# Supplementary Material

# Differential Association of 4E-BP2–Interacting Proteins Is Related to Selective Delayed Neuronal Death after Ischemia

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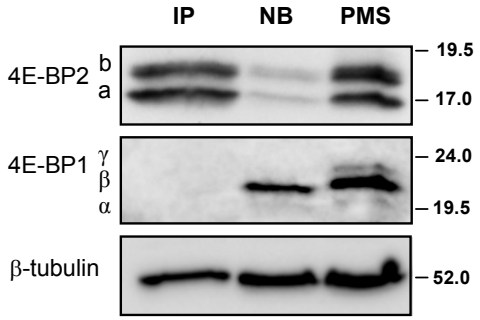



**Figure S1. Specific immunoprecipitation of 4E-BP2 in brain tissue samples.** Postmitochondrial supernatant (PMS) samples of cerebral cortex from 3-day sham control animals (SHC3dC) were immunoprecipitated with anti–4E-BP2 antibody (from Sigma-Aldrich) and subsequent incubation with Protein G-Agarose for 4E-BP2 immunoprecipitation (IP); the non-bound fraction to Protein G-Agarose was recovered as NB fraction. IP and NB samples, and the immunoprecipitated PMS (lanes IP, NB and PMS, respectively) were analyzed by western blotting with anti–4E-BP2 antibody (from Cell Signalling), and after stripping, re-blotted with anti–4E-BP1 antibody. The figure shows representative blots of 4E-BP2 (upper image) and 4E-BP1 (middle image), detecting the subunits *a* and *b*, and α, β and γ, of 4E-BP2 and 4E-BP1 proteins, respectively. No 4E-BP1 was detected in 4E-BP2 immunoprecipitates (lane IP), but was detected in the non-bound fraction (lane NB). On the contrary, 4E-BP2 was almost undetected in the non-bound fraction. Blot developed with anti–β-tubulin antibody is shown as loading control (lower image). Numbers on the right indicate the apparent molecular weight (in kDa). Full original images of the western blots are shown at the end of this Supplementary Material (Figure S6).

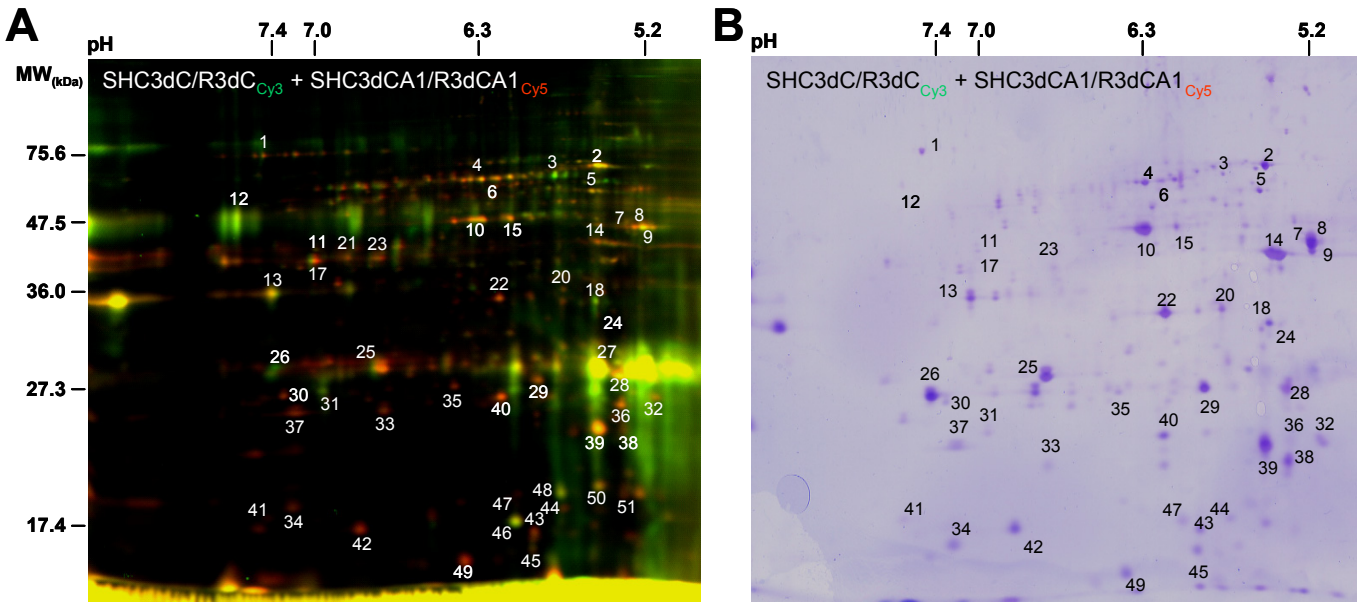



**Figure S2. Identification of 4E-BP2–interacting proteins.** (A) Preparative 2-D DIGE scanned image shows the overlay of the 4E-BP2 immunoprecipitates of PMS samples (1200 μg) from the cerebral cortex (SHC3dC and R3dC), labelled with Cy3 (green), and from the CA1 region (SHC3dCA1 and R3dCA1) labelled with Cy5 (red). After scanning, the gel was stained with Coomassie blue (B). Protein spots were matched between the fluorescence scanned image (A) and the Coomassie stained gel (B). Matched 4E-BP2–associated proteins (spots) were tagged with numbers and subsequently excised for identification by MALDI-TOF/TOF MS. Gel images are full original images; the horizontal axis represents pH and vertical axis represents molecular weight (MW, in kDa).

**Table S1.** 4E-BP2–interacting proteins identified by MALDI-TOF MS without significant changes.

| No. | Protein Identification /Synonyms | Abbreviation[a] | Accession No.[b] | Gene Name | Theoretical / Apparent Mass (Da) | Theoretical/ Apparent pI | Score[c] | Coverage (%) | LIFT (Score)[c,d] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Aconitate hydratase /Aconitase /ACON | Aconitase | Q9ER34 | *Aco2* | 86121/ 75634 | 7.87/ 7.4 | 216 | 31 | 1667.78 (109) |
| 7 | Tubulin alpha-1B chain /Tubulin alpha-2 chain /TBA1B | Tubulin α1B | Q6P9V9 | *Tuba1b* | 50804/ 50970 | 4.94/ 5.2 | 112 | 41 | 2415.17 (97) |
| 14 | Actin, cytoplasmic 1 /Beta-actin/ACTB | β-Actin | P60711 | *Actb* | 42052/ 45931 | 5.29/ 5.4 | 168 | 55 | 2215.05 (144) |
| 20 | L-lactate dehydrogenase B chain /LDH-H /LDHB | LDHB | P42123 | *Ldhb* | 36874/ 37299 | 5.7/ 5.6 | 52 | 18 | 2296.16 (60) |
| 22 | Malate dehydrogenase, cytoplasmic /MDHC | MDHC | O88989 | *Mdh1* | 36631/ 37299 | 6.16/ 6.1 | 70 | 25 | 1706.85 (61) |
| 31 | GTP-binding nuclear protein Ran/Ras–related nuclear protein /RAN | RAN | P62828 | *Ran* | 24579/ 24596 | 7.01/ 7.0 | 113 | 34 | 1784.89 (83) |
| 36 | Peroxiredoxin-2 /PRDX2 | PRDX2 | P35704 | *Prdx2* | 21941/ 22947 | 5.34/ 5.4 | 143 | 68 | 1211.68 (56) |
| 40 | Protein DJ-1 /PARK7 | PARK7 | O88767 | *Park7* | 20190/ 25464 | 6.32/ 6.3 | 125 | 62 | 1230.72 (58) |
| 42 | Nucleoside diphosphate kinase B /NDKB | NDKB | P19804 | *Nme2* | 17386/ 17385 | 6.92/ 6.9 | 136 | 73 | 1175.67 (64) |
| 44 | Stathmin /Prosolin/pp17 /STMN1 | STMN1 | P13668 | *Stmn1* | 17278/ 17998 | 5.76/ 5.7 | 38 | 16 | 1388.70 (55) |
| 49 | Histidine triad nucleotide–binding protein 1 /HINT1 | HINT | P62959 | *Hint1* | 13882/ 14871 | 6.36/ 6.2 | 98 | 47 | 2545.25 (156) |

[a], Abbreviations used in this manuscript. [b], Accession number in the UniProt database (https://www.uniprot.org/, accessed on 24 June 2021). [c], Protein scores > 51 were significant ($p<0.05$) by peptide mass fingerprint in the MASCOT database search algorithm (Matrix Science, London, UK, http://www.matrixscience.com/, accessed on 24 June 2021). [d], MALDI LIFT-TOF/TOF identification mode; scores > 24 were significant ($p<0.05$) by MS/MS ions search performed in MASCOT; the fragmented parental peptide and the score (in parenthesis) are indicated.

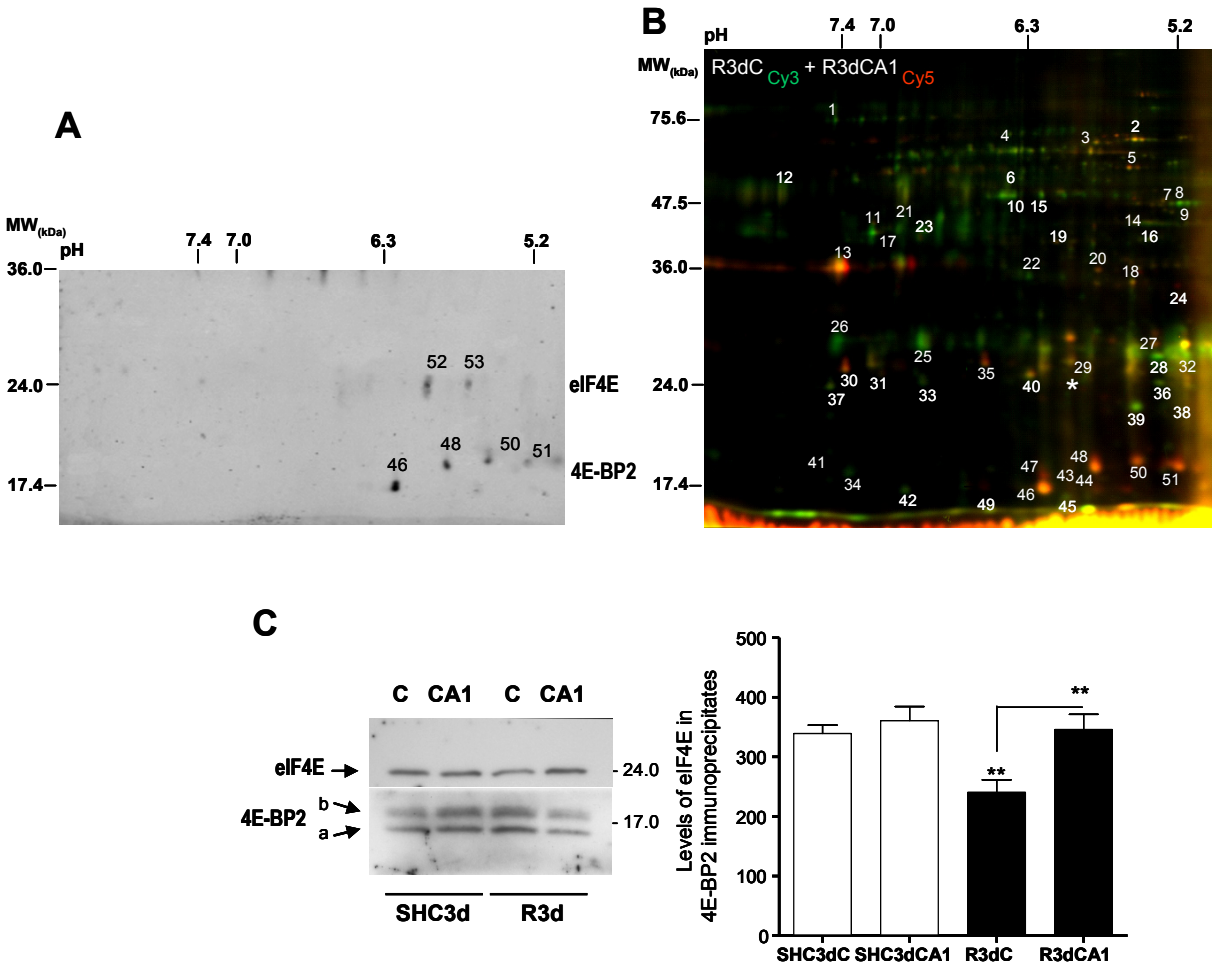



**Figure S3. Detection of 4E-BP2 and eIF4E proteins in 2-D gel electrophoresis of 4E-BP2 immunoprecipitates.** (A) 4E-BP2 immunoprecipitate of the cerebral cortex from control and ischemic samples (SHC3dC and R3dC; 600 μg) were resolved by 2-D gel electrophoresis run in parallel to the experiment shown in Figure 2, and incubated, after gel blotting, with anti–eIF4E antibody and anti–mouse IRDye 680LT-conjugated secondary antibody, and with anti–4E-BP2 antibody and anti–rabbit IRDye 800CW-conjugated antibody, for simultaneous detection of eIF4E and 4E-BP2, respectively. Blotted membrane was cut in 36-kDa region before antibody incubation. Images (A) and (B) are full original images; the horizontal axis represents pH and the vertical axis represents molecular weight (MW, in kDa). 4E-BP2 protein was identified as protein spots 46, 48, 50 and 51 by the exact matching with 2-D DIGE gel from Figure 2A (B). eIF4E protein spots (52 and 53) were identified with the anti–eIF4E antibody (A), and they were absent in the DIGE gel (B). In (B), the position of spot 52 is marked by an asterisk. (C) eIF4E and 4E-BP2 detection by western blotting in 4E-BP2 immunoprecipitates of the cerebral cortex, C, and CA1 region, from SHC3d control and R3d ischemic samples using the same primary antibodies as in (A) and peroxidase-conjugated secondary antibodies. The figure shows representative blots of eIF4E (upper image) and 4E-BP2 (lower image), detecting the subunits *a* and *b* of 4E-BP2. Numbers on the right indicate the apparent molecular weight (in kDa). Full original images of western blots are shown, and they were cut in ~21-kDa region to avoid immunoglobulin light chain signal from immunoprecipitation procedure The bar graph show the levels of eIF4E quantified in the immunoprecipitates. Error bars indicate SE. Statistical significance were performed by Newman-Keuls post-test (**$p < 0.01$) after significant ANOVA ($p < 0.05$), compared with control, or between the cerebral cortex and CA1 samples (line). The vertical axis indicates the quantification values in arbitrary units.

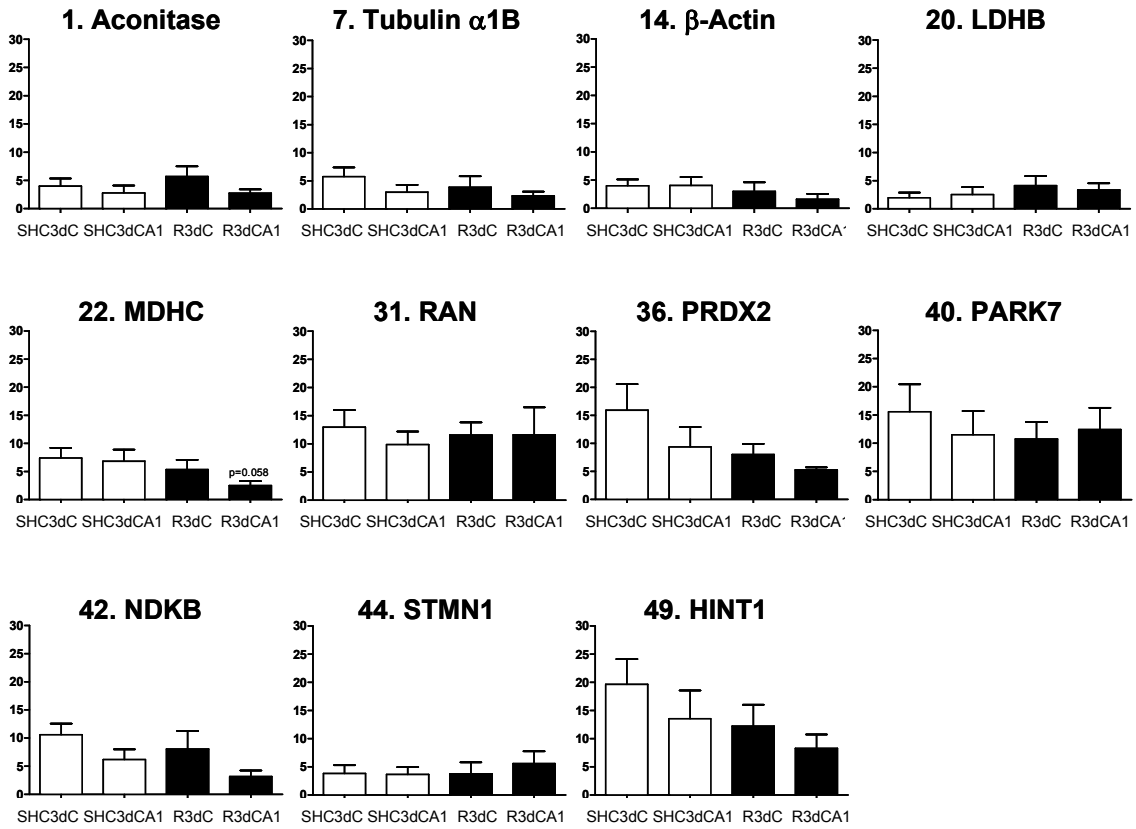



**Figure S4. Levels of identified 4E-BP2–interacting proteins without significant changes.** Bar graphs show the 4E-BP2–associated protein levels as described in Figure S4. Statistical analysis was not significant (ANOVA, $p > 0.05$). The vertical axis indicates the quantification values of the spot intensity in arbitrary units. Graphs are numbered and named according to the proteins listed in Table 1.

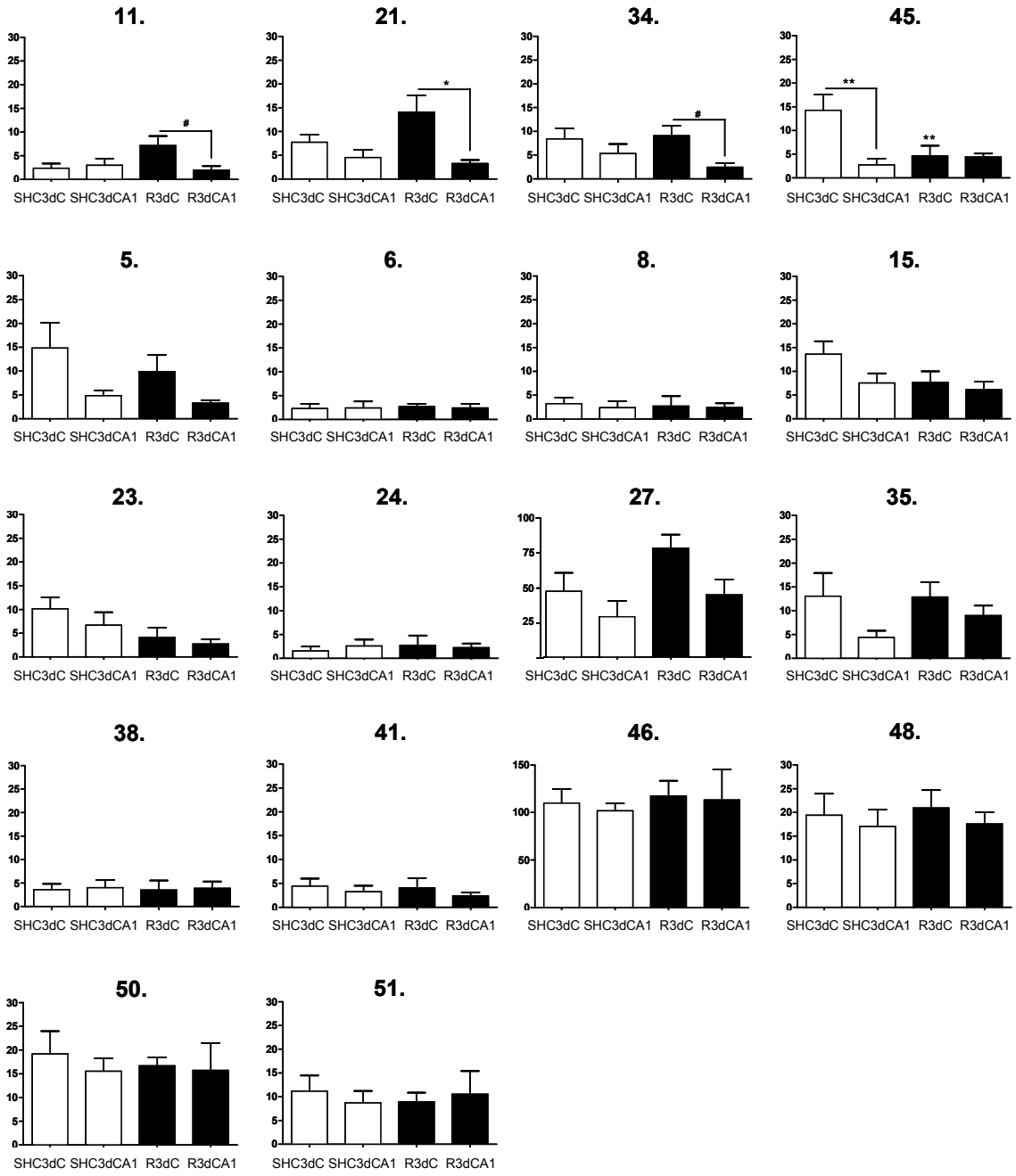



**Figure S5. Levels and statistical analysis of unidentified 4E-BP2–interacting proteins.** Bar graphs show the 4E-BP2–associated protein levels as described in Figure S4. Statistical analysis was performed as in Figure S4. Only proteins 11, 21, 34 and 45 were significant (ANOVA, $p < 0.05$) and post-test were performed by Newman-Keuls (*$p < 0.05$; **$p < 0.01$) or by Student's t-test (#$p < 0.05$; ##$p < 0.01$), compared with their respective control, or between the cerebral cortex and CA1 samples (indicated by lines). The vertical axis indicates the quantification values of the spot intensity in arbitrary units. Graphs are numbered and named according to the proteins listed in Table S1. Note that proteins 46, 48, 50 and 51 were later identified as 4E-BP2 protein (Figure S3).

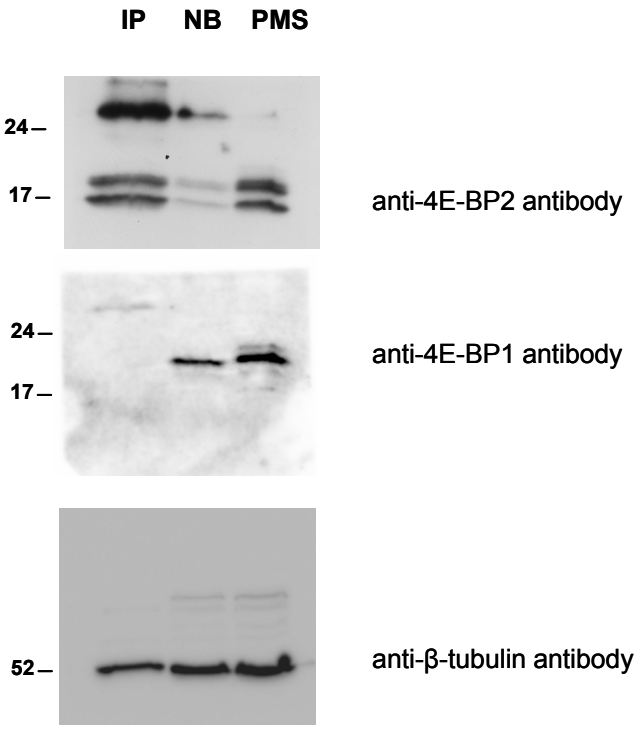
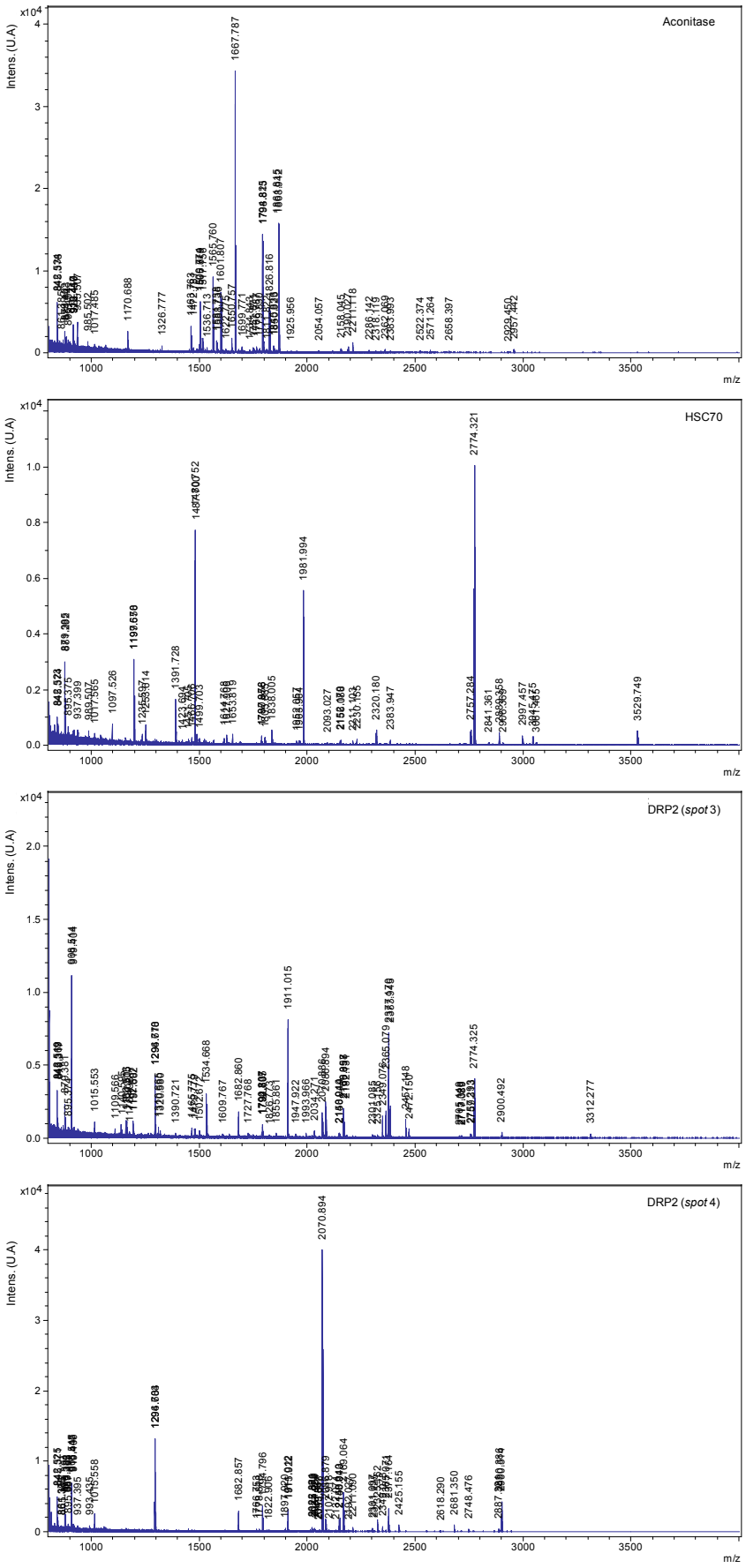
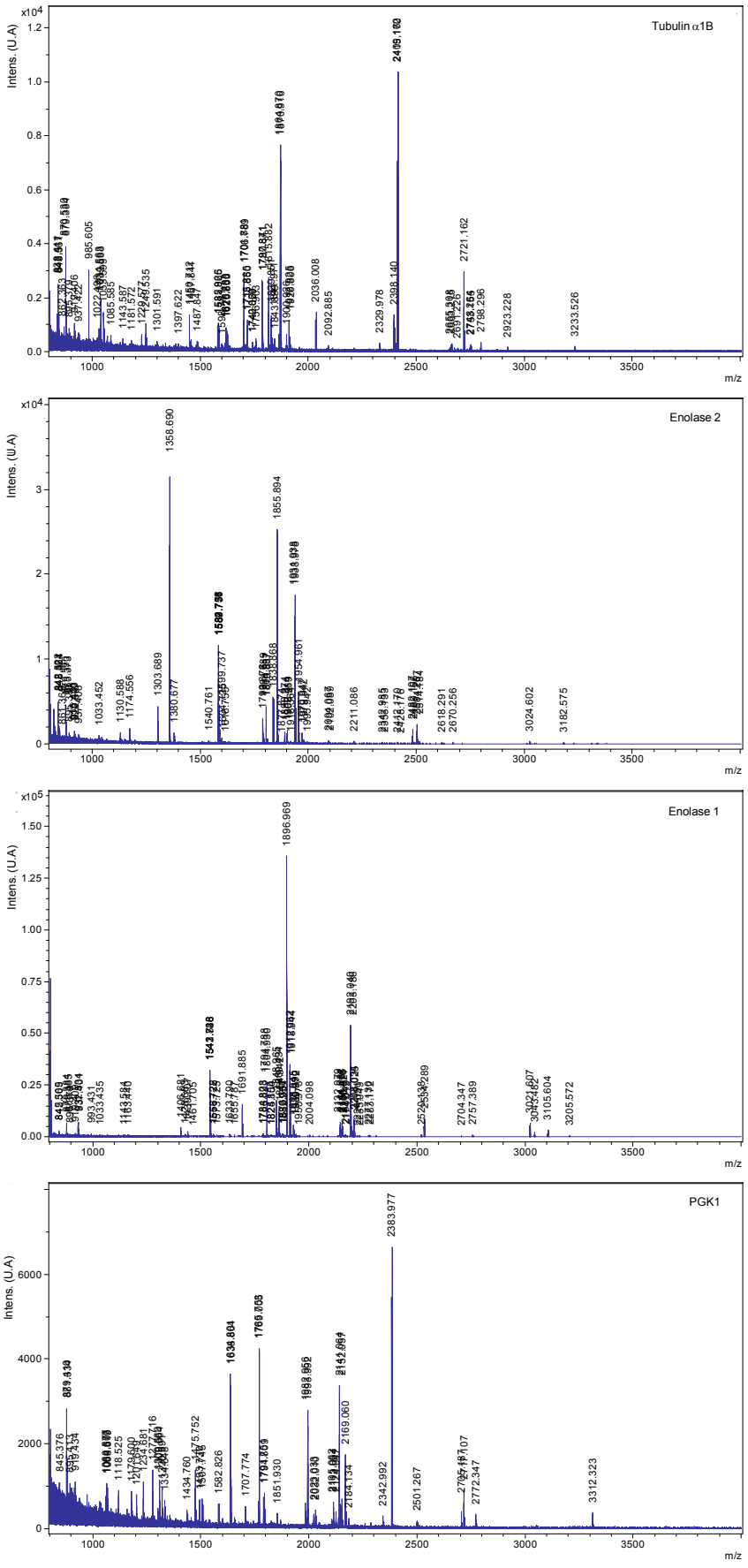



**Figure S6.** Full original images (uncropped) of western blots of the Figure S1, incubated with anti–4E-BP2, anti–4E-BP1 and anti–β-tubulin antibodies. Numbers on the left indicate the apparent molecular weight (in kDa). In the upper image, the band above 24 kDa corresponds to immunoglobulin light chains from immunoprecipitation procedure.

# MALDI-TOF MS spectra

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m/z
Enolase 2
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m/z
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m/z
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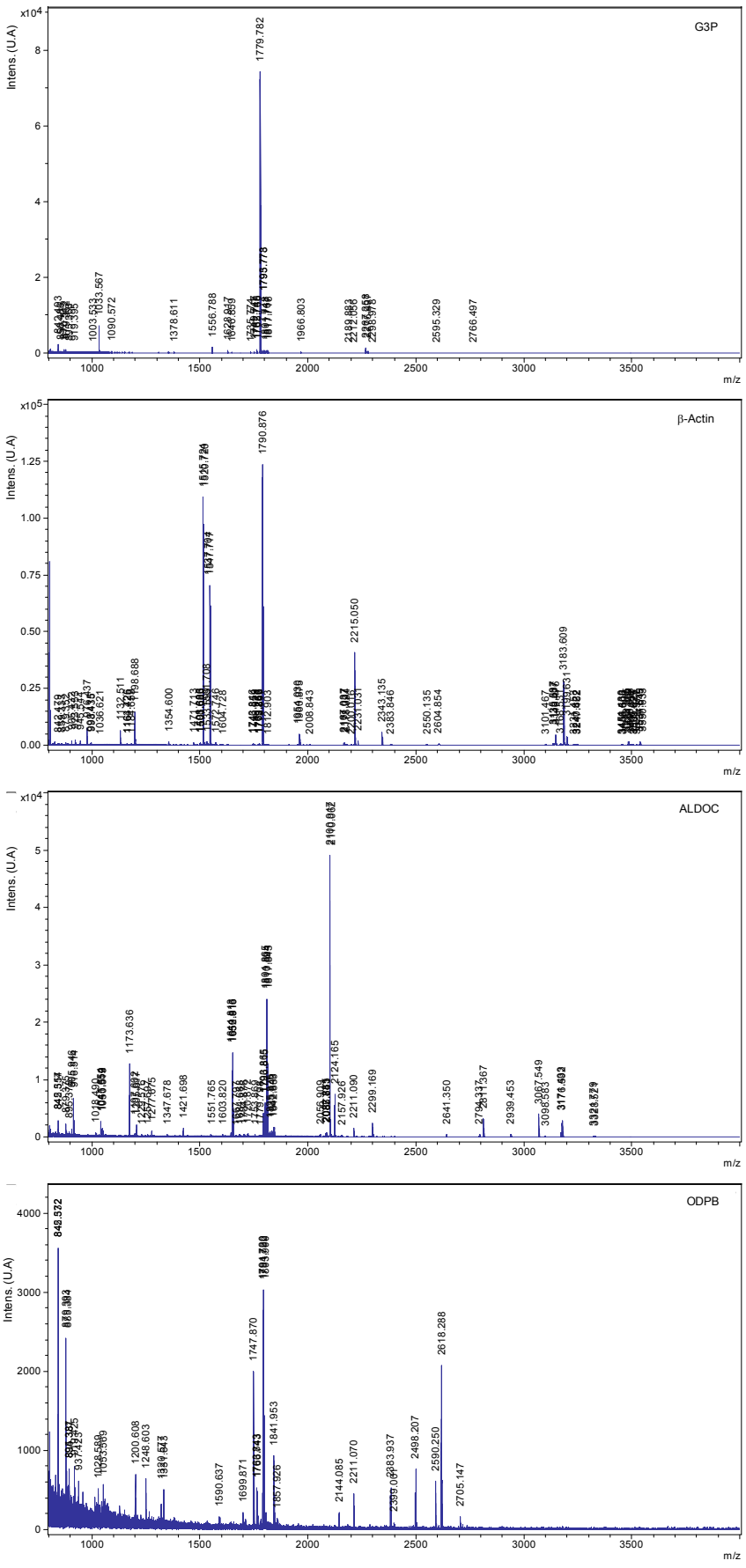
Intens. (U.A)
m/z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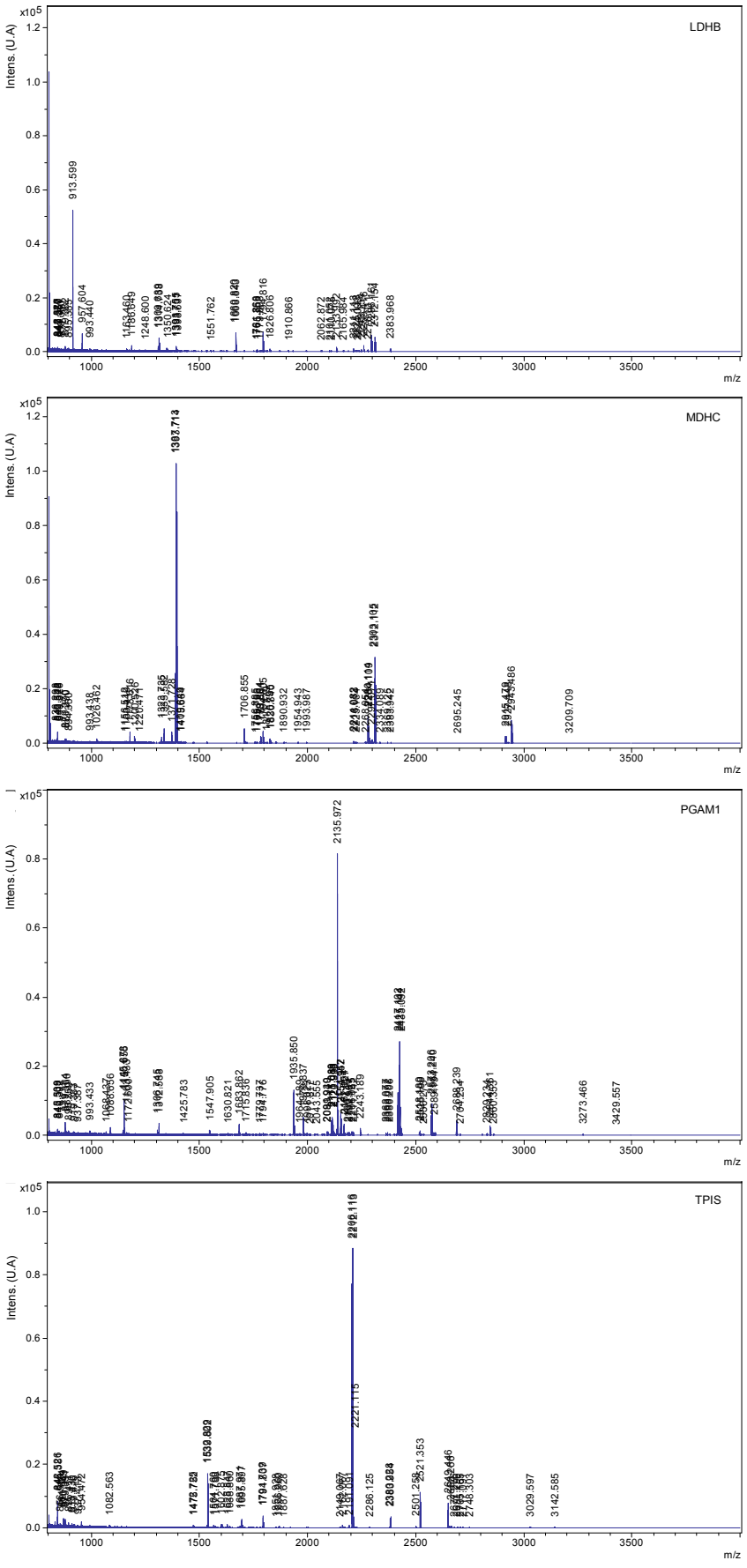
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m/z
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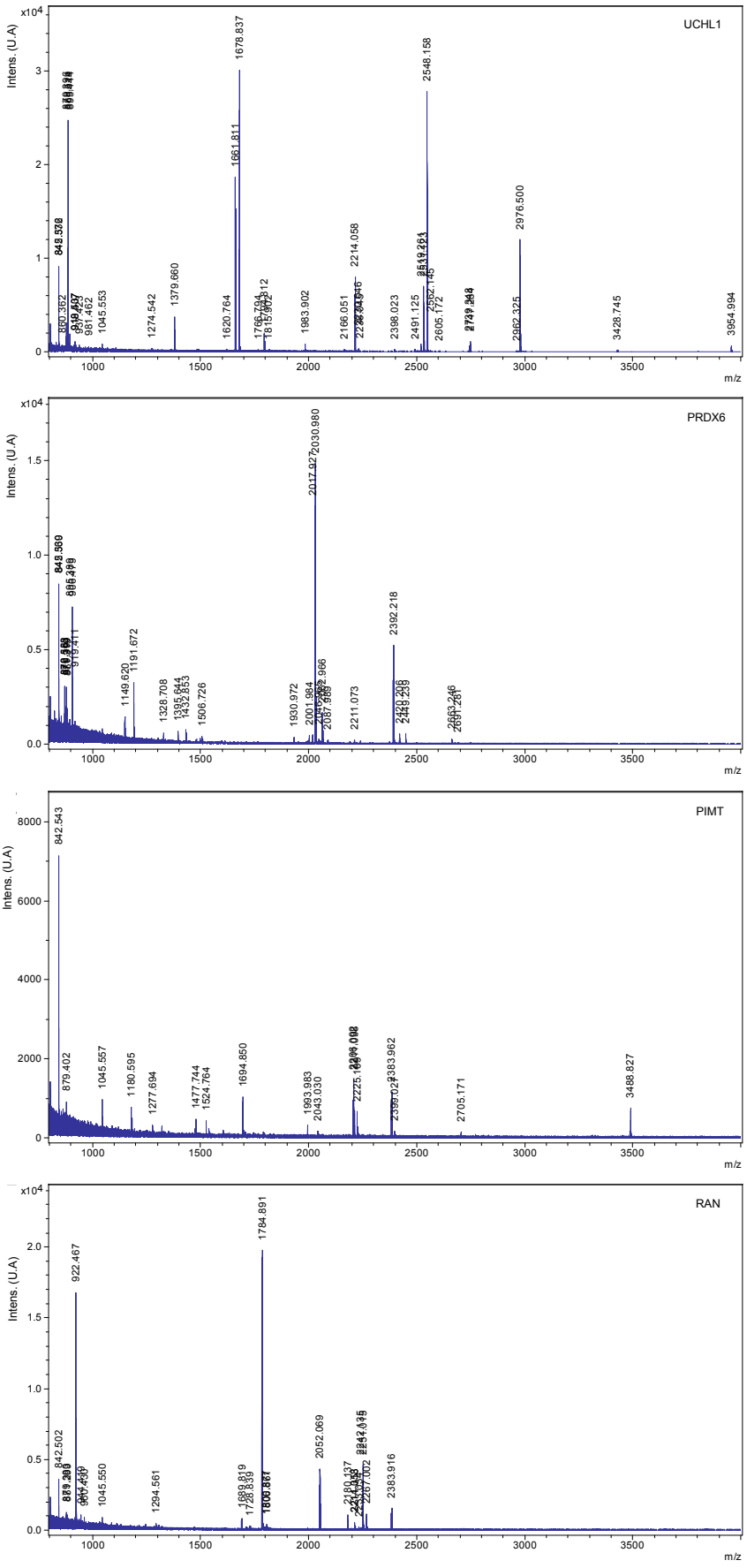
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2491.125
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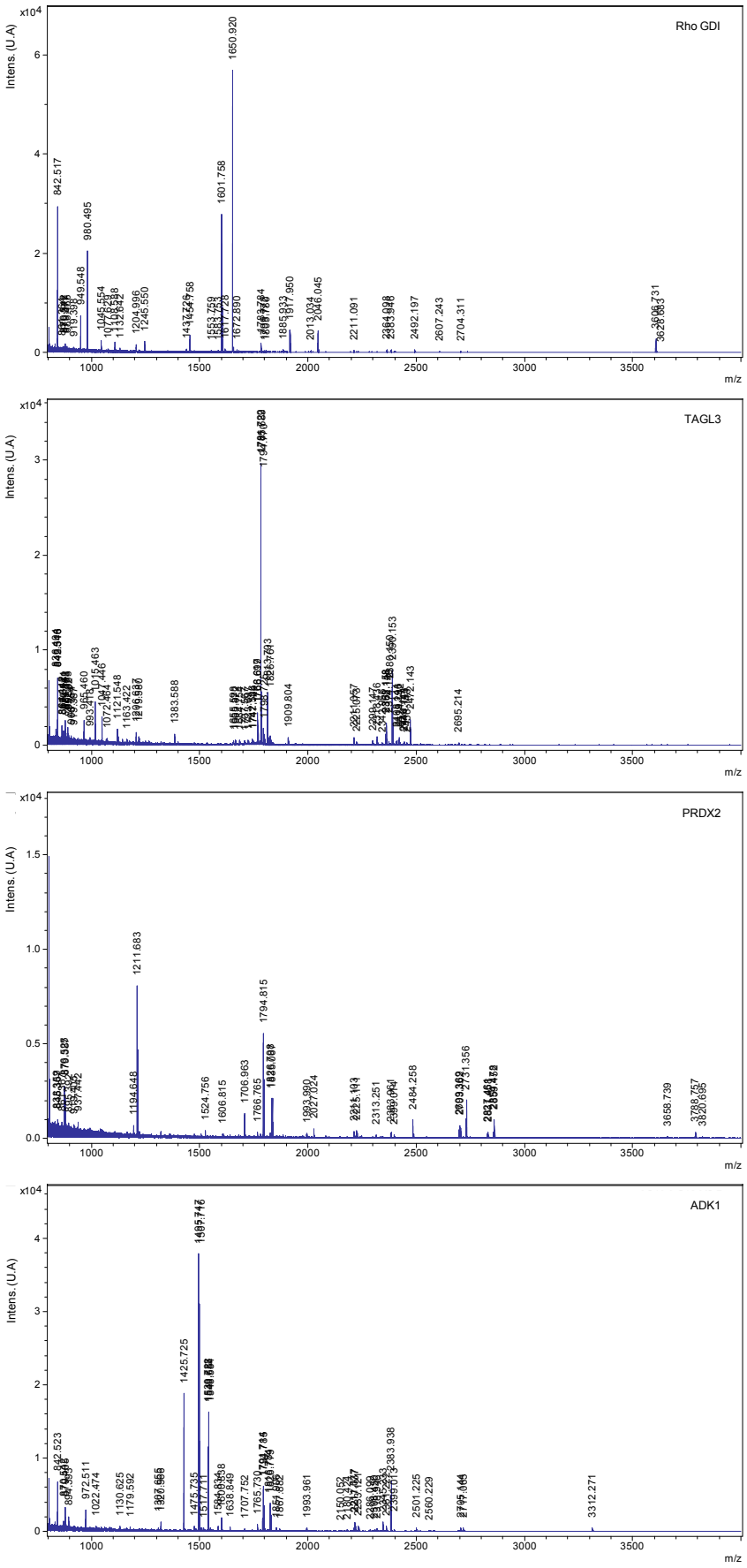
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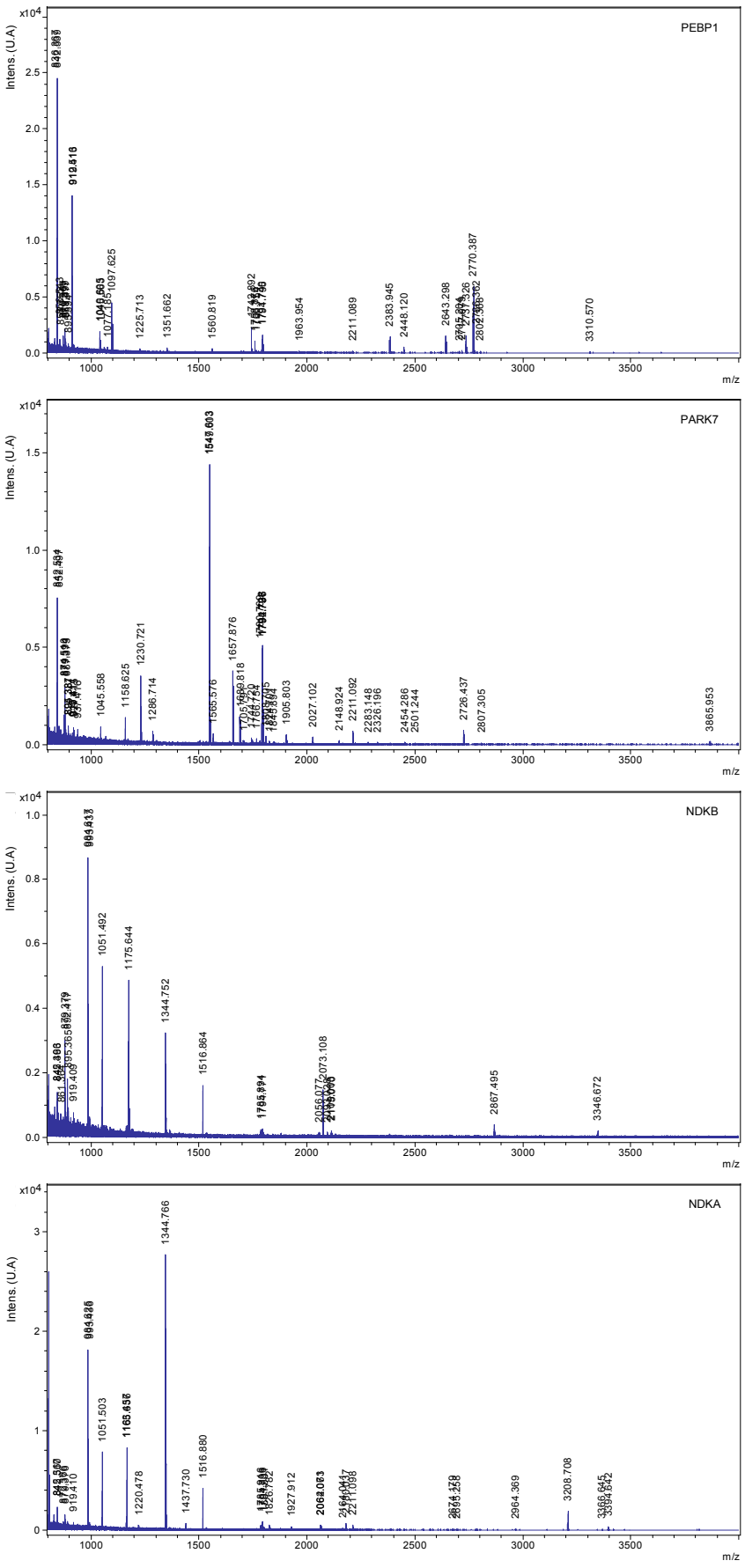
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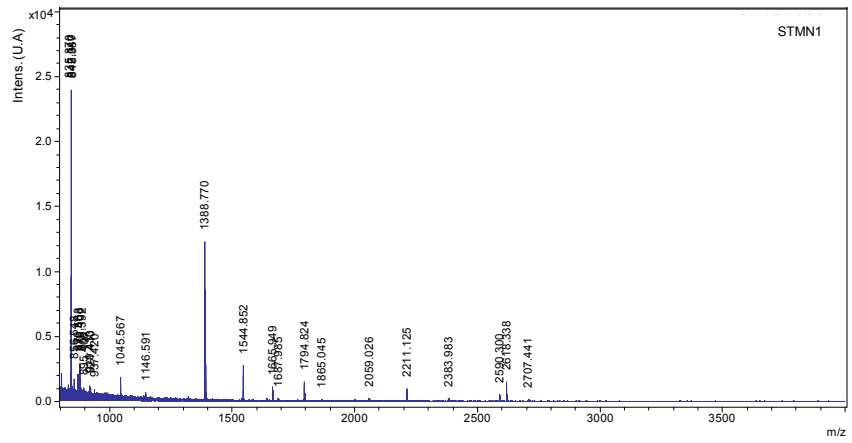
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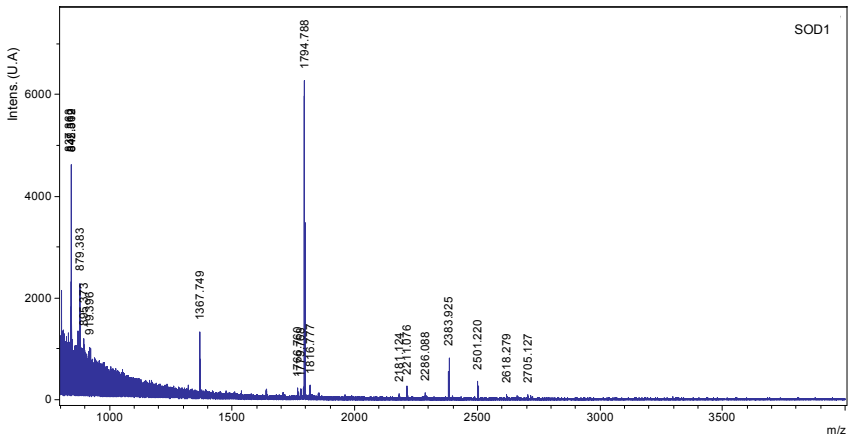
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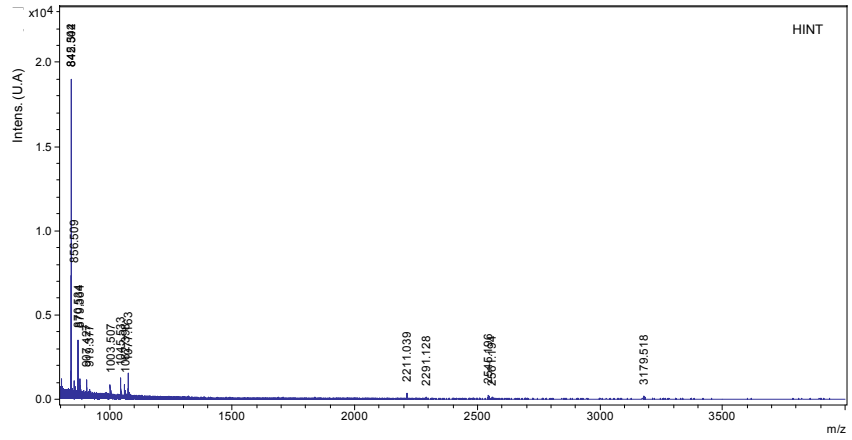
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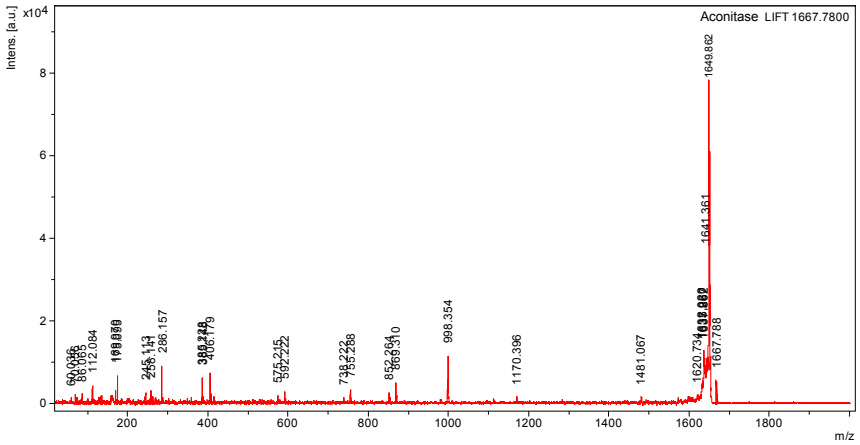
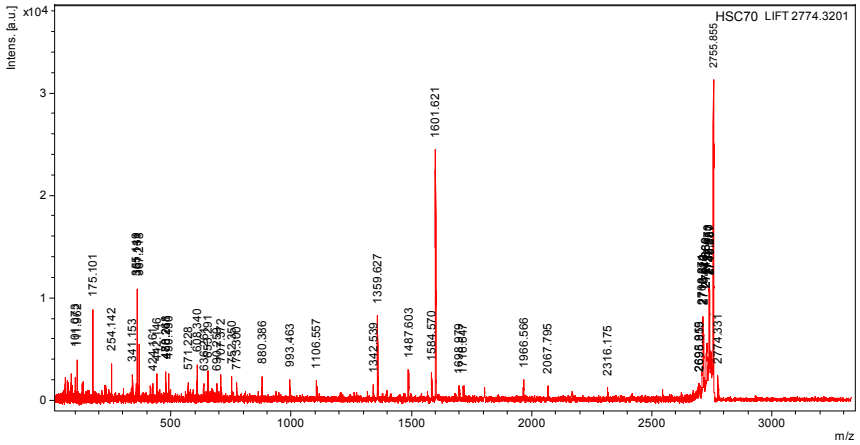
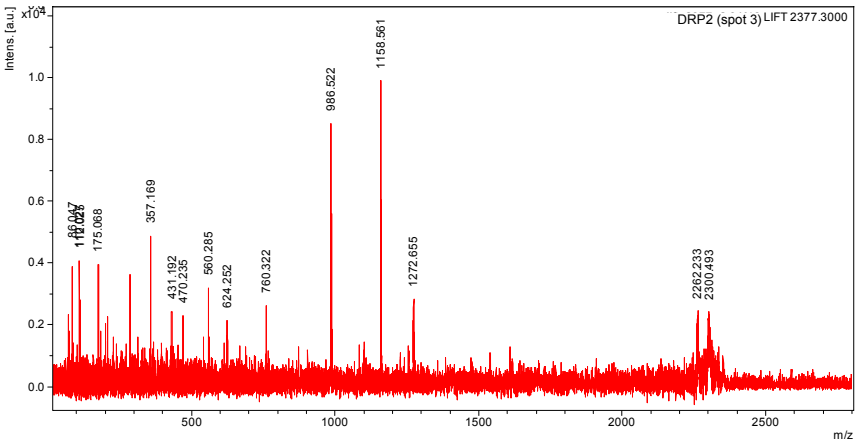
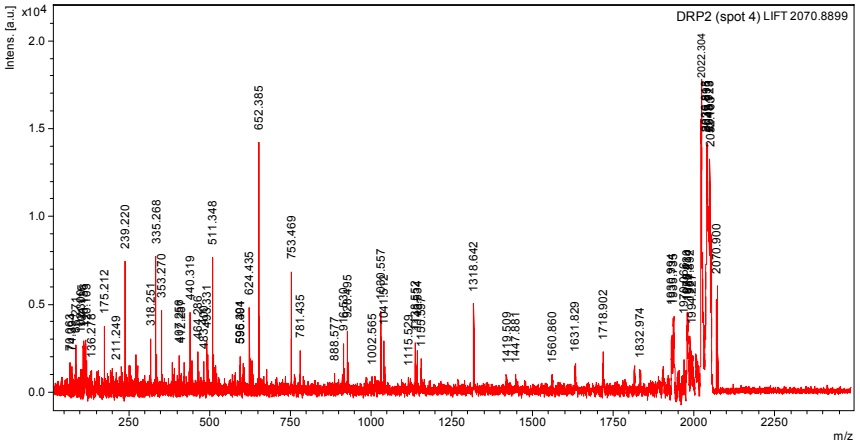
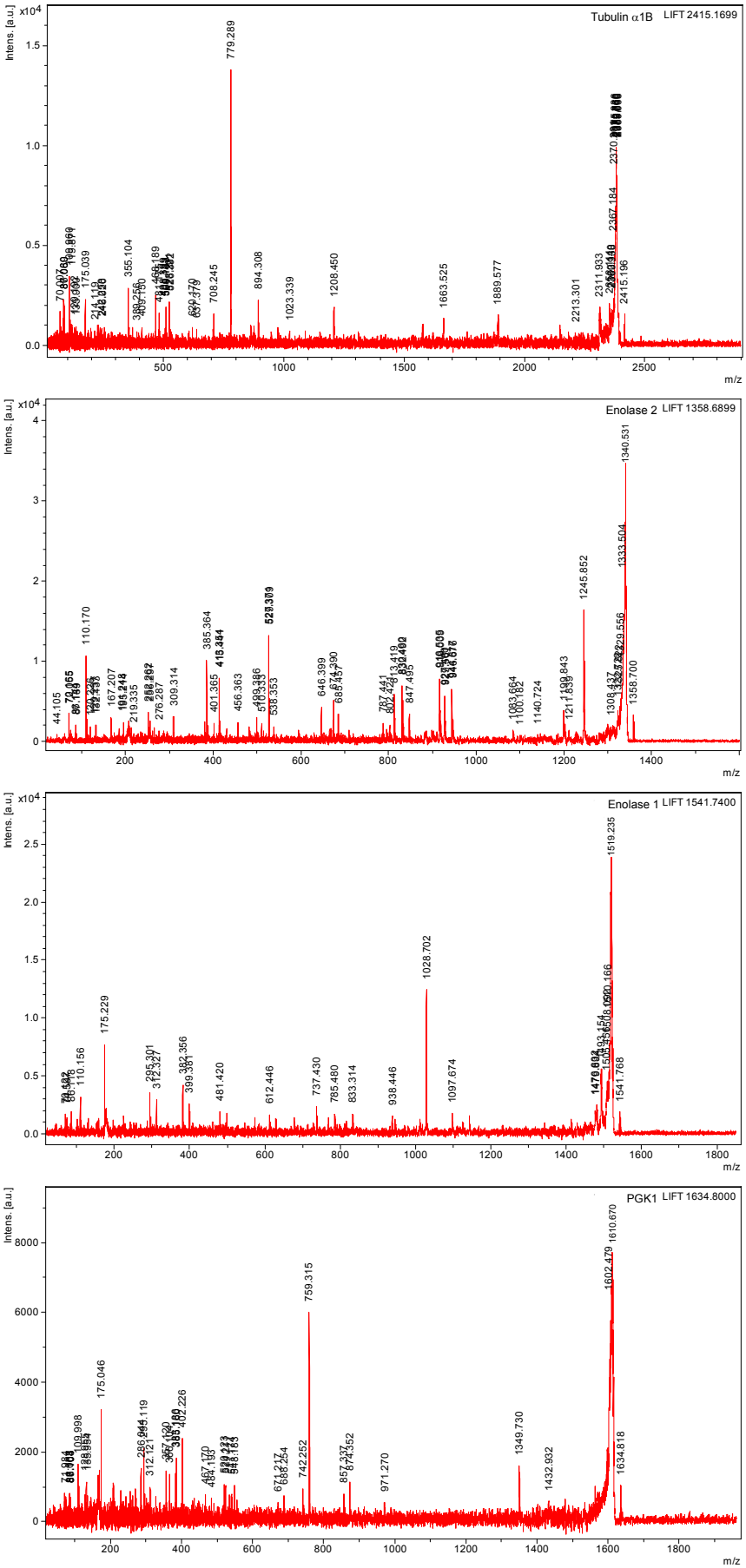
# MALDI LIF-TOF/TOF MS/MS spectra

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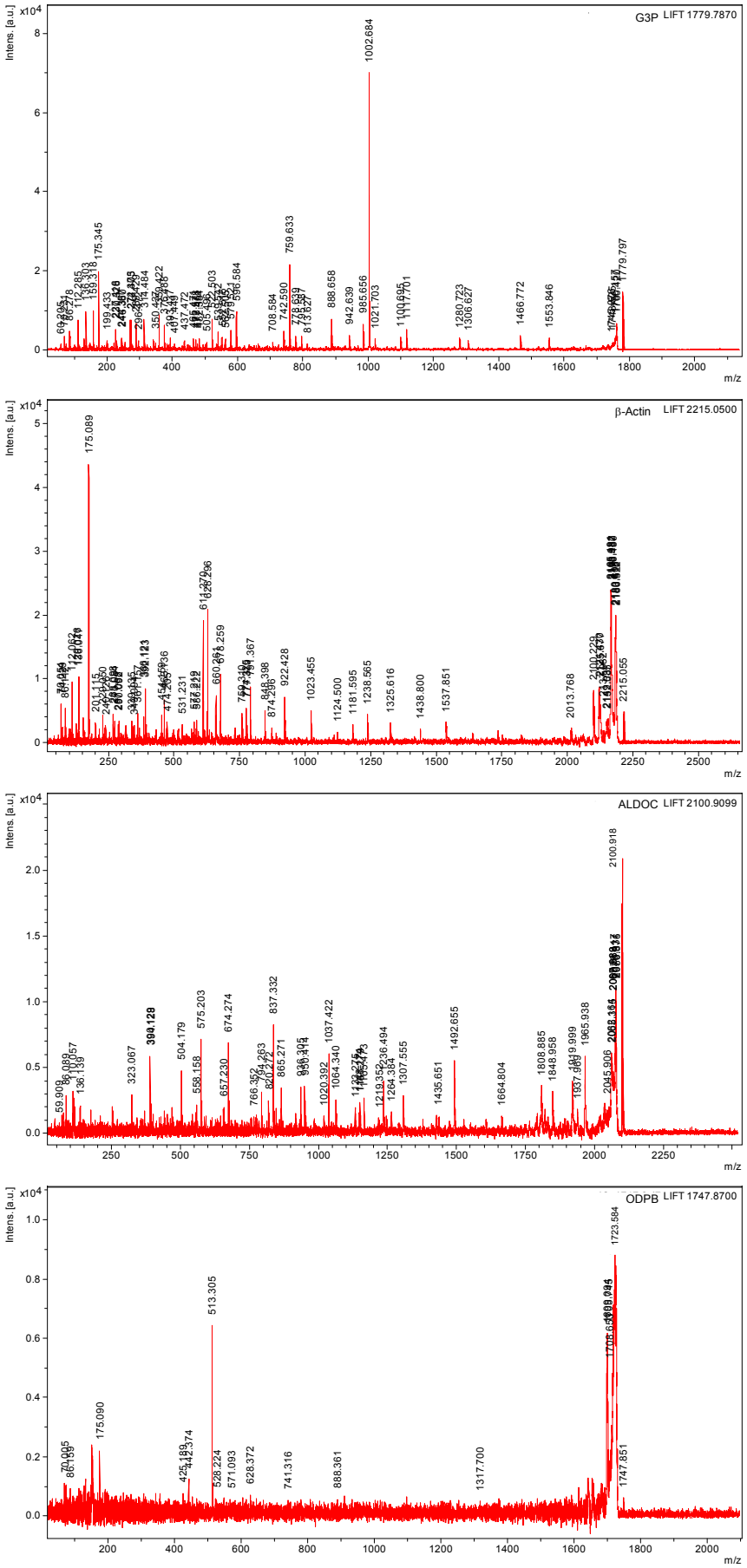
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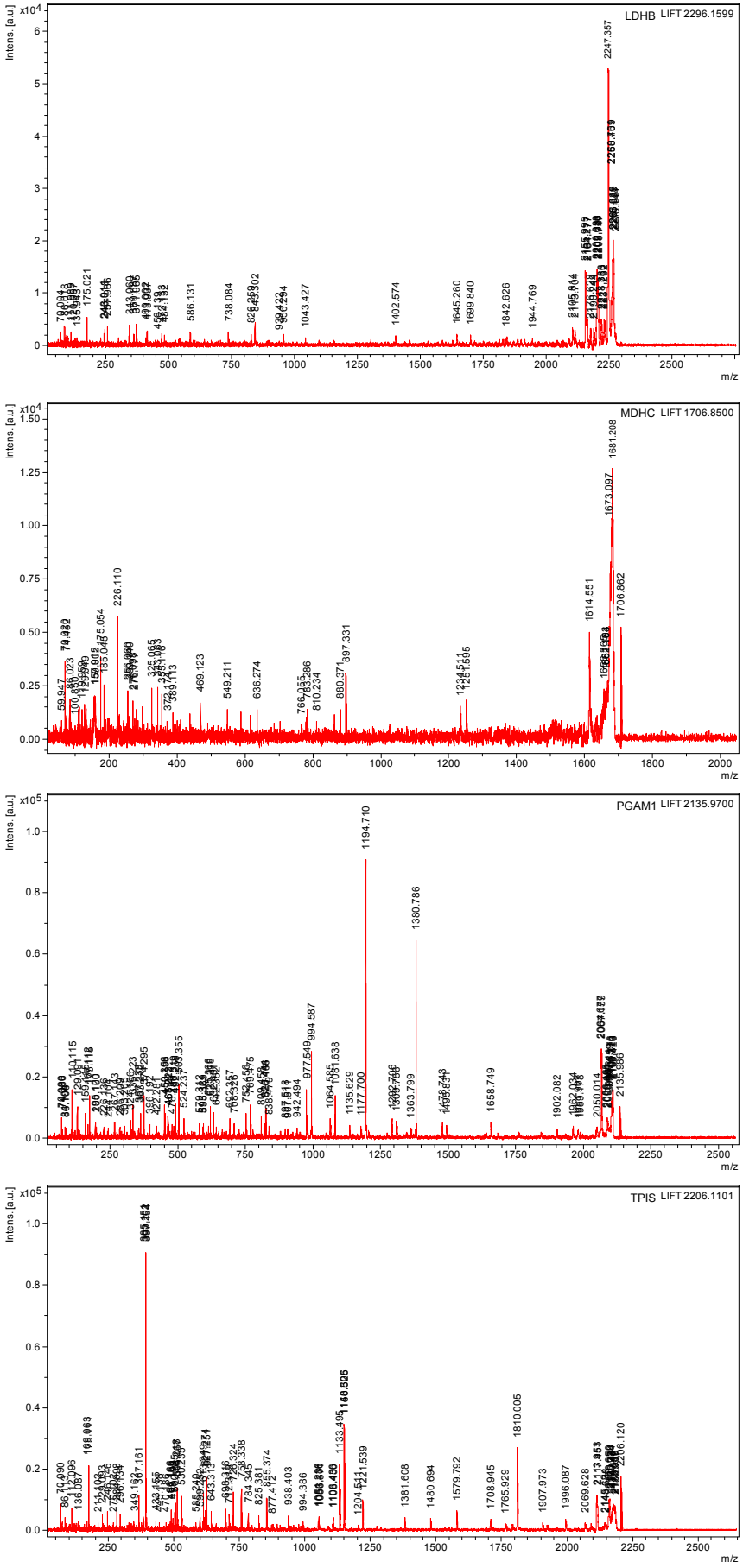
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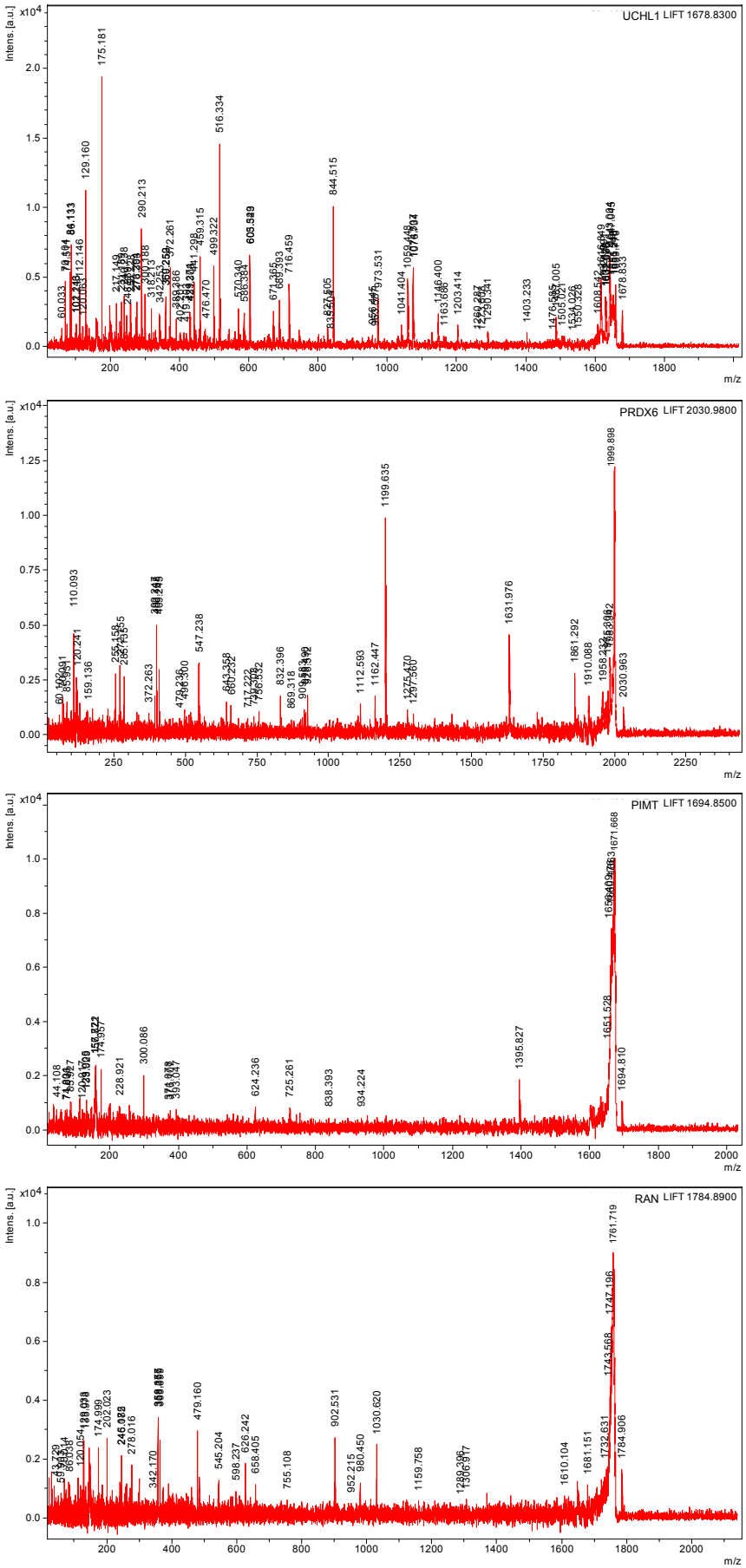
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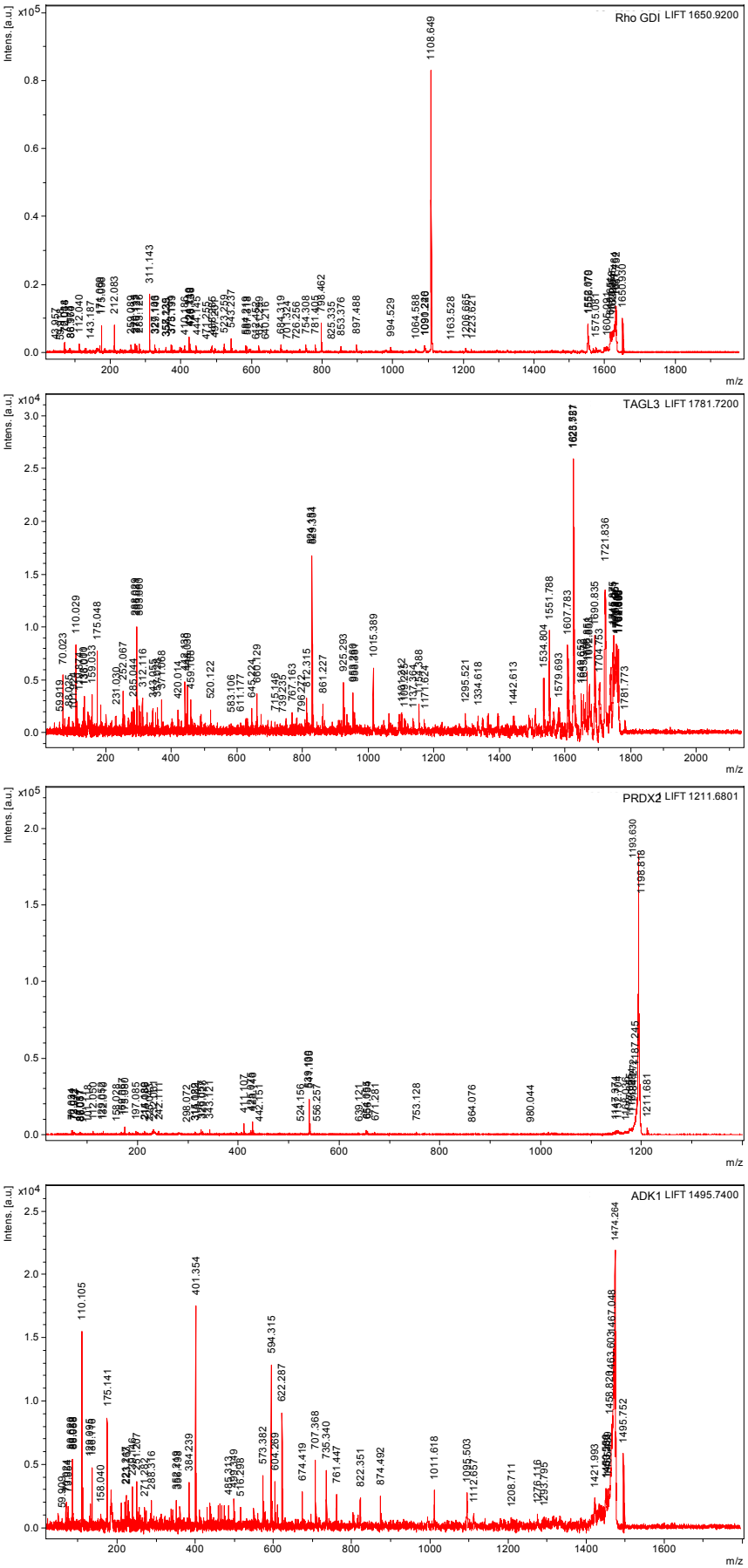
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m/z
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ADK1 LIFT 1495.7400
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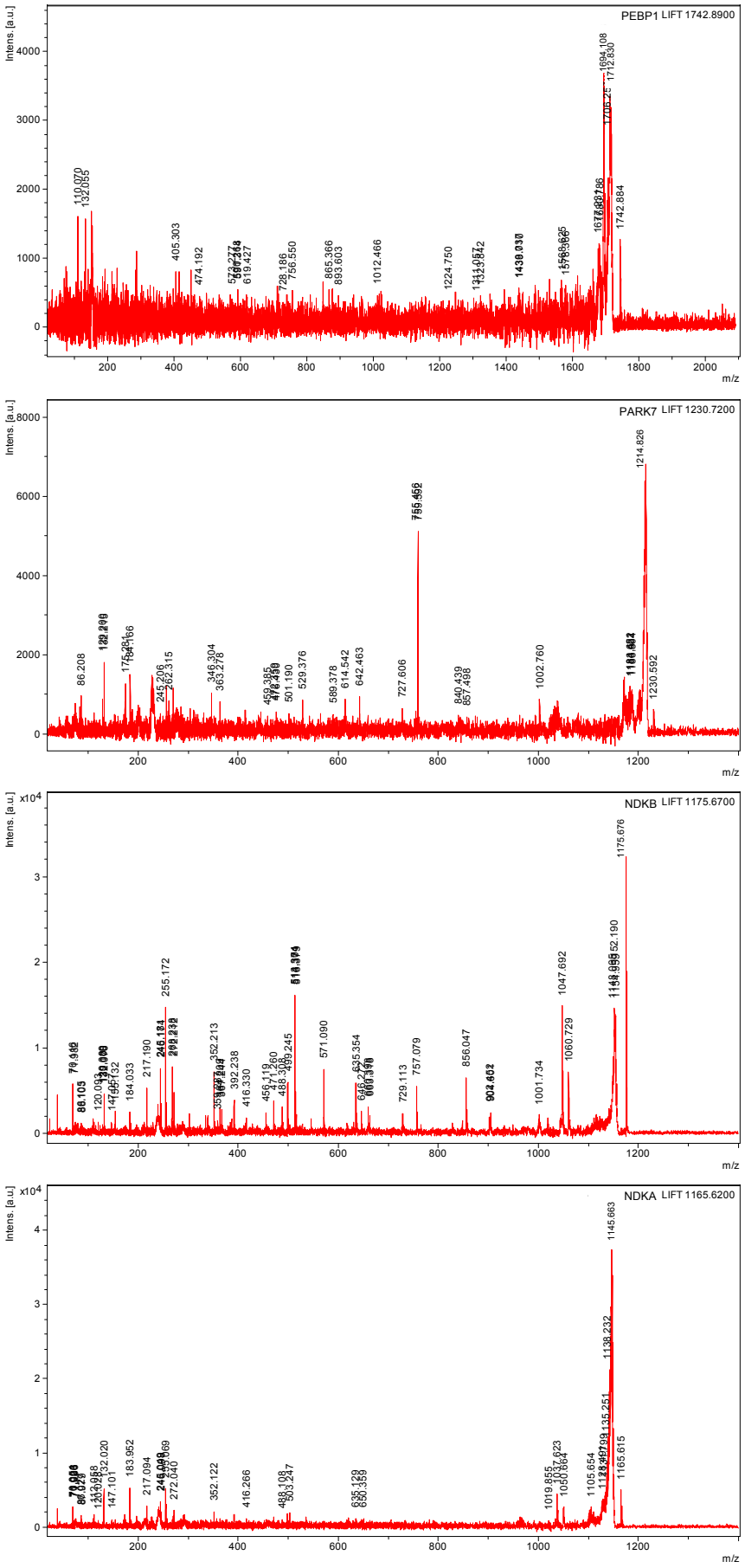
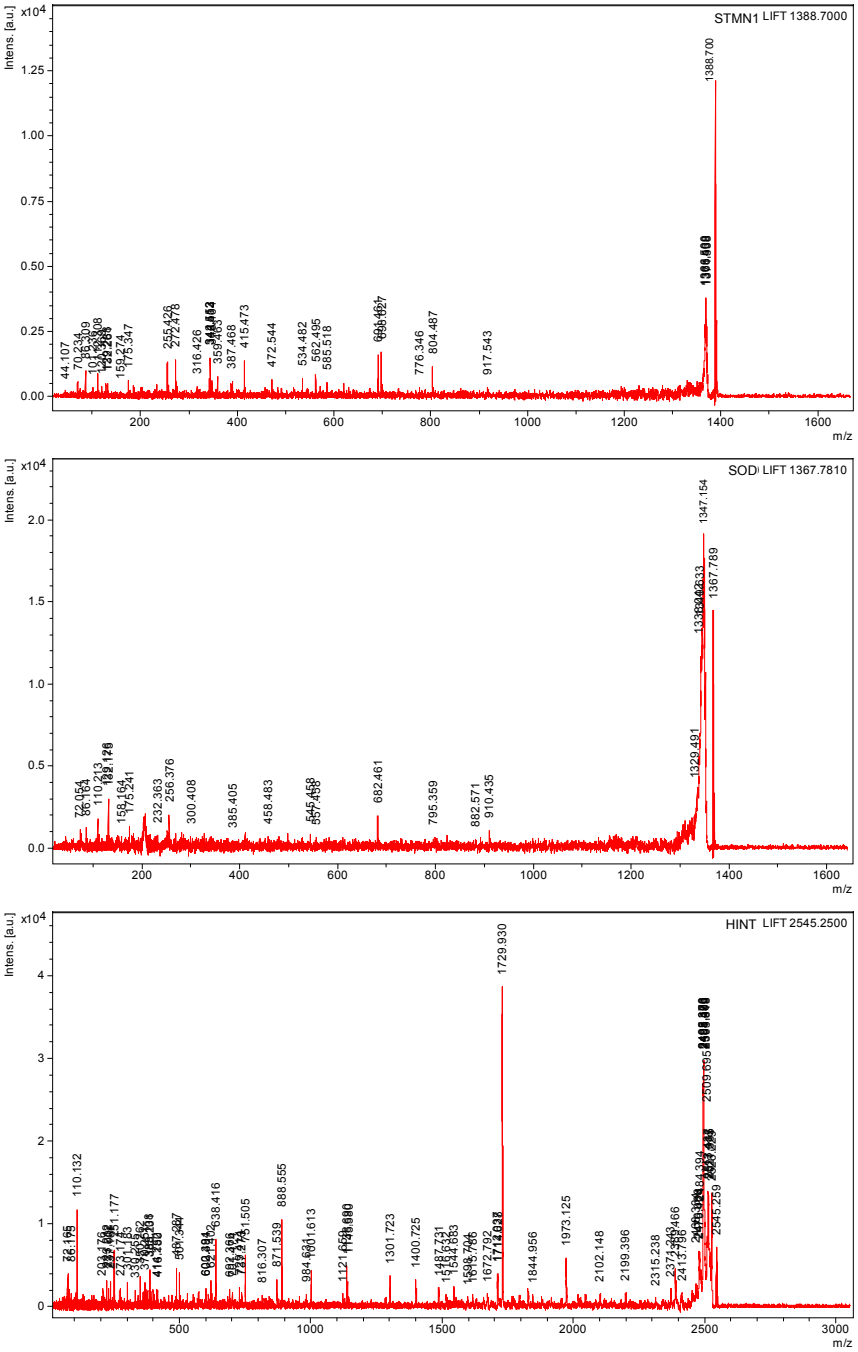
1467.048
1495.752


PEBP1 LIFT 1742.8900
Intens. [a.u.]
m/z
PARK7 LIFT 1230.7200
Intens. [a.u.]
m/z
NDKB LIFT 1175.6700
Intens. [a.u.]
x10^4
m/z
NDKA LIFT 1165.6200
Intens. [a.u.]
x10^4
m/z


STMN1 LIFT 1388.7000
Intens. [a.u.]
x10^4
m/z
1388.700
SOD LIFT 1367.7810
1347.154
1367.789
1329.491
682.461
795.359
882.571
910.435
HINT LIFT 2545.2500
1729.930
110.132
888.555
1301.723
1400.725
1973.125
2102.148
2199.396
2545.259