colloidal Bule (Invitrogen) to demonstrate the protein bands of protein MW markers (B).



Supplemental Figure 5. Time course analysis of the release of signature peptides during tryptic digestion. A mixture of recombinant IIR target proteins was prepared for the time-course digestion. The proteins were denatured with 8 M Guanidine HCl, and reduced and alkylated as described in the Experimental Section. Before trypsin digestion, a mixture of SIS peptides of the signature peptides of IRF3, IkBa, IKKr, RelA, MAVS, IKKa, TBK1, RIG-I, and p100/p52 was added in the mixture. The sample was diluted 10 times with 50 mM ammonium bicarbonate. Trypsin was added in the sample followed by incubation incubation at 37 °C. At 0 s, 5 min, 10 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 5 h, 8 h, 12 h, 16 h, 20 h, 24 h, and 30 h, 10 µL portion of tryptic digest was removed and mixed with 2 µL of trifluoroacetic acid and refrigerated to terminate proteolysis. Each portion was analyzed by SID-SRM-MS twice. The native vs SIS-peptide ratio of each signature peptide was calculated. The error bar is the standard deviation of the two replicates.



Supplemental Figure 6. Schematic representation of the workflow for analysis of IIR in response to the stimulation of ds-RNA. A549 cells are treated with ds-RNA for varying times. The cells were fractionated into cytoplasmic and nuclear fractions. The proteins were extracted and digested with trypsin. The SIS peptides of target proteins were spiked into each tryptic digest. The peptide mixtures were analyzed by LC-SRM-MS. Finally, the temporal profile of each target protein was generated based on the SID-SRM-MS analysis.



Supplemental Figure 7. SRM analysis of Lamin B in cytoplasmic fraction and nuclear fraction.

Supplemental Figure 8.

Cross talk of NFkB and IRF3 pathways. A549 cells were transfected with dsRNA in the absence of specific siRNA (Con siRNA), or siRNA targeting RelA or IRF3 as indicated. A. Effect of RelA and IRF3 knockdown on RelA expression. Note that IRF3 knockdown upregulates RelA expression. B. Effect of RelA and IRF3 knockdown on RelA and IRF3 expression. Note upregulation of RelA with IRF3 knockdown. C. Effect of RelA and IRF3 knockdown on ISG56 expression. ISG56 is almost completely IRF3 dependent. Note upregulation of ISG56 expression with RelA knockdown. D. Effect of RelA and IRF3 knockdown on IL8 expression. IL8 is almost completely RelA dependent. Note upregulation of IL8 with IRF3 knockdown.