

Supporting Information

Ratiometric Mass Spectrometry for Cell Identification and Quantitation

Using Intracellular “Dual-Biomarkers”

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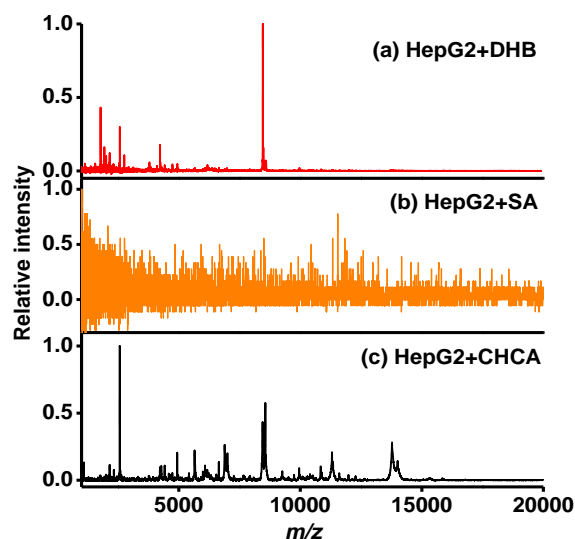


Figure S1. Normalized MALDI mass spectra of HepG2 cells with different organic matrix. (a) DHB (2, 5-Dihydroxybenzoic acid), (b) SA (Sinapic acid), (c) CHCA (α -Cyano-4-hydroxycinnamic acid). All matrix were prepared as saturated solutions whose solvent was a mixture of water, ACN and TFA (50:50:0.1, v/v/v). Spectra have been normalized by dividing the max peak value in each spectrum.

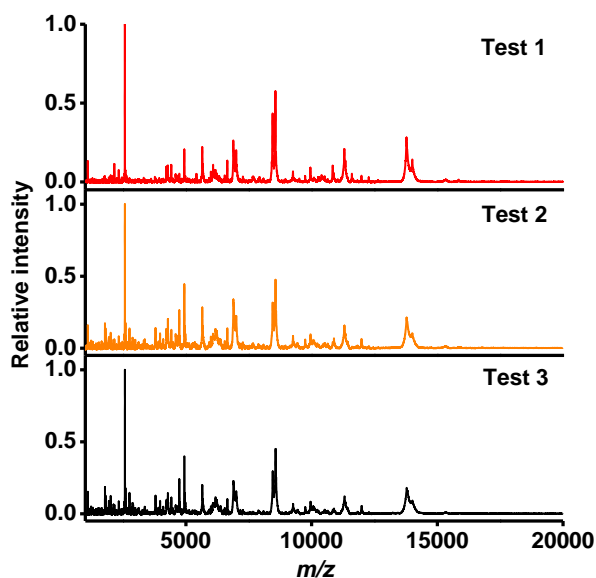


Figure S2. Typical normalized MALDI mass spectra of HepG2 cells acquired in different independent tests. The three tests were completed in three different days and the samples were obtained in the same culture generation. Spectra have been normalized by dividing the max peak value in each spectrum.

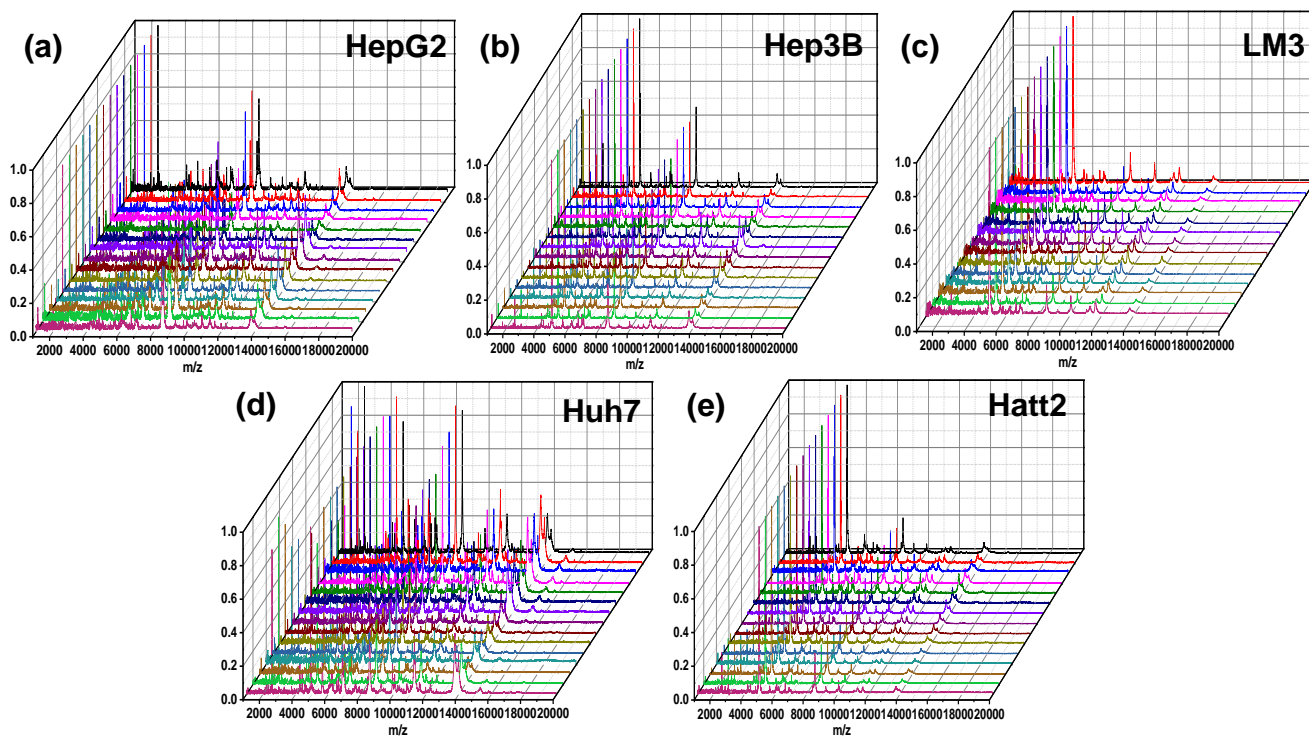


Figure S3. Normalized MALDI mass spectra of five types of hepatocellular cancer cells (HCC cells). (a) HepG2, (b) Hep3B, (c) LM3, (d) Huh7, (e) Hatt2. For each type of cells, spectra were acquired from 15 cell samples, which were gathered from five continuous generations with three replicates in each generation. Spectra have been normalized by dividing the max peak value in each spectrum.

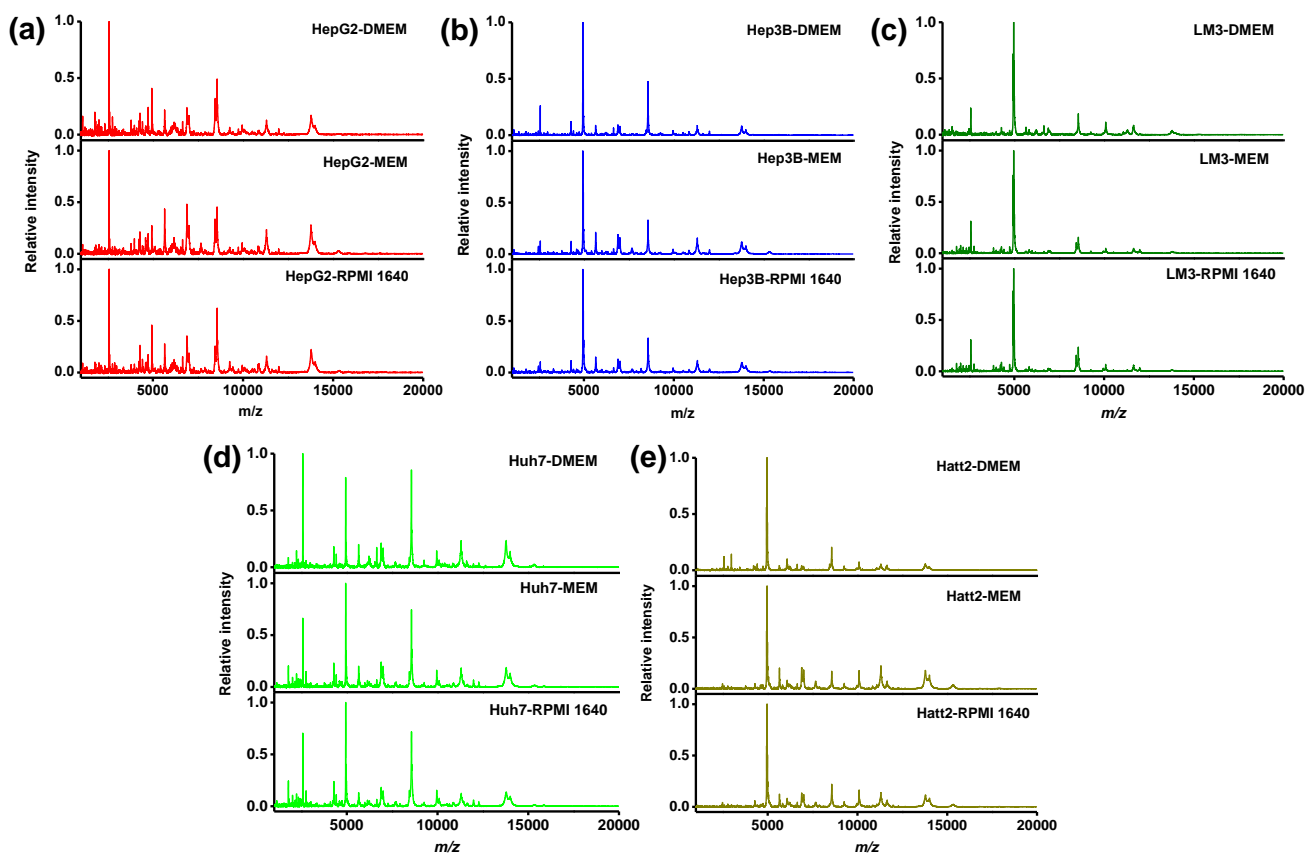


Figure S4. Normalized MALDI mass spectra of five types HCC cells cultured in DMEM, MEM and RPMI 1640 media. (a) HepG2, (b) Hep3B, (c) LM3, (d) Huh7, (e) Hatt2. Mass spectra of cells cultured in DMEM are the same with those in Figure 2.

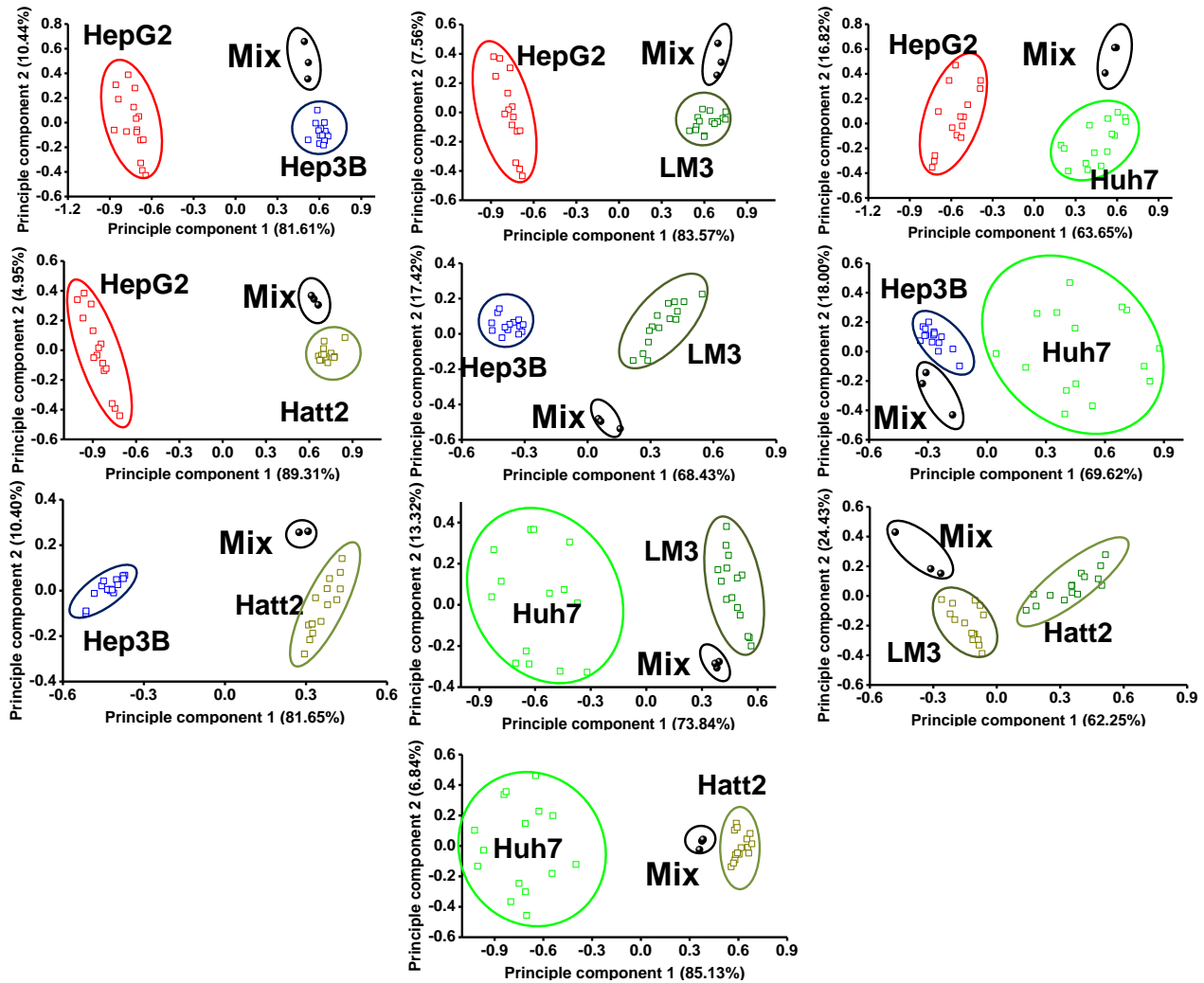


Figure S5. Principle component analysis (PCA) of cell mixture and pure cells. In each graph, “Mix” is referred to the cell mixture composed of two kinds of HCC cells with ratio =1:1. PCA process was all based on the 70 feature peaks selected by ClinproTools ($p < 0.01$). The data of pure cells used in this figure are the same with those in Figure 3b and the culture medium was DMEM.

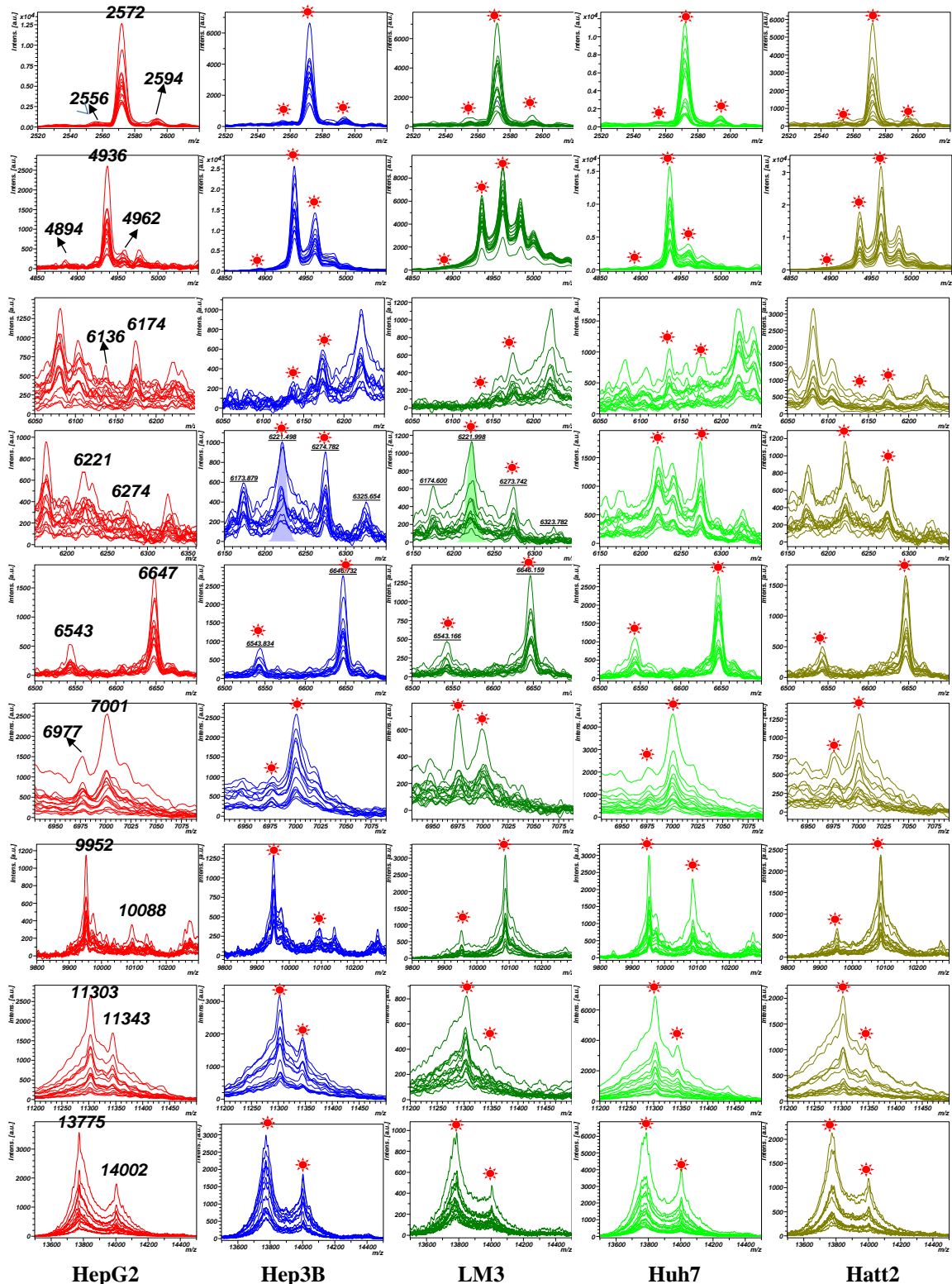


Figure S6. Magnified mass spectra of five type HCC cells at 11 pairs of dual-peaks whose RSD < 25% in each type of cells. The 11 pairs of dual peaks are listed: $m/z = 2556$ vs 2572 , $m/z = 2556$ vs 2594 , $m/z = 4894$ vs 4962 , $m/z = 4936$ vs 4962 , $m/z = 6136$ vs 6174 , $m/z = 6221$ vs 6274 , $m/z = 6543$ vs 6647 , $m/z = 6977$ vs 7001 , $m/z = 9952$ vs 10088 , $m/z = 11303$ vs 11343 , $m/z = 13775$ vs 14002 .

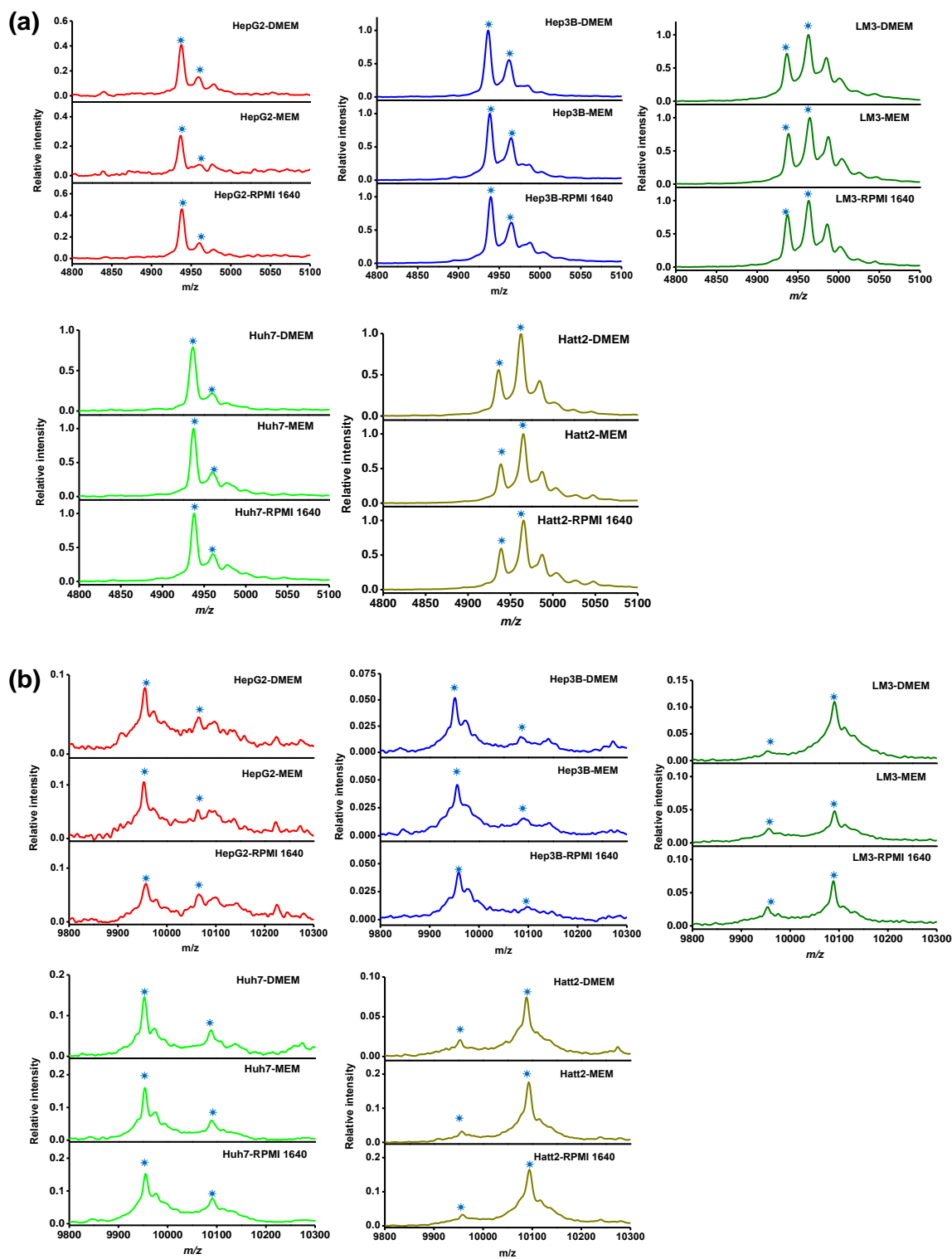


Figure S7. Magnified mass spectra at (a) $m/z = 4962$ vs 4936 , (b) $m/z = 10088$ vs 9952 of five type HCC cells cultured in three different media (DMEM, MEM and RPMI 1640). Mass spectra of cells cultured in DMEM are the same with those in Figure 2.

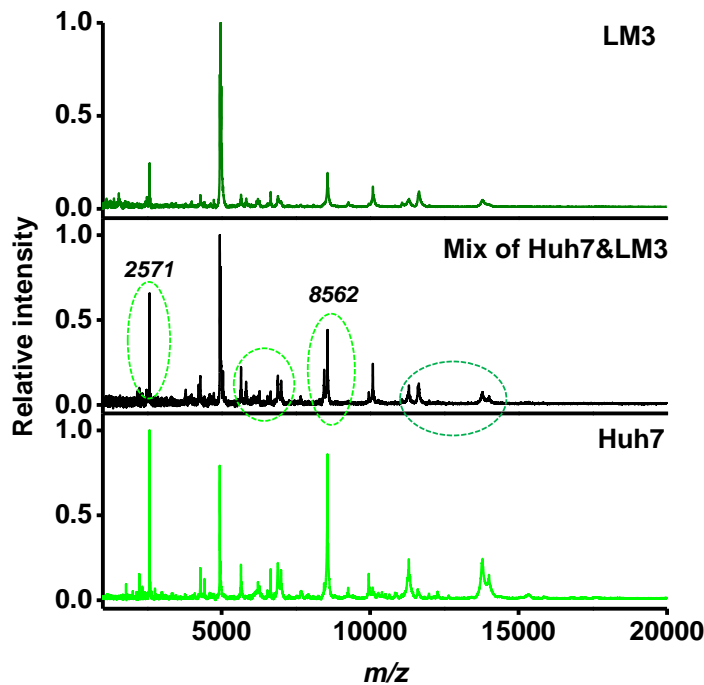


Figure S8. Typical Normalized MALDI mass spectra of (a) LM3, (b) mixture of Huh7 and LM3 with ratio =1:1 and (c) Huh7 cells cultured in DMEM. Mass spectra of Huh7 and LM3 cultured in DMEM are the same with those in Figure 2. The mass fingerprint of cell mixture seemed to overlay the spectra of Huh7 and LM3. For example, the relative intensity of the peak at $m/z = 2571$, $m/z = 8562$ in mixture sample were intermediate of those belonging to LM3 and Huh7 cells.

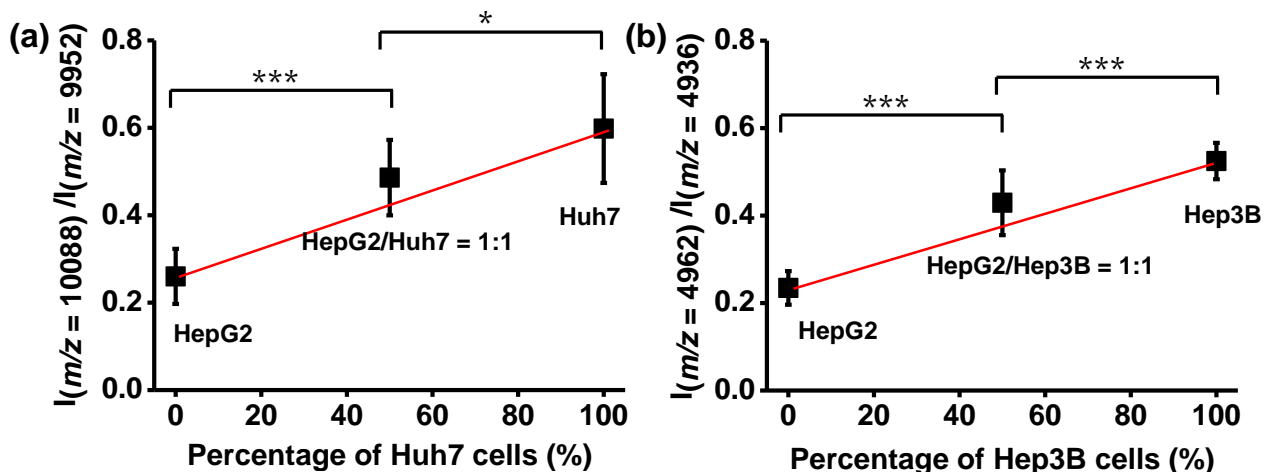
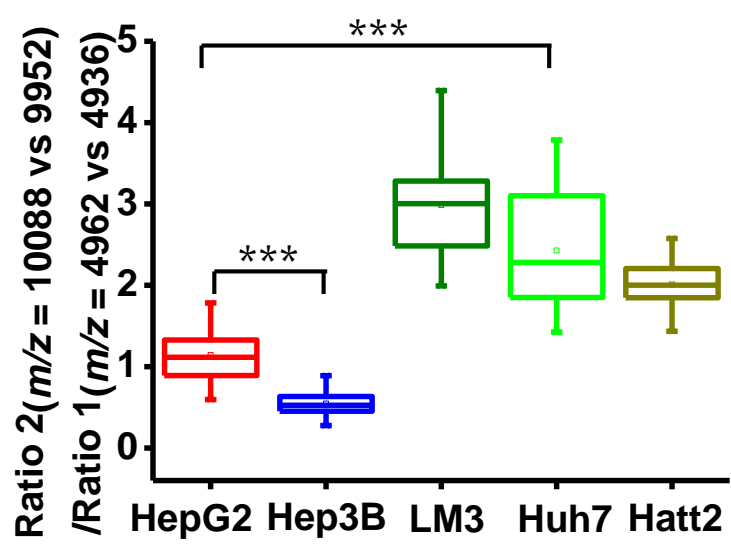


Figure S9. (a) The relationship between peak intensity ratio of $m/z = 10088$ to $m/z = 9952$ and percentage of Huh 7 cell in the cell mixture of Huh7 and HepG2 cells. (b) Relationship between peak intensity ratio of $m/z = 4962$ to $m/z = 4936$ and percentage of Hep3B cell in the cell mixture of Hep3B and HepG2. The ratio of peak pairs for pure cells was an averaged value calculated from 15 replicates while mixture was replicated for 3 tests.



Cell type	P value of Ratio 2/Ratio 1
HepG2 vs Hep3B	2.04E-06
HepG2 vs LM3	7.10E-11
HepG2 vs Huh7	7.28E-07
HepG2 vs Hatt2	3.79E-07
Hep3B vs LM3	7.70E-15
Hep3B vs Huh7	7.86E-11
Hep3B vs Hatt2	7.27E-13
LM3 vs Huh7	0.027341
LM3 vs Hatt2	0.000209
Huh7 vs Hatt2	0.196562

Figure S10. Ratio of two ratio values (Ratio 1 = $I_{(m/z=4962)} / I_{(m/z=4936)}$, Ratio 2 = $I_{(m/z=10088)} / I_{(m/z=9952)}$) in five types of HCC cells and the *P* value results between these cells.

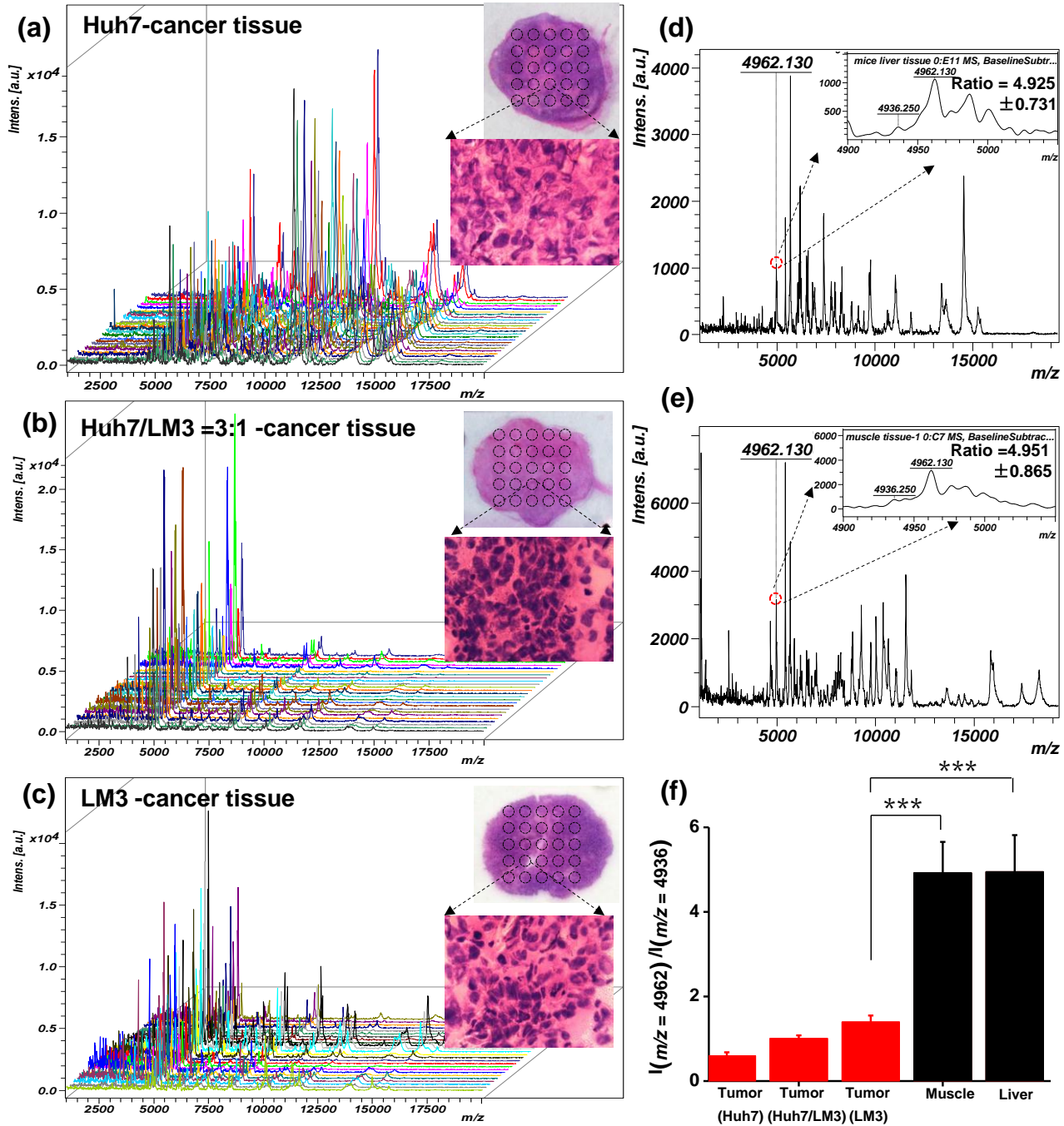


Figure S11. (a~c) MALDI mass spectra of the tissues originate from (a) pure Huh7 cells, (c) pure LM3 cells and (b) mixed cells with a ratio of Huh7/LM3 = 3:1. Insets in (a), (b), (c) are the symbolic images of the 5×5 array for MALDI detection on the tissue section which have been stained by haematoxylin and eosin for visualization. (d~e) MALDI mass spectra of (d) nude mice liver tissue and (e) nude mice muscle tissue. Insets in (d) and (e) are the magnified mass spectra at the $m/z = 4936$ and $m/z = 4962$. (f) Intensity ratio of $m/z = 4962$ to $m/z = 4936$ from different tissues in the model mouse. The ratio values were calculated as $I_{(m/z = 4962)} / I_{(m/z = 4936)}$ from a 5×5 spots array on the tissues. The ratio value for mice liver tissue and mice muscle tissue was 4.925 ± 0.731 and 4.951 ± 0.865 , respectively.

(a)

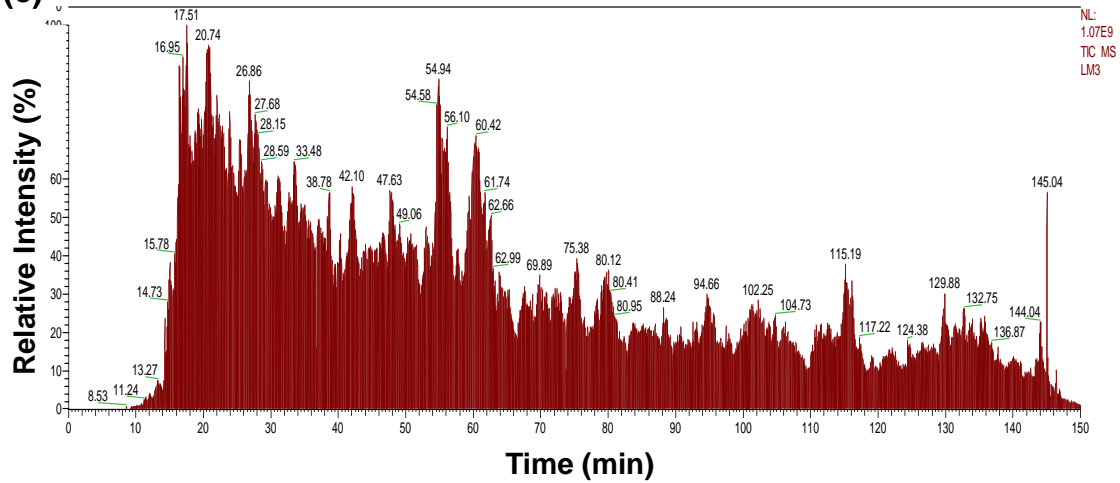
Thymosin β 4 Ac-SDKPDMAEIEKFDKSKLKKKTETQEKNPLPSKETIEQEKQAGES

Thymosin β 10 Ac-ADKPDmGEIASFDKAKLKKKTETQEKNLPTKETIEQEKRSEIS

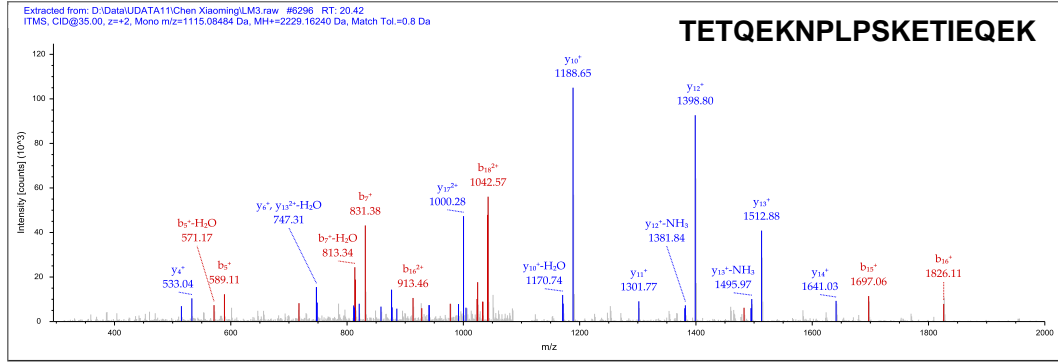
(b)

Protein name	Score	Coverage	# PSMs		
Thymosin-beta 4	94.74	90.70	33		
Sequence	# PSMs	Modifications	MH+ [Da]	Δ M [ppm]	RT [min]
TETQEKNPLPSKETIEQEK	11		2229.16240	16.96	20.42
sDKPDMAEIEKFDK	14	S1(Acetyl)	1694.82529	18.30	62.36
sDKPDMAEIEK	3	S1(Acetyl)	1304.62749	18.05	27.46
NPLPSKETIEQEK	2		1512.81816	18.30	19.48
NPLPSKETIEQEKQAGES	1		1985.02141	19.71	23.50
sDKPDmAEIEKFDK	1	S1(Acetyl); M6(Oxidation)	1710.81736	16.46	34.16
kTETQEknPLPSK	1	K1(GlyGly); K7(GlyGly); N8(Deamidated)	1728.89507	10.84	89.38
Accession	Score	Coverage	# PSMs		
Thymosin-beta 10	42.82	81.40	15		
Sequence	# PSMs	Modifications	MH+ [Da]	Δ M [ppm]	RT [min]
aDKPDMGEIASFDK	7	A1(Acetyl)	1565.74407	18.37	76.36
NLPTKETIEQEK	2		1530.82927	18.44	18.66
NLPTKETIEQEK R	1		1686.92795	15.29	16.42
aDKPDmGEIASFDK	2	A1(Acetyl); M6(Oxidation)	1581.74016	18.93	44.57
nLPTKETIEQEK	1	N1(Deamidated)	1531.81133	17.15	22.85
nLPTKETIEqEK	1	N1(Deamidated); Q11(Deamidated)	1532.74578	-15.20	18.61
kTETQEKNLPTK	1	K1(GlyGly); K7(GlyGly)	1745.89336	-5.45	56.98

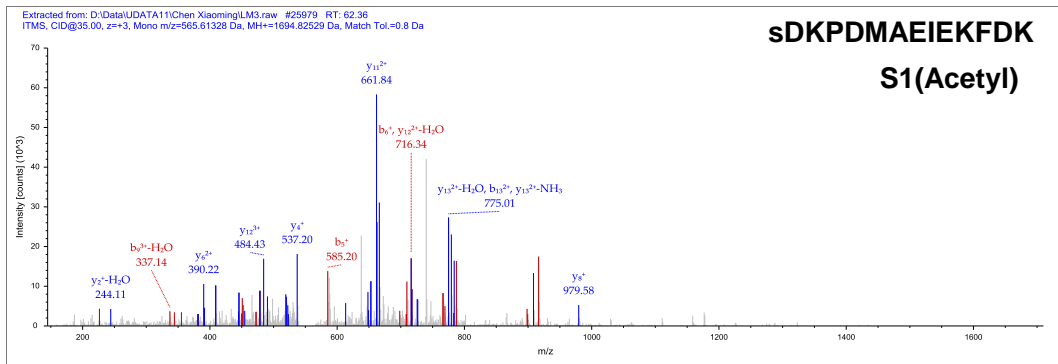
(c)



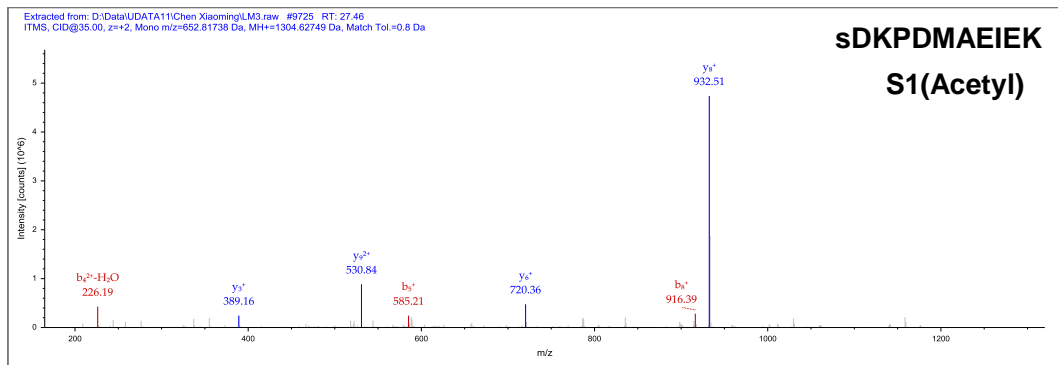
(d-1)



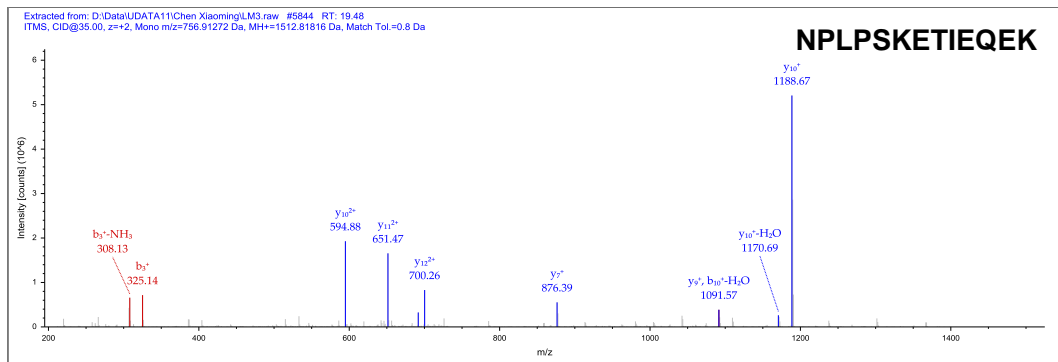
(d-2)



(d-3)



(d-4)



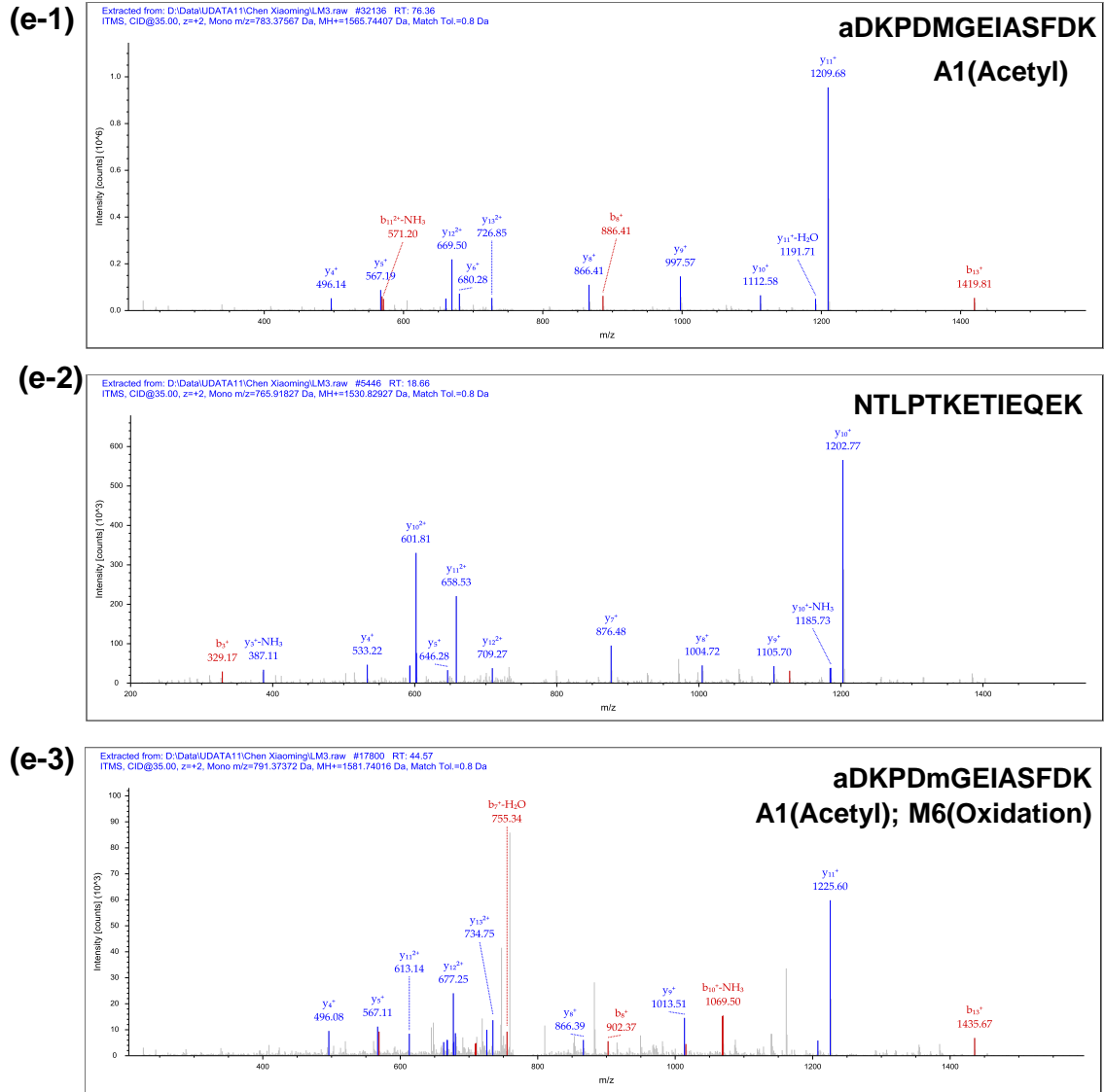


Figure S12. LC-MS/MS identification results of thymosin β 4 and thymosin β 10. (a) Amino acid sequence of thymosin β 4 and thymosin β 10. (b) Information of the peptide sequence identified by LC-MS/MS. (c) Total ion current spectrum of the LM3 cell sample after trypsin lysis procedure. (d-e) MS/MS spectra of the identified peptide sequence belonging to (d) thymosin β 4 and (e) thymosin β 10 with high PSMs, respectively.

Table S1. Normalized intensity of 70 feature peaks from the mass spectra of five types of HCC cells selected by ClinproTools software

m/z of 70 feature peaks	Normalized Intensity (Average value \pm Error)				
	HepG2	Hep3B	LM3	Huh7	Hatt2
1099.13	0.129 \pm 0.029	0.058 \pm 0.022	0.01 \pm 0.011	0.048 \pm 0.023	0.029 \pm 0.016
1779.04	0.052 \pm 0.018	0.018 \pm 0.009	0.012 \pm 0.014	0.046 \pm 0.024	0.005 \pm 0.007
2006.99	0.058 \pm 0.02	0.006 \pm 0.007	0.019 \pm 0.013	0.023 \pm 0.017	0.007 \pm 0.007
2154.19	0.129 \pm 0.027	0.012 \pm 0.006	0.04 \pm 0.018	0.041 \pm 0.018	0.017 \pm 0.007
2227.23	/	/	0.009 \pm 0.01	0.066 \pm 0.044	0.004 \pm 0.006
2328.43	0.075 \pm 0.011	0.019 \pm 0.004	0.043 \pm 0.017	0.053 \pm 0.019	0.017 \pm 0.008
2349.52	0.033 \pm 0.007	0.007 \pm 0.005	0.026 \pm 0.016	0.02 \pm 0.014	0.018 \pm 0.005
2467.37	0.009 \pm 0.014	0.033 \pm 0.007	0.023 \pm 0.015	0.038 \pm 0.006	0.013 \pm 0.007
2482.81	0.019 \pm 0.016	0.022 \pm 0.01	0.029 \pm 0.024	0.019 \pm 0.012	0.022 \pm 0.008
2555.58	0.062 \pm 0.012	0.012 \pm 0.007	0.041 \pm 0.019	0.043 \pm 0.012	0.012 \pm 0.009
2571.90	1 \pm 0	0.221 \pm 0.048	0.508 \pm 0.191	0.658 \pm 0.26	0.132 \pm 0.065
2593.84	0.11 \pm 0.019	0.023 \pm 0.006	0.053 \pm 0.023	0.073 \pm 0.033	0.017 \pm 0.011
2634.26	0.07 \pm 0.023	0.001 \pm 0.003	0.001 \pm 0.005	0.016 \pm 0.015	0.002 \pm 0.004
2754.26	0.031 \pm 0.016	0.008 \pm 0.005	0.021 \pm 0.013	0.035 \pm 0.017	0.011 \pm 0.008
2775.74	0.005 \pm 0.01	0.01 \pm 0.007	0.011 \pm 0.014	0.012 \pm 0.011	0.031 \pm 0.011
2978.37	/	0.03 \pm 0.006	0.002 \pm 0.007	0.024 \pm 0.016	0.109 \pm 0.035
3788.64	0.028 \pm 0.013	0.001 \pm 0.002	0.015 \pm 0.013	0.024 \pm 0.012	0.001 \pm 0.004
3838.72	0.005 \pm 0.01	0.013 \pm 0.008	0.024 \pm 0.016	/	0.019 \pm 0.004
3937.48	0.015 \pm 0.013	0.021 \pm 0.01	0.004 \pm 0.008	0.016 \pm 0.015	0.006 \pm 0.006
4213.78	0.019 \pm 0.027	0.023 \pm 0.01	0.029 \pm 0.016	0.003 \pm 0.007	0.04 \pm 0.006
4225.05	0.1 \pm 0.041	0.017 \pm 0.009	0.023 \pm 0.017	0.033 \pm 0.013	0.033 \pm 0.004
4281.40	0.104 \pm 0.03	0.097 \pm 0.017	0.079 \pm 0.011	0.157 \pm 0.023	0.036 \pm 0.006
4418.30	0.119 \pm 0.038	0.047 \pm 0.006	0.064 \pm 0.025	0.121 \pm 0.029	0.057 \pm 0.009
4735.73	0.05 \pm 0.019	0.033 \pm 0.007	0.049 \pm 0.01	0.039 \pm 0.017	0.038 \pm 0.008
4894.11	0.003 \pm 0.007	0.029 \pm 0.009	0.038 \pm 0.006	0.035 \pm 0.011	0.021 \pm 0.014
4936.25	0.194 \pm 0.053	1 \pm 0	0.711 \pm 0.024	0.972 \pm 0.066	0.588 \pm 0.045
4962.13	0.045 \pm 0.013	0.525 \pm 0.041	1 \pm 0	0.244 \pm 0.026	1 \pm 0
4983.82	0.027 \pm 0.02	0.144 \pm 0.062	0.403 \pm 0.042	0.121 \pm 0.013	0.467 \pm 0.106
4999.71	0.007 \pm 0.011	0.062 \pm 0.034	0.163 \pm 0.052	0.049 \pm 0.026	0.195 \pm 0.052
5044.08	0.002 \pm 0.007	0.007 \pm 0.012	0.058 \pm 0.011	0.032 \pm 0.017	0.06 \pm 0.015
5068.20	0.012 \pm 0.014	0.006 \pm 0.01	0.02 \pm 0.017	0.022 \pm 0.013	0.027 \pm 0.016
5419.21	0.043 \pm 0.013	0.016 \pm 0.008	/	0.01 \pm 0.011	0.001 \pm 0.002

5608.56	0.036±0.01	0.021±0.008	0.02±0.012	0.028±0.02	0.008±0.006
5650.12	0.192±0.063	0.103±0.03	0.08±0.032	0.261±0.107	0.05±0.024
5670.59	0.094±0.041	0.054±0.026	0.014±0.022	0.123±0.06	0.026±0.015
5991.42	0.055±0.028	0.014±0.007	0.015±0.008	0.023±0.011	0.014±0.005
6080.82	0.123±0.048	0.001±0.003	/	0.033±0.022	0.086±0.018
6104.03	0.08±0.045	/	/	0.023±0.02	0.051±0.014
6136.22	0.055±0.023	0.008±0.005	0.003±0.006	0.049±0.017	0.015±0.01
6174.32	0.077±0.024	0.025±0.006	0.029±0.014	0.047±0.02	0.03±0.006
6221.52	0.058±0.024	0.032±0.01	0.052±0.021	0.113±0.017	0.039±0.008
6274.02	0.026±0.022	0.03±0.007	0.033±0.008	0.089±0.021	0.031±0.01
6325.26	0.033±0.017	0.013±0.005	0.001±0.002	0.029±0.017	0.007±0.006
6543.21	0.03±0.019	0.024±0.007	0.022±0.015	0.053±0.015	0.017±0.006
6646.69	0.149±0.072	0.079±0.025	0.088±0.019	0.187±0.038	0.059±0.012
6889.47	0.185±0.079	0.1±0.036	0.053±0.021	0.248±0.107	0.046±0.022
6947.02	0.059±0.042	0.029±0.019	0.018±0.017	0.08±0.044	0.017±0.012
6976.82	0.101±0.029	0.039±0.01	0.027±0.016	0.102±0.032	0.023±0.01
7000.97	0.139±0.058	0.081±0.034	0.038±0.019	0.2±0.085	0.033±0.021
7920.40	0.024±0.012	0.017±0.016	0.003±0.006	0.036±0.034	/
8448.24	0.429±0.187	0.047±0.006	0.09±0.016	0.108±0.017	0.06±0.012
8469.44	0.165±0.091	0.031±0.005	0.053±0.018	0.061±0.018	0.035±0.009
8562.24	0.476±0.138	0.451±0.028	0.506±0.056	0.795±0.083	0.207±0.025
9513.30	0.013±0.011	/	/	0.013±0.014	0±0.001
9743.44	0.032±0.013	/	/	0.004±0.007	/
9952.93	0.079±0.02	0.047±0.009	0.04±0.004	0.141±0.028	0.02±0.003
10088.74	0.02±0.007	0.013±0.003	0.166±0.029	0.086±0.03	0.075±0.024
10274.70	0.022±0.014	0.01±0.004	0.014±0.008	0.033±0.009	0.011±0.003
10396.53	0.036±0.013	/	0.013±0.004	0.027±0.011	/
10517.59	0.034±0.008	0.02±0.003	0.01±0.006	0.012±0.011	0.003±0.003
10838.80	0.078±0.026	0.034±0.01	0.015±0.007	0.04±0.015	0.007±0.003
10885.67	0.047±0.012	0.012±0.005	/	0.032±0.012	0.004±0.003
11069.65	0.004±0.015	/	0.049±0.014	0.029±0.01	0.022±0.009
11302.87	0.151±0.079	0.099±0.041	0.061±0.029	0.273±0.128	0.049±0.029
11343.04	0.095±0.045	0.061±0.031	0.021±0.018	0.147±0.07	0.029±0.015
11603.55	0.038±0.012	0.015±0.004	0.143±0.016	0.051±0.012	0.031±0.007
11981.62	0.03±0.009	0.027±0.007	0.017±0.004	0.031±0.006	0.005±0.003
12273.26	0.021±0.012	0.007±0.001	0.001±0.004	0.048±0.014	0.008±0.002
13774.69	0.208±0.101	0.099±0.038	0.062±0.024	0.259±0.103	0.058±0.027

14002.40	0.1 ±0.05	0.061 ±0.027	0.031 ±0.014	0.162 ±0.073	0.031 ±0.016
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Table S2. *P* values calculated with Student's t test of the 11 pair dual-peaks between each two type of five types of HCC cells

<i>P</i> values of 11 pair dual-peaks											
Cell mixture type	2556 vs 2572	2556 vs 2594	4894 vs 4962	4936 vs 4962	6136 vs 6174	6221 vs 6274	6543 vs 6647	6977 vs 7001	9952 vs 10088	11303 vs 11343	13774 vs 14002
HepG2 vs Hep3B	7.935E-04	8.067E-01	1.678E-13	4.786E-18	4.244E-12	1.333E-05	3.905E-03	2.235E-06	3.832E-01	9.065E-02	1.844E-08
HepG2 vs LM3	1.306E-05	4.270E-01	2.447E-19	1.066E-33	4.913E-15	7.557E-02	8.276E-04	5.518E-03	7.928E-17	2.338E-08	9.494E-02
HepG2 vs Huh7	1.744E-01	9.702E-01	3.176E-07	1.509E-01	2.068E-04	4.547E-04	5.016E-02	4.457E-05	3.734E-10	7.508E-06	1.241E-09
HepG2 vs Hatt2	5.130E-05	8.164E-03	1.521E-10	4.556E-27	9.218E-01	8.300E-04	3.143E-02	6.704E-01	6.475E-17	5.298E-01	1.254E-20
Hep3B vs LM3	2.209E-01	6.989E-01	2.741E-07	6.911E-30	1.408E-03	1.089E-05	6.198E-01	2.561E-10	4.963E-08	1.211E-06	3.656E-06
Hep3B vs Huh7	8.361E-02	7.792E-01	2.372E-11	4.250E-19	6.016E-12	1.134E-02	2.063E-01	3.019E-01	5.243E-04	3.452E-03	4.539E-01
Hep3B vs Hatt2	2.500E-01	1.093E-02	8.543E-05	2.267E-24	2.264E-10	4.523E-03	3.095E-01	1.982E-05	1.873E-07	2.852E-01	1.941E-18
LM3 vs Huh7	7.640E-03	3.878E-01	5.905E-18	4.575E-35	3.485E-14	3.061E-04	6.889E-02	4.774E-09	2.146E-03	2.961E-05	2.769E-07
LM3 vs Hatt2	9.166E-01	1.630E-03	1.368E-01	2.799E-09	2.351E-13	7.588E-04	1.180E-01	3.748E-03	1.005E-03	1.215E-07	4.921E-20
Huh7 vs Hatt2	1.141E-02	7.690E-03	1.280E-09	3.229E-27	5.819E-04	6.076E-01	7.977E-01	3.282E-04	1.059E-05	1.040E-04	2.436E-18

$P < 0.01$ means the significant difference between cell types.

Table S3. Feature peaks information ranking first in the principle component analysis

m/z of feature peaks	Coefficient of the peaks in p1	m/z of feature peaks	Coefficient of the peaks in p2
4962.13	0.59554	4936.25	0.750937
2571.90	-0.5483	8562.24	0.374808
4983.82	0.265733	2571.90	-0.22771
4936.25	0.205614	4962.13	-0.22539
8562.24	-0.19411	8448.24	-0.18991
8448.24	-0.18672	4983.82	-0.15158
6889.47	-0.1386	11302.87	0.144088
4999.71	0.120251	5650.12	0.104848
5650.12	-0.11933	6889.47	0.095461
13774.69	-0.11789	7000.97	0.095435