Supplementary Material

1. High-Throughput in-Gel Digestion—Technical Considerations

Gel-cutting tips generate gel plugs with a standard size and shape which can be handled easily and reliably during the high-throughput tryptic digest protocol. Tips are reusable and both easier and safer than using a blade. The advantages of 96-well microtiter plates are manifold. Twelve channel pipettes virtually eliminate the risk of pipetting errors, and sample error-prone labelling is also eliminated. Autosampler capacity is increased from 120 vials to 288 wells. The typical cost of disposable polypropylene vials and caps is US$1.29 per sample which compares with around US$0.03 per sample for a (full) 96-well plate with seal. A 5 μL pick-up can be reliably performed from a 5 μL sample volume which has been held at 10 °C in a sealed microtiter plate for several days. Plate seals with an adhesion-free zone are essential to prevent the plate lifting when the autosampler needle withdraws. We have found that sample to sample contamination does not occur and we assume that protein remains fully immobilized in the gel plug until trypsin is added. Tips may therefore be re-used throughout every stage of the in-gel digestion protocol excepting the final step following tryptic digestion, representing an additional cost saving. Rainin tips with a narrower orifice than standard 200 μL tips lower the chance of gel plugs fouling the tip during the aspiration cycle. Gentle wiggling of the tips prior to aspiration is also helpful. Close manual inspection of the tips and wells following aspiration is essential, especially following dehydration when the acrylamide plugs become sticky.

2. High-Throughput LC-MSMS—Technical Considerations

LC-MSMS sample runs should always be preceded by a blank run to identify whether significant sample carryover has occurred. In our laboratory, the blank is “spiked” with 1 fmol BSA tryptic digest. This is sufficient to obtain a Mascot hit for BSA reliably without interfering with the blank’s original purpose. This standard/blank injection enables the LC-MSMS performance to be monitored continuously throughout the batch. We have found DVB monolith columns have a useful life of between 5000 and 7000 injections, which equates to $0.29 per sample. Capillary HPLC columns such as this, working at a flow rate of 2.5 μL/min are used with the standard ESI source which can operate continuously for weeks or even months without user intervention. In contrast, nanospray emitter tips have a short life and are a significant consumables cost. The high flow rate allows an injection to injection cycle time of 16 min, compared to a 45 min for the equivalent separation using a 75 μm nano HPLC column. The inclusion of blanks between each sample allows 45 LC-MSMS analyses per day. Although theoretically nano LC-MSMS is more sensitive, in our laboratory we have found the sensitivity of capillary LC-MSMS to be at least as good, if not better.

3. MSMS Data Analysis and Annotation

The optimum MOWSE score for high-throughput analysis is between 200 and 500. Lower scores require more time for manual data inspection to confirm their validity. While scores higher than 500 make no difference to the confidence level of protein identifications, they can cause significant data interpretation difficulties for subsequent samples due to carryover, and reduce the lifetime of the HPLC column. Initial analysis using only 2% of the available digest along with dilution of heavily stained samples is done with this aim in mind. Serial searches of (internal) construct and (external) Uniprot

databases are advantageous and complementary. The MOWSE scores returned from databases of different size require different interpretation. The relatively small constructs database is more sensitive, whereas the much larger Uniprot database is less sensitive but more stringent. Simple annotation and color coding may initially appear unnecessary, however it should be remembered that users with limited experience of mass spectrometry appreciate results in an unambiguous format which can be quickly understood. The use of the proteomics term "hit", where any detected protein is a valid result, is deliberately avoided. This is because here distinction has to be made between identification of the chosen expressed protein (the "target" protein) and any other proteins which may be present.

4. Failed MSMS Analyses

The presence of a visible Coomassie stained band indicates that sufficient protein is present for successful analysis; hence, failure to detect protein is a failure of the analysis. The two most common reasons for this are adhesion of the dehydrated gel plug to the wall of the PCR plate well, such that it fails to contact the trypsin solution; or insufficient protein for detection. The former case is apparent upon visual inspection and re-digestion is carried out. The latter case is apparent when the plate is dried in a rotary evaporator, the samples re-suspended in 5 μL of loading buffer and the remaining digest is reanalysed. "Spent" gel plugs should not be discarded immediately. In-gel tryptic digestion and peptide elution does not generally proceed to completion, such that secondary tryptic or chymotryptic digestion and subsequent LC-MSMS analysis may be performed which generates results almost as good as those from the initial digest.

5. Complete 96-Well Data Set

See Table S1.

Table S1. Combined SDS-PAGE, intact mass and tryptic digest MSMS data for 1 mL test expressions for an entire 96-well experiment. Corresponding gel images for IMAC elutions and total protein are shown in Figure S1.

Sample Description					Intact Mass Analysis				MSMS Analysis Ni Elution		MSMS Analysis Total Protein	
Construct	**N or C Terminal Tag**	**Elution Yield**	**Plate Well**	**Expected Mass**	**Observed Mass**	**Delta M**	**Relative Intensity ×10³**	**Comments**	**MOWSE Score**	**Identity**	**MOWSE Score**	**Identity**
HDAC6 (1–1215)	N	0: None	A01	159,715							788	HDAC6
HDAC6 (1–844)	N	0: None	A02	121,265					105	HDAC6	1036	HDAC6
HDAC6 (1–835)	N	0: None	A03	120,221							1250	HDAC6
HDAC6 (1–801)	N	0: None	A04	116,345							1288	HDAC6
HDAC6 (1–433)	N	3: Medium	A05	75,960.2	not found						273	HDAC6
HDAC6 (1–406)	N	3: Medium	A06	73,260	not found						1158	HDAC6
HDAC6 (85–1215)	N	0: None	A07	150,686							293	HDAC6
HDAC6 (85–844)	N	0: None	A08	112,235							1121	HDAC6
HDAC6 (85–835)	N	0: None	A09	111,191							1145	HDAC6
HDAC6 (85–801)	N	0: None									1134	HDAC6
HDAC6 (85–433)	N	1: Low	A11	66,930.9	not found						915	HDAC6
HDAC6 (85–406)	N	3: Medium			not found						915	HDAC6
HDAC6 (478–1215)	N	0: None	B01	106,990							1039	HDAC6
HDAC6 (478–844)	N	0: None	B02	65,39.7							1104	HDAC6
HDAC6 (478-835)	N	0: None	B03	67,495.5							1104	HDAC6
HDAC6 (478–801)	N	0: None	B04	63,619.9							1079	HDAC6
HDAC7 (483–903)	N	3: Medium	B05	73,747.4	not found						333	HDAC7
HDAC8 (1–286)	N	1: Low	B06	70,036.4	not found						789	HDAC8
GST+peptide	N	5: High	B07	31,264.9	30,345.75	-919.15	500	Truncation?	896	GST	576	GST
NDEL1 (1–345)	N	0: None	B08	68,929.1	29,604		650		777	GST	577	GST
NDEL1 (1–321)	N	0: None	B09	64,519.1					558	Keratin	110	NDEL1
No construct												
NDEL1 (1–310)	N	0: None	B10	63,219.7								
No construct												
NDEL1 (13–345)	N	0: None	B11	67,752.9							1832	NDEL1

Table S1. *Cont.*

Sample Description					Intact Mass Analysis				MSMS Analysis Ni Elution		MSMS Analysis Total Protein	
Construct	**N or C Terminal Tag**	**Elution Yield**	**Plate Well**	**Expected Mass**	**Observed Mass**	**Delta M**	**Relative Intensity ×10^3**	**Comments**	**MOWSE Score**	**Identity**	**MOWSE Score**	**Identity**
NDEL1 (13–321)	N	0: None	B12	63,342.9					94	Keratin	523	GST
NDEL1 (13–310)	N	0: None	C01	62,043.5					715	GST	1584	NDEL1
No construct												
SIRT2 (34–389)	N	1: Low	C03	68,313.8	not found				44	No hit	1470	SIRT2
SIRT2 (34–356)	N	1: Low	C04	64,915.1	not found				207	SIRT2	1670	SIRT2
SIRT2 (38–389)	N	3: Medium	C05	67,810.3	not found				424	SIRT2	1471	SIRT2
SIRT2 (38–356)	N	1: Low	C06	64,411.6	not found				532	SIRT2	1471	SIRT2
BLM (858–1298)	N	5: High	C07	52,750.2	not found				518	BLM	1304	BLM
BLM (994–1298)	N	0: None	C08	37,273.5							967	BLM
BLM (1069–1298)	N	5: High	C09	28,308.3	28309.04	0.74	2400	Intact mass	1221	BLM	92	BLM
LACTB2 (26–288)	N	0: None	C11	32,641.2							877	LACTB2
LACTB2 (29–288)	N	0: None	C12	32,426.9							979	HDAC6
HDAC6 (1–1215)	N	0: None	D01	13,3973							1164	HDAC6
HDAC6 (1–844)	N	0: None	D02	95,522.7							1148	HDAC6
HDAC6 (1–835)	N	0: None	D03	94,478.5							372	HDAC6
HDAC6 (1–801)	N	0: None	D04	90,602.9							742	HDAC6
HDAC6 (1–433)	N	0: None	D05	50,218.2							888	HDAC6
DAC6 (1–406)	N	1: Low	D06	47,518	not found						112	HDAC6
HDAC6 (85–1215)	N	0: None	D07	124,944							900	HDAC6
HDAC6 (85–844)	N	0: None	D08	86,493.4							1172	HDAC6
HDAC6 (85–835)	N	0: None	D09	85,449.2							1035	HDAC6
HDAC6 (85–801)	N	0: None	D10	81,573.6							799	HDAC6
HDAC6 (85–433)	N	3: Medium	D11	41,188.9	not found				240	HDAC6	578	HDAC6
HDAC6 (85–406)	N	3: Medium	D12	38,488.7	not found				58	FKBP Ecoli	561	HDAC6
HDAC6 (478–1215)	N	0: None	E01	81,248.4							519	HDAC6
HDAC6 (478–844)	N	0: None	E02	42,797.7							543	HDAC6
HDAC6 (478–835)	N	0: None	E03	41,753.5							571	HDAC6
HDAC6 (478–801)	N	0: None	E04	37,878							220	HDAC6

Table S1. *Cont.*

Sample Description					Intact Mass Analysis				MSMS Analysis Ni Elution		MSMS Analysis Total Protein	
Construct	N or C Terminal Tag	Elution Yield	Plate Well	Expected Mass	Observed Mass	Delta M	Relative Intensity ×10^3	Comments	MOWSE Score	Identity	MOWSE Score	Identity
HDAC7 (483–903)	N	0: None	E05	48,005.4							314	HDAC7
HDAC8 (1–286)	N	3: Medium	E06	44,294.4	not found				122	HDAC8	564	HDAC8
No construct												
NDEL1 (1–345)	N	5: High	E08	41,900.7	44011.52	2110.82	310	Unidentified	1341	NDEL1	1466	NDEL1
NDEL1 (1–321)	N	3: Medium	E09	38,777.1					697	NDEL1	391	NDEL1
No construct												
NDEL1 (1–310)	N	5: High	E10	37,477.7	not found				1071	NDEL1	1241	NDEL1
NDEL1 (13–345)	N	5: High	E11	40,724.5	42,836	2111.5	7	Unidentified	1487	NDEL1	1306	NDEL1
NDEL1 (13–321)	N	5: High	E12	37,600.9	not found				1089	NDEL1	61	NDEL1
NDEL1 (13–310)	N	5: High	F01	36,301.5	not found				1272	NDEL1		
No construct												
SIRT2 (34–389)	N	1: Low	F03	42,571.9					299	SIRT2	1011	SIRT2
SIRT2 (34–356)	N	1: Low	F04	39,173.1					144	SIRT2	951	SIRT2
SIRT2 (38–389)	N	1: Low	F05	42,068.3					191	SIRT2	1158	SIRT2
SIRT2 (38–356)	N	0: None	F06	38,669.6							841	SIRT2
cobB (1–279)	N	5: High	F07	34,016.9					802	COB1	718	COB1
cobB (1–274)	N	5: High	F08	33,617.4	33,618.47	1.07	260	Intact mass	837	COB1	785	COB1
cobB (40–279)	N	5: High	F09	29,140.1	29,140.27	0.17	4100	Intact mass	756	COB1	609	COB1
cobB (40–274)	N	5: High	F10	2840.6	28,741.33	0.73	2200	Intact mass	662	COB1	655	COB1
RPS27A (1–76)	N	5: High	F11	11,117.6	11,118.01	0.41	26,000	Intact mass	206	RPS27A		no hit
SSBP1 (17–148)	N	5: High	F12	17,879.1	17,879.64	0.54	3600	Intact mass	834	SSBP1	726	SSBP1
cobB (1–279)	N	5: High	G01	59,758.8	not found				1294	COB1		
cobB (1–274)	N	0: None	G02	59,359.4								
cobB (40–279)	N	5: High	G03	54,882.1	54,898.27	16.17	110	Oxide?	911	COB1	1035	COB1
cobB (40–274)	N	5: High	G04	54,482.6	not found				1031	COB1	996	COB1
BLM (858–1298)	C	5: High	G05	52,968.3	not found				569	BLM	1023	BLM
BLM (994–1298)	C	0: None	G06	37,491.6	14,830.26	-22661.3	900	Unidentified				
No construct												
RPS27A (1–76)	C	1: Low	G08	8564.9	not found				236	BLM		

6. SDS-PAGE Analysis of the Remaining Samples from the 96-Well Test Expression

See Figure S1.

Figure S1. SDS-PAGE analysis of samples not shown in Figure 1 of the main text. IMAC elutions (15 µL) and total cell lysates (3 µL) were analysed by electrophoresis on 4%–12% polyacrylamide gradient gels and coomassie staining.