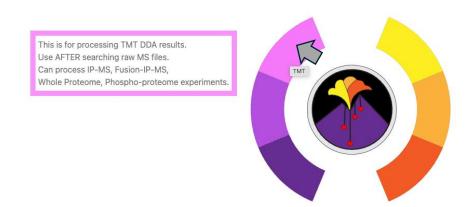
TMT (Phospho Proteome)

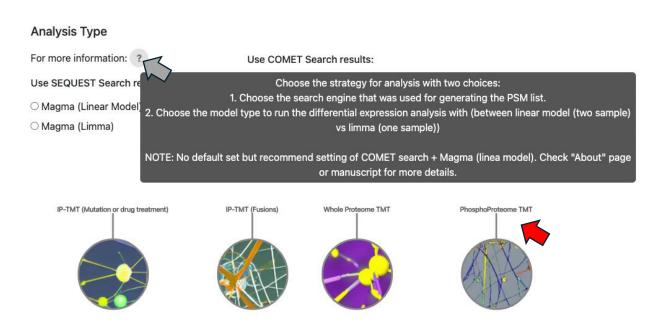
Step 1: Click on the TMT tab on the home page to access all the TMT workflows (as shown in figure below with the grey arrow).



Step 2: Choose the type of analysis to run. You can hover over the question mark to get more details on the different choices and recommended settings (as shown by the grey arrow below). The choices to be made here include:

- The choice of search engine (SEQUEST in Proteome Discoverer vs COMET)
- 2. The choice of analysis type (MAGMa LM for a two-sample t-test and MAGMa Limma for a one sample t-test)

Click on the Phospho-proteome arm (highlighted with red arrow in the figure below) to access to workflow.



Step 3: Upload the annotation file. This is made using the experimental setup.

An example can be seen below taken from a benchmarking two whole proteome (yeast spiked into human) TMT10 phospho-proteome experiment. Here each TMT label is associated with a separate condition. For example, ten implies Human: Yeast of 1:1, four implies Human: Yeast of 1:0.4 and one implied Human: Yeast of 1:0.1. You can also hover over question mark for more details. Click on the "Sample File" in blue to download this example annotation file.



Step 4: Choose the conditions you want to compare in a pairwise fashion. And choose the direction of comparison. So, choosing comparison ('four','ten') and direction (Condition_2/Condition_1) means the following comparison will run – (ten/four).

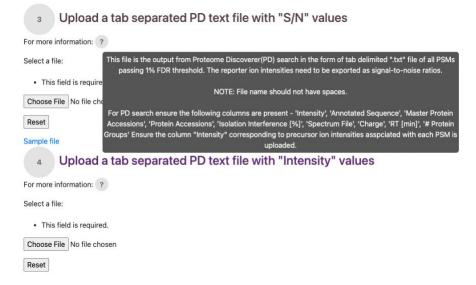
2	Select the Comparison You Would Like to Perform
Choose a	a comparison
○('one', '	four')
○('one', '	ten')
$\bigcirc \text{('four',}$	'ten')
Choose t	the direction for comparison:
○(Condit	ion_1/Condition_2)
○(Condit	ion_2/Condition_1)
Reset	

Step 5: Upload the input files (PSM list) for quantification.

For SEQUEST in Proteome Discoverer search, upload a tab separated file of PSM (filtered at 1% FDR or filter cutoff of choice) and associated reporter ion abundance (per TMT channel). The abundances must be exported as signal-to-noise as well raw reporter intensities. Make sure the files have the following columns – 'Intensity', 'Annotated

Sequence', 'Master Protein Accessions', 'Protein Accessions', 'Isolation Interference [%]', 'Spectrum File', 'Charge', 'RT [min]', '# Protein Groups'<mark>.</mark>

As before hover over question mark for more information. Click on the "Sample File" in blue to download this example PSM files.



The example has been down sampled to 500 proteins from a real benchmarking phosphoproteome experiment and looks like the screenshot below.

Observed	70	C	D			G H		n Donale Base A	K L	-	M	N N	0		P	Q	R	S	D-11-M/	D-14-
Checked	Confidence	Identifying No				Modification # Prote			Missed Cle Charge			DeltaCn	Rank	Sear	cn Engin	m/z [Da]	MH+[Da]	Theo. MH+ [D		
FALSE	High				[K].gRAEGEW		3 Q9NYF8	Q9NYF8-3; Q	1	3	0.1318		0	1	- 1	928.42222	and the same and t		-2.91	- 3
FALSE	High				[K].IENESLPN		1 Q05024	Q05024	0	2	0.437		0	1	- 1		1481.70973		0.75	
FALSE	High				(K).gSLEEIIAE		1 P02400	P02400	0	3	0.5211		0	1	- 1		1731.98862		-0.39	
FALSE	High				[K].dVPPLSET		2 P27816	P27816; P278	0	3	0.3436		0	1	- 1		2231.19827		0.29	0
FALSE	High				[K].aFGPGLQ		2 P21333	P21333; P213	1	3	0.084		0	1	1		2673.38018		0	
FALSE	High				I [K].gGITTGDI:		1 Q06704	Q06704	0	3	0.0516		0	1	- 1		3254.48767		-0.02	
FALSE	High				[K].dNEELQEI		1 P25037	P25037	0	4	0.4519		0	1	1	632.05695			-0.02	
FALSE	High				[K].tQsSESRL		1 P53885	P53885	1	3	0.0355		0	1	- 1		2623.29064		0.08	7.
FALSE	High				[K].gFSWADt		3 Q14847	Q14847-2; Q	1	3	0.2827		0	1	- 1		2198.19942		0.35	
FALSE	High				[K].cTGGGIIL		1 P33401	P33401	0	3	0.0949		0	1	- 1	972.82841	2916.47067		0.55	
FALSE	High				F [K].IEEVLsPEC		2 Q9UEY8	Q9UEY8-2; Q	0	3	0.5768		0	1	- 1		1989.95861	1989.95873	-0.06	
FALSE	High				[K].vQGEAVSI		3 Q13765	Q13765; E9P.	0	4	0.0284		0	1	1	1100.52038	4399.05967	4399.06016	-0.11	-0
FALSE	High				[K].vQISPDsG		1 Q92945	Q92945	1	4	0.0028		0	1	- 1		3176.66263		0.69	
FALSE	High	Sequest HT (Unambiguou	DIDSEDEQU	[K].dIDsEDEC	N-Term(TMT€	1 P40498	P40498	0	3	0.3711		0	1	- 1	1015.12651	3043.36497	3043.36103	1.3	
FALSE	High	Sequest HT (Unambiguou	SPASDTYIVE	([K].sPASDTYI	N-Term(TMT€	3 Q13765	Q13765; E9P.	0	3	0.3956		0	1	- 1	648.35564	1943.05236	1943.05261	-0.13	-8.
FALSE	High	Sequest HT (Unambiguou	VQGEAVSNI	[K].vQGEAVSI	N-Term(TMT6	3 Q13765	Q13765; E9P.	0	4	0.0678		0	1	1	1100.52135	4399.06358	4399.06016	0.78	0
FALSE	High	Sequest HT (Unambiguou	NPDEDEFLIN	[K].nPDEDEF	N-Term(TMT6	1 Q08412	Q08412	0	3	0.5033		0	1	- 1	703.28043	2107.82674	2107.82524	0.71	
FALSE	High	Sequest HT (Unambiguou	SEADPEGLS	[K].sEADPEGI	N-Term(TMT6	1 Q06685	Q06685	0	3	0.0627		0	1	- 1	1047.84003	3141.50553	3141.49892	2.1	
FALSE	High	Sequest HT (Unambiguou	DESEDLINSE	[K].dFSEDLN:	N-Term(TMT€	1 Q04773	Q04773	0	3	0.4485		0	1	- 1	618.95693	1854.85625	1854.8563	-0.03	-2.
FALSE	High	Sequest HT (Unambiguou	RQSSTPSAPE	[K].rQSStPSA	N-Term(TMTE	4 Q93100	Q93100-2; Q	1	4	0.0019		0	1	1	773.89313	3092.55068	3092.54541	1.7	0
FALSE	High	Sequest HT (a	Unambiguou	ALGVISNEQS	[K].aLGVISNF	N-Term(TMT6	2 094876	094876-2; 0	0	3	0.0322		0	1	1	629.34419	1886.01801	1886.01889	-0.46	-0
FALSE	High	Sequest HT (a	Unambiguou	LFESDEEETN	[K].IFEsDEEE	N-Term(TMT€	1 Q04500	Q04500	0	3	0.1309		0	1	1	883.08927	2647.25326	2647.25404	-0.3	-0
FALSE	High	Sequest HT (Unambiguou	IENQQGVNN	[K].iENQQGV	N-Term(TMT€	1 P00549	P00549	0	3	0.509		0	1	1	740.40721	2219.20708	2219.20722	-0.06	-5.
FALSE	High	Sequest HT (a	Unambiguou	TGPFEVGVH	(K].tGPFEVG\	N-Term(TMTE	1 P39954	P39954	0	3	0.6026		0	1	1	640.02466	1918.05941	1918.06036	-0.49	-0
FALSE	High	Sequest HT (Unambiguou	YSDVEVPASV	[K].ySDVEVPA	N-Term(TMT6	6 P35611	P35611-2; P3	1	4	0.0528		0	1	1	1023.22873	4089.89309	4089.89416	-0.26	-0
FALSE	High	Sequest HT (a	Unambiguou	VQGEAVSNI	[K].vQGEAVSI	N-Term(TMT6	3 Q13765	Q13765; E9P.	0	4	0.0603		0	1	- 1	1100.51964	4399.05674	4399.06016	-0.78	-0

For COMET search after Peptide-Prophet and Libra, upload a tab separated file of PSM (filtered at 0.9 probability or the probability associated with FDR cutoff choice) and associated reporter ion abundance (per TMT channel). Make sure the files have the following columns –

"spectrum","precursor_intensity","peptide","assumed_charge","retention_time_sec". The second screenshot is of the down-sampled version of COMET search for the same benchmarking dataset.

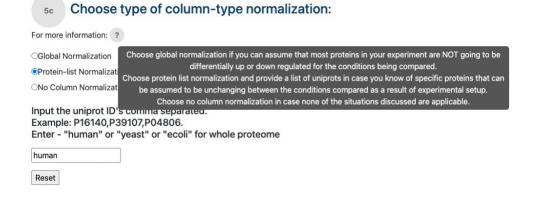
As before hover over question mark for more information. Click on the "Sample File" in blue to download this example PSM files.

Upload a tab separated COMET search output text file after libra quantification

For more information: ? Select a COMET output file: · This field is required. Choose File No file chosen Reset Sample file probability spectrum 0.9448 20171106_LL expect ions peptide protein
0.00218 ######## K.n[230.17]M sp|P07910|F 966.006 3618.04 2007.639 9494.182 2017.693 4901.669 1827.8882 0.9973 20171106_LL 4.43E-05 8/1/40 0:00 K.n[230.17]R. sp | P32583 | 5 606.615 3567.901 367.212 3441.394 709.602 10079.5 0.9288 20171106_LU 1.98 ######## K.n[230.17]C sp | Q07959| 1650.6702 2339.931 5174.733 374.255 2224.98 5097.731 3206.368 6765.972 3335.423 0.9318 20171106 LL 2046.9149 634.643 434.694 2371.54 2003.515 5459 5685 5738 5905 5963 6254 2167.8464 2578.1646 2247.8128 1875.8882 1025.82 2410.963 5687.127 1878.312 3472.387 2859.389 575.893 2886.854 0.9985 20171106 LL 1350.7426 12392.292 23682.845 5090.873 10375.126 29239.514 4806.313 11313.255 31869.045 16375.791 0.9679 20171106 LL 0.381 ######## K.n[230.17]R sp | P42858 | 1452.7856 4799.673 6137.46 4794.658 4784.563 5137.077 4386.058 6006.882 6327.818 4765.245 5223.074 1452.7856 2514.2684 2491.0576 1240.4968 2389.1463 1581.8758 0.9998 20171106 LU 583.999 3556,573 6732.023 576.838 3124.037 6679,552 3071.261 0.9994 20171106_LL 0.9961 20171106_LL 0.9818 20171106_LL 0.9955 20171106_LL 16872.066 21128.106 4258.921 0.9993 20171106 LL 2571.0239 7342.345 9793.976 9070.667 11349.497 9095.361 4871.407 11914.112 11098.983 9975.032 5877.363 14651.612 6995.473 0.8989 20171106 LL 0.228 ######## Ln[230.17]SF sp | O3E752 1252.6016 2382.26 7215.687 12857.378 14052.246 2384.258 8550.36 0.8989 20171106_LL 0.9824 20171106_LL 0.9994 20171106_LL 0.9881 20171106_LL 1 20171106_LL 1 20171106_LL 0.494 9/1/68 0:00 Kn[230.17]G spl [06244] k 6.66E-06 9/1/56 0:00 Kn[230.17]G spl [06512] l 0.15 ####### Kn[230.17]E spl [08945] 2.85E-10 20/56 Kn[230.17]R spl [08I221] F 2114.491 528.652 7002.826 8304.754 2450.1216 411.376 2741.554 2123.989 1735.8184 2121.0621 1733.8126 3262.26 1081.948 5586.897 6018.396 474.057 9287.77 9042.338 K.n[230.17]R sp|Q8IZ21|F K.n[230.17]Si sp|P07910|F 6.91E-06 17/20 71392.832 52282.546 77067.242 64354.653 67517.836 75329.49 68417.097 0.9927 20171106_LL 1.31E-15 36/84 K.n[230.17]Si sp | Q9BXW9 2914.2065 5723.859 6238.138 5279.221 5361.244 5437.253 5679.013 6227.484 5283.838 5149.322 2170.1778 1661.8421 1733.8126 1487.7539 0.9967 20171106 LL 12358.819 11809.062 10859.01 13252.456 15319.129 13949.562 15464.081 13786.604 14788.901 0.9978 20171106 LL 4485 204 8016.051 6629 109 6917.283 6507.02 ark TMT phosproteome COME

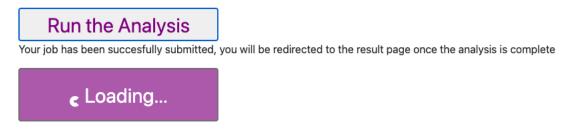
Step 6: You can modify settings (using fractions, doing row normalization and type of column normalization) for your specific analysis. Recommendation is to use the default settings. Hover over question mark for more information.

For the example dataset choose "Protein-list Normalization" for type of column type normalization. Put "Human" in the text box.

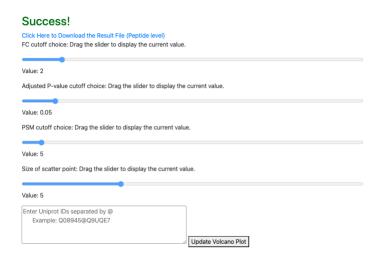


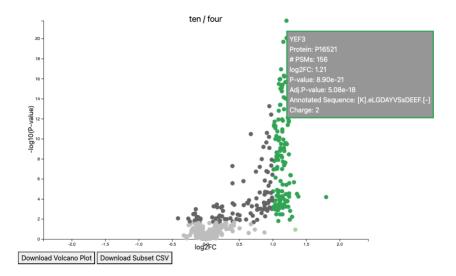
Step 8: Choose whether to do imputation on your data or not. Recommendation is to not to not introduce biases.

A successful start of run with look like this -



The output page (given a successful run) looks like the screenshots below. You can download the protein level and peptide level differential expression analysis results as well as the pdf of resulting volcano plot (set at 5 PSM cutoff). You can highlight specific proteins on the volcano plot by entering the associated Uniprot IDs. You can dynamically set the thresholds and download the resulting file, as well as subset the result file with user-defined thresholds. –





The output file (peptide) looks like this -

A	В	C	D	E	F	G	H	1	J	K	L	М	N	0 P	Q	R	S	T
UniquePeptide	log2FC	FC	pval	adjpval	126	127C	127N	128C	128N	129C	129N	130C	130N	AnnotatedSe Charge	Protein	Proteinnoise	Gene Symbol	# PSMs
K.n[230.17]LSLEGDHSTPPS	-0.0151519	0.98955245	0.69418808	0.80714298	0.00011983	0.000115	0.00011527	0.00010307	0.00010537	0.00010954	0.00011165	0.00010969	0.00011538	K.n[230.17]L5	3 A6NMY6	A6NMY6	ANXA2P2	
K.n[230.17]IEEVLSPEGS[167	-0.0276467	0.98101919	0.54547607	0.73871733	0.00011551	0.00010303	0.00010802	0.00011066	0.00011826	0.00012398	0.00010458	0.00010597	0.00011383	K.n[230.17]IE	3 Q9UEY8	Q9UEY8	ADD3	
K.n[230.17]REAALPPVS[167.	0.14054817	1.10232388	0.23399348	0.53573267	0.00010754	0.00011873	0.0001069	0.00010075	0.00011147	0.00010726	0.00011561	0.00011935	0.00011374	K.n[230.17]R	3 Q04637	Q04637	EIF4G1	
K.n[230.17]YQEDS[167.00]E	-0.0434905	0.97030453	0.62491922	0.76892609	0.00011827	0.00011291	0.00011557	0.00011518	0.00010627	0.00011171	0.00010461	0.00011176	0.00010948	K.n[230.17]YI	3 Q6P6C2	Q6P6C2	ALKBH5	
K.n[230.17]FNADEFEDM[14	0.18205864	1.1345016	0.2871813	0.59232555	9.85E-05	0.00011408	0.00011906	0.00010464	9.46E-05	0.0001137	0.00013262	0.00011743	0.00010667	K.n[230.17]FI	3 P27635	P27635	RPL10	
K.n[230.17]EELQANGSAPAA	-0.1694464	0.88918384	0.12082854	0.36471159	0.0001191	0.00010225	0.00010568	0.00011457	0.00011934	0.00013032	0.00010209	9.40E-05	0.0001166	K.n[230.17]EI	4 P29966	P29966	MARCKS	
K.n[230.17]SPREVPS[167.00	0.0150592	1.01049291	0.28903308	0.59380895	0.00013482	0.00012502	0.00011304	0.00010345	8.87E-05	0.00010447	0.00011433	0.00010653	0.00011227	K.n[230.17]SI	3 Q9Y6X9	Q9Y6X9	MORC2	
K.n[230.17]RINPPSSGGTSS	-0.0629627	0.95729618	0.39463211	0.6695844	0.00011628	0.00010756	0.00011286	0.00011597	0.00011579	0.00011216	0.00010819	0.0001037	0.00011259	K.n[230.17]R	3 P14859	P14859	POU2F1	
K.n[230.17]M[147.04]VIQGF	0.79180724	1.73124181	0.0013531	0.00866213	7.02E-05	0.00017683	9.81E-05	0.00010761	6.65E-05	7.09E-05	0.00015901	0.00016382	9.67E-05	K.n[230.17]M	4 Q12888	Q12888	TP53BP1	
K.n[230.17]ADEPS[167.00]S	0.12345104	1.08933753	0.29377747	0.59979566	0.00010828	0.00010986	0.00010476	0.00011199	0.00011537	0.00010173	0.00011858	0.00012237	0.00010854	K.n[230.17]Al	3 P50502	P50502	ST13	
K.n[230.17]GGPEGVAAQAVA	-0.0069792	0.99517406	0.96111872	0.97112073	0.00011687	0.00010907	0.00010853	0.00010589	0.0001217	9.69E-05	0.00010475	0.0001203	0.00012088	K.n[230.17]G	5 060264	060264	SMARCA5	
K.n[230.17]SS[167.00]ILLDV	0.05615117	1.03968837	0.87647841	0.91527139	0.00010455	9.72E-05	0.00012023	0.00010679	0.00011451	0.00010641	0.00012501	0.00011877	0.00010801	K.n[230.17]St	2 P24534	P24534	EEF1B2	
K.n[230.17]LAAVPAAGPASAG	1.22743626	2.34150523	0.00070191	0.005069	1.96E-05	0.00018328	7.83E-05	0.00010454	4.61E-05	3.48E-05	0.00022231	0.00022871	9.42E-05	K.n[230.17]L/	5 P05319	P05319	RPP2A	- 1
K.n[230.17]RSEDES[167.00]	-0.0196911	0.98644392	0.80075168	0.86763131	0.00011448	0.00010848	0.00010882	0.00011028	0.00011476	0.00012179	0.00010563	0.00011021	0.00010997	K.n[230.17]R	3 P25205	P25205	мсмз	
K.n[230.17]DRTTSFFLNS[16	-0.1387506	0.9083054	0.22924211	0.53129099	0.00012241	0.00010261	0.00011009	0.00010939	0.00012828	0.00011843	9.86E-05	0.00010871	0.0001092	K.n[230.17]D	3 Q8WYP5	Q8WYP5	AHCTF1	
K.n[230.17]GILAADES[167.0	0.16076055	1.1178763	0.406407	0.67535989	0.00011302	9.69E-05	0.00010921	0.00011621	0.00010138	0.00011058	0.00012888	0.00011943	0.00011481	K.n[230.17]G	2 P04075	P04075	ALDOA	
K.n[230.17]ENDTVTIS[167.0	0.02285288	1.01596653	0.77373086	0.85403598	0.00012185	9.55E-05	0.0001071	0.0001167	0.00011859	0.00011933	0.00011731	0.00011295	9.40E-05	K.n[230.17]EI	2 Q9UGU0	Q9UGU0	TCF20	
K.n[230.17]S[167.00]FEDRV	0.15087883	1.11024558	0.16888043	0.44780843	0.00011214	0.00011882	0.00010462	0.00010586	0.00010665	0.00010802	0.00012002	0.00012021	0.00010886	K.n[230.17]S[3 O43399	O43399	TPD52L2	
K.n[230.17]K[357.26]EQT[18	-0.1821911	0.88136338	0.45457262	0.69936832	8.92E-05	0.00011549	0.00013247	0.00015566	0.0001167	8.72E-05	8.16E-05	0.00013971	9.26E-05	K.n[230.17]K]	4 076021	076021	RSL1D1	
K.n[230.17]LYERRGGGSGG	0.03475782	1.02438485	0.53821326	0.73629977	0.00011867	0.00011627	0.00011677	8.49E-05	0.00011015	0.00010837	0.00011375	0.00011424	0.00011541	K.n[230.17]L)	3 Q96QR8	Q96QR8	PURB	
K.n[230.17]ERRT[181.01]PS	-0.0122774	0.99152604	0.71488649	0.8178868	0.00011143	0.00010857	0.00011288	0.00010774	0.00011132	0.00010606	0.00011201	0.00010979	0.00011296	K.n[230.17]EI	3 Q641Q2	Q641Q2	WASHC2A	
K.n[230.17]AM[147.04]ADEI	-0.1129583	0.92469003	0.31689754	0.6171968	0.00010332	0.00010432	0.00011647	0.00011353	0.00011925	0.00011689	0.00010418	0.00011291	0.00011266	K.n[230.17]Al	2 Q03135	Q03135	CAV1	
K.n[230.17]GNAGGS[167.00	1.19063848	2.28253737	0.0001176	0.00166639	3.13E-05	0.00021281	9.81E-05	9.88E-05	2.69E-05	2.87E-05	0.00022908	0.00020556	8.64E-05	K.n[230.17]G	3 P32578	P32578	SIP1	
K.n[230.17]DRSEEERTTEAER	0.13735937	1.09989009	0.55016194	0.74003225	7.50E-05	9.68E-05	7.47E-05	0.0001626	6.38E-05	0.00020068	0.00017884	7.57E-05	7.30E-05	K.n[230.17]D	4 P26038	P26038	MSN	
K.n[230.17]RSYS[167.00]LSI	0.52165869	1.43560484	0.00151251	0.0094971	7.62E-05	0.00015401	0.00010044	0.00010228	8.92E-05	8.22E-05	0.0001442	0.00015219	0.00010956	K.n[230.17]R	3 Q9P0V3	Q9P0V3	SH3BP4	
K.n[230.17]MSQPGS[167.00	-0.0385329	0.97364455	0.73076951	0.82709014	0.00011952	0.00010514	0.00011508	0.00010492	0.00011232	0.00011808	0.00010739	0.00011064	0.0001103	K.n[230.17]M	2 Q14839	Q14839	CHD4	
K.n[230.17]DS[167.00]ASEE	0.31947955	1.2478803	0.26363329	0.56804221	9.72E-05	0.00013755	9.75E-05	0.00010741	0.00010541	0.00010657	0.00012124	0.00012865	0.00010552	K.n[230.17]D	3 Q9NX58	Q9NX58	LYAR	
K.n[230.17]DTIIDVVGAPLT[1	-0.0924194	0.93794848	0.25238634	0.55501758	0.00011566	9.87E-05	0.0001091	0.0001159	0.00011625	0.00010449	0.00011868	0.00011229	0.00011103	K.n[230.17]D	4 Q9NPF5	Q9NPF5	DMAP1	
K.n[230.17]EHEADTANM[14	0.21857751	1.16358573	0.55035635	0.74003225	7.81E-05	0.00012662	8.70E-05	0.00011978	0.00014237	6.54E-05	0.00014322	0.00012203	0.000116	K.n[230.17]EI	3 Q9BXJ9	Q9BXJ9	NAA15	
K.n[230.17]HFELRVNVIDTP	1.17870877	2.26374079	0.00012371	0.00168501	2.99E-05	0.00020668	8.95E-05	9.54E-05	3.12E-05	2.87E-05	0.00021792	0.00021416	9.38E-05	K.n[230.17]H	4 P32468	P32468	CDC12	
K.n[230.17]FAC[160.03]QID	1.21549885	2.32221065	3.74E-05	0.00144962	2.87E-05	0.00021267	9.16E-05	9.08E-05	3.15E-05	2.76E-05	0.00021649	0.00021538	9.80E-05	K.n[230.17]F/	3 P06105	P06105	SCP160	
K.nf230.17lGYSf167.00lPPF	-0.0964048	0.93536103	0.23009148	0.53261352	0.00012883	0.00011444	0.0001223	0.00011225	0.00011085	9.97F-05	9.84F-05	0.00010667	0.00011405	K.n[230.17]G	3 014669-3	014669	TRIP12	