A multi-center study benchmarks software tools for label-free proteome quantification

Supplementary Information

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Supplementary Tables

Supplementary Table 1. Precision and accuracy of relative quantification.

Precision (standard deviation between technical replicates) and accuracy (absolute median deviation from expected log_2(A/B) values) on peptide and protein level for each tertile (1st: lowest intensity (0%-33.3%,), 2nd: medium intensity (33.3%-66.7%) and 3rd: highest intensity (66.7%-100%)). The asterisks at the second iteration panel of HYE124 stand for a significative improvement compared to the first iteration.

Precision and accuracy of the different software tools are compared to each other within the same species (color code: worst value in full red and best value in full green).

The following pages show the precision and accuracy of peptides and proteins for:

- Supplementary Table 1.A: sample set HYE124, TTOF5600_32fix, iteration 1 & 2
- Supplementary Table 1.B: sample set HYE124, TTOF5600_64var, iteration 1 & 2
- Supplementary Table 1.C: sample set HYE124, TTOF6600_32fix, iteration 1 & 2
- Supplementary Table 1.D: sample set HYE124, TTOF6600_64var, iteration 1 & 2
- Supplementary Table 1.E: sample set HYE110, TTOF6600_32fix
- Supplementary Table 1.F: sample set HYE110, TTOF6600_32var
- Supplementary Table 1.G: sample set HYE110, TTOF6600_64fix
- Supplementary Table 1.H: sample set HYE110, TTOF6600_64fix

Precision of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.305	0.448	0.311	0.270	0.562	0.472	0.271*	0.363	0.307	0.508*
		2nd.	0.226	0.286	0.270	0.221	0.362	0.335	0.214*	0.312	0.233	0.367
		3rd.	0.214	0.231	0.230	0.212	0.335	0.267	0.221*	0.248	0.197*	0.288*
	Yeast	1st.	0.430	0.629	0.383	0.464	0.672	0.577	0.277*	0.464	0.496	0.664
		2nd.	0.326	0.434	0.371	0.405	0.414	0.452	0.260*	0.429	0.387	0.358*
		3rd.	0.390	0.404	0.414	0.445	0.393	0.455	0.331*	0.582	0.429	0.405
	E.Coli	1st.	0.524	0.878	0.733	0.772	0.702	0.837	0.557*	0.893	0.796	0.696
		2nd.	0.525	0.641	0.504	0.526	0.690	0.657	0.365*	0.558	0.557	0.430*
		3rd.	0.453	0.440	0.388	0.410	0.539	0.459	0.341*	0.459	0.392	0.401*
Proteins	Human	1st.	0.233	0.271	0.268	0.225	0.356	0.274	0.302	0.311	0.258	0.350
		2nd.	0.207	0.160	0.220	0.177	0.296	0.239	0.235	0.240	0.180	0.236*
		3rd.	0.137	0.180	0.128	0.159	0.216	0.142	0.208	0.150	0.141*	0.246
	Yeast	1st.	0.458	0.393	0.475	0.458	0.419	0.425	0.436	0.495	0.475	0.530
		2nd.	0.386	0.282	0.438	0.490	0.387	0.430	0.319	0.365*	0.537	0.433
		3rd.	0.431	0.350	0.443	0.367	0.329	0.442	0.466	0.492	0.393	0.326
	E.Coli	1st.	0.701	0.632	0.795	0.956	0.657	0.842	0.576	0.863	1.042	0.539
		2nd.	0.468	0.456	0.518	0.544	0.441	0.527	0.489	0.668	0.666	0.560
		3rd.	0.349	0.330	0.346	0.238	0.350	0.318	0.259*	0.375	0.290	0.507

Accuracy of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.014	0.030	0.025	0.011	0.068	0.061	0.018	0.021	0.014	0.062
		2nd.	0.006	0.006	0.007	0.013	0.006	0.014	0.003	0.024*	0.012	0.013
		3rd.	0.005	0.010	0.012	0.003	0.033	0.017*	0.009	0.004	0.000	0.026
	Yeast	1st.	0.386	0.007	0.232	0.278	0.002	0.002	0.088*	0.074	0.055	0.115*
		2nd.	0.442	0.129	0.251	0.342	0.100	0.111	0.104	0.116	0.199	0.167*
		3rd.	0.266	0.111	0.142	0.190	0.164	0.076	0.100	0.111	0.125	0.198*
	E.Coli	1st.	0.936	0.535	0.832	0.800	0.492	0.367*	0.326*	0.487*	0.359*	0.603
		2nd.	0.802	0.302	0.510	0.634	0.310	0.185*	0.266	0.200*	0.357*	0.393
		3rd.	0.480	0.252	0.284	0.399	0.315	0.149*	0.237	0.271*	0.263*	0.360
Proteins	Human	1st.	0.008	0.021	0.006	0.007	0.036	0.008	0.022	0.011	0.007	0.011
		2nd.	0.012	0.012	0.005	0.013	0.008	0.007	0.003	0.030	0.010	0.014
		3rd.	0.006	0.003	0.001	0.006	0.009	0.000	0.003	0.021	0.012	0.010
	Yeast	1st.	0.329	0.070	0.159	0.258	0.006	0.010	0.013	0.051	0.074	0.052
		2nd.	0.376	0.171	0.196	0.326	0.010	0.107	0.094	0.091	0.209	0.030
		3rd.	0.221	0.123	0.123	0.162	0.066	0.072	0.111	0.113	0.096	0.138
	E.Coli	1st.	0.919	0.649	0.785	0.716	0.141	0.393*	0.107*	0.456*	0.365*	0.232
		2nd.	0.713	0.489	0.338	0.527	0.058	0.203*	0.201*	0.195*	0.342*	0.197
		3rd.	0.478	0.286	0.300	0.369	0.164	0.184*	0.209*	0.322	0.254*	0.106

Supplementary Table 1.A: Precision and accuracy in HYE124, TTOF5600_32fix.

Precision of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.306	0.472	0.320	0.301	0.587	0.437	0.272 *	0.414	0.362	0.522 *
		2nd.	0.218	0.266	0.256	0.212	0.401	0.301	0.214 *	0.289	0.230	0.366 *
		3rd.	0.232	0.273	0.249	0.180	0.293	0.243	0.176 *	0.248	0.192	0.271 *
	Yeast	1st.	0.367	0.710	0.414	0.455	0.774	0.543	0.319 *	0.524	0.492	0.575 *
		2nd.	0.304	0.445	0.381	0.382	0.462	0.442	0.266 *	0.464	0.348 *	0.361 *
		3rd.	0.326	0.477	0.422	0.395	0.373	0.382	0.296 *	0.573	0.377 *	0.293 *
	E.Coli	1st.	0.549	0.953	0.763	0.720	0.825	0.770	0.624 *	0.819	0.708	0.712 *
		2nd.	0.452	0.737	0.523	0.429	0.716	0.534	0.319 *	0.498	0.496	0.539 *
		3rd.	0.372	0.457	0.349	0.301	0.582	0.390	0.287 *	0.352	0.331	0.393 *
Proteins	Human	1st.	0.159	0.192	0.229	0.182	0.440	0.218	0.212	0.246	0.210	0.431
		2nd.	0.155	0.166	0.163	0.134	0.280	0.182	0.173	0.233	0.123 *	0.284
		3rd.	0.125	0.101	0.125	0.103	0.209	0.136	0.125	0.141	0.121	0.211
	Yeast	1st.	0.309	0.378	0.432	0.367	0.591	0.372	0.429	0.456	0.382	0.474 *
		2nd.	0.271	0.298	0.353	0.346	0.458	0.267	0.321	0.416	0.336	0.367 *
		3rd.	0.244	0.355	0.379	0.307	0.261	0.253	0.272 *	0.434	0.375	0.272
	E.Coli	1st.	0.548	0.625	0.634	0.602	0.742	0.528	0.570	0.701	0.655	0.613
		2nd.	0.318	0.605	0.553	0.445	0.610	0.433	0.419 *	0.599	0.600	0.527
		3rd.	0.292	0.347	0.287	0.206	0.378	0.255	0.246 *	0.326	0.196	0.338

Accuracy of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.005	0.010	0.010	0.026	0.063	0.021	0.005	0.054	0.030	0.045
		2nd.	0.004	0.002	0.008	0.005	0.009	0.010	0.000	0.018	0.004	0.011
		3rd.	0.001	0.003	0.000	0.009	0.026	0.003	0.002	0.033*	0.009	0.025
	Yeast	1st.	0.305	0.068	0.230	0.185	0.088	0.012	0.104*	0.114	0.069	0.205*
		2nd.	0.325	0.096	0.253	0.220	0.187	0.070	0.095	0.068	0.060	0.198
		3rd.	0.173	0.086	0.130	0.163	0.194	0.056	0.075	0.071	0.082	0.225*
	E.Coli	1st.	0.765	0.427	0.857	0.646	0.607	0.320*	0.326	0.721*	0.209*	0.625
		2nd.	0.635	0.159	0.493	0.444	0.413	0.124*	0.240	0.193*	0.166*	0.415
		3rd.	0.362	0.214	0.286	0.331	0.391	0.135*	0.225	0.082*	0.189*	0.443
Proteins	Human	1st.	0.004	0.007	0.003	0.016	0.043	0.012	0.013	0.077	0.007	0.016
		2nd.	0.009	0.011	0.004	0.001	0.004	0.003	0.014	0.005	0.002	0.010
		3rd.	0.010	0.002	0.001	0.006	0.023	0.007	0.002	0.038*	0.002	0.014
	Yeast	1st.	0.297	0.068	0.185	0.211	0.085	0.049	0.049	0.029	0.003	0.019
		2nd.	0.312	0.142	0.219	0.202	0.088	0.097	0.059	0.038	0.073	0.048
		3rd.	0.146	0.090	0.115	0.139	0.130	0.059	0.082	0.098	0.082	0.123
	E.Coli	1st.	0.712	0.563	0.782	0.557	0.336	0.280*	0.246*	0.517*	0.191*	0.300
		2nd.	0.527	0.367	0.460	0.380	0.203	0.118*	0.161*	0.154*	0.178*	0.227
		3rd.	0.344	0.246	0.285	0.307	0.231	0.161*	0.206*	0.117*	0.201*	0.239

Supplementary Table 1.B: Precision and accuracy in HYE124, TTOF5600_64var.

Precision of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.338	1.180	0.316	0.307	0.666	0.511	0.325*	0.403	0.377	0.583*
		2nd.	0.279	0.388	0.288	0.268	0.449	0.329	0.274*	0.316	0.294	0.400*
		3rd.	0.280	0.286	0.255	0.231	0.347	0.294	0.230*	0.271	0.220*	0.339
	Yeast	1st.	0.439	0.903	0.373	0.458	0.765	0.631	0.300*	0.462	0.492	0.691*
		2nd.	0.344	0.629	0.376	0.436	0.492	0.481	0.274*	0.425	0.400*	0.419*
		3rd.	0.438	0.527	0.462	0.556	0.383	0.447	0.318*	0.536	0.450*	0.375
	E.Coli	1st.	0.652	1.309	0.822	0.878	0.941	0.961	0.600*	0.807	0.803*	0.894
		2nd.	0.629	0.702	0.632	0.636	0.688	0.801	0.384*	0.644	0.624	0.447*
		3rd.	0.529	0.445	0.446	0.441	0.491	0.515	0.303*	0.405*	0.414*	0.393*
Proteins	Human	1st.	0.189	0.457	0.223	0.176	0.400	0.225	0.263*	0.246	0.243	0.421
		2nd.	0.180	0.189	0.168	0.153	0.293	0.191	0.189	0.213	0.156	0.294
		3rd.	0.136	0.153	0.160	0.131	0.239	0.142	0.148	0.122*	0.143	0.243
	Yeast	1st.	0.392	0.451	0.536	0.456	0.667	0.470	0.442	0.459*	0.507	0.557*
		2nd.	0.332	0.400	0.394	0.477	0.446	0.455	0.338*	0.407	0.424*	0.418
		3rd.	0.394	0.444	0.474	0.490	0.289	0.362	0.413	0.482	0.475	0.341
	E.Coli	1st.	0.732	0.657	0.805	0.839	0.782	0.916	0.753	0.772	0.902	0.711
		2nd.	0.528	0.480	0.590	0.598	0.707	0.594	0.411	0.681	0.670	0.661
		3rd.	0.325	0.307	0.277	0.407	0.591	0.349	0.249*	0.284	0.392	0.584

Accuracy of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.007	0.074	0.001	0.018	0.059	0.012	0.003*	0.031	0.028	0.052
		2nd.	0.010	0.020	0.002	0.001	0.012	0.008	0.003	0.000	0.001	0.015
		3rd.	0.008	0.010	0.001	0.007	0.022	0.000*	0.003	0.012*	0.012*	0.019
	Yeast	1st.	0.324	0.058	0.129	0.202	0.078	0.049	0.003*	0.003	0.001	0.015*
		2nd.	0.354	0.064	0.154	0.238	0.024	0.040	0.023	0.074	0.140	0.056*
		3rd.	0.177	0.028	0.038	0.116	0.058	0.008	0.023	0.066*	0.081	0.049
	E.Coli	1st.	0.856	0.438	0.651	0.835	0.283	0.274*	0.196*	0.474*	0.408*	0.258
		2nd.	0.711	0.181	0.383	0.537	0.141	0.143*	0.121*	0.267*	0.346*	0.186
		3rd.	0.328	0.085	0.133	0.246	0.109	0.022*	0.057*	0.131	0.150*	0.105
Proteins	Human	1st.	0.014	0.001	0.001	0.006	0.045	0.018	0.000	0.021	0.011	0.017
		2nd.	0.007	0.005	0.006	0.001	0.009	0.003	0.010	0.005	0.001	0.005
		3rd.	0.013	0.006	0.006	0.002	0.015	0.004	0.004	0.001	0.006	0.000
	Yeast	1st.	0.291	0.052	0.059	0.202	0.221	0.001	0.206	0.024	0.046	0.282
		2nd.	0.317	0.108	0.103	0.240	0.143	0.078	0.070	0.093	0.174	0.158
		3rd.	0.114	0.012	0.013	0.065	0.030	0.030	0.041	0.046*	0.043	0.050
	E.Coli	1st.	0.779	0.505	0.616	0.749	0.045	0.355*	0.027*	0.431*	0.312*	0.174
		2nd.	0.619	0.414	0.296	0.448	0.234	0.136*	0.040*	0.294	0.260*	0.302
		3rd.	0.338	0.164	0.124	0.217	0.089	0.071*	0.016*	0.176	0.127*	0.143

Supplementary Table 1.C: Precision and accuracy in HYE124, TTOF6600_32fix.

Precision of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.329	0.411	0.318	0.309	0.690	0.427	0.299 *	0.380	0.405	0.589 *
		2nd.	0.248	0.307	0.255	0.246	0.470	0.293	0.245 *	0.260	0.262	0.430 *
		3rd.	0.245	0.259	0.257	0.210	0.397	0.282	0.233 *	0.259	0.206	0.349 *
	Yeast	1st.	0.395	0.576	0.376	0.496	0.865	0.537	0.295 *	0.458	0.502	0.708 *
		2nd.	0.317	0.356	0.393	0.393	0.499	0.398	0.275 *	0.395	0.385	0.447 *
		3rd.	0.347	0.439	0.474	0.478	0.518	0.370	0.306 *	0.401 *	0.403 *	0.456 *
	E.Coli	1st.	0.582	0.847	0.760	0.848	1.137	0.789	0.607 *	0.750	0.784 *	1.045 *
		2nd.	0.493	0.632	0.580	0.540	0.837	0.601	0.323 *	0.529 *	0.551	0.559 *
		3rd.	0.419	0.451	0.423	0.363	0.546	0.418	0.335 *	0.380 *	0.349 *	0.375 *
Proteins	Human	1st.	0.166	0.167	0.185	0.147	0.414	0.186	0.192	0.171 *	0.164	0.431
		2nd.	0.162	0.144	0.159	0.107	0.328	0.156	0.148	0.140 *	0.113	0.327
		3rd.	0.169	0.129	0.160	0.106	0.273	0.149 *	0.130	0.148 *	0.116	0.274
	Yeast	1st.	0.289	0.306	0.361	0.333	0.597	0.319	0.343	0.322 *	0.358	0.560
		2nd.	0.273	0.266	0.362	0.361	0.333	0.319	0.350	0.295 *	0.309 *	0.361
		3rd.	0.303	0.429	0.397	0.361	0.627	0.349	0.309 *	0.406	0.320 *	0.483 *
	E.Coli	1st.	0.443	0.652	0.619	0.755	0.914	0.615	0.600	0.658	0.734	0.728 *
		2nd.	0.397	0.438	0.555	0.508	0.736	0.445	0.465	0.490 *	0.487	0.554 *
		3rd.	0.344	0.464	0.393	0.325	0.368	0.312	0.314 *	0.329	0.311	0.483

Accuracy of relative quantification

			Iteration 1					Iteration 2				
		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.016	0.011	0.010	0.018	0.046	0.012	0.007	0.018 *	0.028	0.043
		2nd.	0.000	0.002	0.003	0.001	0.010	0.001	0.003	0.005	0.001	0.008
		3rd.	0.009	0.006	0.008	0.010	0.015	0.005	0.005	0.011	0.011	0.014
	Yeast	1st.	0.264	0.036	0.184	0.171	0.067	0.020	0.029 *	0.041	0.046	0.010 *
		2nd.	0.244	0.018	0.130	0.136	0.054	0.021	0.000	0.052	0.011	0.066 *
		3rd.	0.039	0.035	0.005	0.048	0.061	0.065	0.048	0.021	0.004	0.063
	E.Coli	1st.	0.776	0.354	0.839	0.752	0.230	0.296 *	0.244 *	0.445 *	0.317 *	0.263
		2nd.	0.561	0.110	0.423	0.353	0.164	0.041 *	0.099	0.167 *	0.117 *	0.187
		3rd.	0.135	0.023	0.074	0.132	0.153	0.047 *	0.002 *	0.054 *	0.043 *	0.162
Proteins	Human	1st.	0.014	0.016	0.020	0.010	0.002	0.017	0.010	0.022	0.010	0.016
		2nd.	0.000	0.001	0.000	0.002	0.005	0.005	0.002	0.004	0.000	0.003
		3rd.	0.009	0.006	0.008	0.006	0.006	0.008	0.005	0.009	0.006	0.003
	Yeast	1st.	0.312	0.044	0.162	0.163	0.145	0.037	0.056	0.077	0.004	0.126
		2nd.	0.201	0.029	0.119	0.114	0.047	0.002	0.043	0.041	0.027	0.072
		3rd.	0.008	0.041	0.028	0.022	0.013	0.058	0.058	0.028	0.002	0.038
	E.Coli	1st.	0.675	0.487	0.736	0.616	0.046	0.233 *	0.107 *	0.394 *	0.133 *	0.142
		2nd.	0.498	0.271	0.463	0.334	0.048	0.095 *	0.059 *	0.182 *	0.120 *	0.115
		3rd.	0.128	0.044	0.076	0.109	0.057	0.015 *	0.025 *	0.075	0.031 *	0.051

Supplementary Table 1.D: Precision and accuracy in HYE124, TTOF6600_64var.

Precision of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.553	0.354	0.598	0.444	0.579
		2nd.	0.427	0.324	0.444	0.340	0.444
		3rd.	0.404	0.297	0.399	0.331	0.407
	Yeast	1st.	1.027	0.821	1.046	0.937	1.240
		2nd.	1.149	1.125	1.461	1.197	0.996
		3rd.	1.776	1.592	2.206	1.793	1.396
	E.Coli	1st.	1.219	1.172	1.675	1.339	1.273
		2nd.	0.774	0.690	0.841	0.689	0.569
		3rd.	0.611	0.479	0.638	0.551	0.656
Proteins	Human	1st.	0.313	0.321	0.423	0.293	0.501
		2nd.	0.287	0.300	0.318	0.226	0.393
		3rd.	0.256	0.207	0.240	0.242	0.353
	Yeast	1st.	0.812	0.644	0.939	0.906	0.783
		2nd.	1.239	0.946	1.208	1.186	1.238
		3rd.	1.786	1.399	1.775	1.538	1.050
	E.Coli	1st.	1.069	0.922	1.433	1.194	1.129
		2nd.	0.506	0.468	0.712	0.692	0.633
		3rd.	0.421	0.413	0.343	0.387	0.695

Accuracy of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.065	0.045	0.047	0.041	0.056
		2nd.	0.009	0.005	0.040	0.014	0.000
		3rd.	0.033	0.035	0.007	0.011	0.051
	Yeast	1st.	0.000	0.177	0.043	0.252	0.435
		2nd.	0.007	0.067	0.027	0.347	0.201
		3rd.	0.238	0.227	0.135	0.029	0.179
	E.Coli	1st.	0.697	0.527	0.750	0.815	0.642
		2nd.	0.089	0.124	0.035	0.376	0.177
		3rd.	0.216	0.234	0.167	0.004	0.064
Proteins	Human	1st.	0.001	0.028	0.023	0.009	0.055
		2nd.	0.001	0.019	0.045	0.008	0.002
		3rd.	0.002	0.003	0.031	0.000	0.036
	Yeast	1st.	0.096	0.393	0.000	0.189	0.829
		2nd.	0.053	0.082	0.034	0.301	0.479
		3rd.	0.179	0.034	0.442	0.073	0.069
	E.Coli	1st.	0.427	0.149	0.604	0.608	0.174
		2nd.	0.003	0.216	0.124	0.317	0.627
		3rd.	0.284	0.312	0.198	0.086	0.619

Supplementary Table 1.E: Precision and accuracy in HYE110, TTOF6600_32fix.

Precision of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.533	0.360	0.493	0.448	0.545
		2nd.	0.407	0.313	0.393	0.369	0.439
		3rd.	0.378	0.331	0.432	0.340	0.408
	Yeast	1st.	0.854	0.856	1.048	0.874	1.379
		2nd.	1.174	1.195	1.108	1.125	1.134
		3rd.	1.610	1.733	1.933	1.821	1.474
	E.Coli	1st.	1.065	1.150	1.567	1.319	1.414
		2nd.	0.711	0.640	0.809	0.643	0.661
		3rd.	0.616	0.454	0.588	0.586	0.972
Proteins	Human	1st.	0.285	0.286	0.315	0.285	0.486
		2nd.	0.235	0.252	0.224	0.202	0.389
		3rd.	0.250	0.280	0.389	0.289	0.373
	Yeast	1st.	0.652	0.708	0.854	0.847	0.855
		2nd.	0.983	0.617	0.988	1.103	1.081
		3rd.	1.514	1.649	1.677	1.613	0.986
	E.Coli	1st.	0.906	0.623	1.489	1.074	0.936
		2nd.	0.409	0.560	0.739	0.695	0.511
		3rd.	0.478	0.332	0.524	0.374	0.914

Accuracy of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.065	0.034	0.036	0.047	0.057
		2nd.	0.010	0.004	0.010	0.006	0.005
		3rd.	0.025	0.021	0.009	0.022	0.037
	Yeast	1st.	0.055	0.132	0.082	0.137	0.420
		2nd.	0.068	0.076	0.121	0.221	0.180
		3rd.	0.143	0.208	0.191	0.060	0.129
	E.Coli	1st.	0.568	0.489	0.578	0.644	0.642
		2nd.	0.133	0.120	0.129	0.246	0.221
		3rd.	0.192	0.154	0.150	0.064	0.026
Proteins	Human	1st.	0.023	0.017	0.010	0.018	0.082
		2nd.	0.001	0.010	0.016	0.001	0.040
		3rd.	0.010	0.005	0.019	0.009	0.011
	Yeast	1st.	0.001	0.008	0.162	0.100	0.584
		2nd.	0.131	0.265	0.069	0.269	0.196
		3rd.	0.043	0.609	0.024	0.126	0.212
	E.Coli	1st.	0.401	0.149	0.227	0.410	0.093
		2nd.	0.072	0.040	0.127	0.204	0.416
		3rd.	0.246	0.254	0.101	0.124	0.386

Supplementary Table 1.F: Precision and accuracy in HYE110, TTOF6600_32var.

Precision of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.493	0.356	0.561	0.636	0.545
		2nd.	0.385	0.320	0.445	0.430	0.443
		3rd.	0.376	0.335	0.413	0.396	0.446
	Yeast	1st.	0.791	0.814	1.175	1.141	1.201
		2nd.	0.961	1.206	1.397	1.583	0.787
		3rd.	1.602	1.671	2.144	2.250	1.254
	E.Coli	1st.	1.025	1.116	1.553	1.752	0.929
		2nd.	0.551	0.585	0.850	0.669	0.643
		3rd.	0.565	0.498	0.637	0.579	0.612
Proteins	Human	1st.	0.277	0.250	0.343	0.373	0.448
		2nd.	0.266	0.259	0.332	0.273	0.390
		3rd.	0.205	0.199	0.257	0.302	0.454
	Yeast	1st.	0.612	0.742	0.997	1.120	0.838
		2nd.	1.065	0.714	1.273	1.455	0.663
		3rd.	1.502	1.472	1.786	2.176	1.091
	E.Coli	1st.	1.004	0.800	1.428	1.498	0.910
		2nd.	0.483	0.442	0.904	0.505	0.621
		3rd.	0.445	0.413	0.361	0.318	0.728

Accuracy of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.062	0.047	0.091	0.224	0.086
		2nd.	0.001	0.006	0.004	0.013	0.002
		3rd.	0.038	0.042	0.041	0.101	0.057
	Yeast	1st.	0.110	0.225	0.019	0.500	0.447
		2nd.	0.010	0.022	0.317	0.123	0.336
		3rd.	0.151	0.154	0.138	0.126	0.280
	E.Coli	1st.	0.579	0.556	0.675	0.415	0.904
		2nd.	0.114	0.230	0.303	0.148	0.380
		3rd.	0.208	0.174	0.419	0.334	0.124
Proteins	Human	1st.	0.026	0.022	0.059	0.110	0.069
		2nd.	0.001	0.012	0.001	0.011	0.004
		3rd.	0.017	0.021	0.024	0.041	0.056
	Yeast	1st.	0.003	0.015	0.342	0.183	0.354
		2nd.	0.028	0.132	0.206	0.133	0.289
		3rd.	0.017	0.145	1.123	0.064	0.079
	E.Coli	1st.	0.230	0.157	0.116	0.393	0.116
		2nd.	0.002	0.038	0.362	0.142	0.246
		3rd.	0.286	0.268	0.386	0.327	0.511

Supplementary Table 1.G: Precision and accuracy in HYE110, TTOF6600_64fix.

Precision of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.480	0.369	0.536	0.578	0.556
		2nd.	0.371	0.289	0.397	0.396	0.418
		3rd.	0.316	0.300	0.373	0.293	0.376
	Yeast	1st.	0.723	0.868	1.066	1.005	1.127
		2nd.	0.684	1.000	1.220	1.307	0.961
		3rd.	1.200	1.605	2.060	1.926	1.304
	E.Coli	1st.	0.890	1.209	1.468	1.733	1.241
		2nd.	0.607	0.462	0.809	0.732	0.608
		3rd.	0.468	0.426	0.554	0.498	0.619
Proteins	Human	1st.	0.246	0.229	0.309	0.278	0.490
		2nd.	0.249	0.197	0.209	0.216	0.373
		3rd.	0.258	0.270	0.223	0.204	0.330
	Yeast	1st.	0.482	0.780	0.770	0.761	0.813
		2nd.	0.671	0.744	1.019	1.124	0.599
		3rd.	1.126	1.087	1.847	1.923	1.361
	E.Coli	1st.	0.738	1.249	1.266	1.226	0.991
		2nd.	0.443	0.414	0.993	0.558	0.727
		3rd.	0.333	0.375	0.500	0.346	0.621

Accuracy of relative quantification

		Intensity tertile	OpenSWATH	SWATH 2.0	Skyline	Spectronaut	DIAumpire
Peptides	Human	1st.	0.027	0.014	0.026	0.066	0.038
		2nd.	0.005	0.001	0.016	0.011	0.002
		3rd.	0.010	0.010	0.005	0.018	0.027
	Yeast	1st.	0.063	0.273	0.013	0.554	0.339
		2nd.	0.013	0.017	0.247	0.248	0.253
		3rd.	0.256	0.227	0.248	0.229	0.227
	E.Coli	1st.	0.443	0.509	0.532	0.098	0.702
		2nd.	0.083	0.173	0.300	0.217	0.380
		3rd.	0.163	0.095	0.266	0.193	0.161
Proteins	Human	1st.	0.002	0.002	0.008	0.001	0.040
		2nd.	0.013	0.003	0.002	0.005	0.007
		3rd.	0.007	0.005	0.008	0.001	0.030
	Yeast	1st.	0.049	0.014	0.376	0.397	0.344
		2nd.	0.041	0.089	0.195	0.039	0.451
		3rd.	0.323	0.282	0.076	0.250	0.064
	E.Coli	1st.	0.294	0.291	0.153	0.085	0.086
		2nd.	0.039	0.041	0.286	0.092	0.189
		3rd.	0.207	0.207	0.216	0.205	0.521

Supplementary Table 1.H: Precision and accuracy in HYE110, TTOF6600_64var.

Supplementary Table 2. Metrics summary

Number of protein and peptide identifications, number of valid quantification ratios, and calculated raw and AUQC values for separation between (yeast vs. human) and (E.coli vs. human). AUQC values are also displayed as the arctangent hyperbolics (arctanh) for an easier interpretation of the values. The arctanh reflects that the difficulty of improving the AUQC value is not linear. AUQC values of the different software tools were compared to each other (color code: worst value in full red and best value in full green).

The following pages show the metrics summary for:

- Supplementary Table 2.A: sample set HYE124, TTOF5600_32fix, iteration 1 & 2
- Supplementary Table 2.B: sample set HYE124, TTOF5600_64var, iteration 1 & 2
- Supplementary Table 2.C: sample set HYE124, TTOF6600_32fix, iteration 1 & 2
- Supplementary Table 2.D: sample set HYE124, TTOF6600_64var, iteration 1 & 2
- Supplementary Table 2.E: sample set HYE110, TTOF6600_32fix
- Supplementary Table 2.F: sample set HYE110, TTOF6600_32var
- Supplementary Table 2.G: sample set HYE110, TTOF6600_64fix
- Supplementary Table 2.H: sample set HYE110, TTOF6600_64fix

Iteration 1

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	5%	26,225	19,611	0.94	0.96	1.73	1.97
	SWATH2.0	6%	17,988	17,988	0.94	0.96	1.76	1.95
	Skyline	7%	25,090	16,502	0.94	0.95	1.74	1.82
	Spectronaut	6%	30,361	21,037	0.94	0.95	1.72	1.82
	DIA-Umpire	10%	12,584	10,668	0.95	0.99	1.79	2.58
proteins	OpenSWATH	4%	3,296	2,764	0.92	0.95	1.58	1.82
	SWATH2.0	4%	2,671	2,671	0.96	0.98	1.94	2.29
	SWATH2.0 (built-in)	5%	4,213	4,213	0.93	0.95	1.66	1.80
	Skyline	6%	2,872	2,359	0.95	0.96	1.82	1.91
	Spectronaut	4%	3,379	2,829	0.94	0.95	1.78	1.79
	DIA-Umpire	9%	1,668	1,495	0.97	1.00	2.11	3.34
	DIA-Umpire (built-in)	12%	2,244	2,079	0.95	0.99	1.86	2.59

Iteration 2

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	9%	26,767	20,029	0.93	0.96	1.70	1.97
	SWATH2.0	4%	17,962	10,997	0.98	0.98	2.21	2.35
	Skyline	7%	26,341	17,116	0.95	0.96	1.80	1.89
	Spectronaut	6%	30,052	20,497	0.96	0.97	1.92	2.06
	DIA-Umpire	10%	12,598	8,978	0.94	0.99	1.70	2.57
proteins	OpenSWATH	6%	3,339	2,817	0.95	0.96	1.78	2.01
	SWATH2.0	5%	2,125	1,680	0.97	0.99	2.18	2.93
	SWATH2.0 (built-in)							
	Skyline	6%	2,878	2,399	0.95	0.97	1.83	2.07
	Spectronaut	4%	3,332	2,748	0.95	0.96	1.84	1.95
	DIA-Umpire	10%	1,566	1,264	0.98	1.00	2.26	4.26
	DIA-Umpire (built-in)							

Supplementary Table 2.A: Metrics summary in HYE124, TTOF5600_32fix.

Iteration 1

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	5%	34,154	26,992	0.96	0.98	1.96	2.31
	SWATH2.0	6%	27,750	27,750	0.95	0.96	1.83	1.96
	Skyline	7%	35,508	26,267	0.94	0.95	1.72	1.81
	Spectronaut	7%	34,335	25,012	0.95	0.97	1.87	2.03
	DIA-Umpire	11%	15,346	12,499	0.93	0.97	1.65	2.09
proteins	OpenSWATH	4%	4,056	3,518	0.98	0.98	2.27	2.35
	SWATH2.0	4%	3,651	3,651	0.98	0.98	2.20	2.32
	SWATH2.0 (built-in)	5%	5,448	5,448	0.95	0.95	1.86	1.88
	Skyline	5%	3,931	3,420	0.96	0.96	1.96	2.00
	Spectronaut	4%	3,751	3,209	0.96	0.98	1.99	2.38
	DIA-Umpire	10%	1,880	1,643	0.96	0.98	2.01	2.43
	DIA-Umpire (built-in)	12%	2,588	2,357	0.94	0.97	1.72	2.12

Iteration 2

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	8%	34,476	27,290	0.95	0.98	1.88	2.25
	SWATH2.0	5%	27,701	18,444	0.98	0.98	2.33	2.33
	Skyline	9%	34,206	24,455	0.94	0.97	1.72	2.03
	Spectronaut	7%	33,598	24,038	0.97	0.98	2.07	2.25
	DIA-Umpire	10%	15,235	10,656	0.94	0.98	1.76	2.35
proteins	OpenSWATH	5%	4,086	3,545	0.98	0.99	2.38	2.62
	SWATH2.0	4%	3,119	2,593	0.98	0.98	2.20	2.39
	SWATH2.0 (built-in)							
	Skyline	6%	3,629	3,148	0.96	0.97	1.94	2.06
	Spectronaut	4%	3,669	3,120	0.97	0.98	2.12	2.32
	DIA-Umpire	10%	1,781	1,445	0.97	0.99	2.11	2.73
	DIA-Umpire (built-in)							

Supplementary Table 2.B: Metrics summary in HYE124, TTOF5600_64var.

Iteration 1

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	8%	34,851	28,392	0.95	0.96	1.79	1.94
	SWATH2.0	9%	23,904	23,904	0.87	0.93	1.33	1.67
	Skyline	8%	32,042	23,201	0.95	0.95	1.88	1.79
	Spectronaut	5%	38,974	31,250	0.94	0.94	1.71	1.73
	DIA-Umpire	14%	25,042	20,013	0.95	0.98	1.81	2.19
proteins	OpenSWATH	7%	4,030	3,617	0.95	0.97	1.87	2.03
	SWATH2.0	7%	3,269	3,269	0.95	0.98	1.82	2.44
	SWATH2.0 (built-in)	8%	4,959	4,959	0.91	0.95	1.54	1.81
	Skyline	8%	3,582	3,044	0.96	0.97	1.96	2.07
	Spectronaut	3%	4,293	3,799	0.95	0.95	1.78	1.85
	DIA-Umpire	12%	2,852	2,517	0.97	0.99	2.15	2.72
	DIA-Umpire (built-in)	13%	3,643	3,344	0.96	0.98	1.91	2.40

Iteration 2

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	10%	35,110	28,548	0.95	0.96	1.79	1.99
	SWATH2.0	7%	23,883	14,020	0.98	0.99	2.36	2.47
	Skyline	10%	37,299	29,036	0.95	0.96	1.86	1.96
	Spectronaut	5%	38,443	29,934	0.95	0.97	1.88	2.10
	DIA-Umpire	13%	24,872	17,404	0.95	0.98	1.88	2.33
proteins	OpenSWATH	8%	4,035	3,624	0.96	0.97	1.99	2.11
	SWATH2.0	8%	2,656	2,141	0.98	0.99	2.34	2.51
	SWATH2.0 (built-in)							
	Skyline	9%	4,046	3,613	0.96	0.96	1.91	2.00
	Spectronaut	3%	4,226	3,704	0.95	0.97	1.86	2.05
	DIA-Umpire	12%	2,729	2,250	0.98	1.00	2.35	3.72
	DIA-Umpire (built-in)							

Supplementary Table 2.C: Metrics summary in HYE124, TTOF6600_32fix.

Iteration 1

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	6%	40,726	36,098	0.97	0.98	2.12	2.24
	SWATH2.0	7%	35,517	35,517	0.97	0.97	2.14	2.11
	Skyline	7%	40,804	34,103	0.96	0.95	1.91	1.85
	Spectronaut	6%	42,439	37,120	0.96	0.96	1.97	1.90
	DIA-Umpire	13%	36,332	28,785	0.94	0.96	1.74	1.98
proteins	OpenSWATH	5%	4,632	4,343	0.98	0.99	2.30	2.56
	SWATH2.0	6%	4,323	4,323	0.98	0.98	2.37	2.36
	SWATH2.0 (built-in)	6%	6,178	6,178	0.98	0.97	2.23	2.03
	Skyline	6%	4,518	4,140	0.97	0.97	2.03	2.15
	Spectronaut	3%	4,692	4,346	0.97	0.97	2.13	2.18
	DIA-Umpire	12%	3,795	3,379	0.97	0.98	2.12	2.30
	DIA-Umpire (built-in)	13%	4,849	4,489	0.95	0.96	1.78	1.94

Iteration 2

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	8%	40,728	35,944	0.97	0.98	2.10	2.26
	SWATH2.0	6%	35,489	26,303	0.99	0.99	2.49	2.52
	Skyline	7%	42,517	37,977	0.97	0.97	2.13	2.14
	Spectronaut	6%	42,325	36,292	0.97	0.98	2.11	2.26
	DIA-Umpire	13%	36,249	25,677	0.95	0.97	1.82	2.18
proteins	OpenSWATH	6%	4,636	4,352	0.99	0.99	2.51	2.60
	SWATH2.0	6%	3,946	3,371	0.98	0.99	2.42	2.56
	SWATH2.0 (built-in)							
	Skyline	5%	4,692	4,456	0.98	0.98	2.37	2.43
	Spectronaut	3%	4,675	4,300	0.98	0.99	2.31	2.50
	DIA-Umpire	12%	3,673	3,111	0.97	0.99	2.13	2.85
	DIA-Umpire (built-in)							

Supplementary Table 2.D: Metrics summary in HYE124, TTOF6600_64var.

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	15%	30,848	16,788	0.94	0.98	1.77	2.21
	SWATH2.0	13%	20,742	9,685	0.95	0.98	1.79	2.21
	Skyline	17%	27,236	13,430	0.91	0.95	1.51	1.78
	Spectronaut	7%	35,576	18,858	0.93	0.97	1.65	2.04
	DIA-Umpire	15%	25,209	11,836	0.95	0.97	1.88	2.15
proteins	OpenSWATH	13%	3,700	2,382	0.94	0.99	1.74	2.72
	SWATH2.0	13%	2,421	1,431	0.97	0.99	2.13	2.87
	Skyline	16%	2,878	1,886	0.93	0.96	1.68	1.98
	Spectronaut	5%	3,955	2,512	0.94	0.98	1.75	2.21
	DIA-Umpire	16%	2,696	1,602	0.99	0.99	2.47	2.63

Supplementary Table 2.E: Metrics summary in HYE110, TTOF6600_32fix.

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	9%	33,462	18,848	0.96	0.99	1.89	2.46
	SWATH2.0	5%	24,151	11,706	0.94	0.98	1.74	2.21
	Skyline	8%	31,761	16,933	0.94	0.96	1.76	1.98
	Spectronaut	6%	36,771	19,796	0.94	0.97	1.74	2.10
	DIA-Umpire	11%	28,221	13,534	0.94	0.98	1.77	2.20
proteins	OpenSWATH	6%	3,988	2,585	0.98	0.99	2.21	2.75
	SWATH2.0	5%	2,840	1,721	0.95	1.00	1.80	4.85
	Skyline	6%	3,461	2,321	0.96	0.98	1.92	2.24
	Spectronaut	4%	4,100	2,636	0.95	0.98	1.88	2.28
	DIA-Umpire	11%	3,032	1,844	0.98	1.00	2.40	3.18

Supplementary Table 2.F: Metrics summary in HYE110, TTOF6600_32var.

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	11%	32,479	17,774	0.97	0.99	2.03	2.45
	SWATH2.0	8%	24,268	11,804	0.95	0.98	1.81	2.23
	Skyline	14%	28,937	14,567	0.92	0.97	1.59	2.03
	Spectronaut	12%	33,297	16,931	0.89	0.96	1.43	1.93
	DIA-Umpire	12%	28,781	13,295	0.96	0.98	2.00	2.36
proteins	OpenSWATH	10%	3,844	2,467	0.98	0.99	2.30	2.50
	SWATH2.0	9%	2,813	1,714	0.98	1.00	2.19	3.25
	Skyline	12%	3,019	2,009	0.94	0.98	1.72	2.20
	Spectronaut	9%	3,653	2,332	0.91	0.98	1.51	2.22
	DIA-Umpire	13%	3,010	1,820	0.98	1.00	2.38	3.11

Supplementary Table 2.G: Metrics summary in HYE110, TTOF6600_64fix.

		Median CV of Human	Identifications	Valid quantification ratios	Overlap Yeast-Human	Overlap E.coli-Human	Overlap Yeast-Human (arctanh)	Overlap E.coli-Human (arctanh)
peptides	OpenSWATH	8%	37,133	21,721	0.98	0.99	2.34	2.79
	SWATH2.0	6%	29,483	15,224	0.95	0.98	1.85	2.31
	Skyline	10%	35,014	19,786	0.94	0.97	1.70	2.09
	Spectronaut	8%	37,242	20,139	0.93	0.97	1.68	2.09
	DIA-Umpire	12%	37,167	17,238	0.96	0.98	1.92	2.26
proteins	OpenSWATH	6%	4,307	2,881	0.99	1.00	2.50	3.09
	SWATH2.0	5%	3,438	2,151	0.99	0.99	2.51	2.65
	Skyline	7%	3,787	2,631	0.95	0.97	1.83	2.09
	Spectronaut	4%	4,115	2,672	0.95	0.99	1.85	2.55
	DIA-Umpire	13%	3,641	2,233	0.97	1.00	2.11	3.80

Supplementary Table 2.H: Metrics summary in HYE110, TTOF6600_64var.

Supplementary Table 3. Number of identified peptides and proteins

Numbers of peptides and proteins identified by each software tool in the samples HYE124 (iteration 1 and 2) and HYE110 across the different datasets are listed. The number of proteins is estimated after protein filters were applied at the FSWE module (first module of LFQbench): a protein must have a quantification value in at least two replicates of at least one of the samples (A or B). The change from 32 windows to 64 windows is estimated by taking the highest number of each of the respective modes (either fixed or variable).

The following pages show the numbers of identified peptides and proteins for:

- Supplementary Table 3.A: sample set HYE124 iteration 1 and 2
- Supplementary Table 3.B: sample set HYE110

		Iteration 1					
		peptides			proteins		
		32fix	64var	change	32fix	64var	change
OpenSWATH	5600	26,225	34,154	30%	3,296	4,056	23%
	6600	34,851	40,726	17%	4,030	4,632	15%
	change	33%	19%		22%	14%	
SWATH 2.0	5600	17,988	27,750	54%	2,671	3,651	37%
	6600	23,904	35,517	49%	3,269	4,323	32%
	change	33%	28%		22%	18%	
Skyline	5600	25,090	35,508	42%	2,872	3,931	37%
	6600	32,042	40,804	27%	3,582	4,518	26%
	change	28%	15%		25%	15%	
Spectronaut	5600	30,361	34,335	13%	3,379	3,751	11%
	6600	38,974	42,439	9%	4,293	4,692	9%
	change	28%	24%		27%	25%	
DIA-Umpire	5600	12,584	15,346	22%	1,668	1,880	13%
	6600	25,042	36,332	45%	2,852	3,795	33%
	change	99%	137%		71%	102%	

		Iteration 2					
		peptides			proteins		
		32fix	64var	change	32fix	64var	change
OpenSWATH	5600	26,767	34,476	29%	3,339	4,086	22%
	6600	35,110	40,728	16%	4,035	4,636	15%
	change	31%	18%		21%	13%	
SWATH 2.0	5600	17,962	27,701	54%	2,125	3,119	47%
	6600	23,883	35,489	49%	2,656	3,946	49%
	change	33%	28%		25%	27%	
Skyline	5600	26,341	34,206	30%	2,878	3,629	26%
	6600	37,299	42,517	14%	4,046	4,692	16%
	change	42%	24%		41%	29%	
Spectronaut	5600	30,052	33,598	12%	3,332	3,669	10%
	6600	38,443	42,325	10%	4,226	4,675	11%
	change	28%	26%		27%	27%	
DIA-Umpire	5600	12,598	15,235	21%	1,566	1,781	14%
	6600	24,872	36,249	46%	2,729	3,673	35%
	change	97%	138%		74%	106%	

Supplementary Table 3.A: Number of peptides and proteins identified in HYE124.

		peptides			proteins		
		#	change (fix vs var)	change (32 vs 64)	#	change (fix vs var)	change (32 vs 64)
OpenSWATH	**32fix**	30,848	8%	11%	3,700	8%	8%
	32var	33,462			3,988		
	64fix	32,479	14%		3,844	12%	
	64var	37,133			4,307		
SWATH 2.0	**32fix**	20,742	16%	22%	2,421	17%	21%
	32var	24,151			2,840		
	64fix	24,268	21%		2,813	22%	
	64var	29,483			3,438		
Skyline	**32fix**	27,236	17%	10%	2,878	20%	9%
	32var	31,761			3,461		
	64fix	28,937	21%		3,019	25%	
	64var	35,014			3,787		
Spectronaut	**32fix**	35,576	3%	1%	3,955	4%	0%
	32var	36,771			4,100		
	64fix	33,297	12%		3,653	13%	
	64var	37,242			4,115		
DIA-Umpire	**32fix**	25,209	12%	32%	2,696	12%	20%
	32var	28,221			3,032		
	64fix	28,781	29%		3,010	21%	
	64var	37,167			3,641		

Supplementary Table 3.B: Number of peptides and proteins identified in HYE110.

Supplementary Table 4. Average R^2 regression values of between technical replicates

The quality of the single measurements was assessed by pairing technical replicates of the different samples (i.e.: A_1 vs A_2, A_1 vs A_3, and A_2 vs A_3) and estimate their correlation. The following tables display the average and standard deviation of R^2 of A vs A, A vs B, and B vs B pairs. Note that quantifications of A and B should only correlate for human proteins, and thus lower values of R^2 for A vs B are reported.

The following pages show the Average R^2 regression values of technical replicates pairing for:

- Supplementary Table 4.A: sample set HYE124 iteration 1 and 2
- Supplementary Table 4.B: sample set HYE110

OpenSWATH

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE124_TTOF5600_32fix	0.983	0.00430	0.693	0.00732	0.987	0.00115
HYE124_TTOF5600_64var	0.981	0.00728	0.704	0.01105	0.969	0.00359
HYE124_TTOF6600_32fix	0.966	0.01735	0.686	0.02684	0.931	0.02806
HYE124_TTOF6600_64var	0.988	0.00340	0.716	0.00841	0.989	0.00391

Skyline

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE124_TTOF5600_32fix	0.986	0.00456	0.764	0.00399	0.991	0.002132
HYE124_TTOF5600_64var	0.990	0.00192	0.759	0.00372	0.987	0.002047
HYE124_TTOF6600_32fix	0.987	0.00157	0.771	0.00427	0.991	0.000971
HYE124_TTOF6600_64var	0.909	0.06484	0.702	0.05421	0.983	0.013755

Spectronaut

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE124_TTOF5600_32fix	0.931	0.054651	0.791	0.04927	0.978	0.01679
HYE124_TTOF5600_64var	0.995	0.000259	0.735	0.11007	0.851	0.12259
HYE124_TTOF6600_32fix	0.991	0.003228	0.787	0.00771	0.982	0.01094
HYE124_TTOF6600_64var	0.995	0.002177	0.799	0.00759	0.997	0.00144

SWATH 2.0

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE124_TTOF5600_32fix	0.918	0.04608	0.713	0.0330	0.946	0.01466
HYE124_TTOF5600_64var	0.985	0.00615	0.751	0.0110	0.979	0.00627
HYE124_TTOF6600_32fix	0.833	0.10626	0.655	0.0690	0.861	0.05089
HYE124_TTOF6600_64var	0.833	0.07657	0.672	0.0863	0.912	0.06577

DIA-Umpire

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE124_TTOF5600_32fix	0.929	0.00768	0.772	0.0124	0.950	0.0247
HYE124_TTOF5600_64var	0.934	0.03512	0.749	0.0222	0.956	0.0021
HYE124_TTOF6600_32fix	0.943	0.03079	0.766	0.0171	0.941	0.0264
HYE124_TTOF6600_64var	0.939	0.01481	0.768	0.0145	0.942	0.0190

Supplementary Table 4.A: R^2 regression values of technical replicates in HYE124.

OpenSWATH

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE110_TTOF6600_32fix	0.994	0.00260	0.549	0.1409	0.987	0.003706
HYE110_TTOF6600_32var	0.996	0.00161	0.756	0.0198	0.969	0.024200
HYE110_TTOF6600_64fix	0.989	0.00752	0.748	0.0347	0.997	0.000247
HYE110_TTOF6600_64var	0.994	0.00218	0.513	0.0329	0.993	0.000682

Skyline

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE110_TTOF6600_32fix	0.862	0.11449	0.663	0.0973	0.968	0.020531
HYE110_TTOF6600_32var	0.988	0.00872	0.729	0.0597	0.886	0.083625
HYE110_TTOF6600_64fix	0.896	0.06159	0.740	0.0723	0.997	0.000516
HYE110_TTOF6600_64var	0.944	0.03799	0.405	0.0225	0.990	0.001988

Spectronaut

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE110_TTOF6600_32fix	0.845	0.130729	0.660	0.10114	0.854	0.123848
HYE110_TTOF6600_32var	0.999	0.000419	0.806	0.00926	0.999	0.000471
HYE110_TTOF6600_64fix	0.993	0.005015	0.852	0.00638	0.997	0.001602
HYE110_TTOF6600_64var	0.992	0.002992	0.478	0.00398	0.995	0.002471

SWATH 2.0

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE110_TTOF6600_32fix	0.684	0.2680	0.471	0.1369	0.841	0.1342
HYE110_TTOF6600_32var	0.883	0.1004	0.676	0.0864	0.841	0.1308
HYE110_TTOF6600_64fix	0.964	0.0282	0.720	0.0804	0.853	0.1160
HYE110_TTOF6600_64var	0.946	0.0433	0.358	0.0254	0.960	0.0238

DIA-Umpire

	avg_R2_AA	avg_R2_error_AA	avg_R2_AB	avg_R2_error_AB	avg_R2_BB	avg_R2_error_BB
HYE110_TTOF6600_32fix	0.962	0.0097	0.606	0.0658	0.850	0.0933
HYE110_TTOF6600_32var	0.895	0.1135	0.626	0.0984	0.812	0.1102
HYE110_TTOF6600_64fix	0.891	0.1130	0.507	0.1010	0.720	0.1033
HYE110_TTOF6600_64var	0.896	0.0433	0.551	0.0445	0.903	0.0447

Supplementary Table 4.B: R^2 regression values of technical replicates in HYE110.

Supplementary Table 5. Configuration parameters for all software tools

Detailed configuration parameters for all software tools investigated.

The following pages show the configuration parameters for:

- Supplementary Table 5.A: Most common parameters for library-based tools
- Supplementary Table 5.B: parameters of OpenSWATH
- Supplementary Table 5.C: parameters of SWATH2.0
- Supplementary Table 5.D: parameters of Skyline
- Supplementary Table 5.E: parameters of Spectronaut
- Supplementary Table 5.F: parameters of DIA-Umpire

Library-based Software tools critical parameters setup							
		Iteration 1					
		m/z window	Resolving Power	XIC extraction window (minutes)	# transitions	FDR threshold	Q-Value threshold
TripleTOF 5600	OpenSWATH	50	-	10	6	0.01	
	PeakView	50	-	10	6	0.01	-
	Skyline	-	40000	10	3	-	0.01
	Spectronaut	(dynamically estimated)	(dynamically estimated)	(dynamically estimated)	6	-	0.01
		Iteration 2					
		m/z window	Resolving Power	XIC extraction window (minutes)	# transitions	FDR threshold	Q-Value threshold
	OpenSWATH	50	-	10	6	0.01	
	PeakView	50	-	10	6	0.01	-
	Skyline	-	60000	10	3	-	0.01
	Spectronaut	(dynamically estimated)	(dynamically estimated)	(dynamically estimated)	6	-	0.01
		Iteration 1					
		m/z window	Resolving Power	XIC extraction window (minutes)	# transitions	FDR threshold	Q-Value threshold
TripleTOF 6600	OpenSWATH	30	-	10	6	0.01	
	PeakView	30	-	10	6	0.01	-
	Skyline	-	66660	10	3	-	0.01
	Spectronaut	(dynamically estimated)	(dynamically estimated)	(dynamically estimated)	6	-	0.01
		Iteration 2					
		m/z window	Resolving Power	XIC extraction window (minutes)	# transitions	FDR threshold	Q-Value threshold
	OpenSWATH	30	-	10	6	0.01	
	PeakView	30	-	10	6	0.01	-
	Skyline	-	100000	10	3	-	0.01
	Spectronaut	(dynamically estimated)	(dynamically estimated)	(dynamically estimated)	6	-	0.01

Supplementary Table 5.A: Most common parameters for library-based tools.

OpenSWATH

	Iteration 1			
	TripleTOF 5600		TripleTOF 6600	
	32 windows	64 windows	32 windows	64 windows
MIN_RSQ	0.95			
ALIGNER_DSCORE_CUTOFF	1			
WORKFLOW	openswath_2014-12-01-154112__2015-05-22-152035 imsbtools/20140808 applicake@6059b97 msproteomicstools@7527c7b openms@7c408dd			
ALIGNER_REALIGN_METHOD	lowess			
WINDOW_UNIT	ppm			
TRAML	traml32file*	traml64file*	traml32file*	traml64file*
ALIGNER_TARGETFDR	0.01			
MIN_UPPER_EDGE_DIST	1			
ALIGNER_MAX_RT_DIFF	30			
IRTTRAML	/cluster/apps/imsbtools/stable/files/hroest_DIA_iRT.TraML			
MPR_MAINVAR	xx_swath_prelim_score			
COMMENT	IRR_Lib_5600_32SW	IRR_Lib_5600_64SW		
MIN_COVERAGE	0.6			
PARENT-DATA-SET-CODES	** corresponding internal codes in server for the files.			
RT_EXTRACTION_WINDOW	600			
MPR_VARS	library_corr yseries_score xcorr_coelution_weighted massdev_score norm_rt_score library_rmsd bseries_score intensity_score xcorr_coelution log_sn_score isotope_overlap_score massdev_score_weighted xcorr_shape_weighted isotope_correlation_score xcorr_shape			
ALIGNER_FRACSELECTED	0			
EXTRACTION_WINDOW	50			
MPR_NUM_XVAL	10			
ALIGNER_METHOD	global_best_overall			
DO_CHROMML_REQUANT	FALSE			

	Iteration 2			
	TripleTOF 5600		TripleTOF 6600	
	32 windows	64 windows	32 windows	64 windows
MIN_RSQ	0.95			
ALIGNER_DSCORE_CUTOFF	1			
WORKFLOW	openswath__2015-05-22-154105 imsbtools/20140808 applicake@6059b97 msproteomicstools@7527c7b openms@7c408dd			
ALIGNER_REALIGN_METHOD	lowess			
WINDOW_UNIT	ppm			
TRAML	traml32file*	traml64file*	traml32file*	traml64file*
ALIGNER_TARGETFDR	0.01			
MIN_UPPER_EDGE_DIST	1			
ALIGNER_MAX_RT_DIFF	30			
IRTTRAML	/cluster/apps/imsbtools/stable/files/hroest_DIA_iRT.TraML			
MPR_MAINVAR	xx_swath_prelim_score			
COMMENT	IRR_Lib_5600_32SW_BG_CORR			
MIN_COVERAGE	0.6			
PARENT-DATA-SET-CODES	** corresponding internal codes in server for the files.			
RT_EXTRACTION_WINDOW	600			
MPR_VARS	library_corr yseries_score xcorr_coelution_weighted massdev_score norm_rt_score library_rmsd bseries_score intensity_score xcorr_coelution log_sn_score isotope_overlap_score massdev_score_weighted xcorr_shape_weighted isotope_correlation_score xcorr_shape			
ALIGNER_FRACSELECTED	0			
EXTRACTION_WINDOW	50			
MPR_NUM_XVAL	10			
ALIGNER_METHOD	global_best_overall			
DO_CHROMML_REQUANT	FALSE			

*

traml32file	./ecolihumanyeast_concat_mayu_IRR_cons_openswath_32sw_curated_decoy.TraML
traml64file	./ecolihumanyeast_concat_mayu_IRR_cons_openswath_64var_curated_decoy.TraML

**

codes_5600_32w	20150211221143737-1038504, 20150210205123604-1038204, 20150211161643511-1038423, 20150211021822894-1038280, 20150211232943800-1038518, 20150210202322907-1038187, PDB-LGILLET-ECOLIHUMANYEASTCONCATMAYUIR-20150522131005
codes_5600_64w	20150212083943868-1038587, 20150212103343910-1038642, 20150212121843915-1038648, 20150212171344465-1038846, 20150211174243600-1038444, 20150211182443601-1038455, PDB-LGILLET-ECOLIHUMANYEASTCONCATMAYUIR-20150522122202
codes_6600_32w	20150213113244196-1039325, 20150224023055874-1042500, 20150213033545820-1039270, 20150213224644148-1039490, 20150224012055854-1042497, 20150213172344252-1039380, PDB-LGILLET-ECOLIHUMANYEASTCONCATMAYUIR-20150522131005
codes_6600_64w	20150223142355786-1042242, 20150221040854005-1041481, 20150218233953981-1040703, 20150221050954009-1041502, 20150223132455770-1042209, 20150221053954494-1041507, PDB-LGILLET-ECOLIHUMANYEASTCONCATMAYUIR-20150522122202

Supplementary Table 5.B: Parameters of OpenSWATH.

SWATH 2.0

Iteration 1		
	TripleTOF 5600	**TripleTOF 6600**
Number of Peptides per Protein	2000	
Number of Transitions per Peptide	6	
Peptide Confidence Threshold % (0-99)	99	
False Discovery Rate Threshold % (0-100)	1	
Exclude Modified Peptides	unchecked	
Exclude Shared Peptides	unchecked	
Fix Rank	unchecked	
XIC Extraction Window (min)	10	
XIC width (ppm)	50	30
XIC width (Da)	unchecked	

Iteration 2		
	TripleTOF 5600	**TripleTOF 6600**
Number of Peptides per Protein	2000	
Number of Transitions per Peptide	6	
Peptide Confidence Threshold % (0-99)	99	
False Discovery Rate Threshold % (0-100)	1	
Exclude Modified Peptides	unchecked	
Exclude Shared Peptides	unchecked	
Fix Rank	unchecked	
XIC Extraction Window (min)	10	
XIC width (ppm)	50	30
XIC width (Da)	unchecked	

Supplementary Table 5.C: Parameters of SWATH2.0.

Skyline

Common Parameters to all iterations

Integrate all			checked
Peptide settings	*Digestion*	*Enzyme*	Trypsin [KR|P]
		Max missed cleavages	0
		Background proteome	None
	Prediction	*use measured retention times when present*	checked
		Time window (min)	3
		Drift time predictor	None
		Use spectral library drift times when present	unchecked
		Resolving power	(blank)
		Min length	7
	Filter	*Max length*	36
		Exclude N-terminal Aas	36
		Exclude potential ragged ends	unchecked
		Exclude peptides containing	(blank)
		Auto-select all matching peptides	checked
		Pick peptides matching	Library
	Library	*Rank peptides by*	(blank)
		Limit peptides per protein	(blank)
		Peptides	(blank)
		Structural modifications	Carbamidomethyl (C), Oxidation (M)
		Max variable mods	3
	Modifications	*Max neutral losses*	1
		Isotope label type	heavy
		Isotope modifications	Label: 13C(6)15N(2)(C-term K), Label: 13C(6)15N(4)(C-term R)
		Internal standard type	heavy
		Precursor mass	Monoisotopic
		Product ion mass	Monoisotopic
Transition settings	*Prediction*	*Collision energy*	ABI 5500 Q Trap
		Declustering potential	None
		Optimization library	None
		Compensation voltage	None
		Use optimization values when present	unchecked
		Precursor charges	2, 3
		Ion charges	1, 2
	Filter	*Ion types*	y, b
		Product ions - from	ion 3
		Product ions - to	last ion - 1
		Product ions - special ions	(blank)
		Product ions - Use DIA window for exclusion	checked
		Auto-select all matching transitions	checked
		Ion match tolerance	0.1
		If a library spectrum is available, pick its most intense ions	checked
	Library	*Pick XX product ions*	3
		From filtered ion charges and types	unchecked
		From filtered ion charges and types plus filtered product ions	unchecked
		From filtered product ions	checked
		Min m/z	50
		Max m/z	2000
	Instrument	*dynamic min product m/z*	unchecked
		Method match tolerance m/z	0.01
		Firmware transition limit	(blank)
		Firmware inclusion limit	(blank)
		Min time (min)	(blank)
		Max time (min)	(blank)
		MS1 filtering - Isotope peaks included	None
		MS1 filtering - Precursor mass analyzer	(blank)
	Full-Scan	*MS1 filtering - Peaks*	(blank)
		MS1 filtering - Resolution (m/z)	(blank)
		MS1 filtering - Isotope labeling enrichment	(blank)
		MS/MS filtering - Acquisition method	DIA
		MS/MS filtering - Product mass analyzer	TOF
		Retention time filtering - use scans within XX minutes of MS/MS Ids	unchecked
		Retention time filtering - Use only scans within XX minutes of predicted RT	10
		Include all matching scans	unchecked
		annotations list	annotation_Qvalue
		group comparisons list	(blank)
		name	give any name to the model
		Choose model	mProphet
Document settings	*Annotations*	*Training - Use decoys*	checked
	Group Comparisons	*Training - Use second best peaks*	unchecked
Reintegrate	*Integrate all peaks*		checked
	Only integrate significant Q values		unchecked
	Q value cutoff		(blank)
	Add q value annotation		checked
	Overwrite manual integration		checked

Skyline users iteration

			TripleTOF 5600		TripleTOF 6600	
			32 windows	**64 windows**	**32 windows**	**64 windows**
File Import	Results	Raw type	raw (wiff file)			
Peptide settings	Prediction	Retention time predictor	TTOF_32w_iRT-C18	TTOF_64w_iRT-C18	TTOF_32w_iRT-C18	TTOF_64w_iRT-C18
	Library	Libraries	TTOF_32w-assay	TTOF_64w-assay	TTOF_32w-assay	TTOF_64w-assay
Transition settings	Full-Scan	MS/MS filtering - Isolation scheme	SWATH_32fixed	SWATH_64windows	SWATH_32fixed	SWATH_64windows
		MS/MS filtering - Resolving power	20000		33330	
Reintegrate	Peak scoring model		All values are automatically assigned when the model is trained			

Iteration 1

			TripleTOF 5600		TripleTOF 6600	
			32 windows	**64 windows**	**32 windows**	**64 windows**
File Import	Results	Raw type	raw (wiff file)			
Peptide settings	Prediction	Retention time predictor	TTOF_32w_iRT-C18	TTOF_64w_iRT-C18	TTOF_32w_iRT-C18	TTOF_64w_iRT-C18
	Library	Libraries	TTOF_32w-assay	TTOF_64w-assay	TTOF_32w-assay	TTOF_64w-assay
Transition settings	Full-Scan	MS/MS filtering - Isolation scheme	SWATH_32fixed	SWATH_64windows	SWATH_32fixed	SWATH_64windows
		MS/MS filtering - Resolving power	40000		66660	
Reintegrate	Peak scoring model		All values are automatically assigned when the model is trained			

Iteration 2

			TripleTOF 5600		TripleTOF 6600	
			32 windows	**64 windows**	**32 windows**	**64 windows**
File Import	Results	Raw type	centroid (mzML file)			
Peptide settings	Prediction	Retention time predictor	TTOF_32w_iRT-C18	TTOF_64w_iRT-C18	TTOF_32w_iRT-C18	TTOF_64w_iRT-C18
	Library	Libraries	TTOF_32w-assay	TTOF_64w-assay	TTOF_32w-assay	TTOF_64w-assay
Transition settings	Full-Scan	MS/MS filtering - Isolation scheme	SWATH_32fixed	SWATH_64windows	SWATH_32fixed	SWATH_64windows
		MS/MS filtering - Resolving power	60000		100000	
Reintegrate	Peak scoring model		All values are automatically assigned when the model is trained			

Supplementary Table 5.D: Parameters of Skyline.

Spectronaut		
both iterations		
Peak Detection	*XIC Extraction Window*	Dynamic Window
	Correction Factor	1
Calibration	*Force Calibration*	FALSE
	iRT Calibration Strategy	Non-Linear iRT Calibration
Identification	*Dynamic Score Refinement*	TRUE
	Pvalue Estimator	Normal Distribution Estimator
	Include MS1 Scoring	TRUE
Quantitation	*Interference Correction*	TRUE
	Cross Run Normalization	TRUE
	Normalization Base	Total Peak Area
Workflow	*Profiling Strategy*	iRT Profiling
	Profiling Row Selection	Minimum Qvalue Row Selection
	Qvalue Threshold	0.01
	Profiling Target Selection	Automatic Selection
	Default Labeling Type Assumed	LABEL
Post Analysis	*Regulation Analysis*	Student's T-Test
	Quantity Base	MS2 Peak Area
	Row Selection Strategy	Qvalue Based Row Selection
	Row Filter	Sparse Profiles
	Qvalue threshold	0.01
	NA Recovery	unchecked
	Peptide Grouping	Protein Grouping
Reporting	*Pipeline Report Schema*	SWATHbenchmark (Normal)
	Pipeline Report	Experiment
	Generate SNE File	TRUE

Supplementary Table 5.E: Parameters of Spectronaut.

DIA-Umpire					
		both iterations			
		TripleTOF 5600		TripleTOF 6600	
		32 windows	64 windows	32 windows	64 windows
Signal extraction	RPmax	25			
	RFmax	300			
	CorrThreshold	0.2			
	DeltaApex	0.6			
	RTOverlap	0.3			
	AdjustFragIntensity	TRUE			
	BoostComplementaryIon	TRUE			
	MS1PPM	30			
	MS2PPM	40			
	SN	1.5			
	MS2SN	1.5			
	MinMSIntensity	300	500	300	400
	MinMSMSIntensity	30	50	30	40
	MaxCurveRTRange	1.5			
	StartCharge	1			
	EndCharge	5			
	MS2StartCharge	2			
	MS2EndCharge	4			
	NoMissedScan	2	2	1	1
	EstimateBG	FALSE			
	IsoPattern	0.5			
Database search	X! Tandem	tandem.param			
	PeptideProphet(X!Tandem)	"-OpdEAP -PPM -dreverse -p0.1"			
	Comet	comet.param			
	PeptideProphet(Comet)	"-OpdAP -PPM -dreverse -p0.1"			
	MSGF+	-s *.mzXML -d			
	PeptideProphet(MSGF+)	"-OpdEAP -PPM -dreverse -p0.1"			
Quantification	TopNFrag	6			
	TopNPep	6			
	Freq	0.5			
	FilterWeight	GW			
	MinWeight	0.9			
	Peptide FDR	0.01			
	Protein FDR	0.01			
	ProbThreshold	0.5			

Supplementary Table 5.F: Parameters of DIA-Umpire.

Supplementary Table 6. Analysis of the dynamic ranges on peptide and protein level

Dynamic ranges of peptide and protein quantification values are expressed as orders of magnitude and were estimated by using the intensity range between 1% and 99% percentiles of quantification values.

The following pages show the dynamic ranges for:

- Supplementary Table 6.A: sample set HYE124 iteration 1 and 2
- Supplementary Table 6.B: sample set HYE110

Peptides

		TripleTOF 5600		TripleTOF 6600	
		Iteration 1	Iteration 2	Iteration 1	Iteration 2
32 windows	OpenSWATH	2.81	3.18	3.40	3.67
	SWATH2.0	3.03	2.92	3.57	3.23
	Skyline	2.94	2.96	3.53	3.40
	Spectronaut	3.29	3.60	3.75	3.93
	DIA-Umpire	3.14	3.05	3.69	3.64
64 windows	OpenSWATH	3.00	3.23	3.60	3.78
	SWATH2.0	3.22	3.06	3.83	3.73
	Skyline	3.13	3.09	3.77	3.73
	Spectronaut	3.31	3.47	3.90	4.04
	DIA-Umpire	3.18	3.10	3.78	3.72

Proteins

		TripleTOF 5600		TripleTOF 6600	
		Iteration 1	Iteration 2	Iteration 1	Iteration 2
32 windows	OpenSWATH	2.02	2.45	2.54	2.79
	SWATH2.0	2.47	2.36	2.84	2.68
	Skyline	2.36	2.35	2.71	2.73
	Spectronaut	2.63	2.73	2.92	2.99
	DIA-Umpire	2.41	2.21	2.98	2.91
64 windows	OpenSWATH	2.37	2.58	2.93	3.15
	SWATH2.0	2.57	2.45	3.03	2.92
	Skyline	2.47	2.55	2.99	2.99
	Spectronaut	2.68	2.77	3.11	3.21
	DIA-Umpire	2.42	2.26	3.08	2.99

Supplementary Table 6.A: Dynamic ranges in HYE124.

Peptides

	32 windows		64 windows	
	fixed	**variable**	**fix**	**var**
OpenSWATH	4.16	4.26	4.26	4.10
SWATH2.0	3.88	4.03	4.02	3.61
Skyline	3.90	4.05	4.14	3.78
Spectronaut	4.36	4.46	4.67	4.35
DIA-Umpire	3.58	3.67	3.84	3.78

Proteins

	32 windows		64 windows	
	fixed	**variable**	**fix**	**var**
OpenSWATH	3.44	3.42	3.54	3.24
SWATH2.0	3.06	3.18	3.22	3.01
Skyline	3.02	3.16	3.25	3.07
Spectronaut	3.42	3.50	3.75	3.31
DIA-Umpire	2.89	2.86	3.00	2.99

Supplementary Table 6.B: Dynamic ranges in HYE110.

Supplementary Table 7. SWATH isolation windows setups

Isolation m/z ranges applied on each SWATH configuration handled in this study.

32 fixed windows			32 variable windows			64 fixed windows			64 variable windows		
	mz.start	mz.end		mz.start	mz.end		mz.start	mz.end		mz.start	mz.end
swath.1	399	425	swath.1	399.5	415.8	swath.1	399	412.5	swath.1	399.5	408.2
swath.2	424	450	swath.2	414.8	429.7	swath.2	411.5	425	swath.2	407.2	415.8
swath.3	449	475	swath.3	428.7	444.8	swath.3	424	437.5	swath.3	414.8	422.7
swath.4	474	500	swath.4	443.8	458.7	swath.4	436.5	450	swath.4	421.7	429.7
swath.5	499	525	swath.5	457.7	473.4	swath.5	449	462.5	swath.5	428.7	437.3
swath.6	524	550	swath.6	472.4	485.4	swath.6	461.5	475	swath.6	436.3	444.8
swath.7	549	575	swath.7	484.4	497.7	swath.7	474	487.5	swath.7	443.8	451.7
swath.8	574	600	swath.8	496.7	511.2	swath.8	486.5	500	swath.8	450.7	458.7
swath.9	599	625	swath.9	510.2	525.3	swath.9	499	512.5	swath.9	457.7	466.7
swath.10	624	650	swath.10	524.3	540.3	swath.10	511.5	525	swath.10	465.7	473.4
swath.11	649	675	swath.11	539.3	554.5	swath.11	524	537.5	swath.11	472.4	478.3
swath.12	674	700	swath.12	553.5	568.3	swath.12	536.5	550	swath.12	477.3	485.4
swath.13	699	725	swath.13	567.3	582.3	swath.13	549	562.5	swath.13	484.4	491.2
swath.14	724	750	swath.14	581.3	595.8	swath.14	561.5	575	swath.14	490.2	497.7
swath.15	749	775	swath.15	594.8	608.9	swath.15	574	587.5	swath.15	496.7	504.3
swath.16	774	800	swath.16	607.9	624.8	swath.16	586.5	600	swath.16	503.3	511.2
swath.17	799	825	swath.17	623.8	640.8	swath.17	599	612.5	swath.17	510.2	518.2
swath.18	824	850	swath.18	639.8	654.8	swath.18	611.5	625	swath.18	517.2	525.3
swath.19	849	875	swath.19	653.8	670.3	swath.19	624	637.5	swath.19	524.3	533.3
swath.20	874	900	swath.20	669.3	687.8	swath.20	636.5	650	swath.20	532.3	540.3
swath.21	899	925	swath.21	686.8	706.9	swath.21	649	662.5	swath.21	539.3	546.8
swath.22	924	950	swath.22	705.9	726.2	swath.22	661.5	675	swath.22	545.8	554.5
swath.23	949	975	swath.23	725.2	746.6	swath.23	674	687.5	swath.23	553.5	561.8
swath.24	974	1000	swath.24	745.6	767.9	swath.24	686.5	700	swath.24	560.8	568.3
swath.25	999	1025	swath.25	766.9	792.9	swath.25	699	712.5	swath.25	567.3	575.7
swath.26	1024	1050	swath.26	791.9	820	swath.26	711.5	725	swath.26	574.7	582.3
swath.27	1049	1075	swath.27	819	849.4	swath.27	724	737.5	swath.27	581.3	588.8
swath.28	1074	1100	swath.28	848.4	884.4	swath.28	736.5	750	swath.28	587.8	595.8
swath.29	1099	1125	swath.29	883.4	919	swath.29	749	762.5	swath.29	594.8	601.8
swath.30	1124	1150	swath.30	918	971.6	swath.30	761.5	775	swath.30	600.8	608.9
swath.31	1149	1175	swath.31	970.6	1053	swath.31	774	787.5	swath.31	607.9	616.9
swath.32	1174	1200	swath.32	1052	1200.5	swath.32	786.5	800	swath.32	615.9	624.8
						swath.33	799	812.5	swath.33	623.8	632.2
						swath.34	811.5	825	swath.34	631.2	640.8
						swath.35	824	837.5	swath.35	639.8	647.9
						swath.36	836.5	850	swath.36	646.9	654.8
						swath.37	849	862.5	swath.37	653.8	661.5
						swath.38	861.5	875	swath.38	660.5	670.3
						swath.39	874	887.5	swath.39	669.3	678.8
						swath.40	886.5	900	swath.40	677.8	687.8
						swath.41	899	912.5	swath.41	686.8	696.9
						swath.42	911.5	925	swath.42	695.9	706.9
						swath.43	924	937.5	swath.43	705.9	715.9
						swath.44	936.5	950	swath.44	714.9	726.2
						swath.45	949	962.5	swath.45	725.2	737.4
						swath.46	961.5	975	swath.46	736.4	746.6
						swath.47	974	987.5	swath.47	745.6	757.5
						swath.48	986.5	1000	swath.48	756.5	767.9
						swath.49	999	1012.5	swath.49	766.9	779.5
						swath.50	1011.5	1025	swath.50	778.5	792.9
						swath.51	1024	1037.5	swath.51	791.9	807
						swath.52	1036.5	1050	swath.52	806	820
						swath.53	1049	1062.5	swath.53	819	834.2
						swath.54	1061.5	1075	swath.54	833.2	849.4
						swath.55	1074	1087.5	swath.55	848.4	866
						swath.56	1086.5	1100	swath.56	865	884.4
						swath.57	1099	1112.5	swath.57	883.4	899.9
						swath.58	1111.5	1125	swath.58	898.9	919
						swath.59	1124	1137.5	swath.59	918	942.1
						swath.60	1136.5	1150	swath.60	941.1	971.6
						swath.61	1149	1162.5	swath.61	970.6	1006
						swath.62	1161.5	1175	swath.62	1005	1053
						swath.63	1174	1187.5	swath.63	1052	1110.6
						swath.64	1186.5	1200	swath.64	1109.6	1200.5

Supplementary Table 7: Isolation m/z ranges applied on each swath configuration.

Supplementary Table 8. Ion Libraries Statistics

The library used by OpenSWATH, SWATH2.0, Skyline, and Spectronaut consists of identifications from separate, triplicate DDA acquisitions of samples from each species (human, yeast, and E.coli). A consensus of the search results of two different database search engines (Comet and Mascot) was used to build the ion library. Fragment ions with an m/z within the range of the SWATH precursor isolation window were excluded from the library due to interferences with unfragmented precursor ions. Since the different SWATH windows configurations (64 variable, 64 fixed, 32 variable, and 32 fixed) have different precursor isolation windows, a library for each mode (with different excluded ions) was built. The number of proteins counts all proteins present in the library including proteins that share their sequence with other proteins. Protein groups were defined by iProphet, and any reference to the number of proteins identified by a software tool is referred to protein groups.

Biognosys AG provided a dedicated library of Orbitrap/Fusion assays of human peptides including 6,826 peptides, which were exclusively detected by DIA-Umpire. The other 2,556 (a total of 9,382 peptides are present in the library) were excluded from the study.

32 fixed windows assay library statistics

Species	Proteins	Protein groups	Peptides	Precursors	Sequences	Transitions
ECOLI	1,564	1,594	12,753	13,304	12,514	79,824
HUMAN	3,136	3,148	16,405	16,995	16,353	101,970
YEAST	2,005	2,105	14,272	14,885	14,172	89,310
Total	6,705	6,847	43,430	45,184	43,039	271,104

32 variable windows assay library statistics

Species	Proteins	Protein groups	Peptides	Precursors	Sequences	Transitions
ECOLI	1,571	1,602	12,897	13,448	12,656	80,772
HUMAN	3,153	3,191	16,621	17,219	16,569	104,112
YEAST	2,017	2,128	14,569	15,186	14,467	91,116
Total	6,741	6,921	44,087	45,853	43,692	276,000

64 fixed windows assay library statistics

Species	Proteins	Protein groups	Peptides	Precursors	Sequences	Transitions
ECOLI	1,572	1,603	12,938	13,490	12,694	80,940
HUMAN	3,161	3,199	16,788	17,387	16,736	104,364
YEAST	2,020	2,131	14,473	15,093	14,370	91,398
Total	6,753	6,933	44,199	45,970	43,800	276,702

64 variable windows assay library statistics

Species	Proteins	Protein groups	Peptides	Precursors	Sequences	Transitions
ECOLI	1,575	1,605	12,999	13,550	12,754	81,300
HUMAN	3,159	3,176	16,755	17,349	16,703	104,094
YEAST	2,021	2,122	14,540	15,153	14,440	90,918
Total	6,755	6,903	44,294	46,052	43,897	276,312

DIA-Umpire dedicated assay library statistics

Species	Proteins	Protein groups	Peptides	Precursors	Sequences	Transitions
HUMAN	2,734	2,734	9,382	14,007	7,641	82,626

Supplementary Table 8: Ion Libraries Statistics.

Supplementary Table 9. log_2 ratios shift corrections

In order to correctly assess the accuracy of relative quantification it is necessary to shift all quantification values (expressed in log_2 ratios) to a reference. We estimate the reference as the average of all quantification values (in log_2 ratios) of the human proteins (or peptides). Since human proteins (and peptides) are expected to be in the same proportion in both samples, A and B, all quantitation values are shifted by subtracting the estimated reference value (average of human proteins or peptides). Note that by doing this shift the accuracy of the human peptides (or proteins) at the Supplementary Table 4 is artificially set to zero.

The following pages show the log_2 ratios shift corrections of quantified peptides and proteins for:

- Supplementary Table 9.A: sample set HYE124 iteration 1 and 2
- Supplementary Table 9.B: sample set HYE110

Peptides

		TripleTOF 5600		TripleTOF 6600	
		Iteration 1	Iteration 2	Iteration 1	Iteration 2
32 windows	OpenSWATH	-0.024	-0.005	0.048	0.066
	SWATH2.0	-0.005	-0.003	0.069	0.056
	Skyline	-0.007	-0.031	0.063	0.056
	Spectronaut	-0.015	-0.006	-0.025	-0.012
	DIA-Umpire	0.032	0.029	0.061	0.056
64 windows	OpenSWATH	-0.009	0.000	0.048	0.055
	SWATH2.0	-0.006	-0.006	0.056	0.057
	Skyline	-0.004	0.064	0.053	0.047
	Spectronaut	-0.015	-0.006	-0.029	-0.019
	DIA-Umpire	0.002	-0.005	0.066	0.063

Proteins

		TripleTOF 5600		TripleTOF 6600	
		Iteration 1	Iteration 2	Iteration 1	Iteration 2
32 windows	OpenSWATH	-0.025	-0.011	0.038	0.054
	SWATH2.0	-0.005	0.002	0.052	0.049
	SWATH2.0 (built-in)	-0.010	NA	0.056	NA
	Skyline	-0.012	-0.040	0.057	0.042
	Spectronaut	-0.011	-0.010	-0.032	-0.019
	DIA-Umpire	0.064	0.058	0.053	0.054
	DIA-Umpire (built-in)	0.088	NA	0.051	NA
64 windows	OpenSWATH	-0.003	0.003	0.045	0.049
	SWATH2.0	-0.003	0.001	0.051	0.055
	SWATH2.0 (built-in)	-0.007	NA	0.044	NA
	Skyline	0.003	0.066	0.050	0.049
	Spectronaut	-0.017	-0.009	-0.033	-0.024
	DIA-Umpire	0.008	-0.001	0.056	0.049
	DIA-Umpire (built-in)	0.010	NA	0.055	NA

Supplementary Table 9.A: log_2 ratios shift corrections in HYE124.

Peptides

	32 windows		64 windows	
	fixed	**variable**	**fix**	**var**
OpenSWATH	-0.092	-0.071	-0.127	0.035
SWATH2.0	-0.090	-0.072	-0.116	0.039
Skyline	-0.181	-0.102	-0.110	0.028
Spectronaut	0.005	-0.008	-0.037	-0.002
DIA-Umpire	-0.051	-0.068	-0.105	0.040

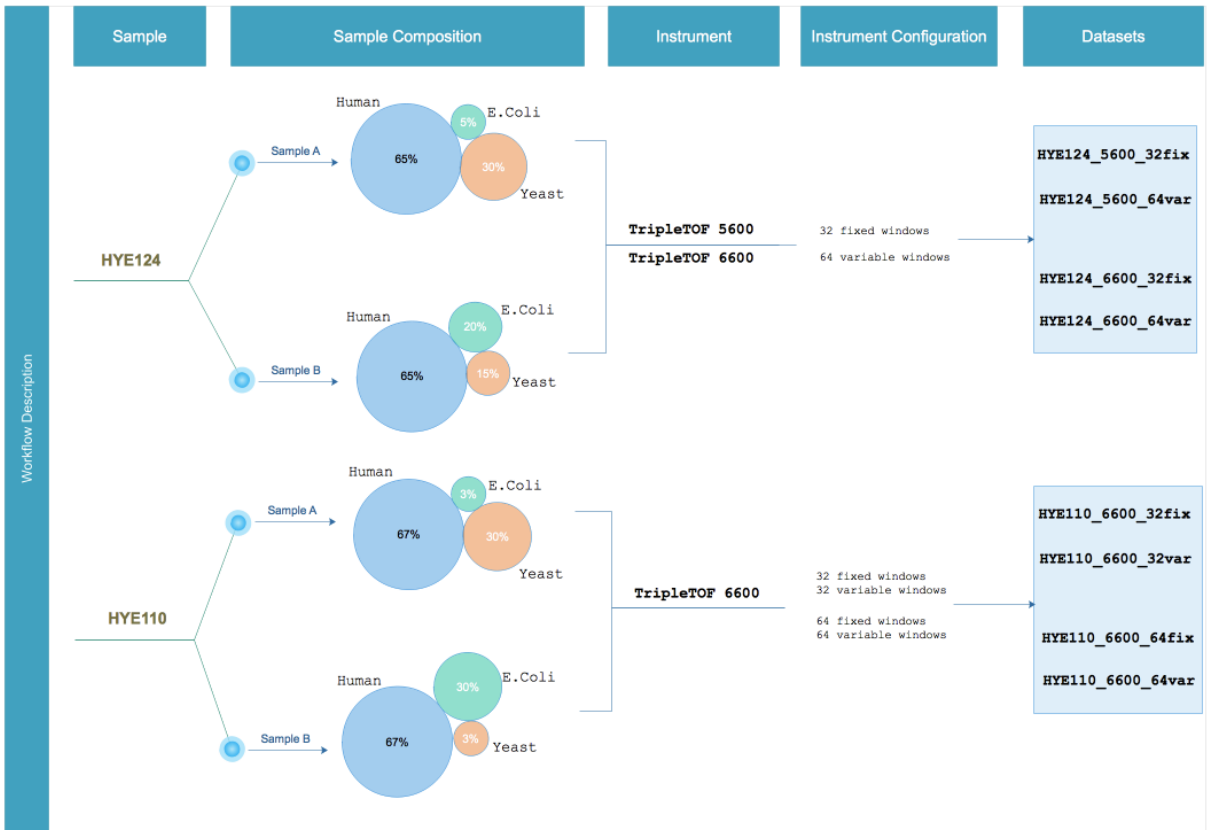
Proteins

	32 windows		64 windows	
	fixed	**variable**	**fix**	**var**
OpenSWATH	-0.124	-0.094	-0.159	0.026
SWATH2.0	-0.102	-0.091	-0.134	0.032
Skyline	-0.177	-0.118	-0.131	0.027
Spectronaut	-0.007	-0.026	-0.107	-0.020
DIA-Umpire	-0.081	-0.115	-0.152	0.028

Supplementary Table 9.B: log_2 ratios shift corrections in HYE110.

Supplementary Figures

Supplementary Figure 1. Schematic overview of the datasets acquired and analyzed in this study.

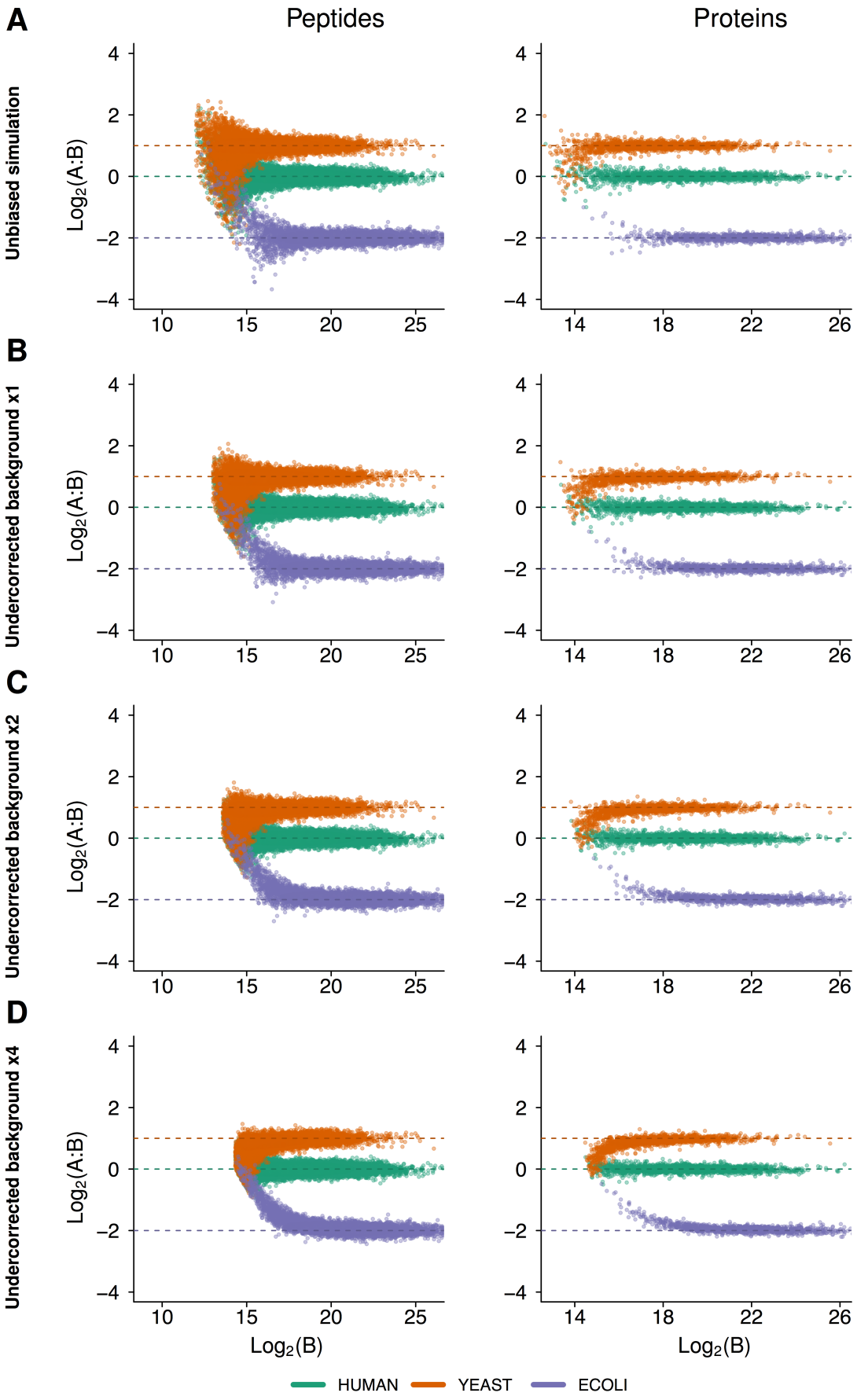


Supplementary Figure 1: Schematic overview of the datasets acquired and analyzed in this study.

Supplementary Figure 2. Simulated quantification data.

A LFQ data simulator is provided with LFQbench. This simulator helps to visualize how ideal or biased LFQ data look in LFQbench plots. Panel A shows an ideal (unbiased) simulated experiment. Panels B, C, and D show the same simulated experiment as in panel A with an increasing quantity of background signal (1-, 2-, and 3- times the minimum intensity detected in the dataset) added to all peptide signals.

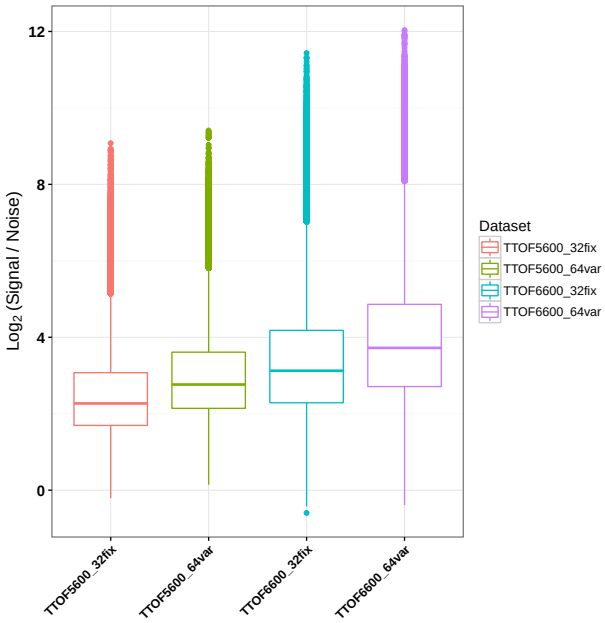
LFQ data simulation was performed by simulating the log_2 ratios of 2000 human proteins (expected log_2 ratio = 0), 1500 yeast proteins (expected log_2 ratio = 1), and 1000 E.coli proteins (expected log_2 ratio = -2) from normal distributions centered at the expected log_2 ratios and standard deviations of $\sigma = 0.1$. For each protein, the number of peptides is simulated by a Poisson distribution with $\lambda = 6$ (with a minimum value of 1). The protein intensity is distributed among its peptides, for which ionization efficiencies were simulated by a standard normal distribution.
Three technical replicates for each peptide and sample were simulated by choosing three random values from a normal distribution centered at the peptide intensity value and $\sigma = 0.01$. A random noise factor was added to each peptide intensity using random values from a normal distribution of $\mu = 0$ and $\sigma = 2^{13}$. Finally, in order to simulate missing values, 1% of randomly chosen peptides from the total simulated peptide signals were removed.



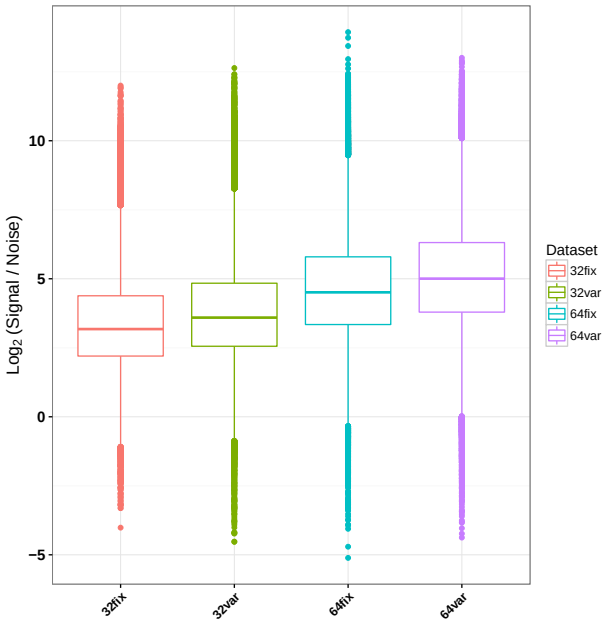
Supplementary Figure 2: Simulated quantification data with perfect background substraction (A) and undercorrected background levels (B, C, D).

Supplementary Figure 3. Signal-to-noise ratios for all datasets.

Distribution of the signal-to-noise ratio of all detected peaks (FDR < 0.01) as detected in Spectronaut for samples HYE124 and HYE110. The sample HYE124 allows to compare both instruments (TripleTOF 5600 vs TripleTOF 6600), whereas the sample HYE110 allows to compare the four different SWATH modes tested (32 fixed windows, 32 variable windows, 64 fixed windows, and 64 variable windows). For both, 32 fixed windows and 64 variable windows setups, the TripleTOF 6600 platform displayed a higher median signal-to-noise ratio as compared to the TripleTOF 5600 platform. For each instrument platform, the calculated median signal-to-noise ratio was higher for the 64 window setup.



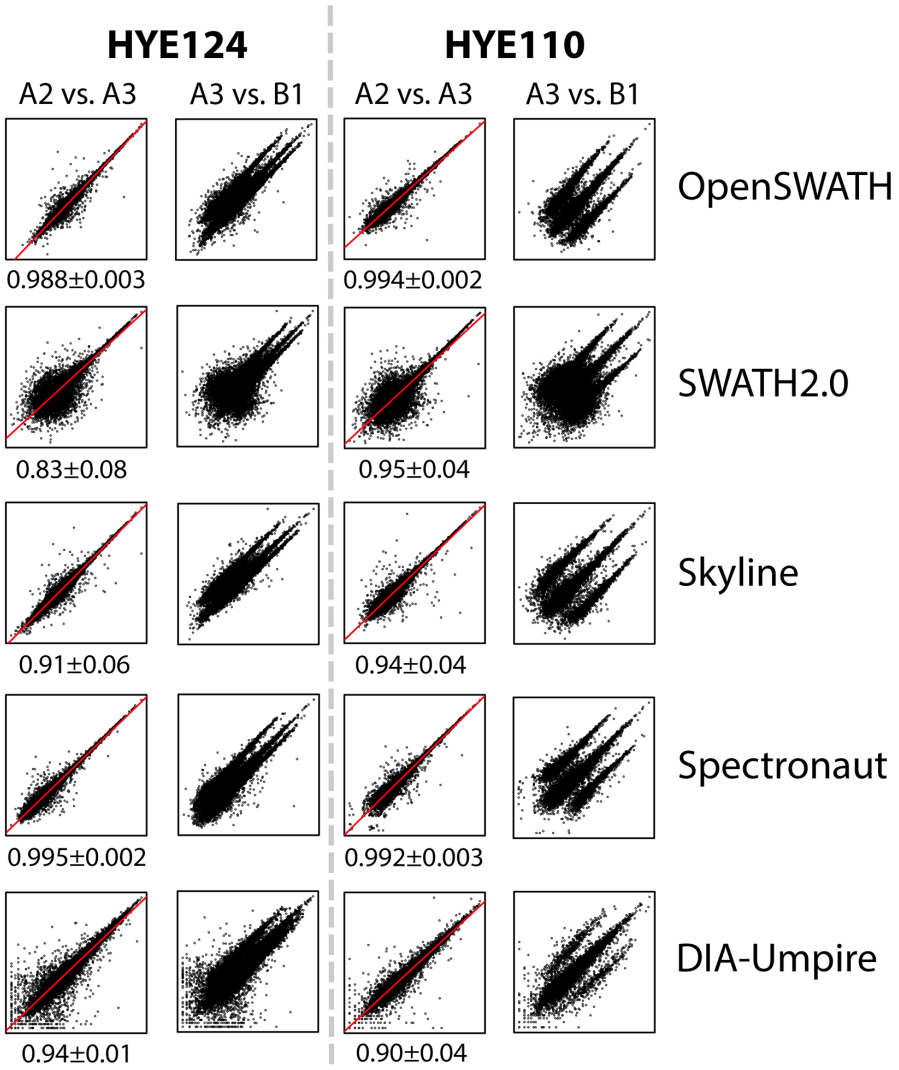
Supplementary Figure 3.A: Signal-to-Noise ratios for all datasets in HYE124 sample set.



Supplementary Figure 3.B: Signal-to-Noise ratios for all datasets in HYE110 sample set.

Supplementary Figure 4. Precursor intensity correlation between technical replicates.

Intensity signal correlations of detected precursors (FDR < 0.01) of technical replicates are exemplarily shown for A_1 vs A_3 and A_3 vs B_1. The other possible pairs (e.g. A_1 vs A_2, A_1 vs B_3) display a very similar pattern. For A_1 vs A_3 a linear regression trend line is shown in red and the average R^2 regression parameter (average of all possible pairs of sample A) is displayed below. A higher correlation is observed within technical replicates of the same sample (A_1 vs A_3), and the patterns of expression change of Yeast and E.coli are visible at the A_3 vs B_1 pairs.

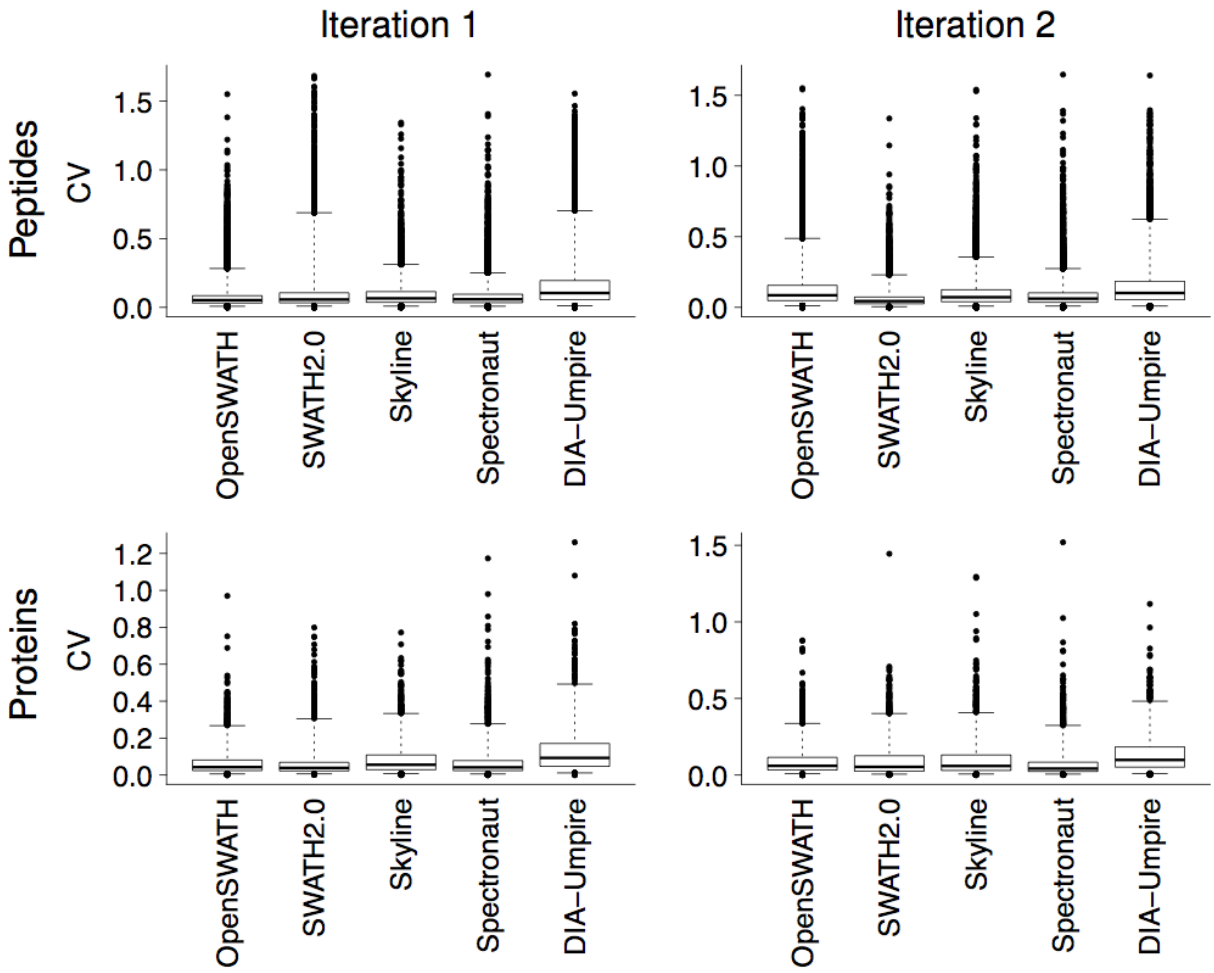


Supplementary Figure 4: Precursor intensity correlation between technical replicates.

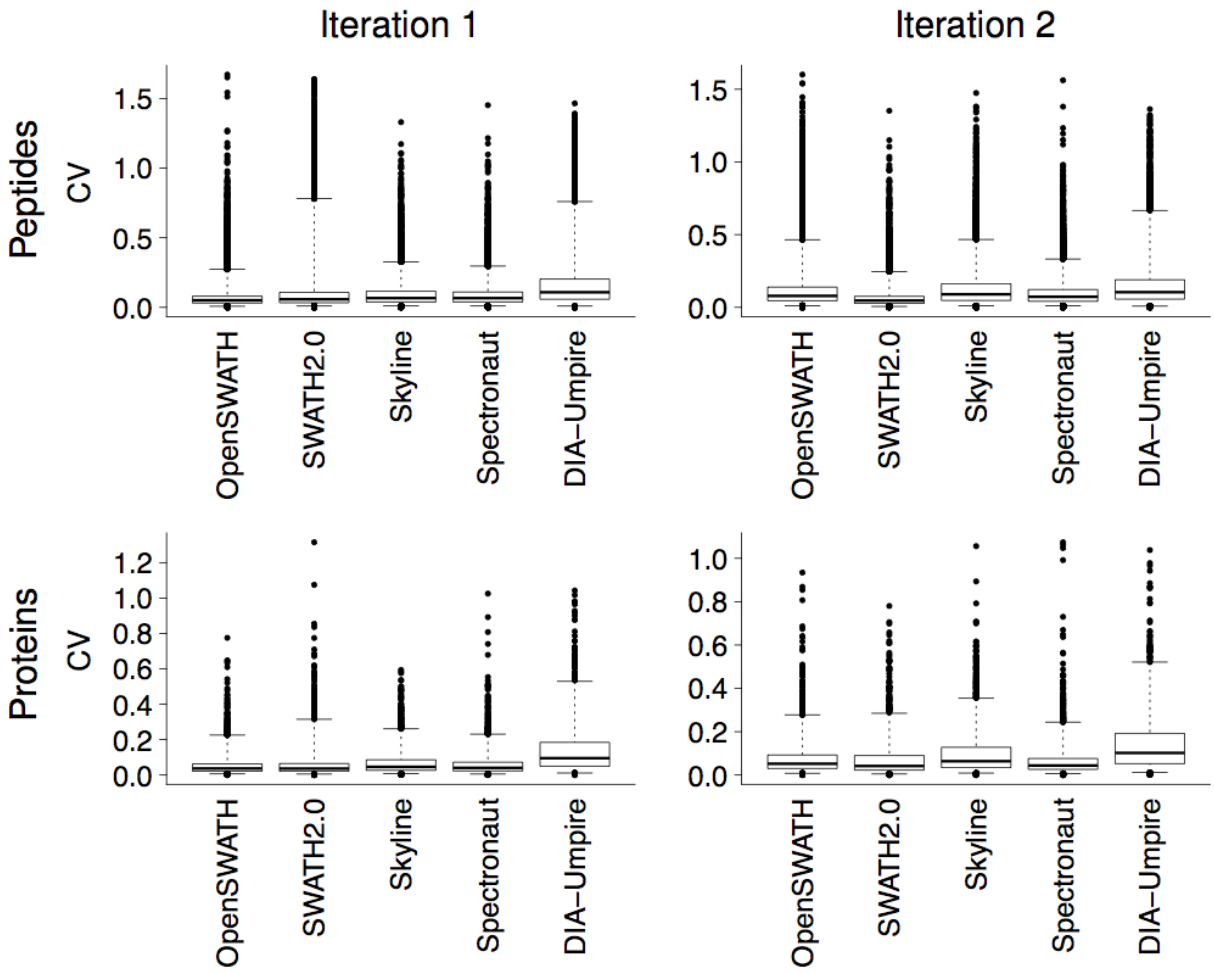
Supplementary Figure 5. Coefficients of Variance (CVs) between technical replicates for human proteins and peptides.

The following pages show the CVs between technical replicates for human proteins and peptides for:

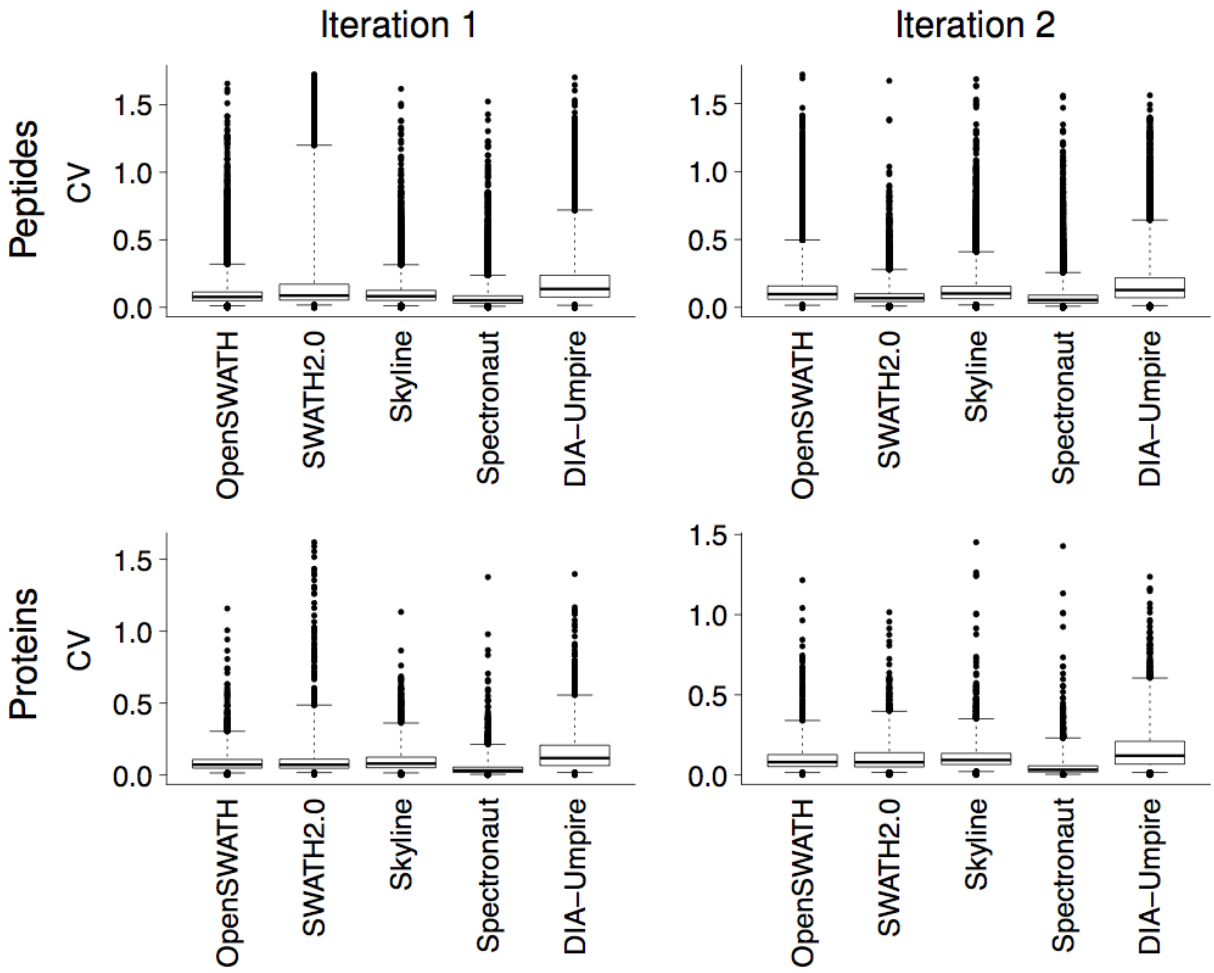
- Supplementary Figure 5.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 5.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 5.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 5.D: HYE 124, TripleTOF 6600, 64var setup
- Supplementary Figure 5.E: HYE 110, TripleTOF 6600, 32fix setup
- Supplementary Figure 5.F: HYE 110, TripleTOF 6600, 32var setup
- Supplementary Figure 5.G: HYE 110, TripleTOF 6600, 64fix setup
- Supplementary Figure 5.H: HYE 110, TripleTOF 6600, 64var setup



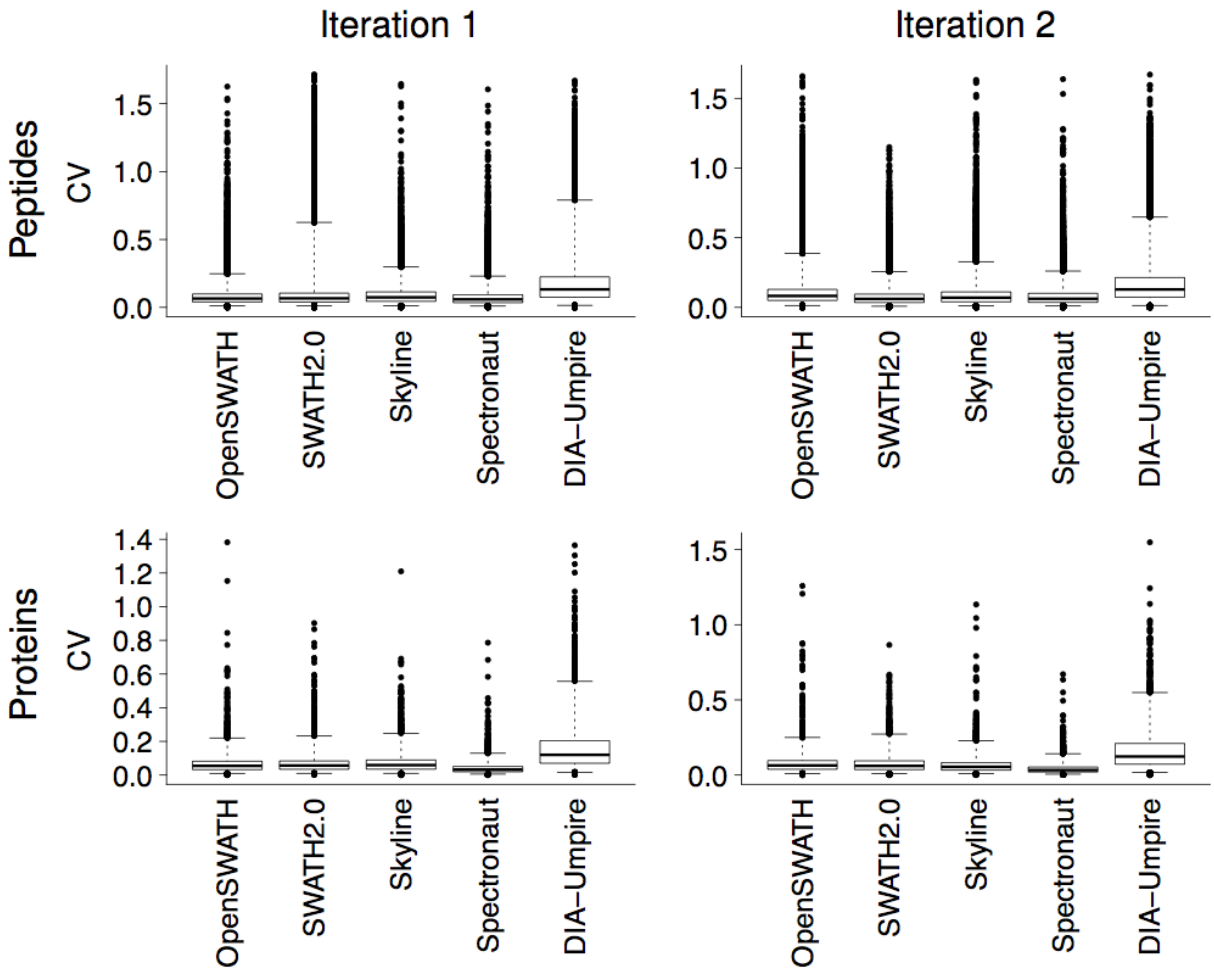
Supplementary Figure 5.A: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 124, TripleTOF 5600, 32fix setup.



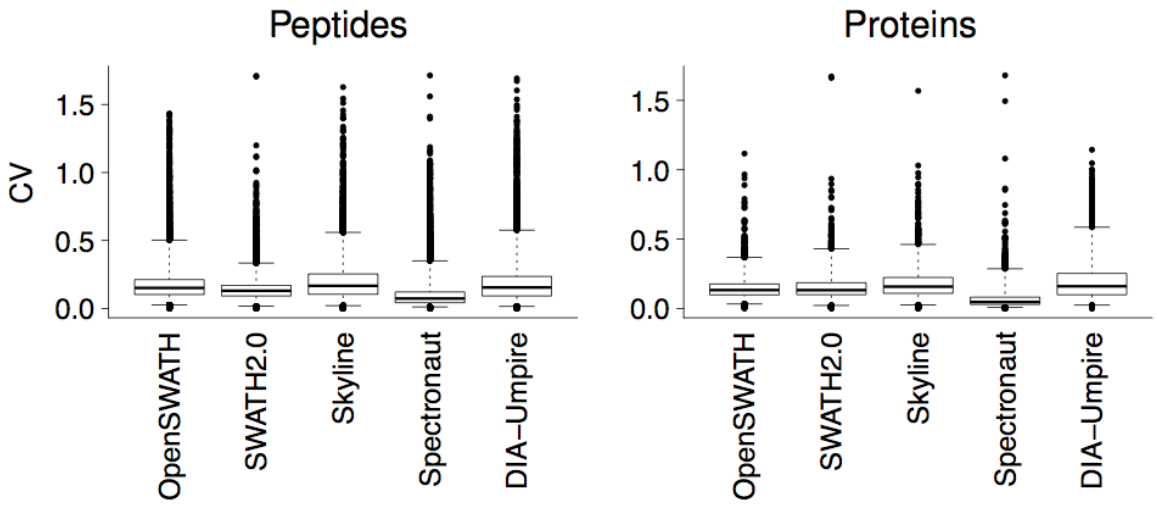
Supplementary Figure 5.B: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 124, TripleTOF 5600, 64var setup.



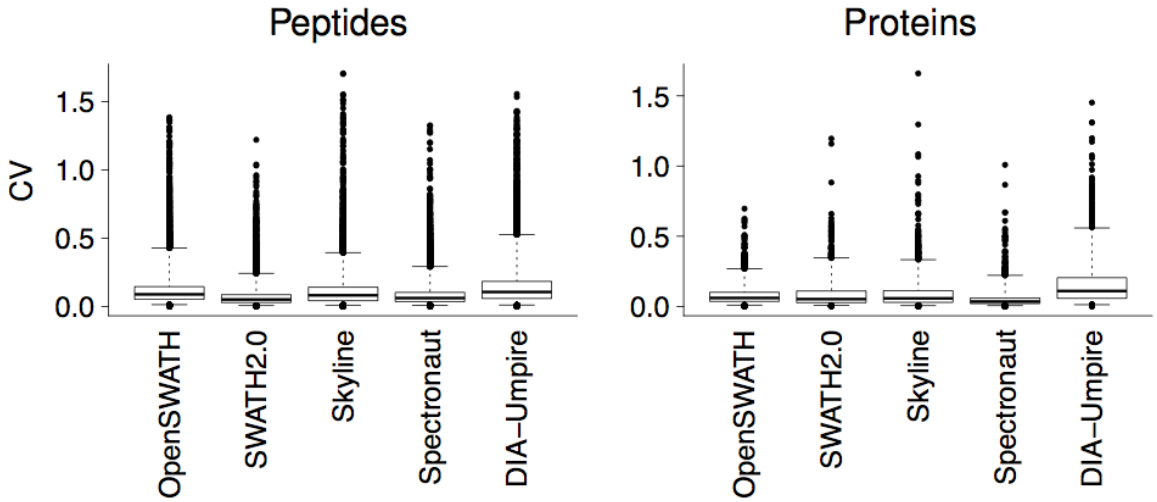
Supplementary Figure 5.C: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 124, TripleTOF 6600, 32fix setup.



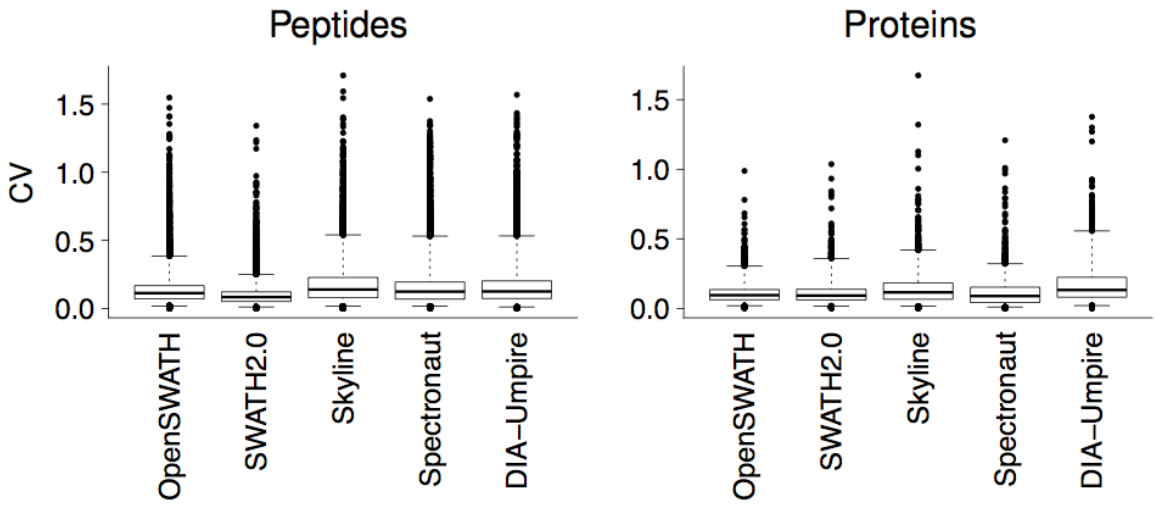
Supplementary Figure 5.D: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 124, TripleTOF 6600, 64var setup.



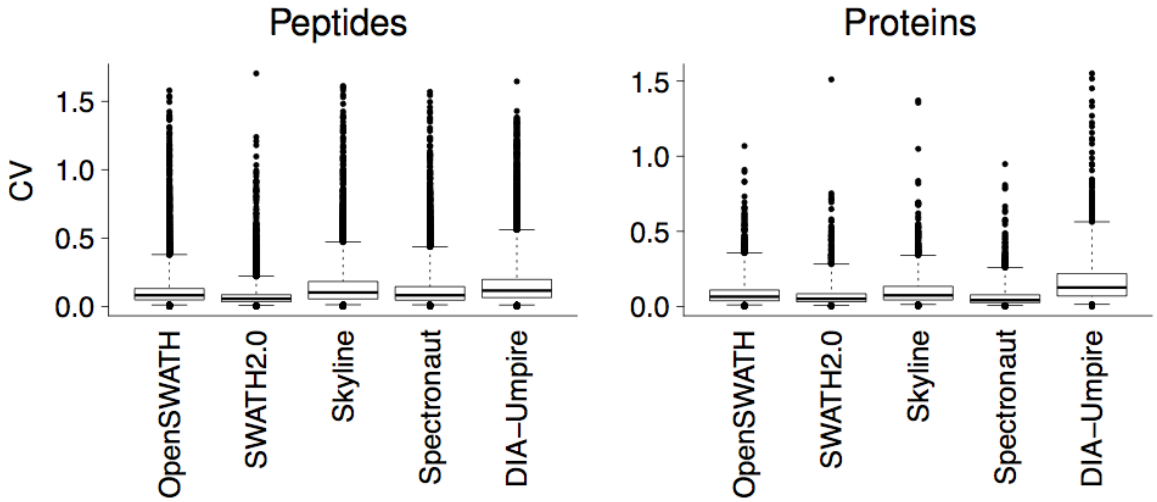
Supplementary Figure 5.E: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 110, TripleTOF 6600, 32fix setup.



Supplementary Figure 5.F: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 110, TripleTOF 6600, 32var setup.



Supplementary Figure 5.G: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 110, TripleTOF 6600, 64fix setup.



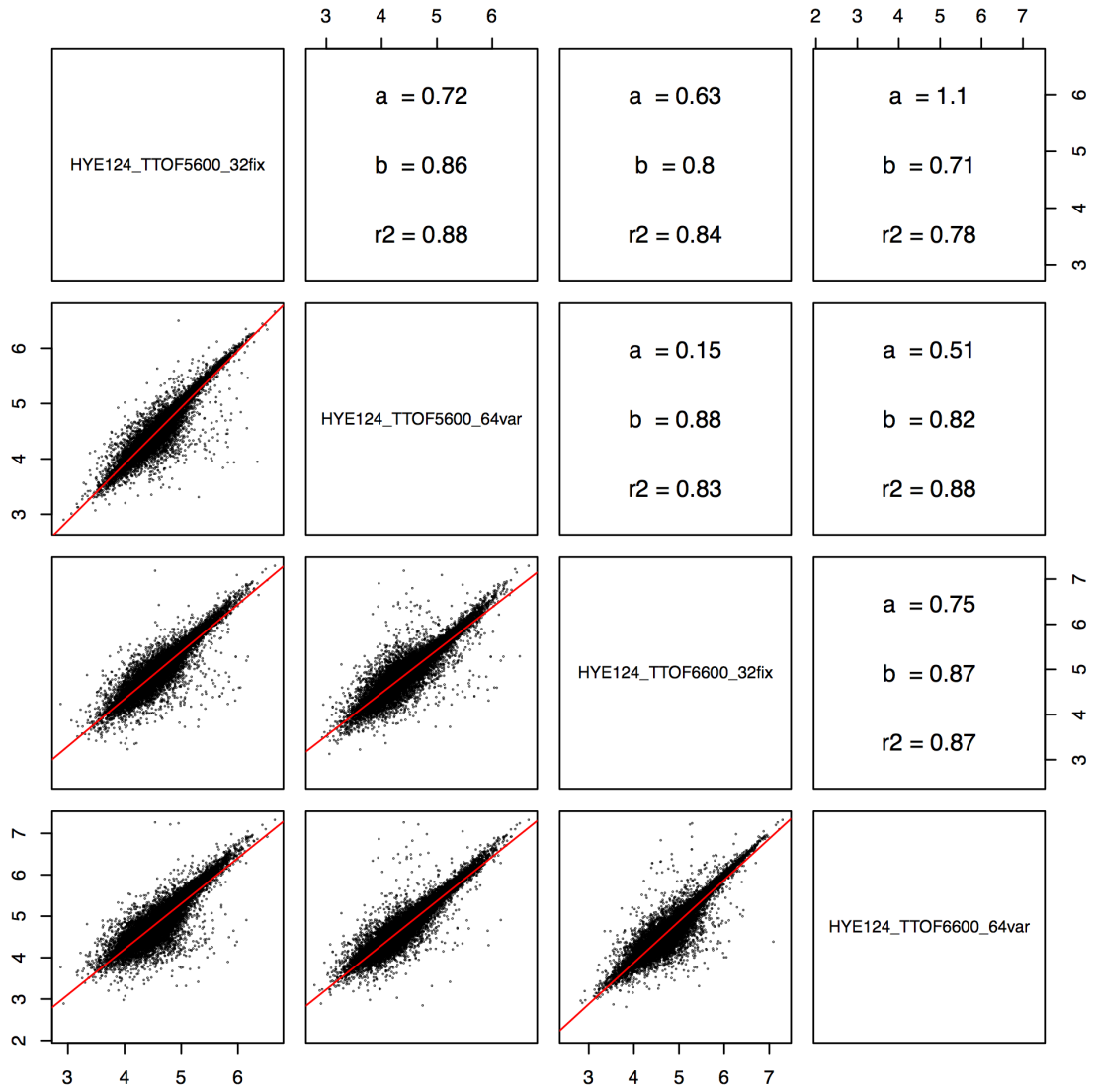
Supplementary Figure 5.H: Coefficients of Variance (CVs) between technical replicates for human proteins and peptides for HYE 110, TripleTOF 6600, 64var setup.

Supplementary Figure 6. Precursor intensity correlation between the different datasets.

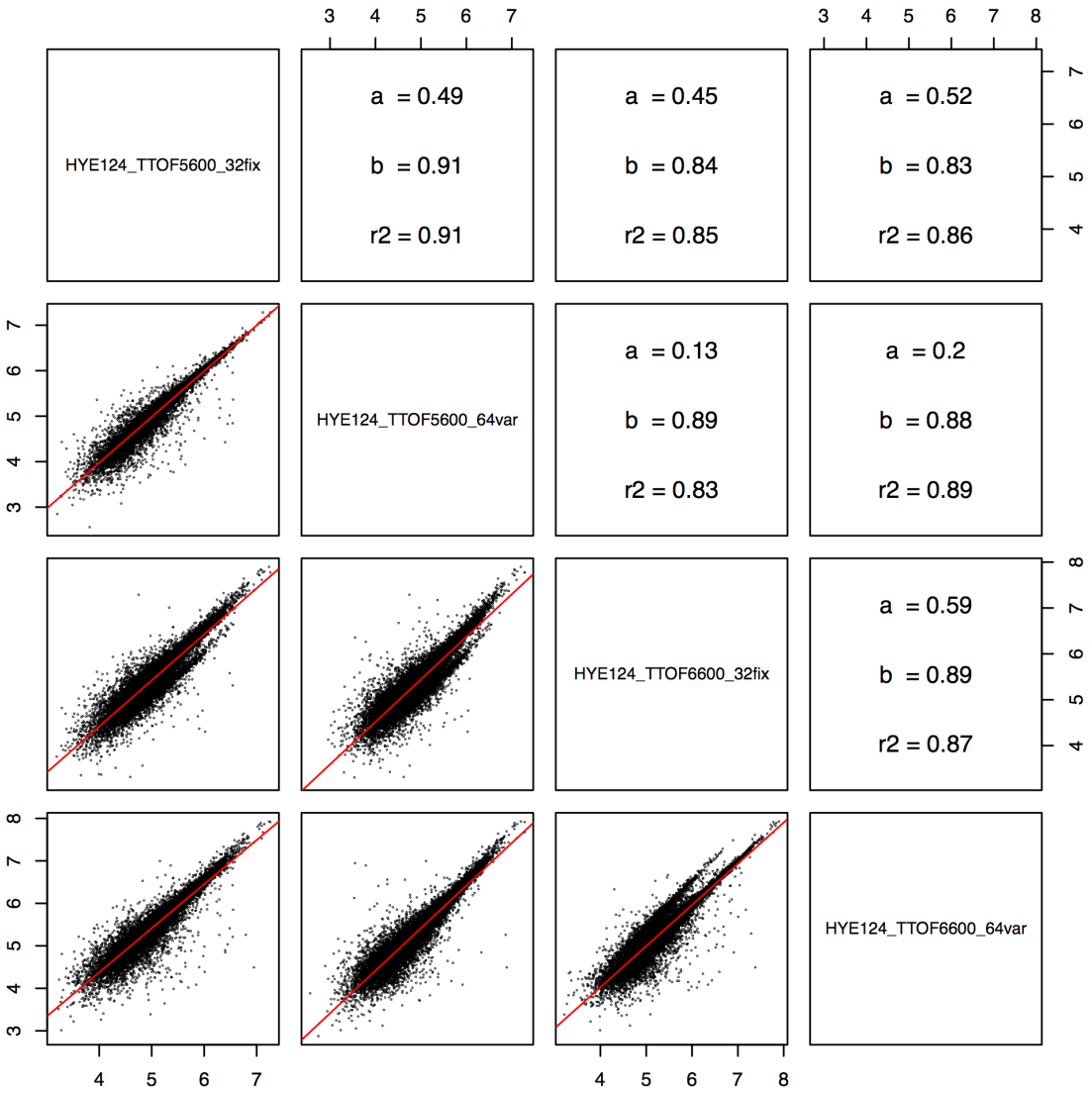
Datasets were compared pair-wise correlating total precursor intensities. Precursor intensities were calculated summing the intensity of all detected precursors (FDR < 0.01) within a dataset (i.e. across all technical replicates of both sample A and B).

The following pages show precursor intensity correlations between the different workflows for:

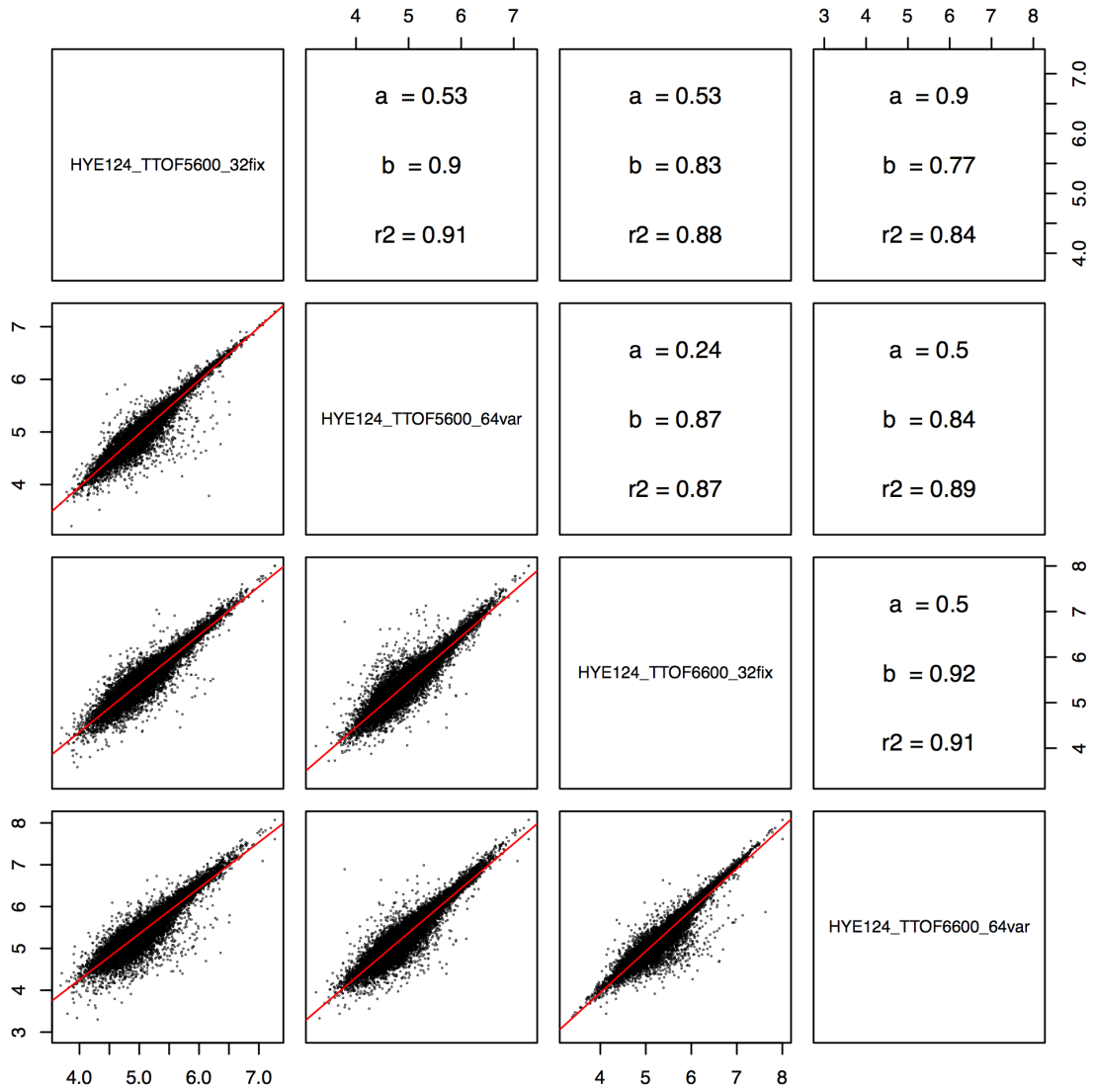
- Supplementary Figure 6.A: HYE124, OpenSWATH
- Supplementary Figure 6.B: HYE124, SWATH 2.0
- Supplementary Figure 6.C: HYE124, Skyline
- Supplementary Figure 6.D: HYE124, Spectronaut
- Supplementary Figure 6.E: HYE124, DIA-Umpire
- Supplementary Figure 6.F: HYE110, OpenSWATH
- Supplementary Figure 6.G: HYE110, SWATH 2.0
- Supplementary Figure 6.H: HYE110, Skyline
- Supplementary Figure 6.I: HYE110, Spectronaut
- Supplementary Figure 6.J: HYE110, DIA-Umpire



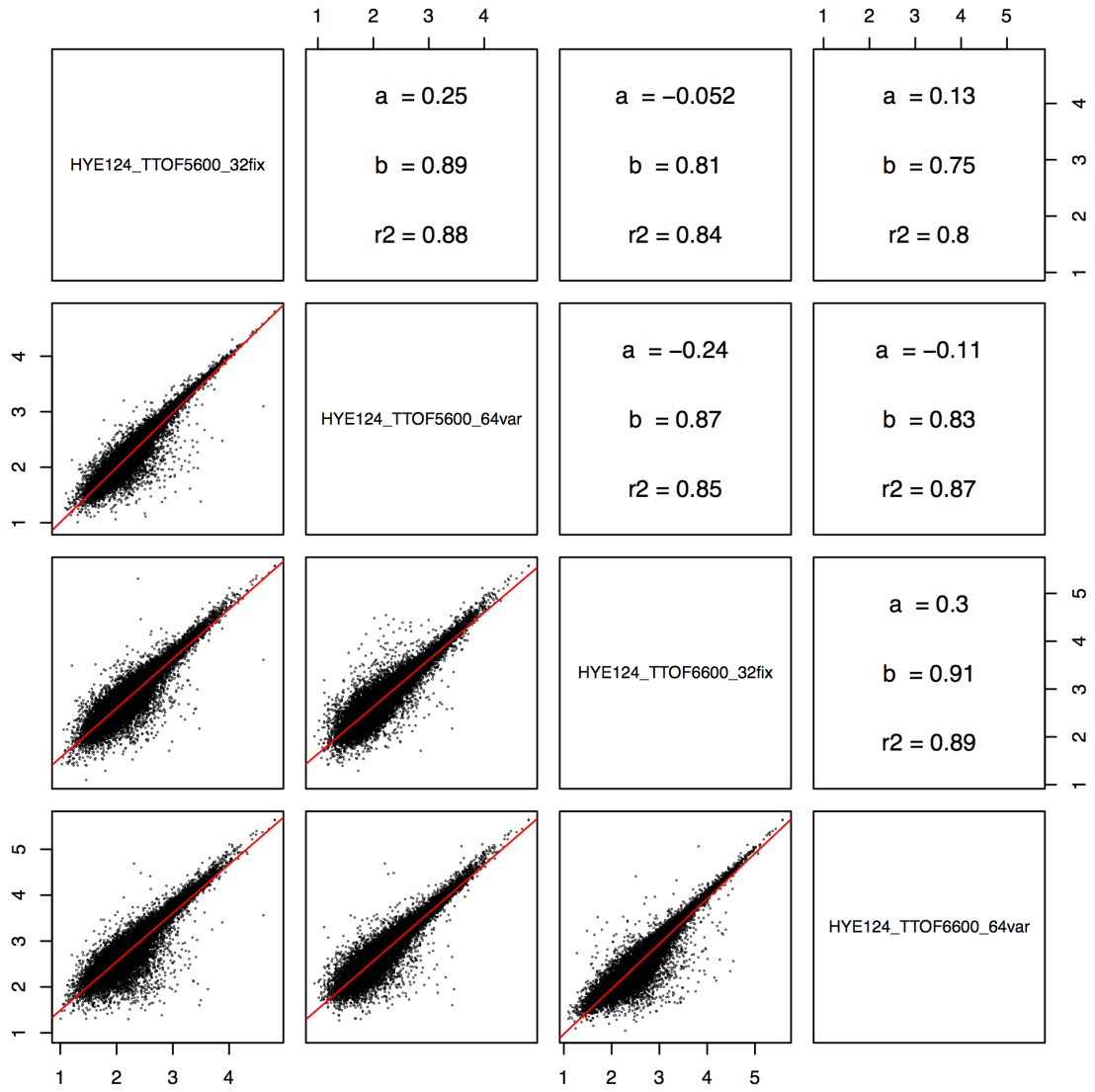
Supplementary Figure 6.A: Precursor intensity correlation between workflows for HYE124, OpenSWATH.



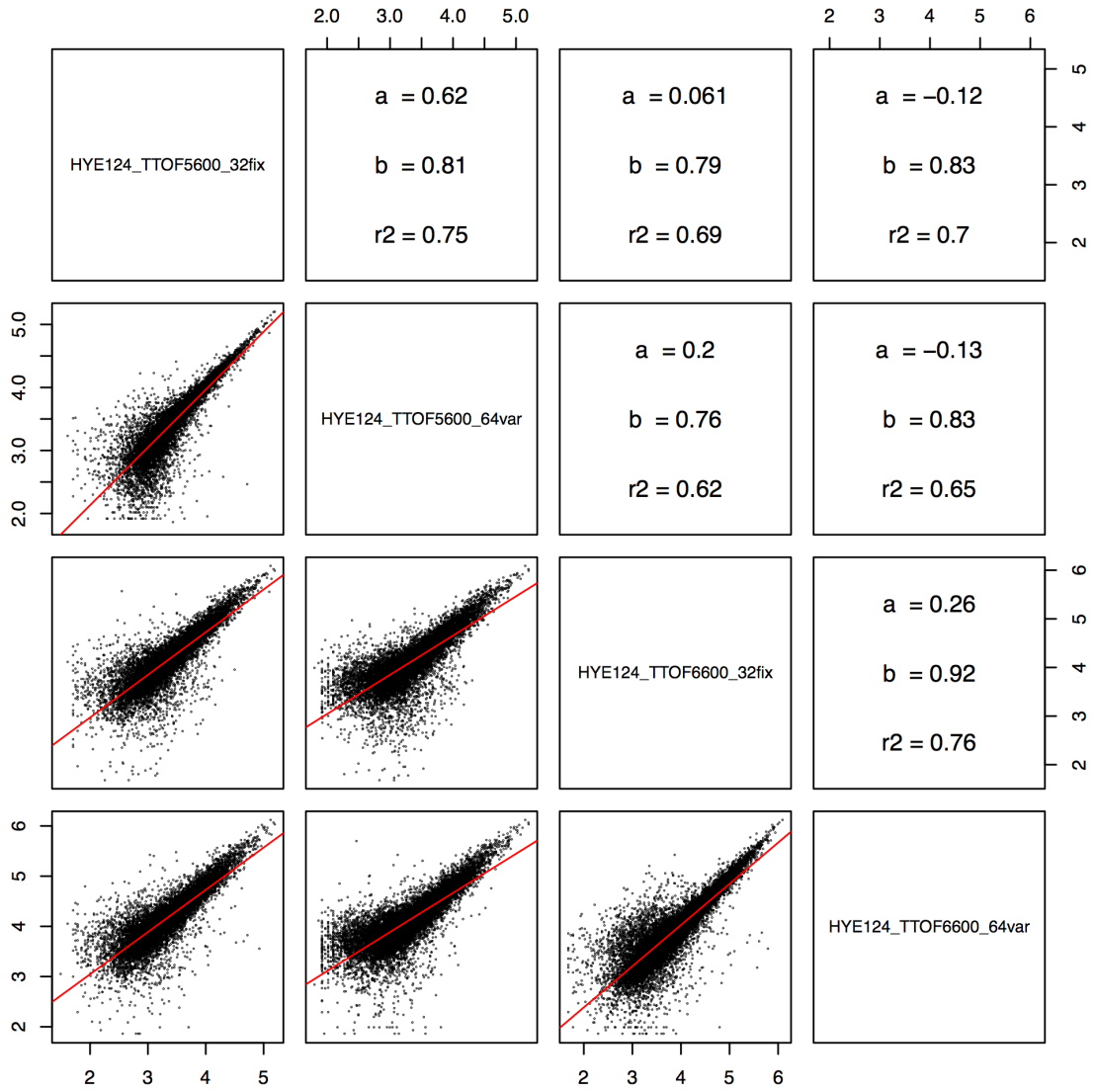
Supplementary Figure 6.B: Precursor intensity correlation between workflows for HYE124, SWATH 2.0.



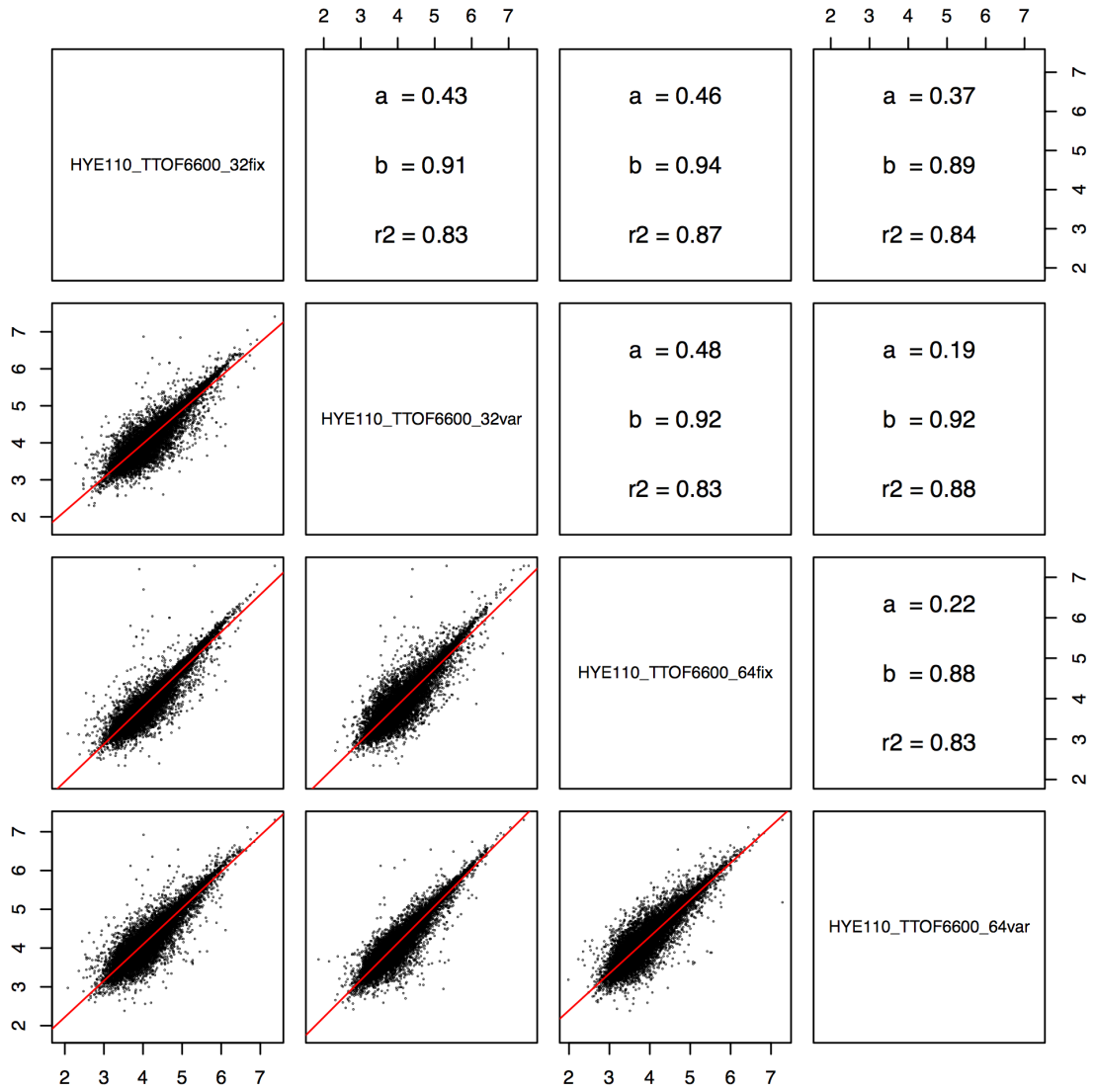
Supplementary Figure 6.C: Precursor intensity correlation between workflows for HYE124, Skyline.



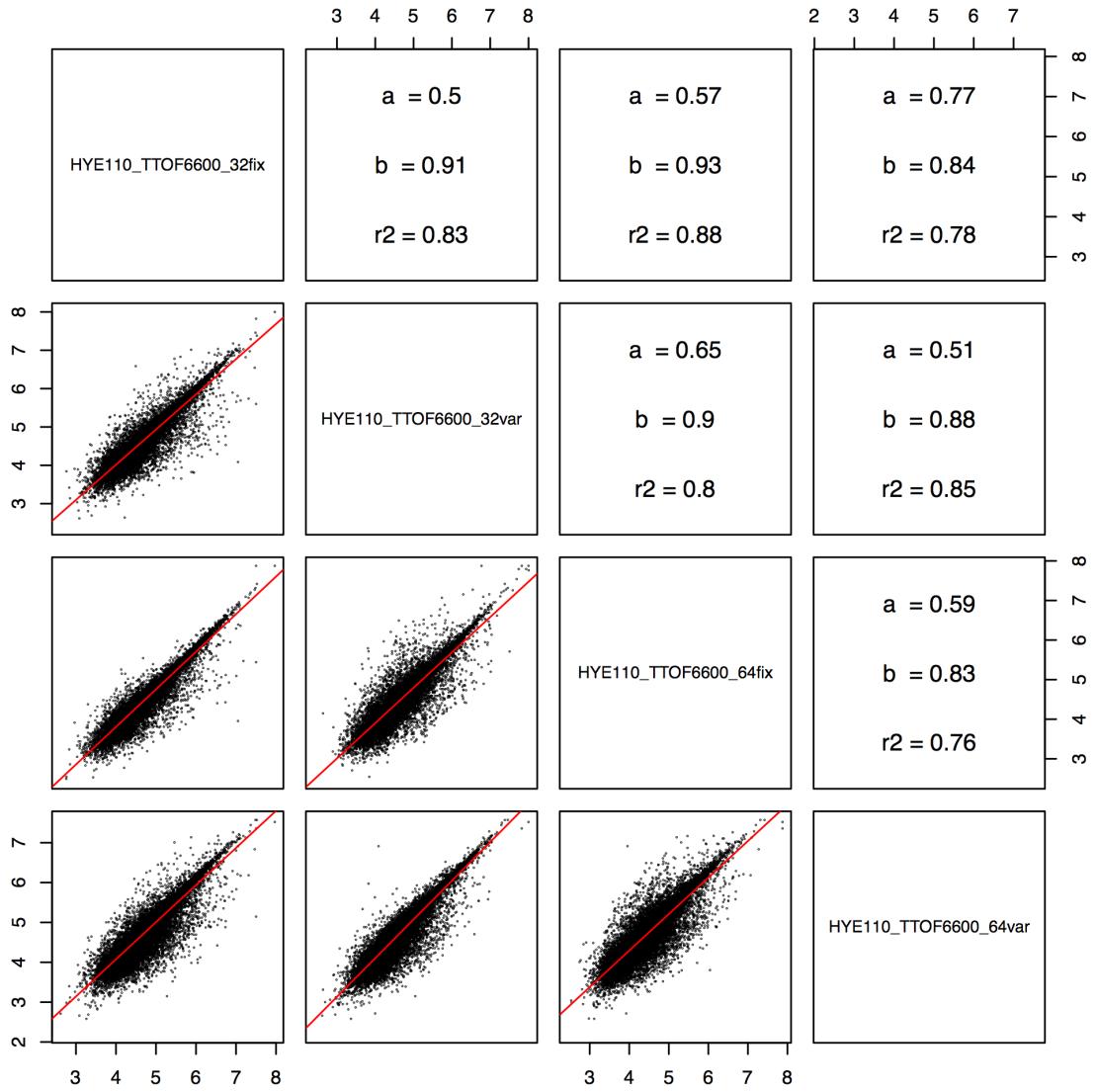
Supplementary Figure 6.D: Precursor intensity correlation between workflows for HYE124, Spectronaut.



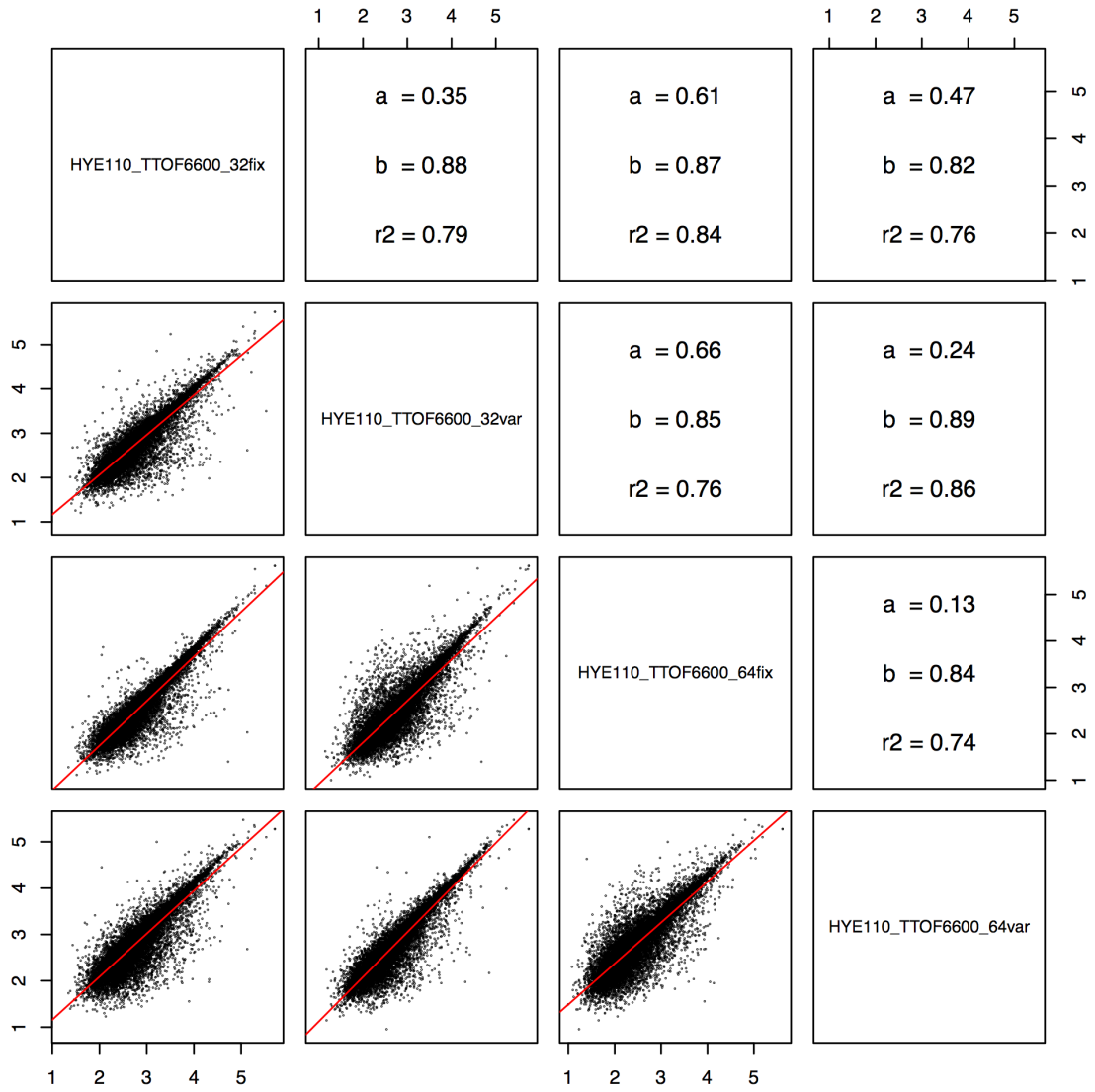
Supplementary Figure 6.E: Precursor intensity correlation between workflows for HYE124, DIA-Umpire.



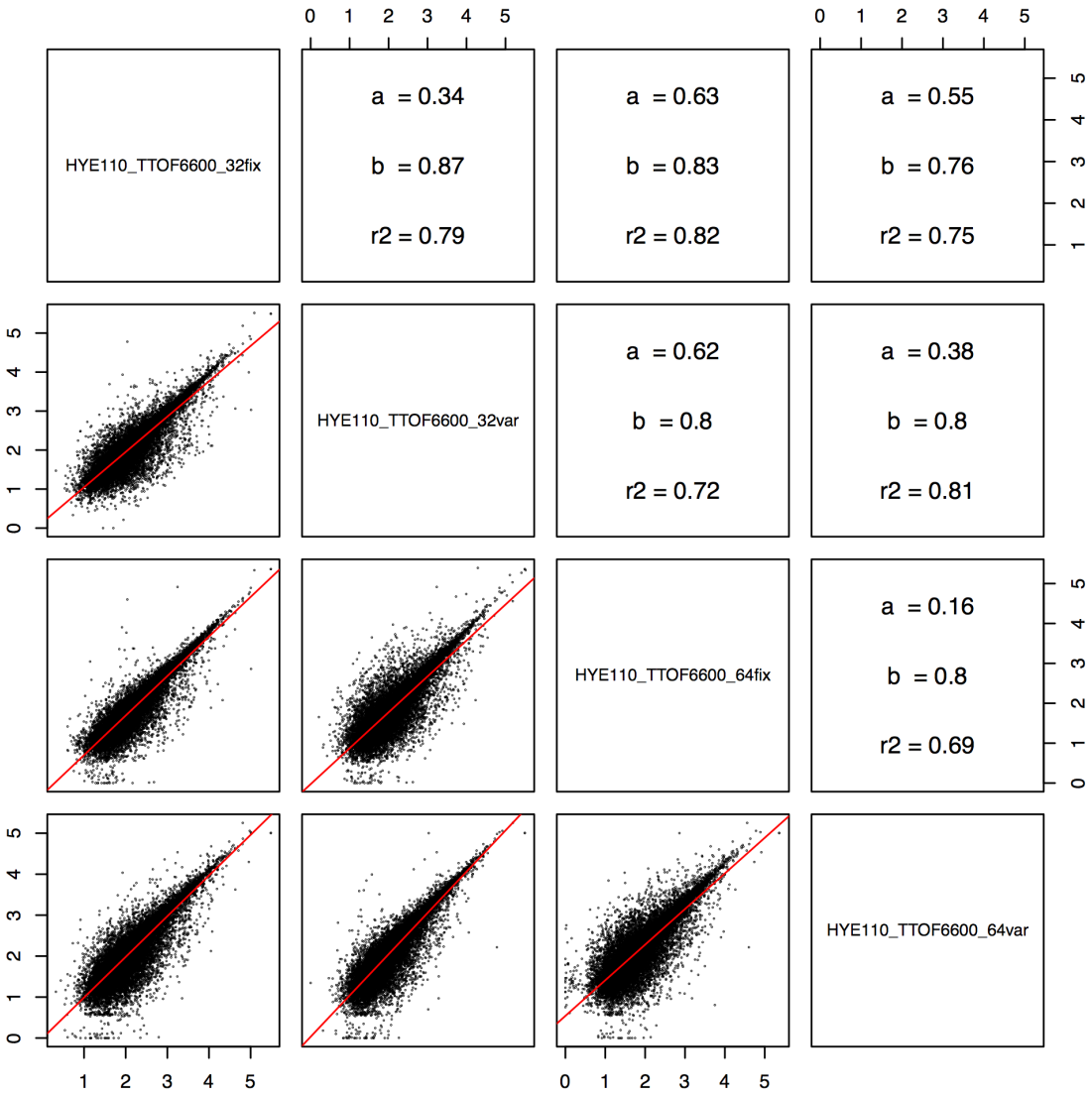
Supplementary Figure 6.F: Precursor intensity correlation between workflows for HYE110, OpenSWATH.



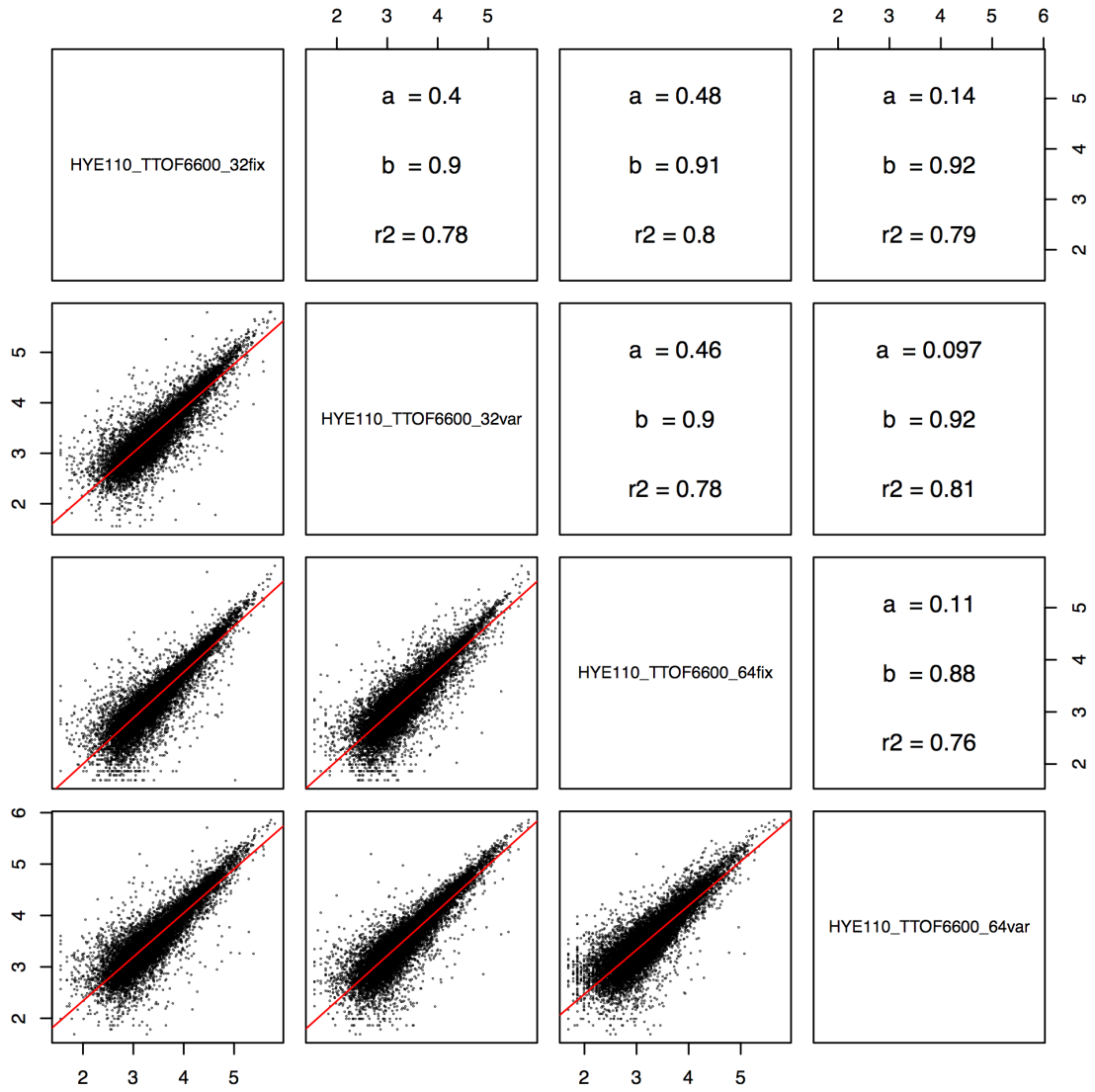
Supplementary Figure 6.G: Precursor intensity correlation between workflows for HYE110, SWATH 2.0.



Supplementary Figure 6.H: Precursor intensity correlation between workflows for HYE110, Skyline.



Supplementary Figure 6.I: Precursor intensity correlation between workflows for HYE110, Spectronaut.



Supplementary Figure 6.J: Precursor intensity correlation between workflows for HYE110, DIA-Umpire.

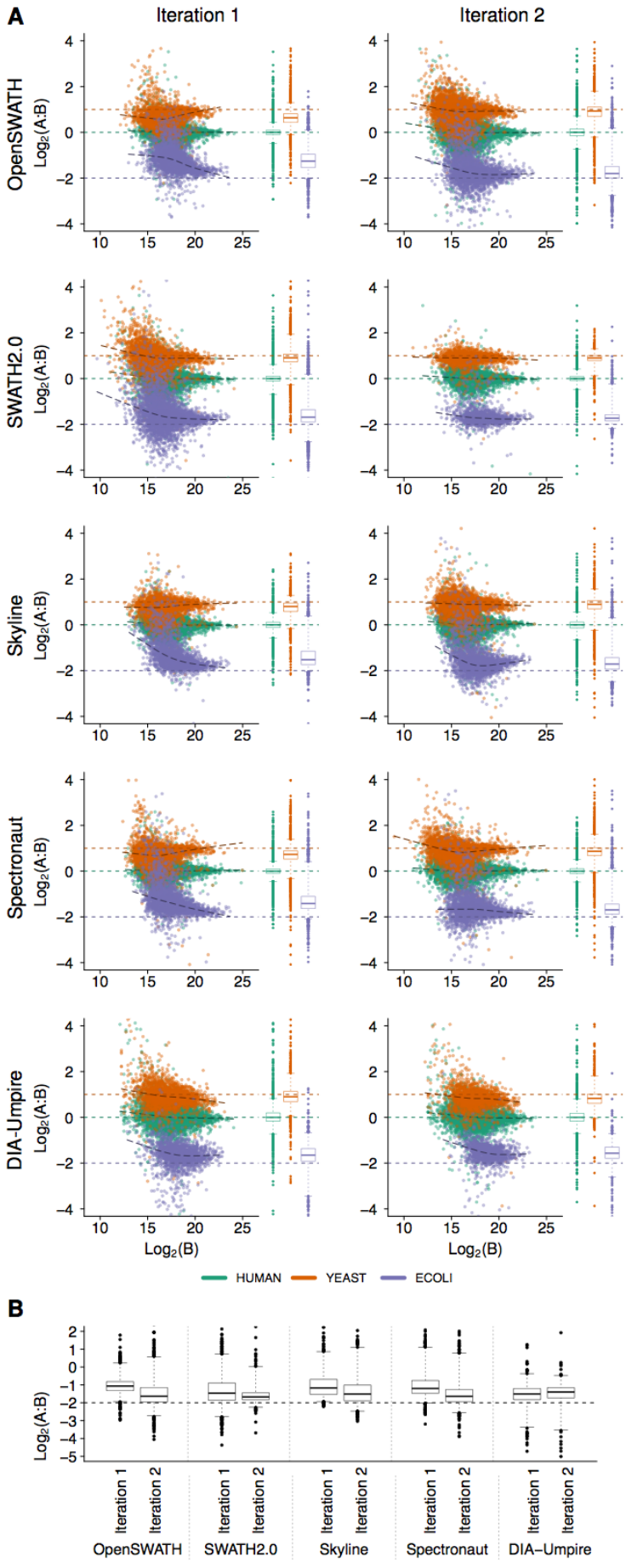
Supplementary Figure 7. LFQbench peptide level benchmarks.

Log-transformed ratios (log_2(A/B)) of peptides were plotted for each benchmarked software tool over the log-transformed scaled intensity of sample B. Dashed colored lines represent the expected log_2(A/B) values for human, yeast, and E.coli peptides. Black dashed lines represent the local trend along the x-axis of experimental log-transformed ratios of each population (human, yeast, and E.coli). In case of sample HYE124, panel A shows both iterations for all software tools, and panel B shows the log_2(A/B) of the averages between technical replicates of A and B for E.coli peptides in the lowest intensity tertile. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

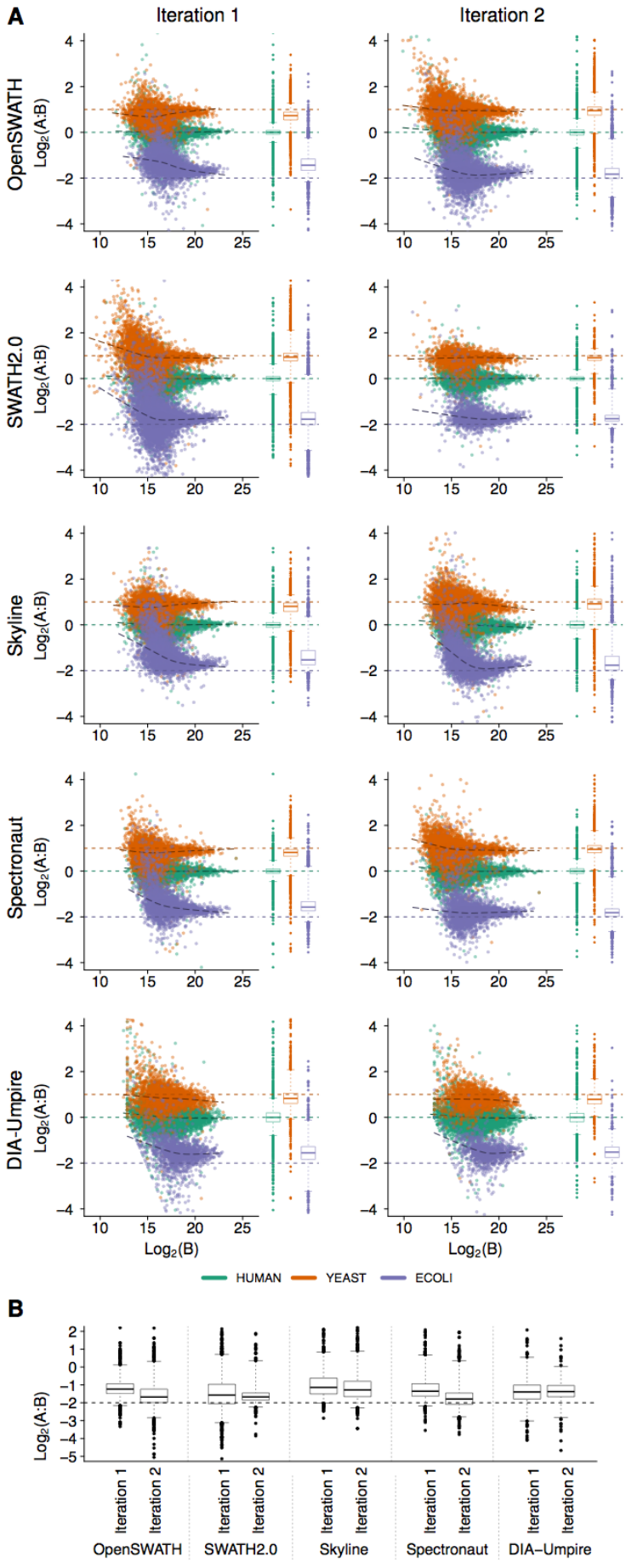
Intensities reported by each software tool were scaled to SWATH 2.0 intensity scale using a linear model fixed in the origin. Intensities of multiply charged precursors were summed up, and averaged between technical replicates of each sample.

The following pages show LFQbench peptide level benchmarks for:

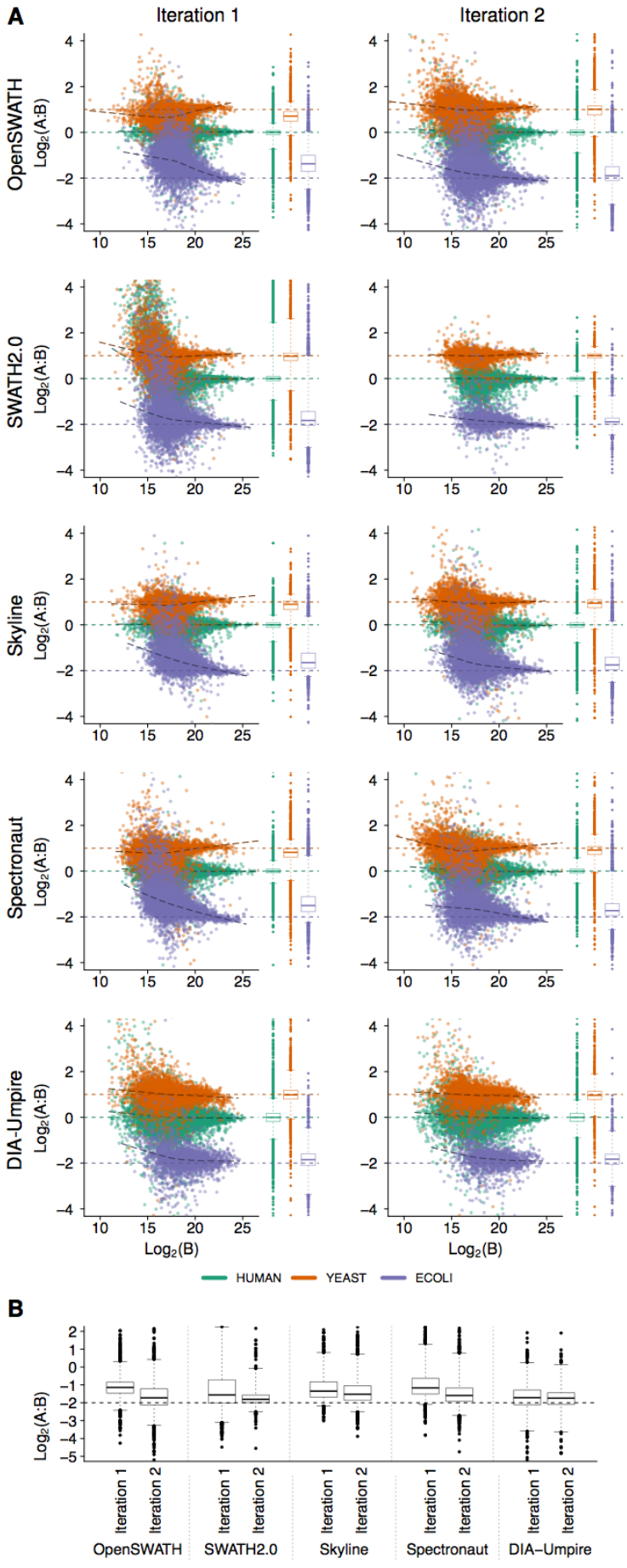
- Supplementary Figure 7.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 7.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 7.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 7.D: HYE 124, TripleTOF 6600, 64var setup
- Supplementary Figure 7.E: HYE 110, TripleTOF 6600, 32fix setup
- Supplementary Figure 7.F: HYE 110, TripleTOF 6600, 32var setup
- Supplementary Figure 7.G: HYE 110, TripleTOF 6600, 64fix setup
- Supplementary Figure 7.H: HYE 110, TripleTOF 6600, 64var setup



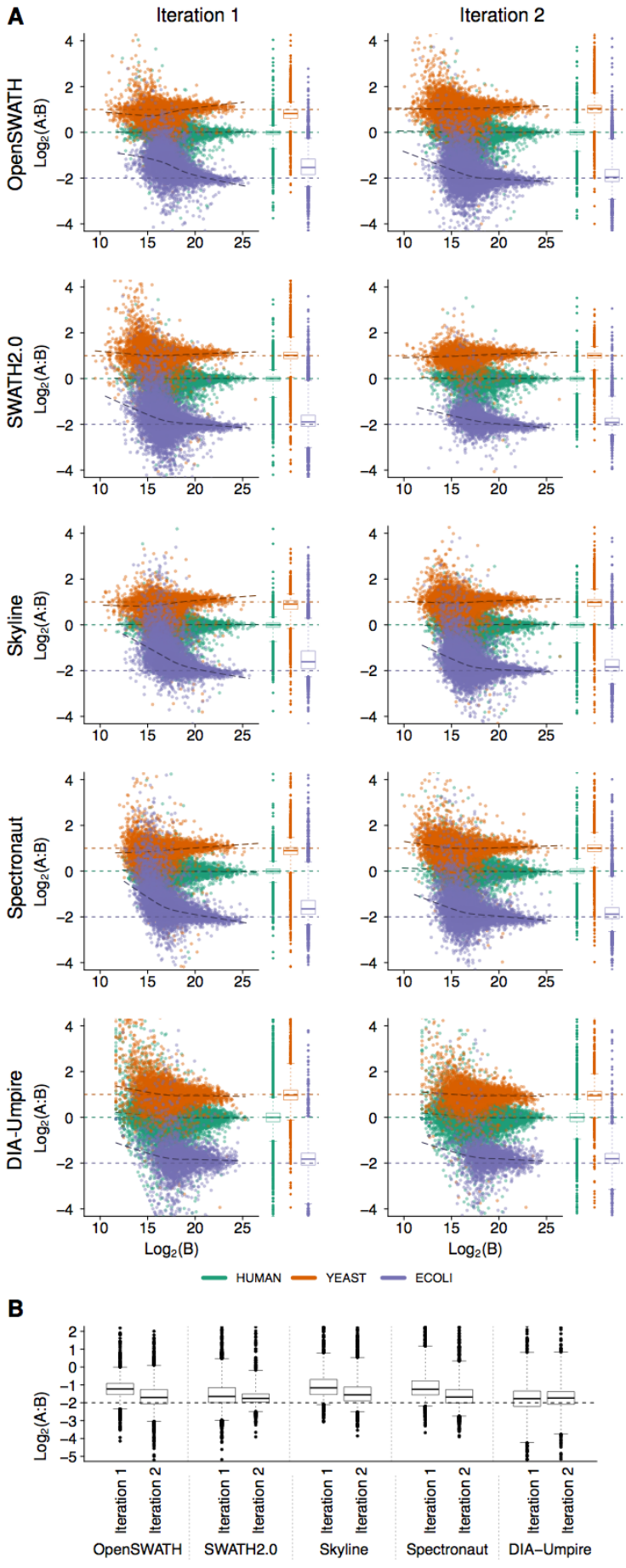
Supplementary Figure 7.A: LFQbench peptide level benchmarks for HYE 124, TripleTOF 5600, 32fix setup.



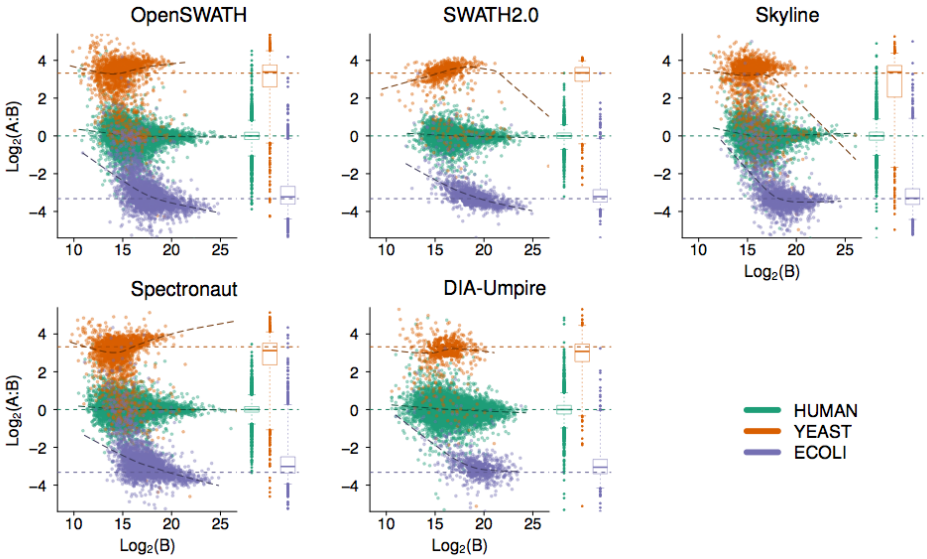
Supplementary Figure 7.B: LFQbench peptide level benchmarks for HYE 124, TripleTOF 5600, 64var setup.



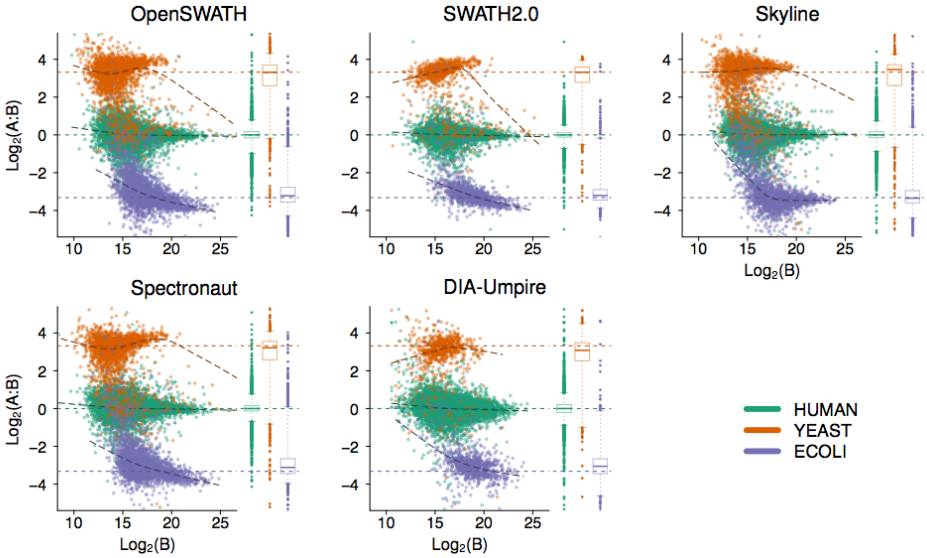
Supplementary Figure 7.C: LFQbench peptide level benchmarks for HYE 124, TripleTOF 6600, 32fix setup.



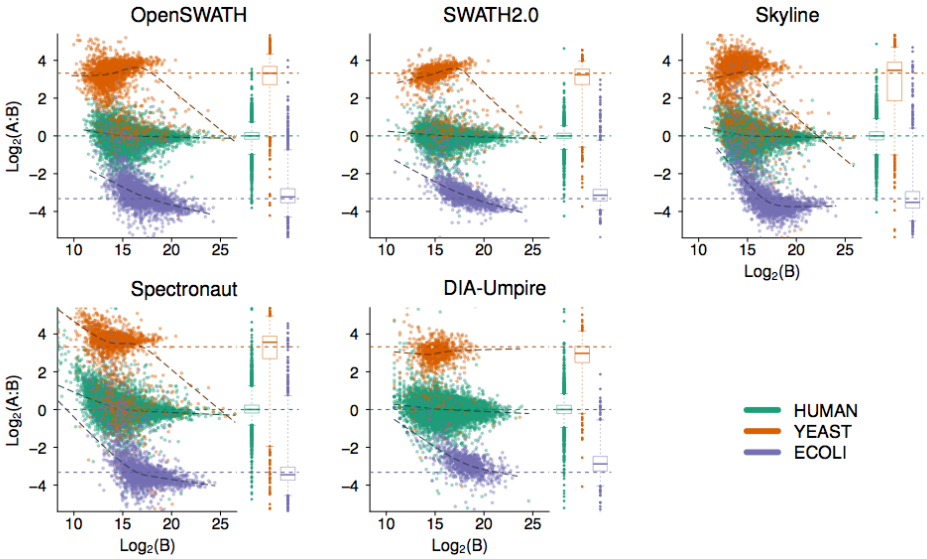
Supplementary Figure 7.D: LFQbench peptide level benchmarks for HYE 124, TripleTOF 6600, 64var setup.



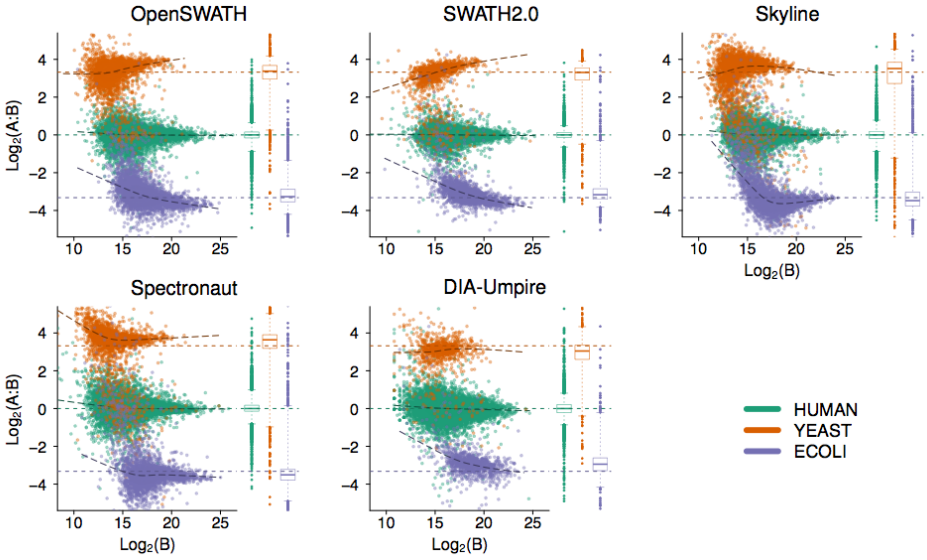
Supplementary Figure 7.E: LFQbench peptide level benchmarks for HYE 110, TripleTOF 6600, 32fix setup.



Supplementary Figure 7.F: LFQbench peptide level benchmarks for HYE 110, TripleTOF 6600, 32var setup.



Supplementary Figure 7.G: LFQbench peptide level benchmarks for HYE 110, TripleTOF 6600, 64fix setup.



Supplementary Figure 7.H: LFQbench peptide level benchmarks for HYE 110, TripleTOF 6600, 64var setup.

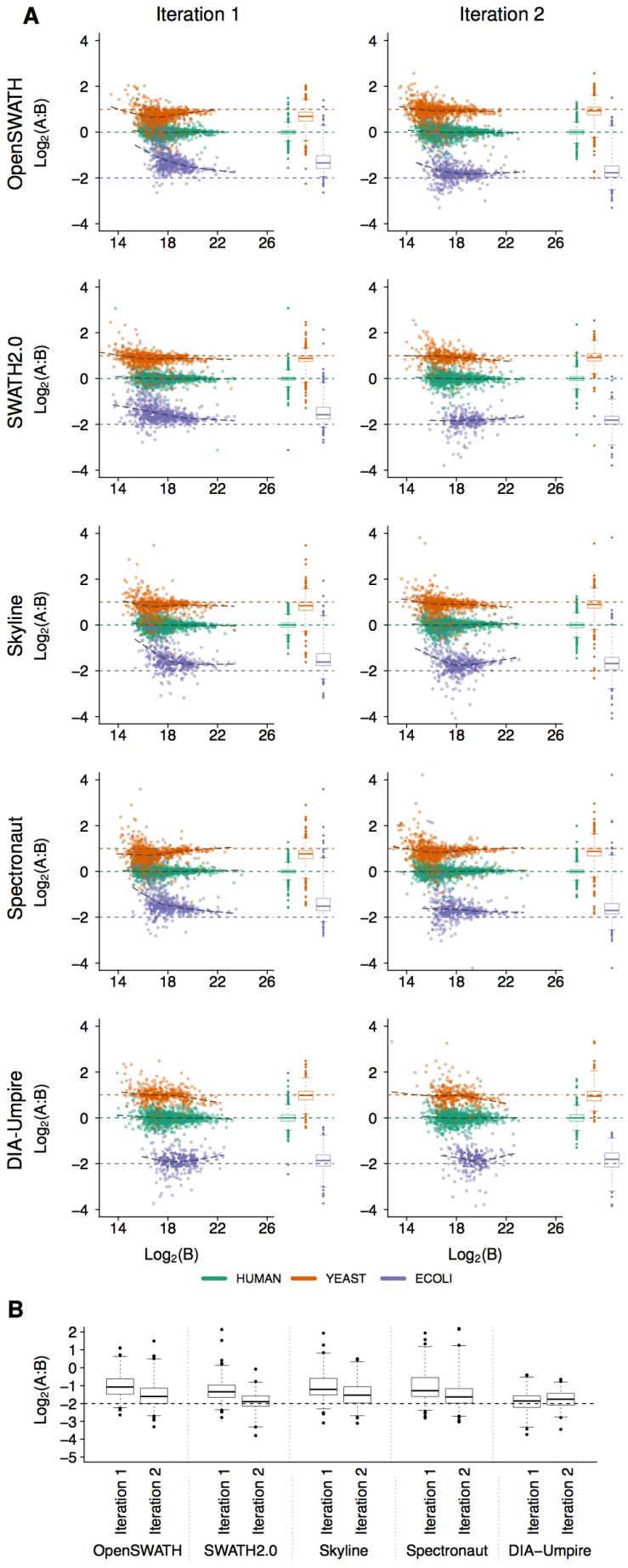
Supplementary Figure 8. LFQbench protein level benchmarks.

Log-transformed ratios (log_2(A/B)) of proteins were plotted for each benchmarked software tool over the log-transformed scaled intensity of sample B. Dashed colored lines represent the expected log_2(A/B) values for human, yeast, and E.coli peptides. Black dashed lines represent the local trend along the x-axis of experimental log-transformed ratios of each population (human, yeast, and E.coli). In case of sample HYE124, panel A shows both iterations for all software tools, and panel B shows the log_2(A/B) of the averages between technical replicates of A and B for E.coli peptides in the lowest intensity tertile. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

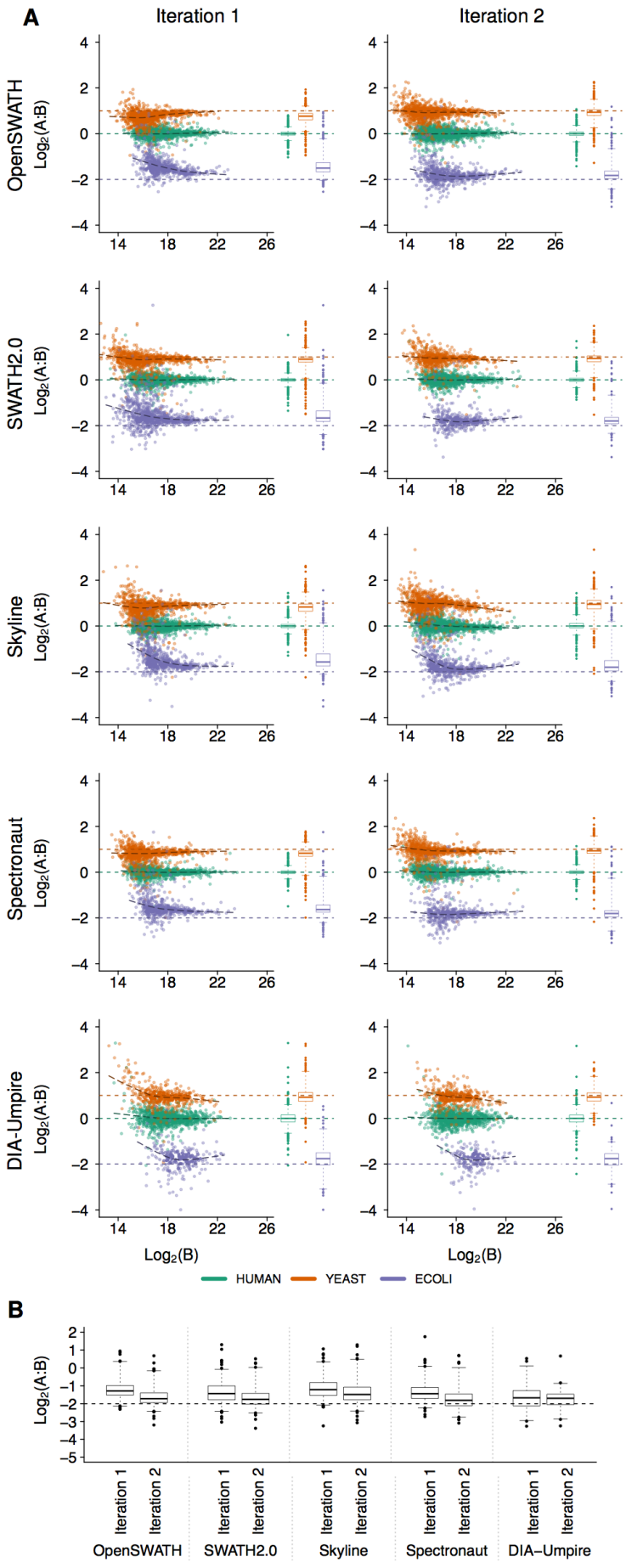
Intensities of multiply charged precursors were summed up, and averaged between technical replicates of each sample. Protein quantities were estimated in each technical replicate by the average of the most 3 intense peptides reported for each protein. Single hit proteins (a single peptide detected in a protein) and proteins detected in less than two injections in both samples A and B were discarded. The protein intensities of each software tool were then scaled to SWATH 2.0 intensity scale using a linear model fixed in the origin.

The following pages show LFQbench protein level benchmarks for:

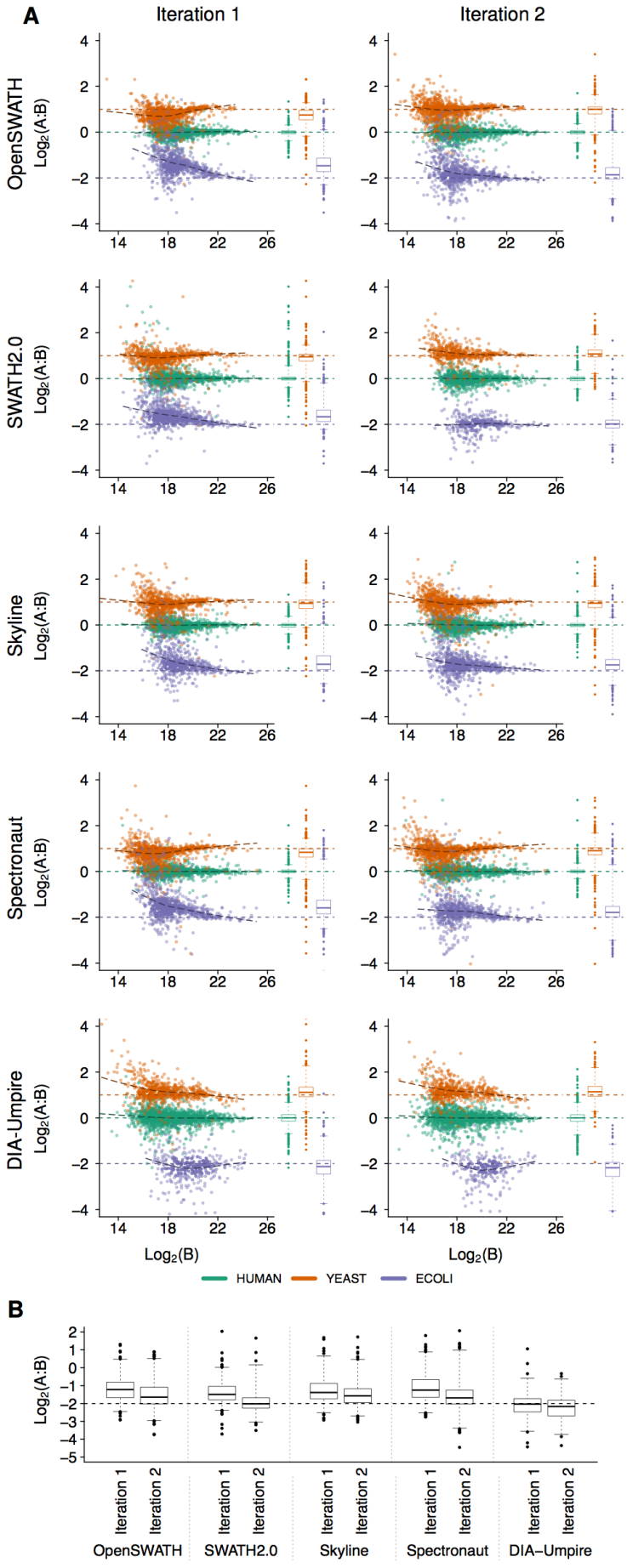
- Supplementary Figure 8.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 8.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 8.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 8.D: HYE 124, TripleTOF 6600, 64var setup
- Supplementary Figure 8.E: HYE 110, TripleTOF 6600, 32fix setup
- Supplementary Figure 8.F: HYE 110, TripleTOF 6600, 32var setup
- Supplementary Figure 8.G: HYE 110, TripleTOF 6600, 64fix setup
- Supplementary Figure 8.H: HYE 110, TripleTOF 6600, 64var setup



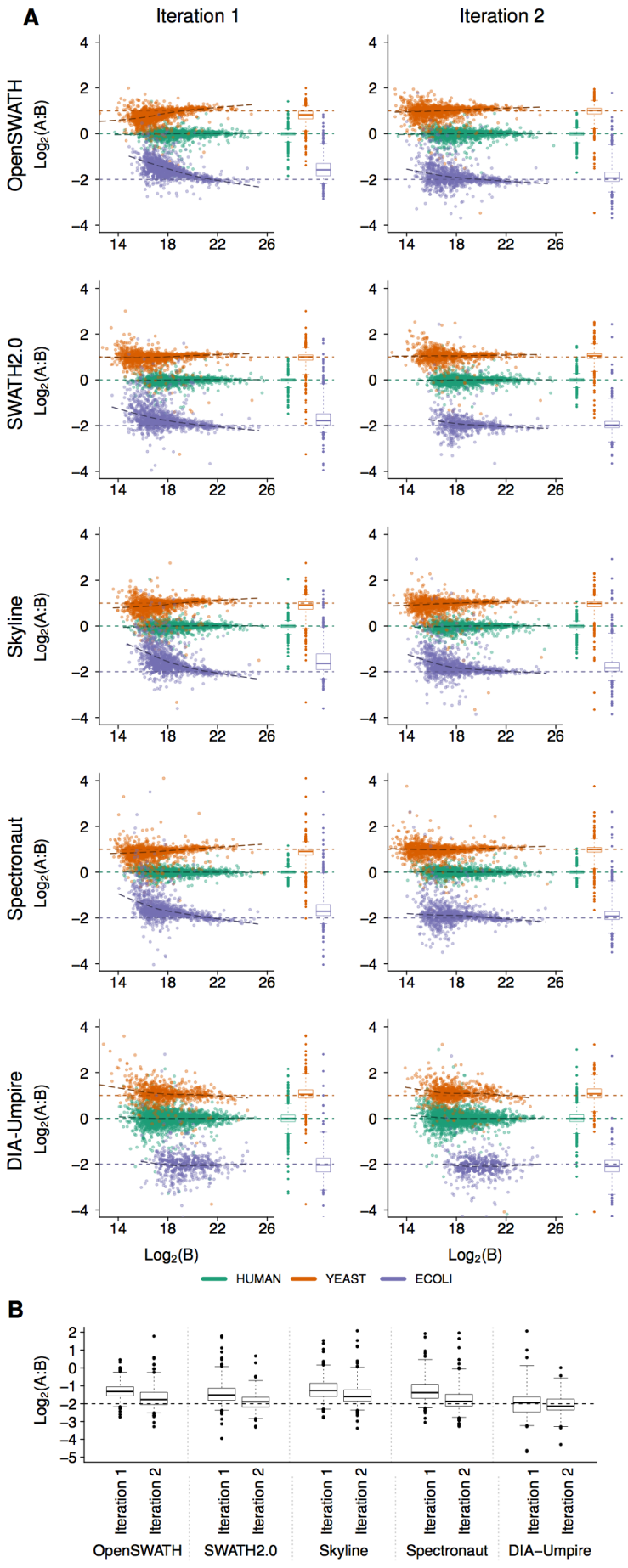
Supplementary Figure 8.A: LFQbench protein level benchmarks for HYE 124, TripleTOF 5600, 32fix setup.



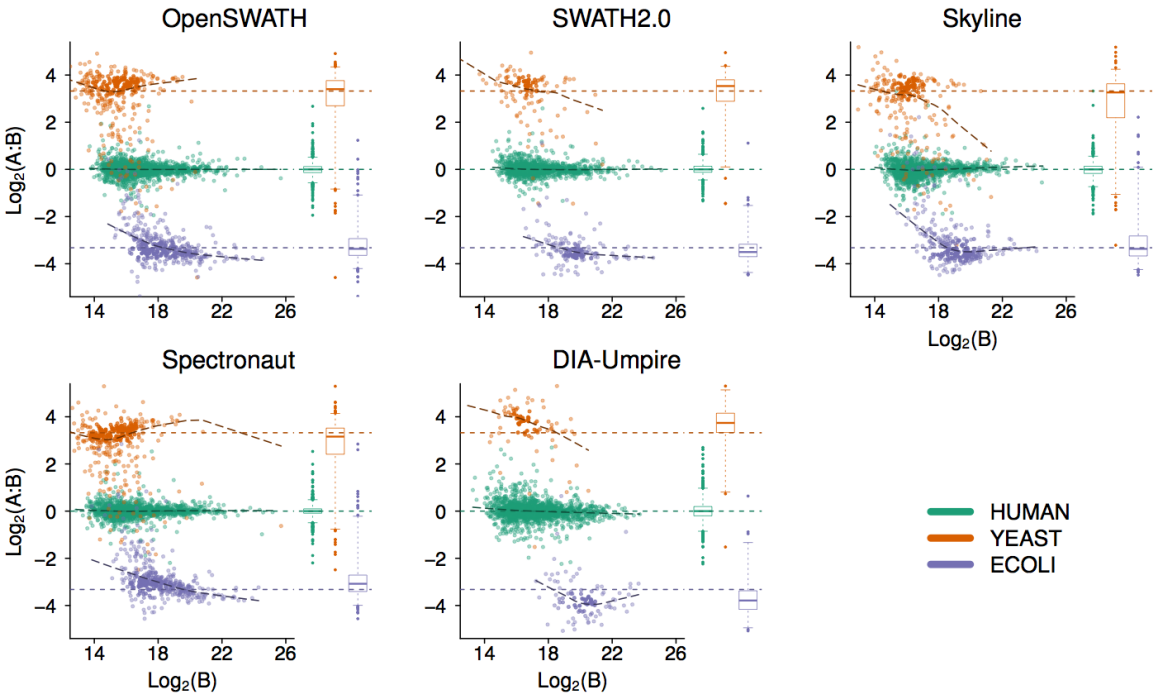
Supplementary Figure 8.B: LFQbench protein level benchmarks for HYE 124, TripleTOF 5600, 64var setup.



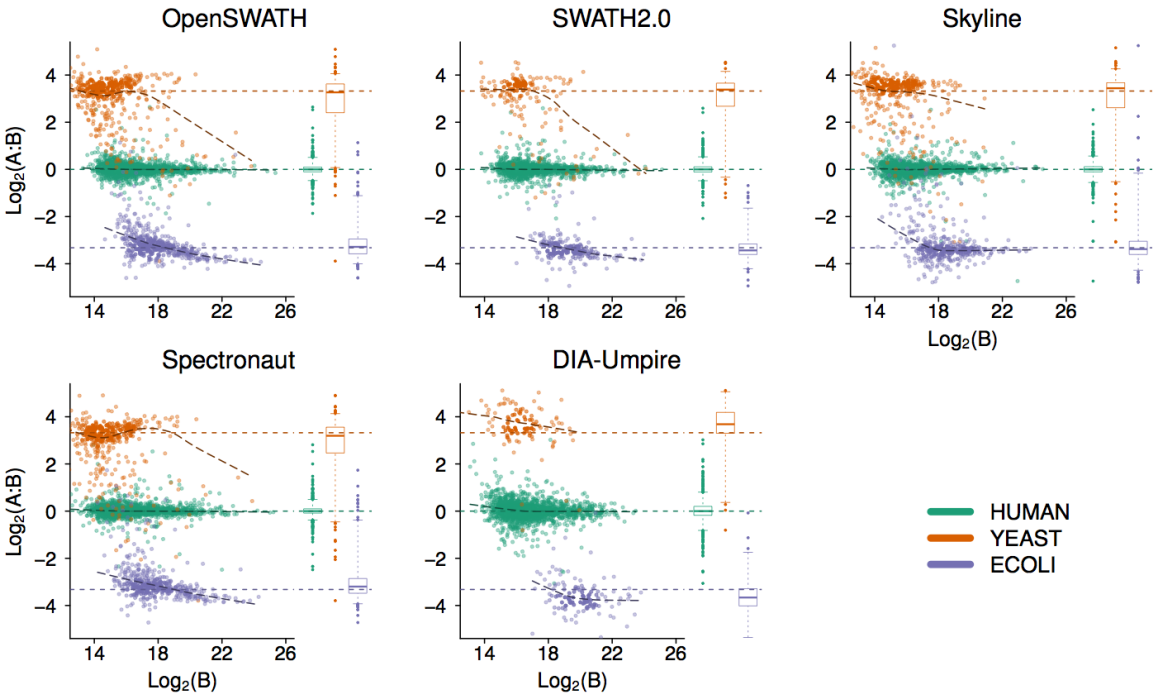
Supplementary Figure 8.C: LFQbench protein level benchmarks for HYE 124, TripleTOF 6600, 32fix setup.



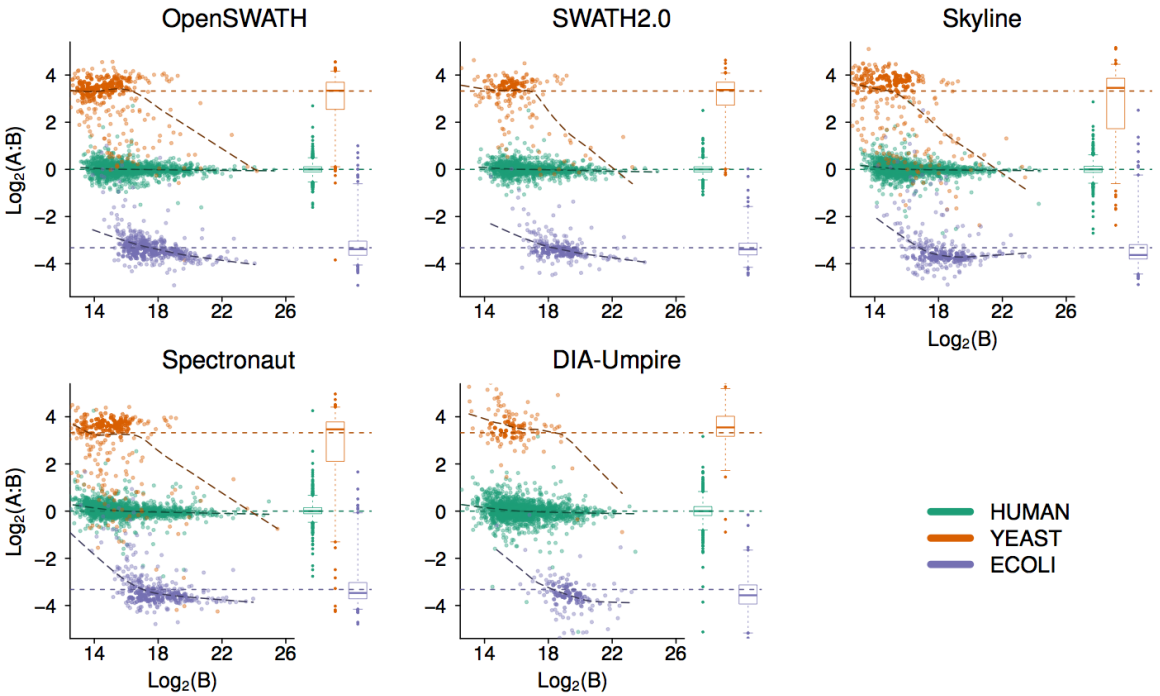
Supplementary Figure 8.D: LFQbench protein level benchmarks for HYE 124, TripleTOF 6600, 64var setup.



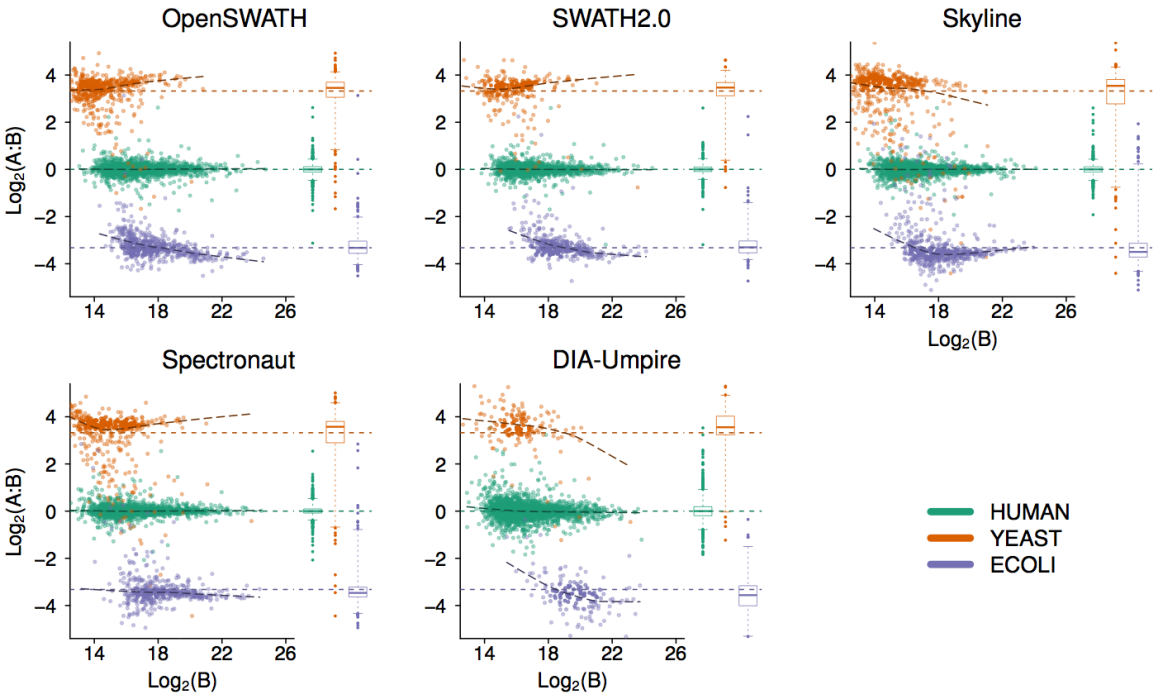
Supplementary Figure 8.E: LFQbench protein level benchmarks for HYE 110, TripleTOF 6600, 32fix setup.



Supplementary Figure 8.F: LFQbench protein level benchmarks for HYE 110, TripleTOF 6600, 32var setup.



Supplementary Figure 8.G: LFQbench protein level benchmarks for HYE 110, TripleTOF 6600, 64fix setup.



Supplementary Figure 8.H: LFQbench protein level benchmarks for HYE 110, TripleTOF 6600, 64var setup.

Supplementary Figure 9. LFQbench protein level benchmarks for single-hit proteins for all instrument/workflow combinations.

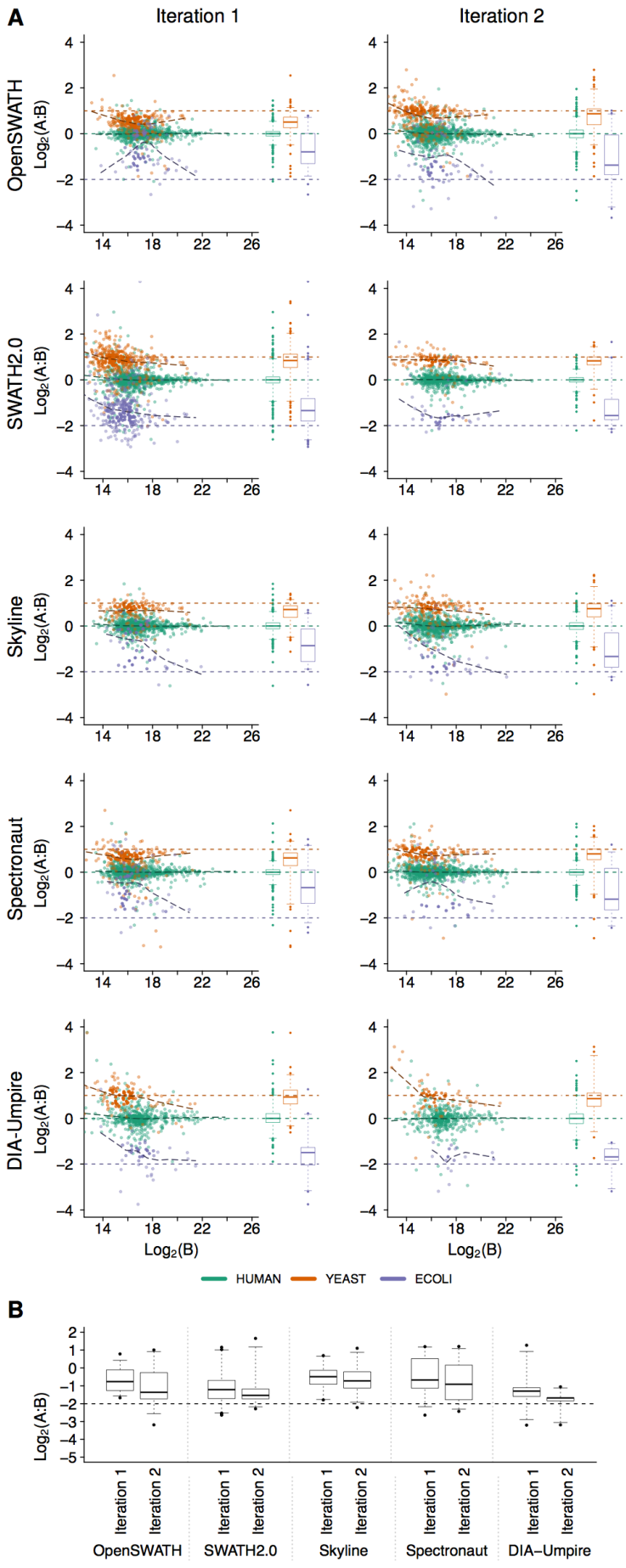
Protein single hits are proteins identified by only one peptide, which are discarded by LFQbench.

Log-transformed ratios (log_2(A/B)) of proteins were plotted for each benchmarked software tool over the log-transformed scaled intensity of sample B. Dashed colored lines represent the expected log_2(A/B) values for human, yeast, and E.coli peptides. Black dashed lines represent the local trend along the x-axis of experimental log-transformed ratios of each population (human, yeast, and E.coli). In case of sample HYE124, panel A shows both iterations for all software tools, and panel B shows the log_2(A/B) of the averages between technical replicates of A and B for E.coli peptides in the lowest intensity tertile. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

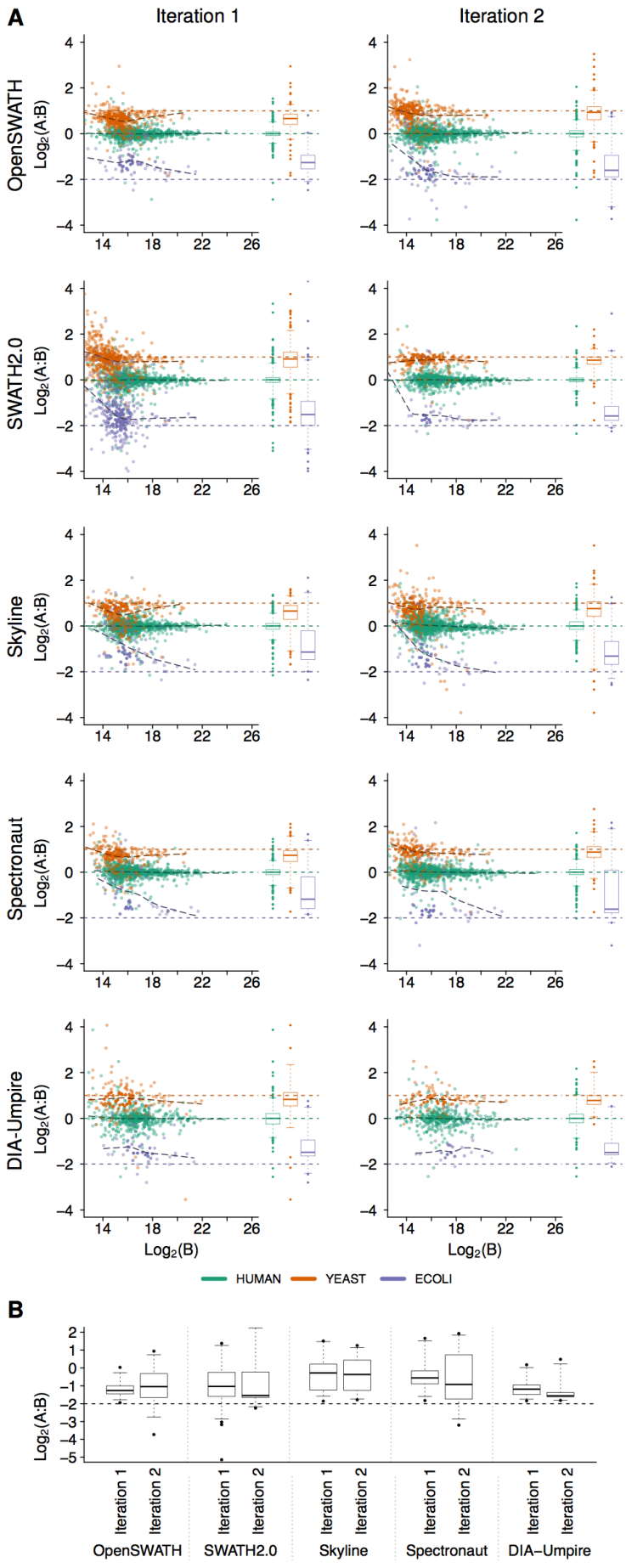
Intensities of multiply charged precursors were summed up, and averaged between technical replicates of each sample. Protein quantities were estimated by the average intensity (three technical replicates) of the single peptide identified of the protein (single hit). The protein intensities of each software tool were then scaled to SWATH 2.0 intensity scale by using a linear model fixed in the origin.

The following pages show:

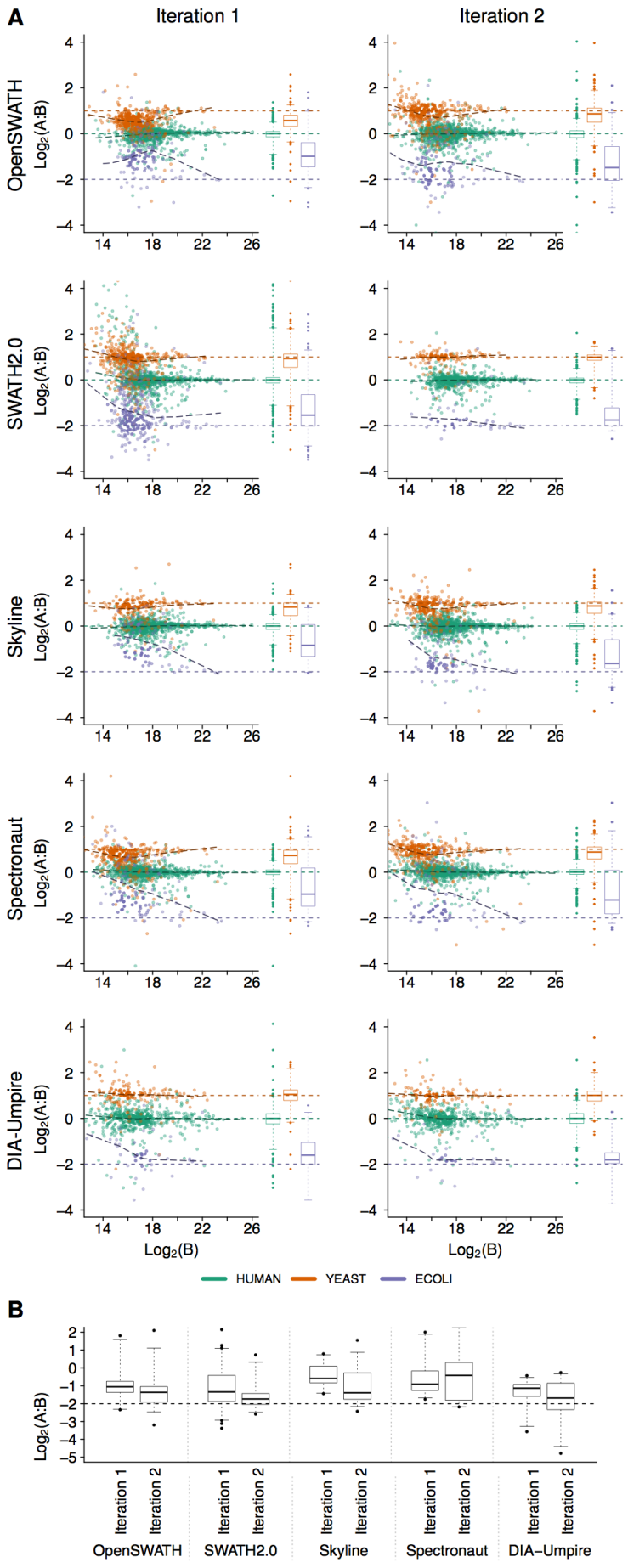
- Supplementary Figure 9.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 9.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 9.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 9.D: HYE 124, TripleTOF 6600, 64var setup



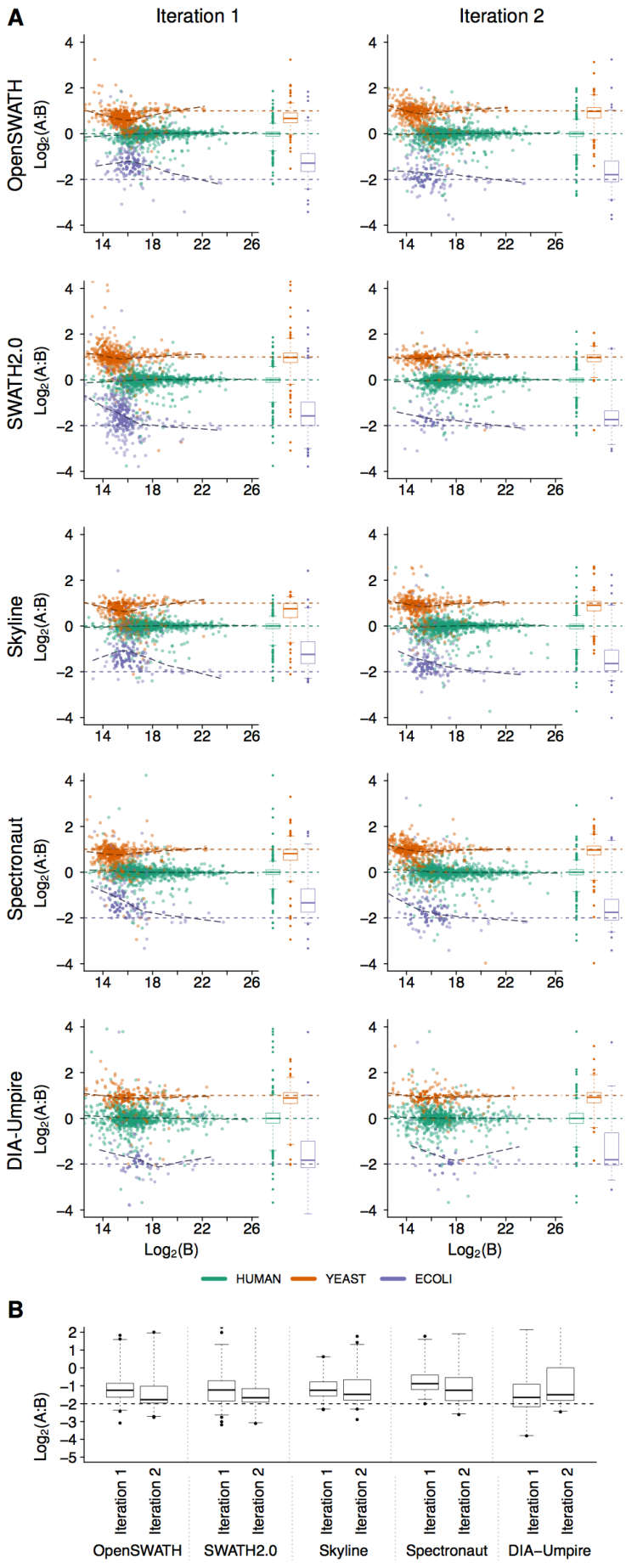
Supplementary Figure 9.A: LFQbench single-hits protein level benchmarks for HYE 124, TripleTOF 5600, 32fix setup.



Supplementary Figure 9.B: LFQbench single-hits protein level benchmarks for HYE 124, TripleTOF 5600, 64var setup.



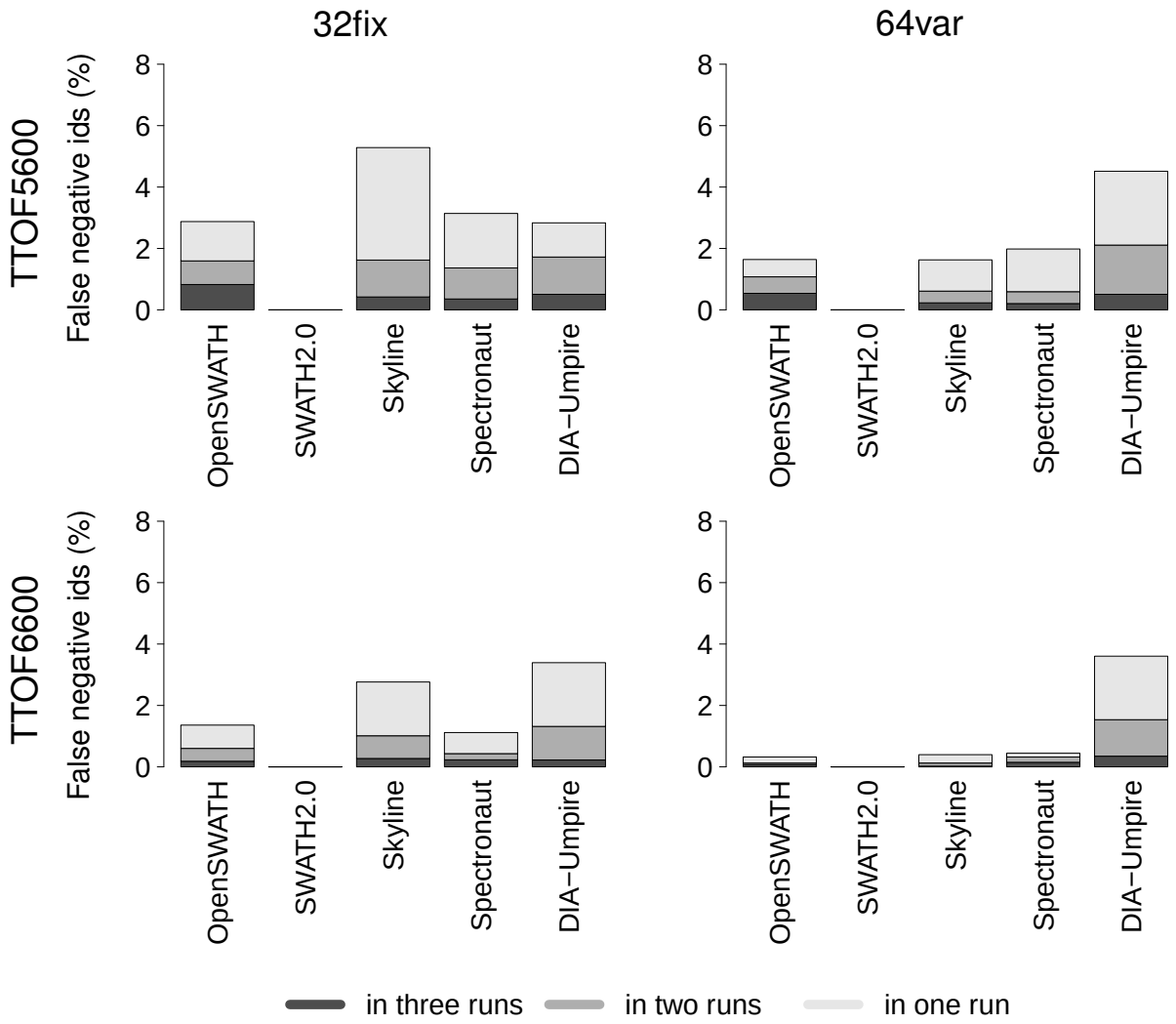
Supplementary Figure 9.C: LFQbench single-hits protein level benchmarks for HYE 124, TripleTOF 6600, 32fix setup.



Supplementary Figure 9.D: LFQbench single-hits protein level benchmarks for HYE 124, TripleTOF 6600, 64var setup.

Supplementary Figure 10. Percentage of false negative human proteins (human proteins detected in only one of the samples) without replication rate filtering.

According to sample composition, human proteins should always be detectable in both samples (A and B). Human proteins that are false-negative in one of the samples (and thus falsely reported as "exclusively detected" in the other samples) are highly problematic from a biological perspective. The data indicate that requiring a protein to be quantified in at least two biological replicates (bar plots group by number of technical replicates – runs – the peptide or protein is detected in the other sample) reduces the false negative rate by up to 70%, depending on instrument, software and SWATH window setup.



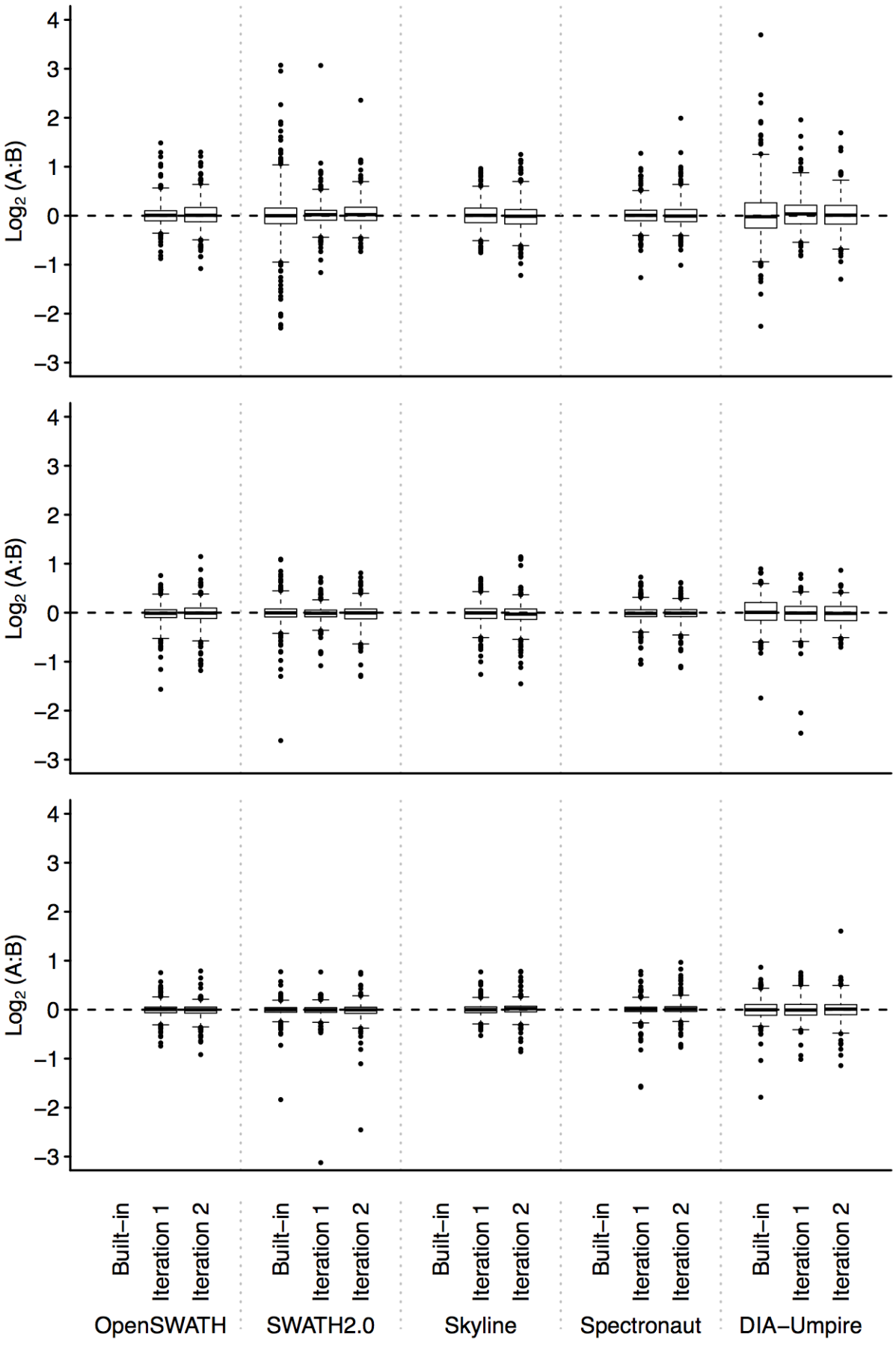
Supplementary Figure 10: Percentage of false negative human proteins (human proteins detected in only one of the samples) without replication rate filtering.

Supplementary Figure 11. Box-and-whisker plots of log_2 ratio distributions in different intensity tertiles for human proteins.

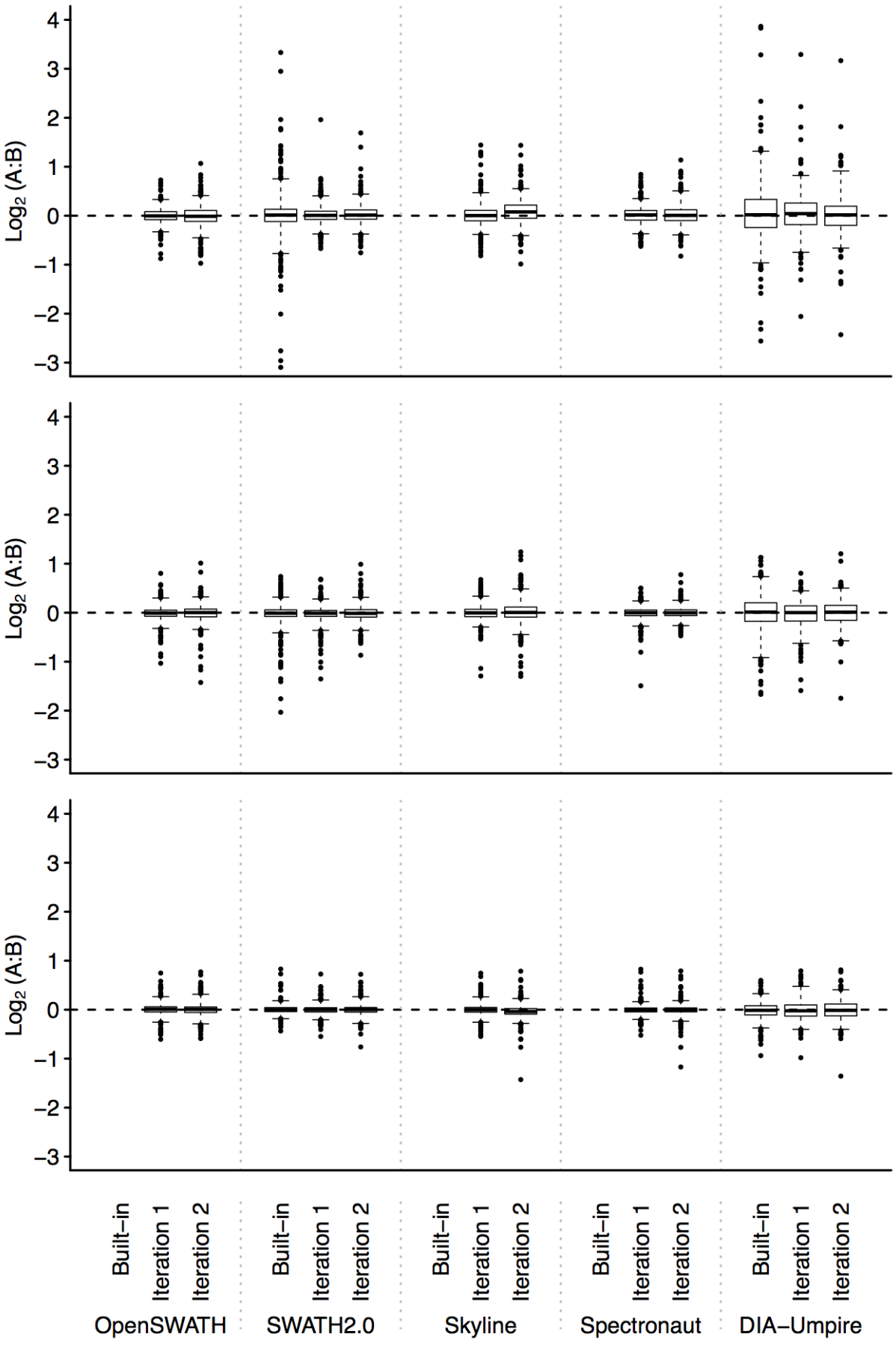
The present figure displays the log_2(A/B) of the averages between technical replicates of A and B for human proteins in the lowest intensity (0%-33.3%, top panel), medium (33.3%-66.7%) and highest (66.7%-100%) tertiles. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

The following pages show log_2 ratio distributions for:

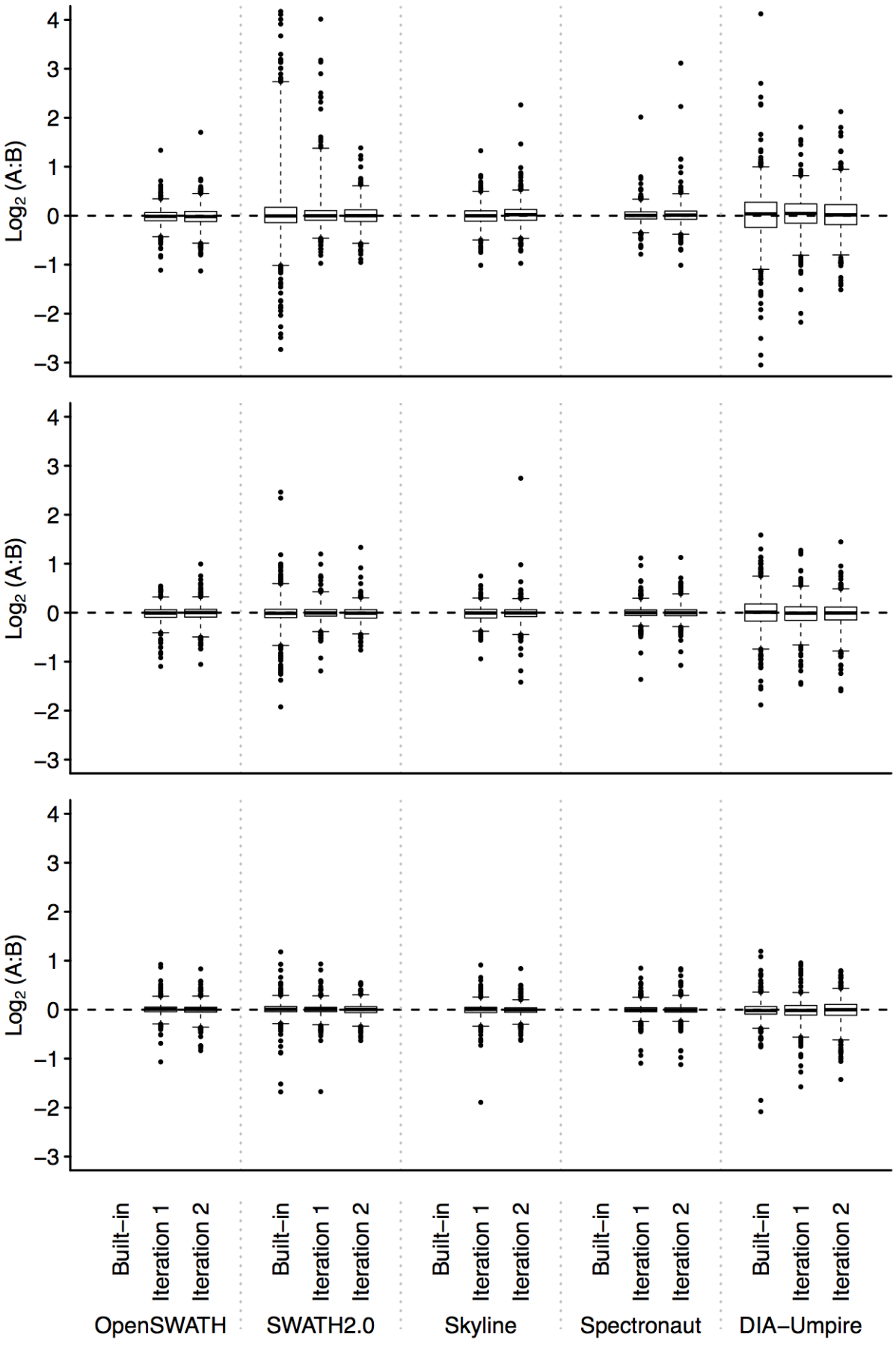
- Supplementary Figure 11.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 11.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 11.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 11.D: HYE 124, TripleTOF 6600, 64var setup



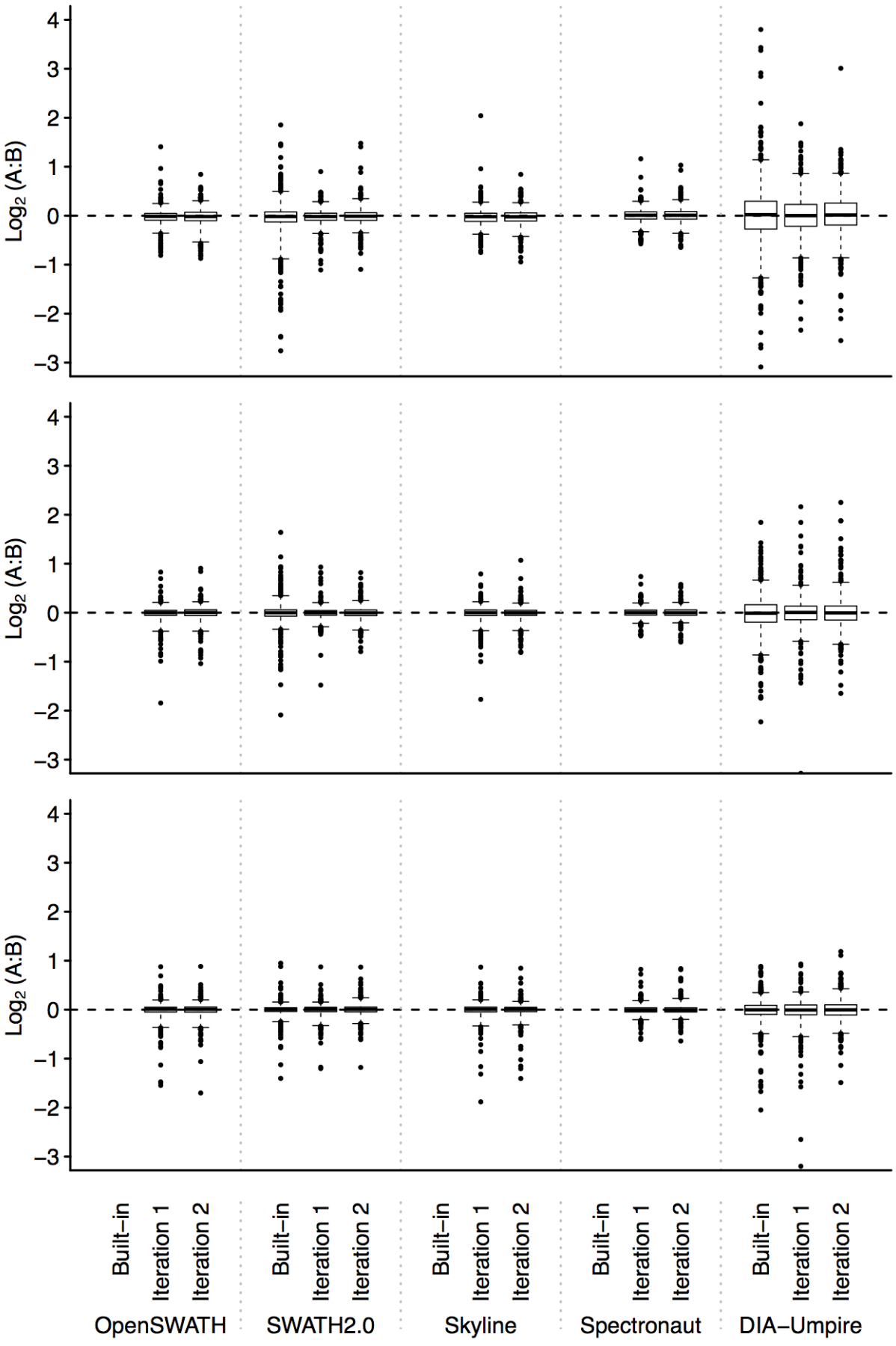
Supplementary Figure 11.A: log_2 ratio distributions in different intensity tertiles for human proteins for HYE 124, TripleTOF 5600, 32fix setup.



Supplementary Figure 11.B: log_2 ratio distributions in different intensity tertiles for human proteins for HYE 124, TripleTOF 5600, 64var setup.



Supplementary Figure 11.C: log_2 ratio distributions in different intensity tertiles for human proteins for HYE 124, TripleTOF 6600, 32fix setup.



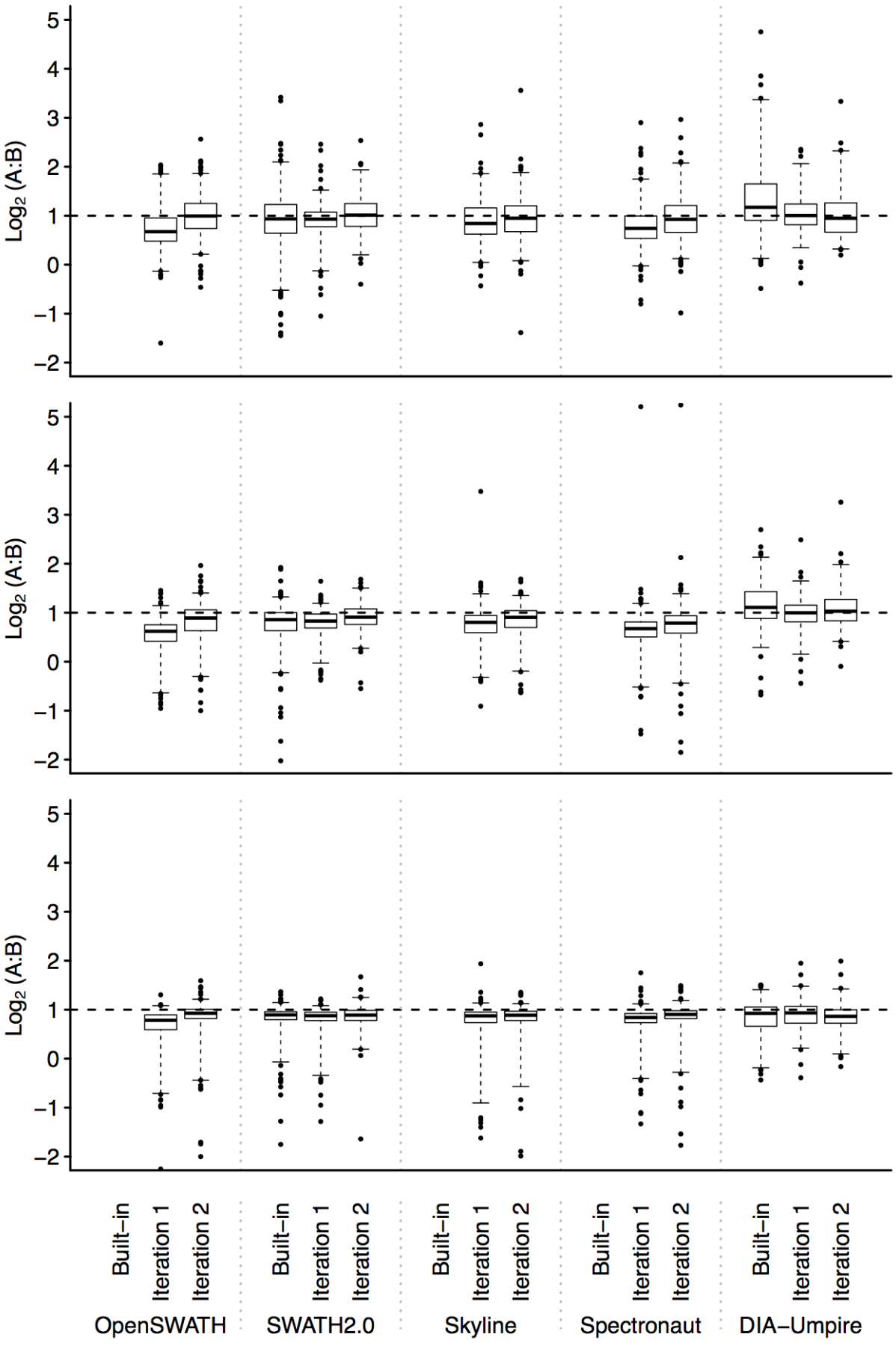
Supplementary Figure 11.D: log_2 ratio distributions in different intensity tertiles for human proteins for HYE 124, TripleTOF 6600, 64var setup.

Supplementary Figure 12. Box-and-whisker plots of log_2 ratio distributions in different intensity tertiles for yeast proteins.

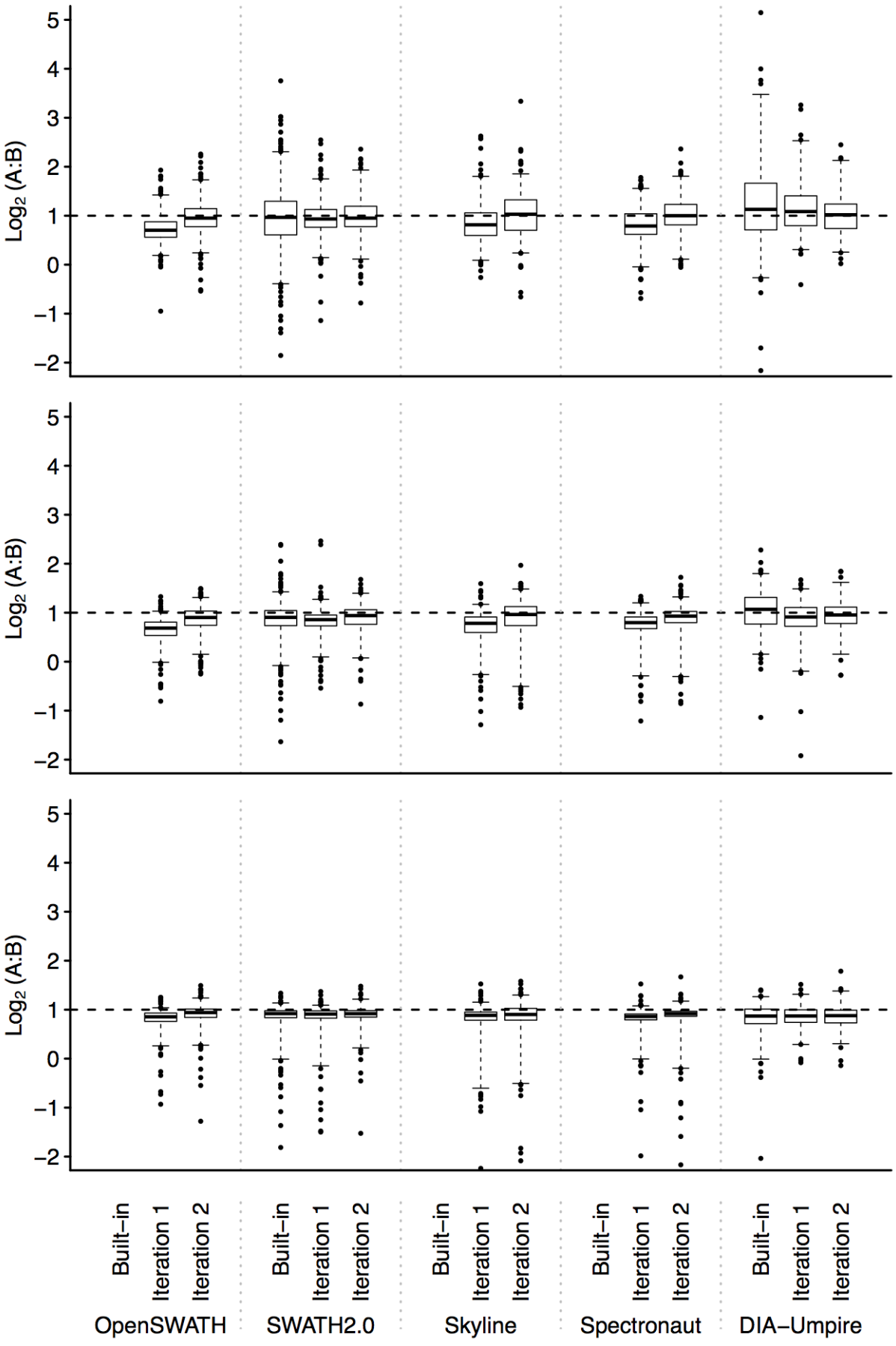
The present figure displays the log_2(A/B) of the averages between technical replicates of A and B for yeast proteins in the lowest intensity (0%-33.3%, top panel), medium (33.3%-66.7%) and highest (66.7%-100%) tertiles. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

The following pages show log_2 ratio distributions for:

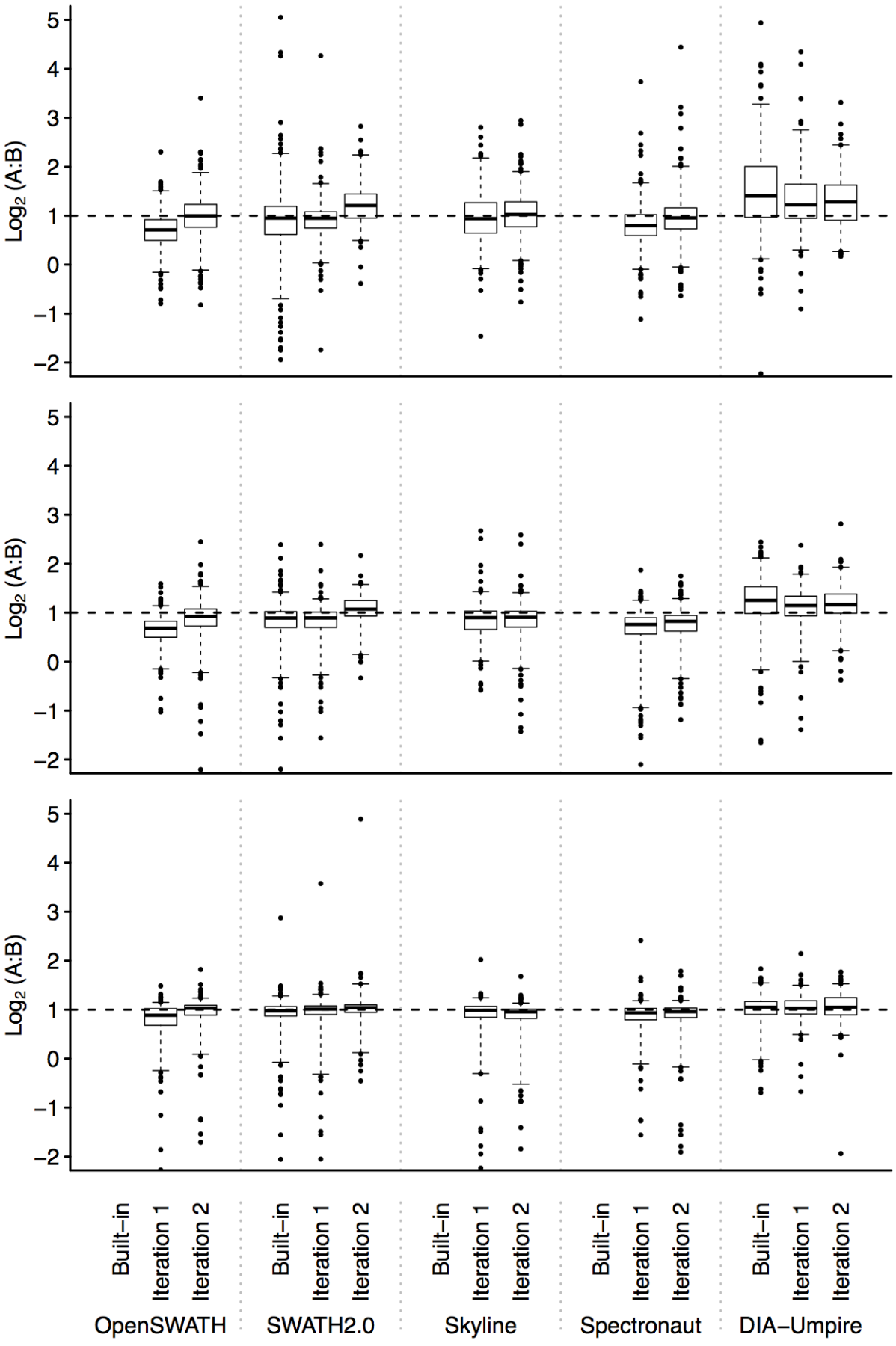
- Supplementary Figure 12.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 12.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 12.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 12.D: HYE 124, TripleTOF 6600, 64var setup



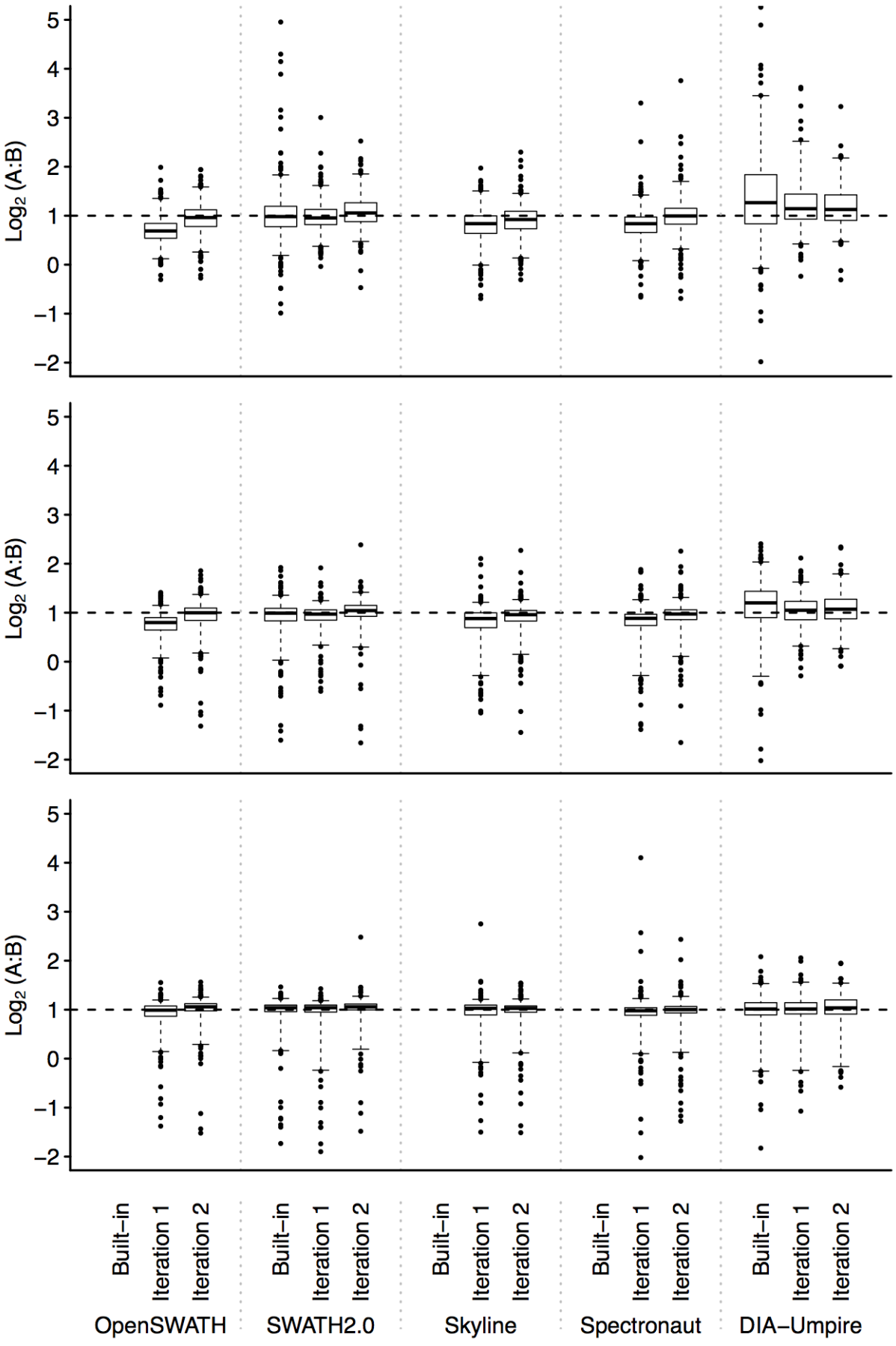
Supplementary Figure 12.A: log_2 ratio distributions in different intensity tertiles for yeast proteins for HYE 124, TripleTOF 5600, 32fix setup.



Supplementary Figure 12.B: log_2 ratio distributions in different intensity tertiles for yeast proteins for HYE 124, TripleTOF 5600, 64var setup.



Supplementary Figure 12.C: log_2 ratio distributions in different intensity tertiles for yeast proteins for HYE 124, TripleTOF 6600, 32fix setup.



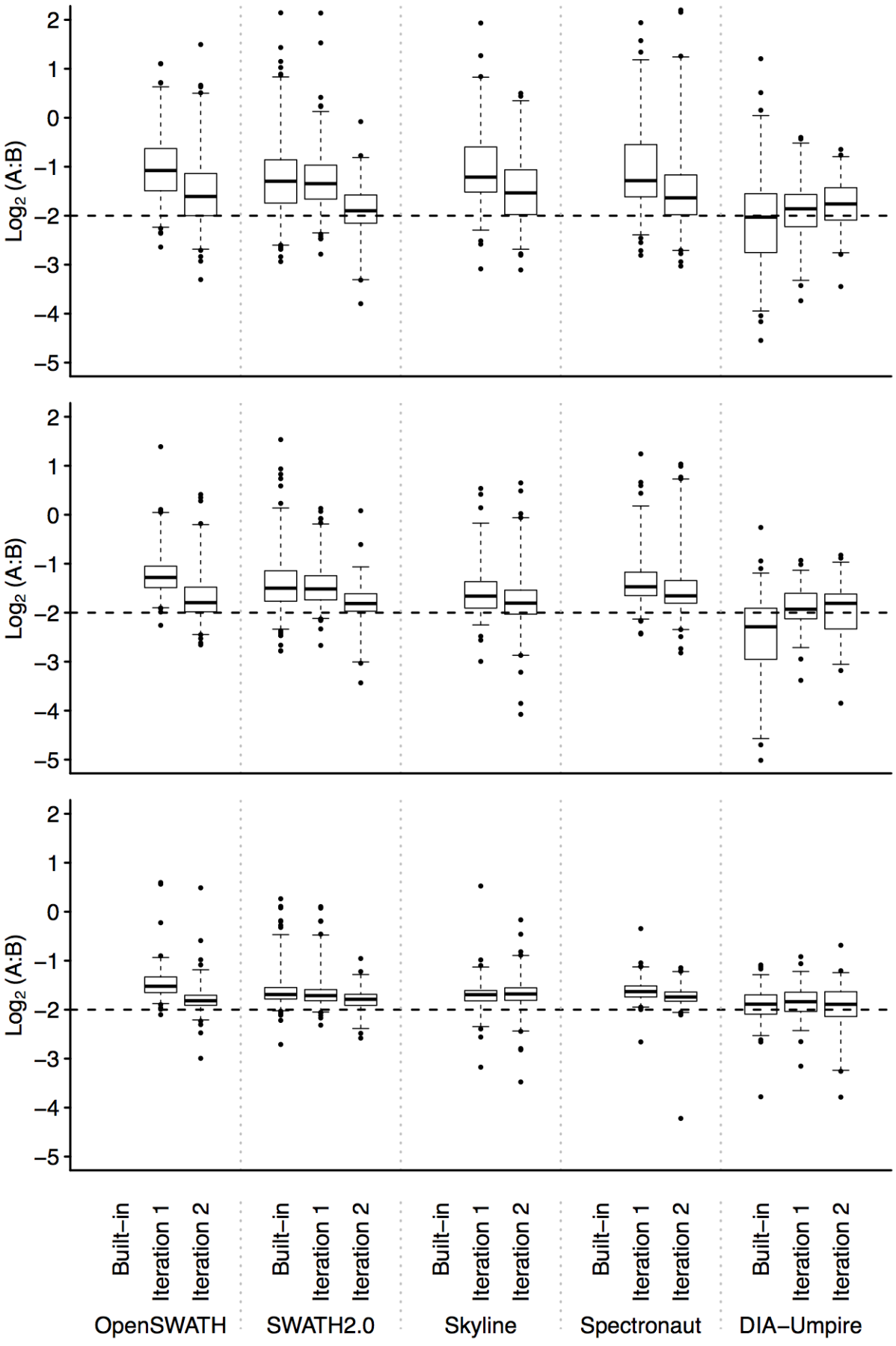
Supplementary Figure 12.D: log_2 ratio distributions in different intensity tertiles for yeast proteins for HYE 124, TripleTOF 6600, 64var setup.

Supplementary Figure 13. Box-and-whisker plots of log_2 ratio distributions in different intensity tertiles for E.coli proteins.

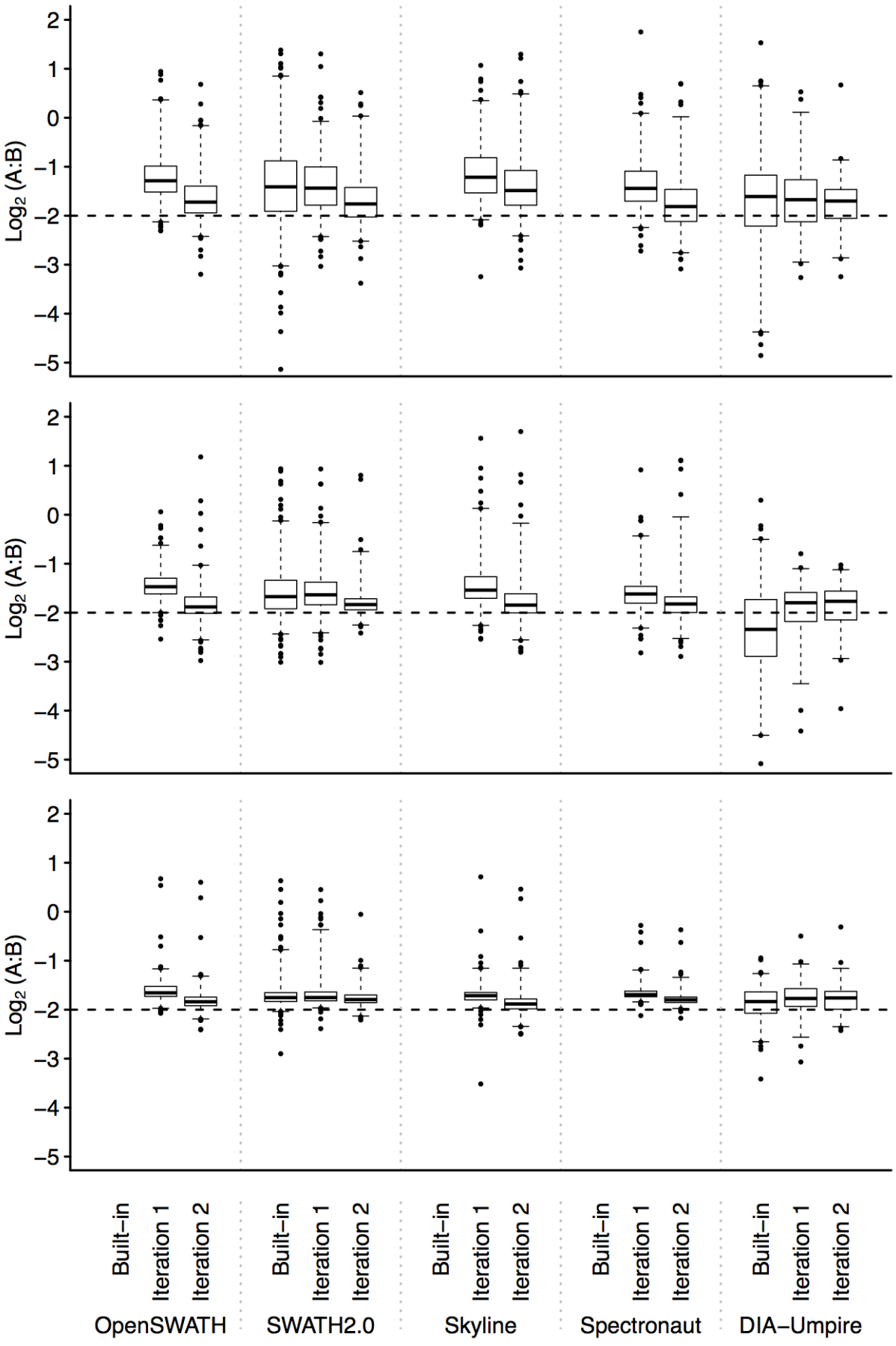
The present figure displays the log_2(A/B) of the averages between technical replicates of A and B for E.coli proteins in the lowest intensity (0%-33.3%, top panel), medium (33.3%-66.7%) and highest (66.7%-100%) tertiles. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

The following pages show log_2 ratio distributions for:

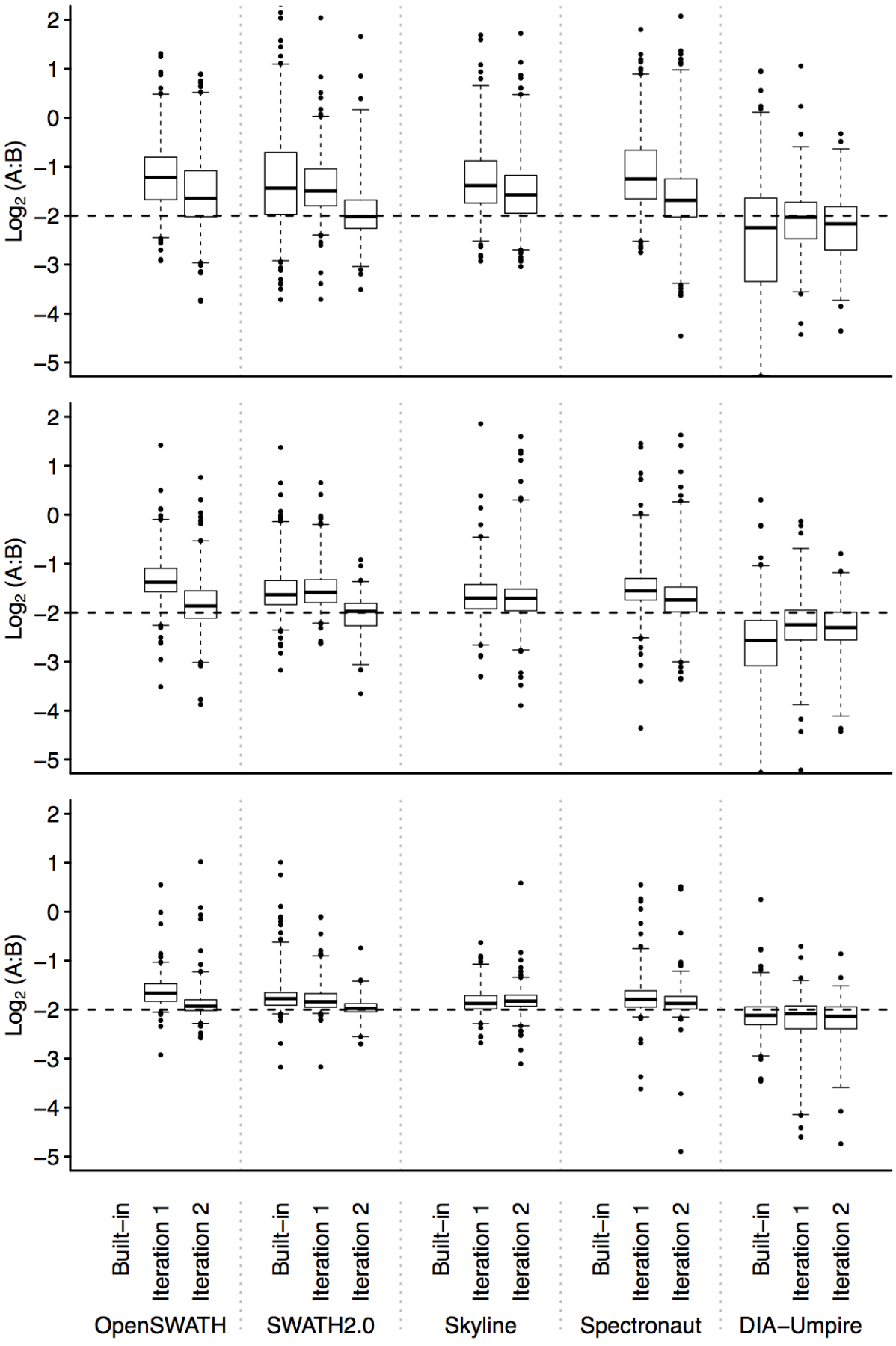
- Supplementary Figure 13.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 13.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 13.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 13.D: HYE 124, TripleTOF 6600, 64var setup



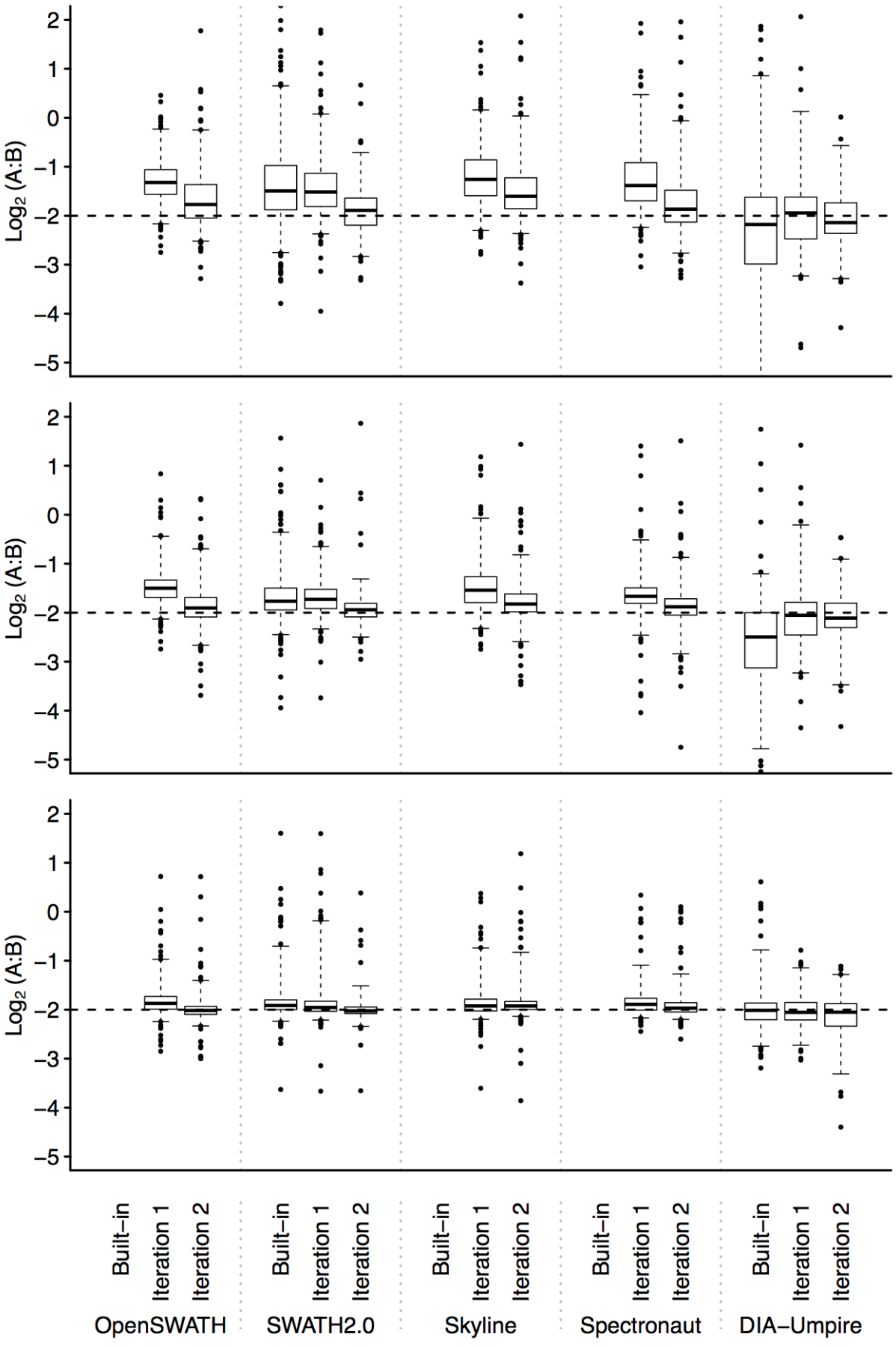
Supplementary Figure 13.A: log_2 ratio distributions in different intensity tertiles for E.coli proteins for HYE 124, TripleTOF 5600, 32fix setup.



Supplementary Figure 13.B: log_2 ratio distributions in different intensity tertiles for E.coli proteins for HYE 124, TripleTOF 5600, 64var setup.



Supplementary Figure 13.C: log_2 ratio distributions in different intensity tertiles for E.coli proteins for HYE 124, TripleTOF 6600, 32fix setup.



Supplementary Figure 13.D: log_2 ratio distributions in different intensity tertiles for E.coli proteins for HYE 124, TripleTOF 6600, 64var setup.

Supplementary Figure 14. LFQbench protein level benchmarks for built-in protein level reports.

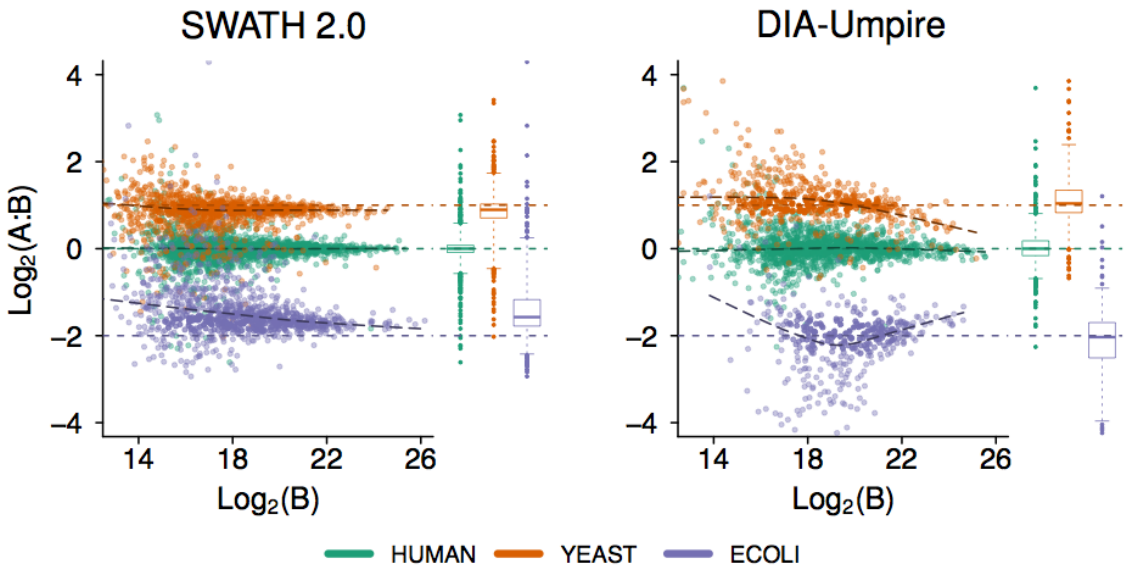
Protein quantities were estimated using built-in protein level quantification workflows in SWATH2.0 (panel A) and DIA-Umpire (panel B).

Log-transformed ratios (log_2(A/B)) of proteins were plotted for each benchmarked software tool over the log-transformed scaled intensity of sample B. Dashed colored lines represent the expected log_2(A/B) values for human, yeast, and E.coli peptides. Black dashed lines represent the local trend along the x-axis of experimental log-transformed ratios of each population (human, yeast, and E.coli). In case of sample HYE124, panel A shows both iterations for all software tools, and panel B shows the log_2(A/B) of the averages between technical replicates of A and B for E.coli peptides in the lowest intensity tertile. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

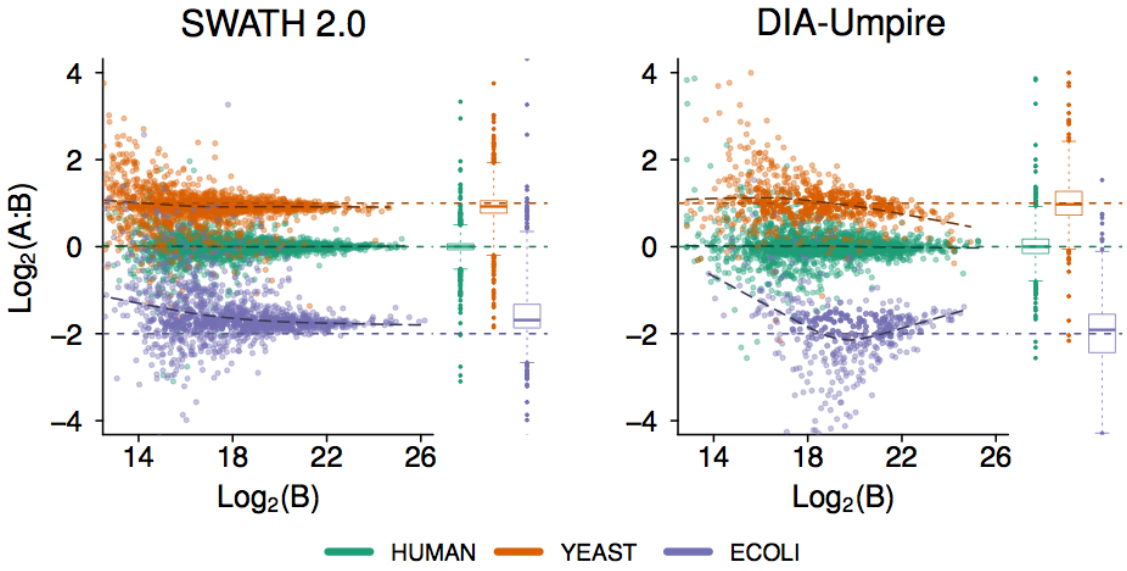
Intensities of multiply charged precursors were summed up, and averaged between technical replicates of each sample. Protein quantities were estimated in each technical replicate by the average of the most 3 intense peptides reported for each protein. Single hit proteins (a single peptide detected in a protein) and proteins detected in less than two injections in both samples A and B were discarded.

The following pages show LFQbench protein level benchmarks based on built-in protein level reports for:

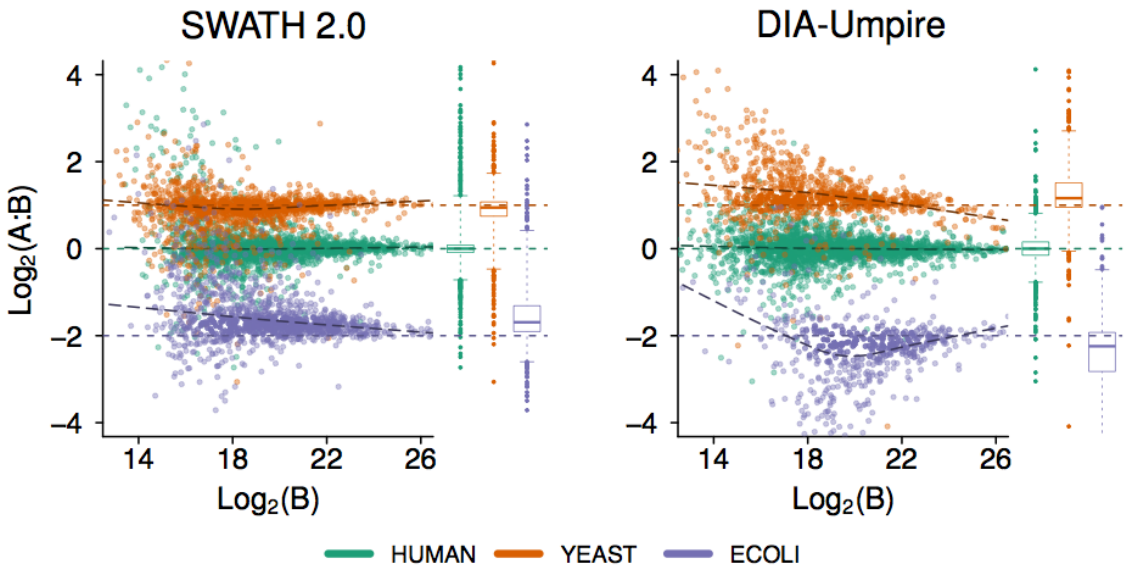
- Supplementary Figure 14.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 14.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 14.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 14.D: HYE 124, TripleTOF 6600, 64var setup



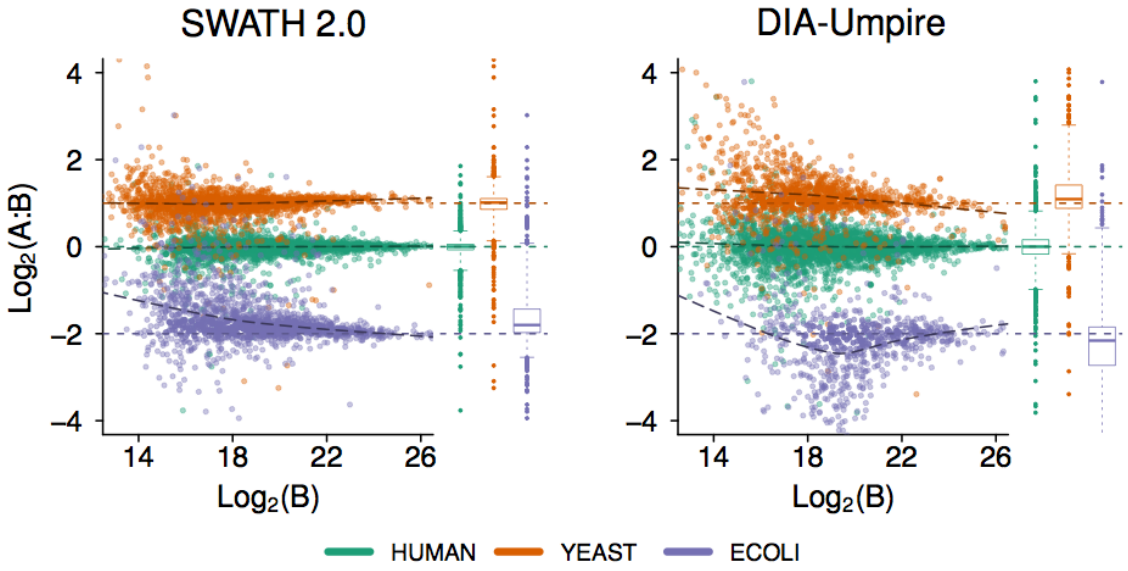
Supplementary Figure 14.A: LFQbench built-in protein level benchmarks for HYE 124, TripleTOF 5600, 32fix setup.



Supplementary Figure 14.B: LFQbench built-in protein level benchmarks for HYE 124, TripleTOF 5600, 64var setup.



Supplementary Figure 14.C: LFQbench built-in protein level benchmarks for HYE 124, TripleTOF 6600, 32fix setup.

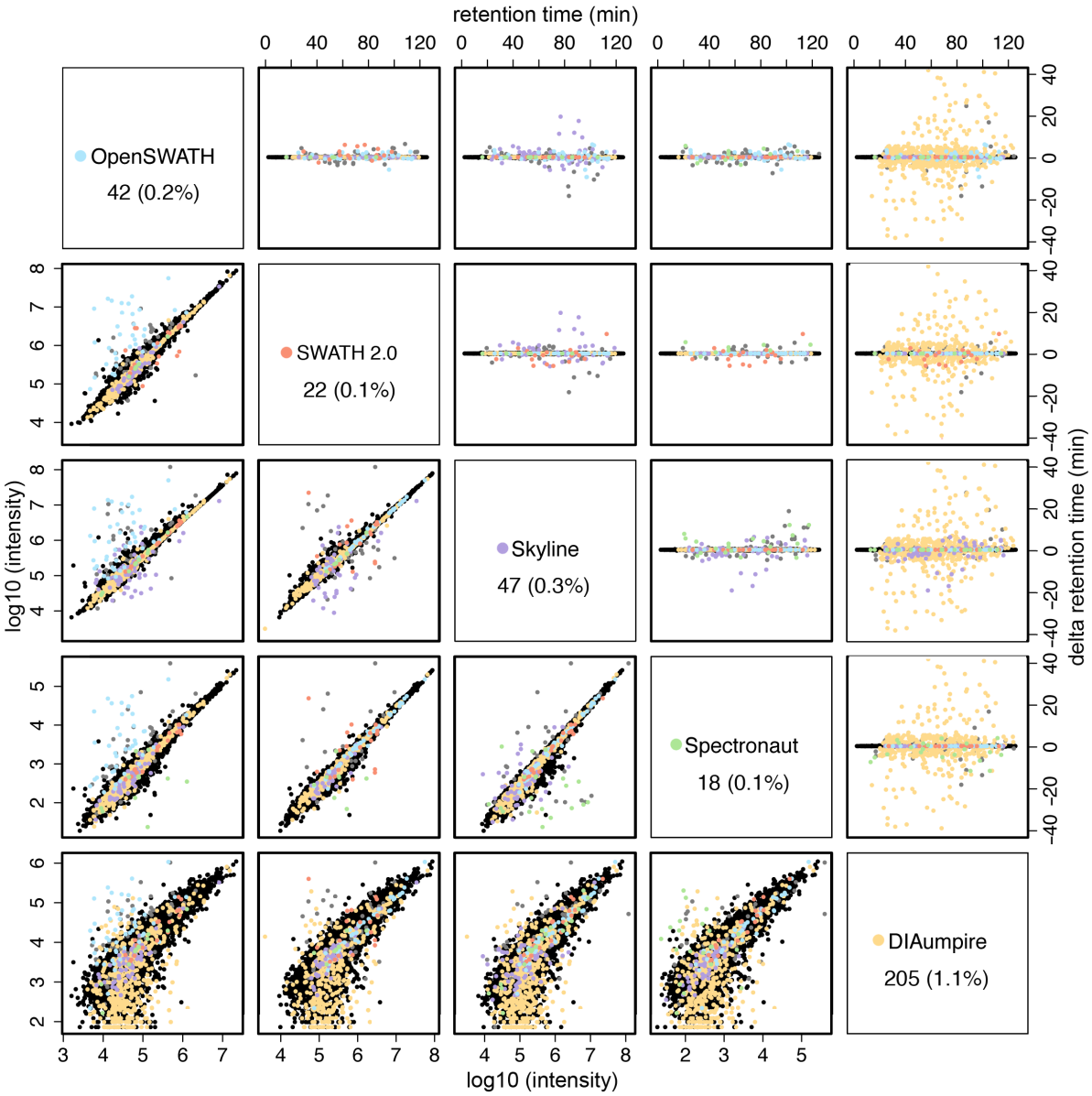


Supplementary Figure 14.D: LFQbench built-in protein level benchmarks for HYE 124, TripleTOF 6600, 64var setup.

Supplementary Figure 15. Retention time differences and correlation of reported peak intensities between all software tools for the first iteration.

The present figure shows the retention time differences (upper right panels) and correlation of reported peak intensities (lower left panels) for the respective matching precursors between all software tools for the first iteration. Peak intensities and retention times reported by all software tools in HYE124 (64 variable windows) were compared pair-wise.

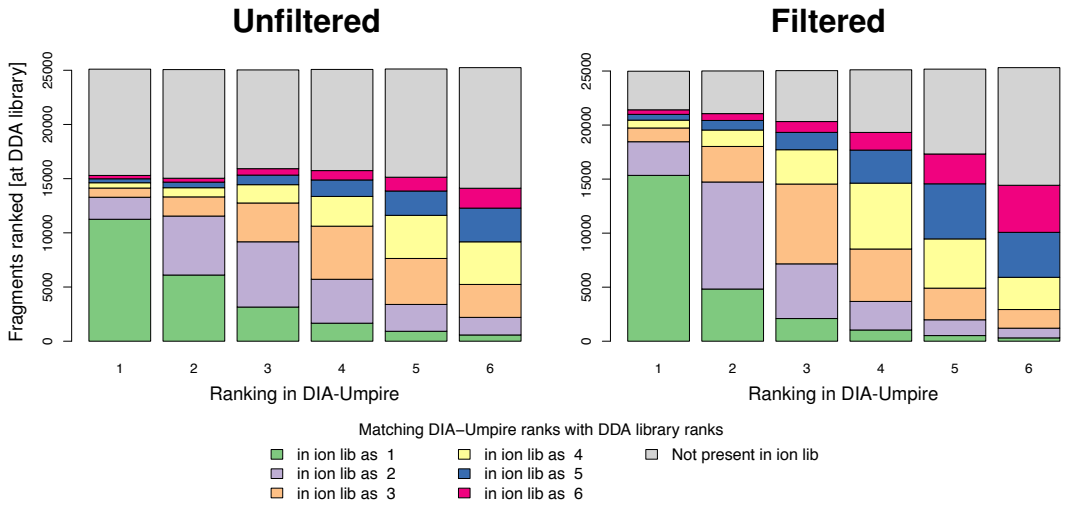
Outliers are plotted in the color of the outlier software tool (see color legend in the diagonal panels). Diagonal panels show the total number and percentage (to the total number of common detected peptides) of outliers of each respective software tool. Outliers have been defined as producing a standard deviation of the peak retention time greater than 0.2 minutes relative to all other software tools detecting that precursor, after removing ambiguous cases, in which more than one software tool produce a greater standard deviation in the peak retention time.



Supplementary Figure 15: Retention time differences (upper right panels) and correlation of reported peak intensities (lower left panels) between all software tools for the respective matching precursors for the first iteration of all tools.

Supplementary Figure 16. Comparative analysis of fragment intensity rankings between DIA-Umpire and the library.

For each fragment reported by DIA-Umpire the respective intensity ranking in the spectral library was determined. In the first iteration (left panel), 39% of the fragment ions reported by DIA-Umpire are not part of the library (grey). The agreement between the other DIA-Umpire's fragment ions and the spectral library is good (70% of the three most intense fragment ions of DIA-Umpire match with the three most intense fragment ions of the spectral library). After removing low mass fragments (<350 m/z) the percentage of unmatched ion fragments to the spectral library was reduced to 24%.



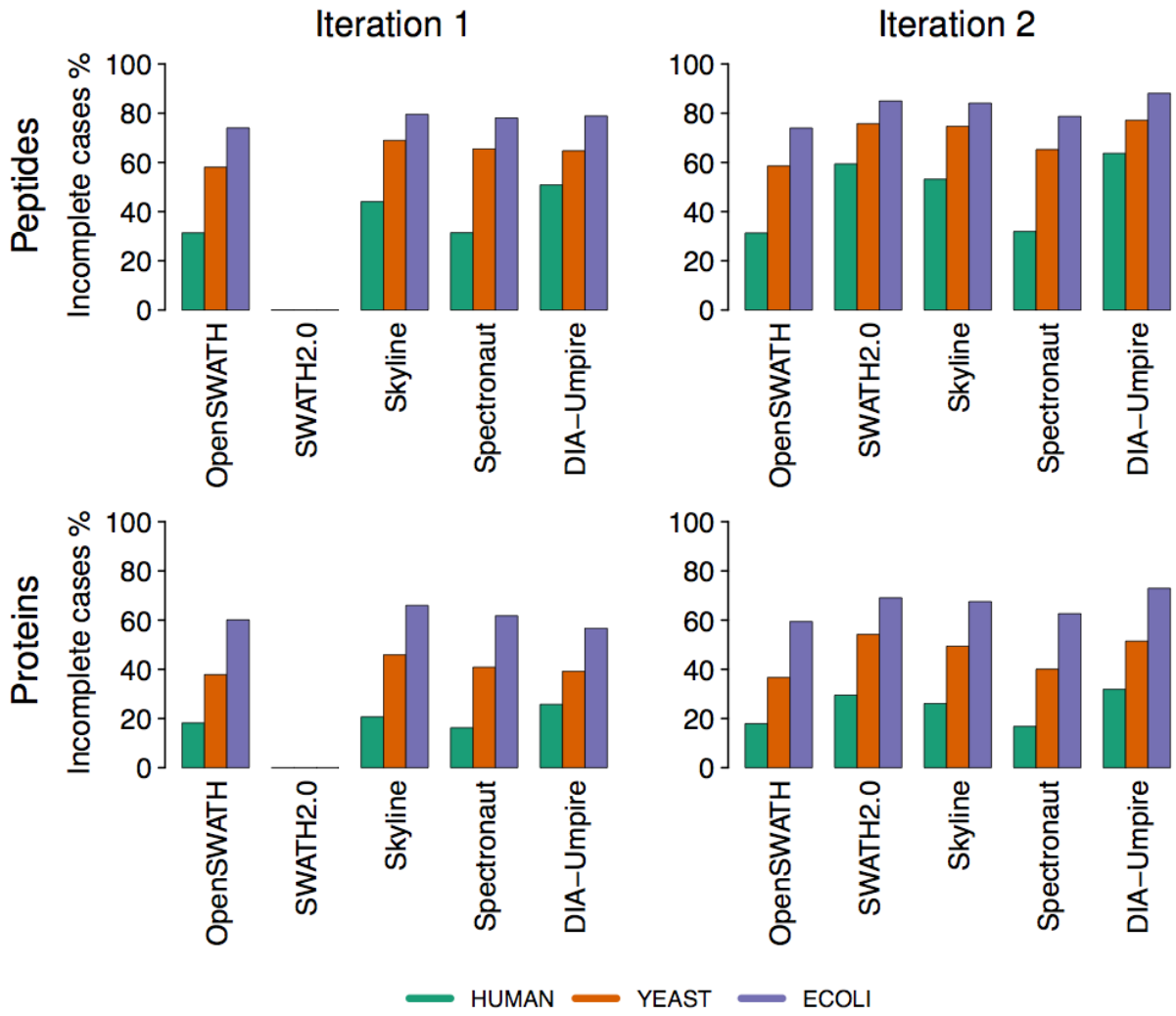
Supplementary Figure 16: Comparative analysis of fragment intensity ranking between DIA-Umpire and the library.

Supplementary Figure 17. Number of incomplete cases.

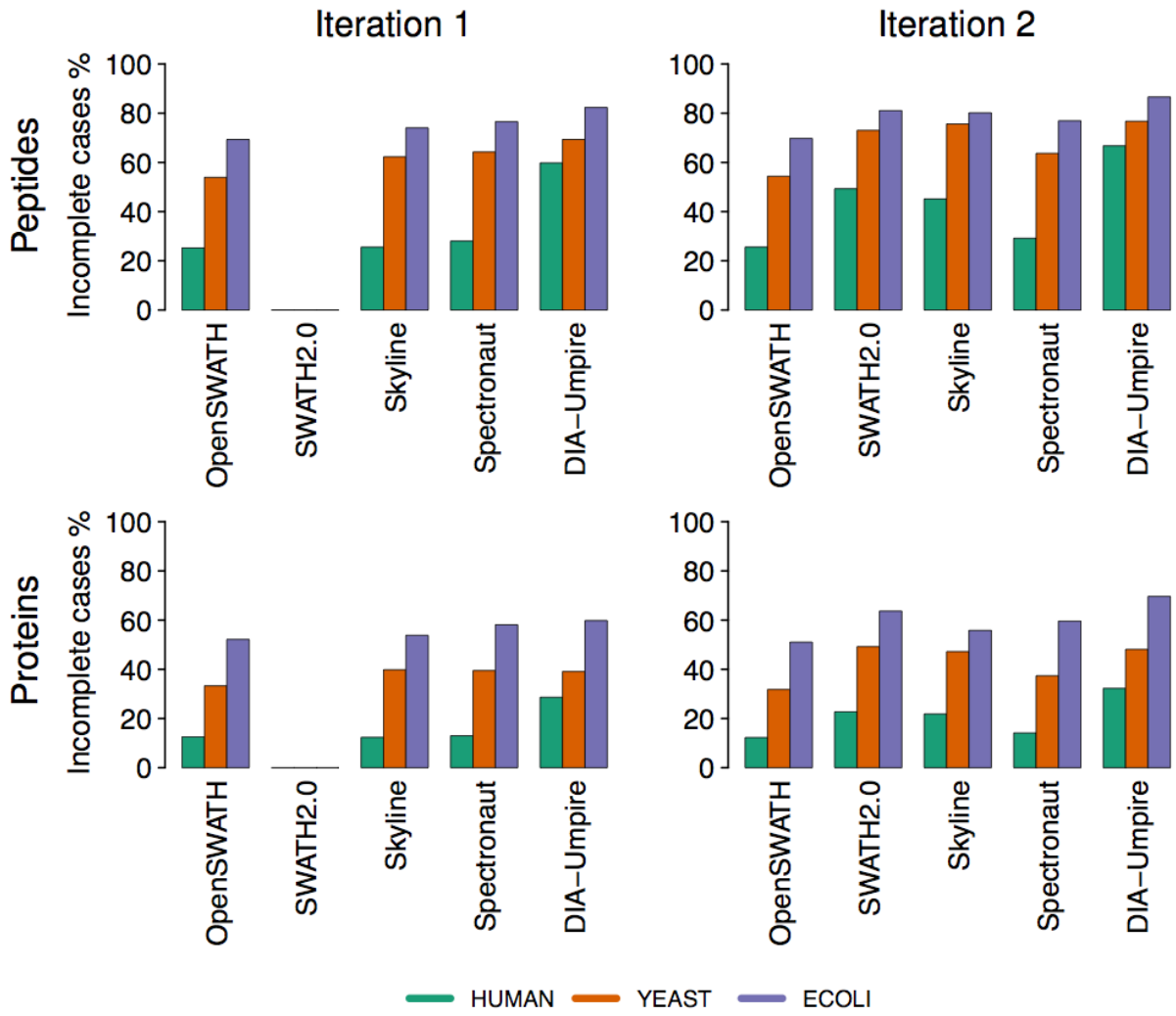
The percentage of the number of peptides or proteins with less than six quantification values among the six injections (three technical replicates by sample) is shown in the present figure. Of note, yeast and E.coli proteins reported more incomplete cases in HYE110 than in HYE124 due to its more challenging composition (1:10 ratios instead of 1:2 or 1:4 ratios).

The following pages show number of incomplete cases for:

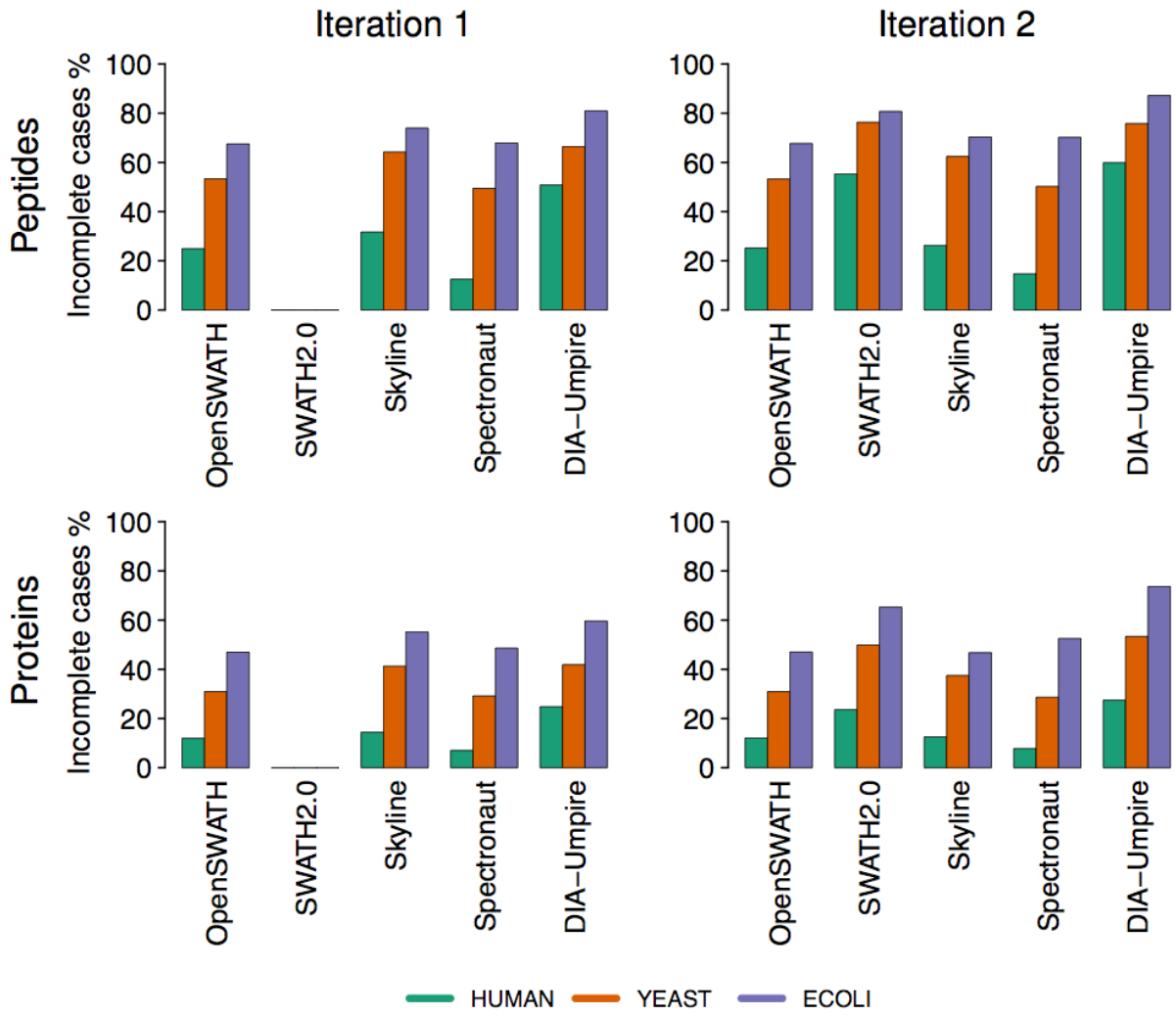
- Supplementary Figure 17.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 17.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 17.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 17.D: HYE 124, TripleTOF 6600, 64var setup
- Supplementary Figure 17.E: HYE 110, TripleTOF 6600, 32fix setup
- Supplementary Figure 17.F: HYE 110, TripleTOF 6600, 32var setup
- Supplementary Figure 17.G: HYE 110, TripleTOF 6600, 64fix setup
- Supplementary Figure 17.H: HYE 110, TripleTOF 6600, 64var setup



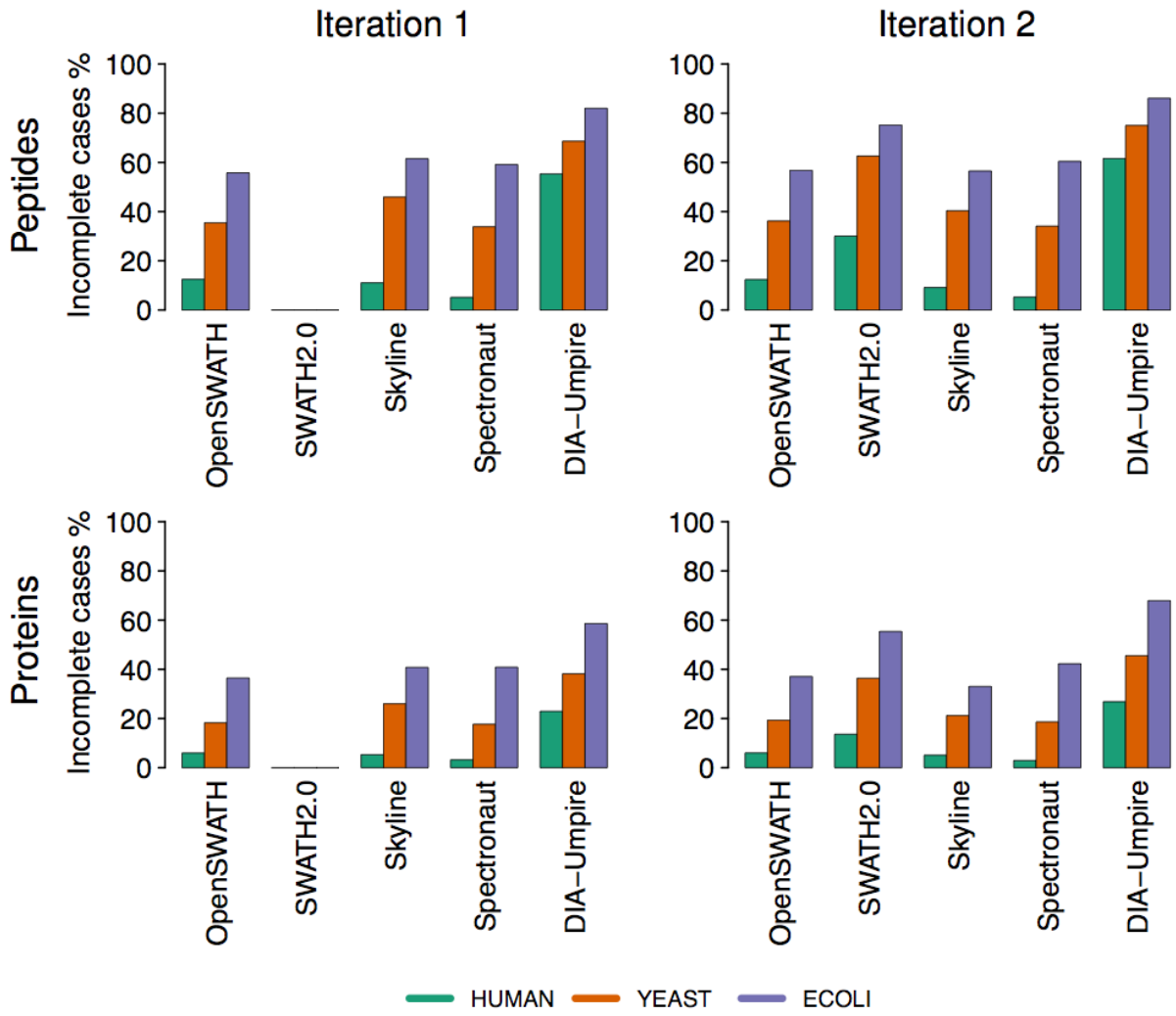
Supplementary Figure 17.A: Number of incomplete cases for HYE 124, TripleTOF 5600, 32fix setup.



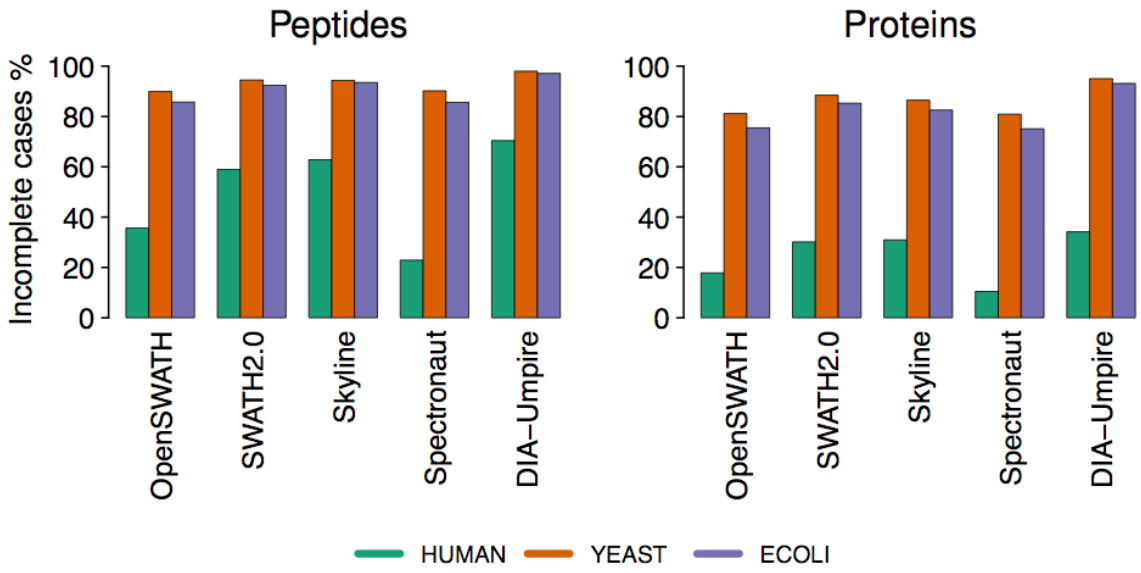
Supplementary Figure 17.B: Number of incomplete cases for HYE 124, TripleTOF 5600, 64var setup.



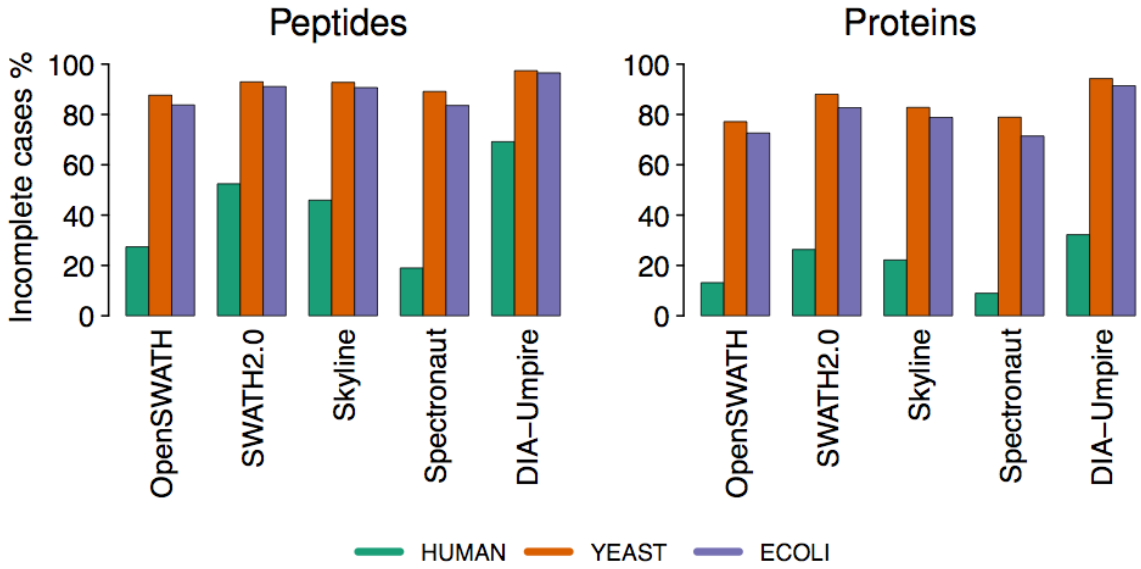
Supplementary Figure 17.C: Number of incomplete cases for HYE 124, TripleTOF 6600, 32fix setup.



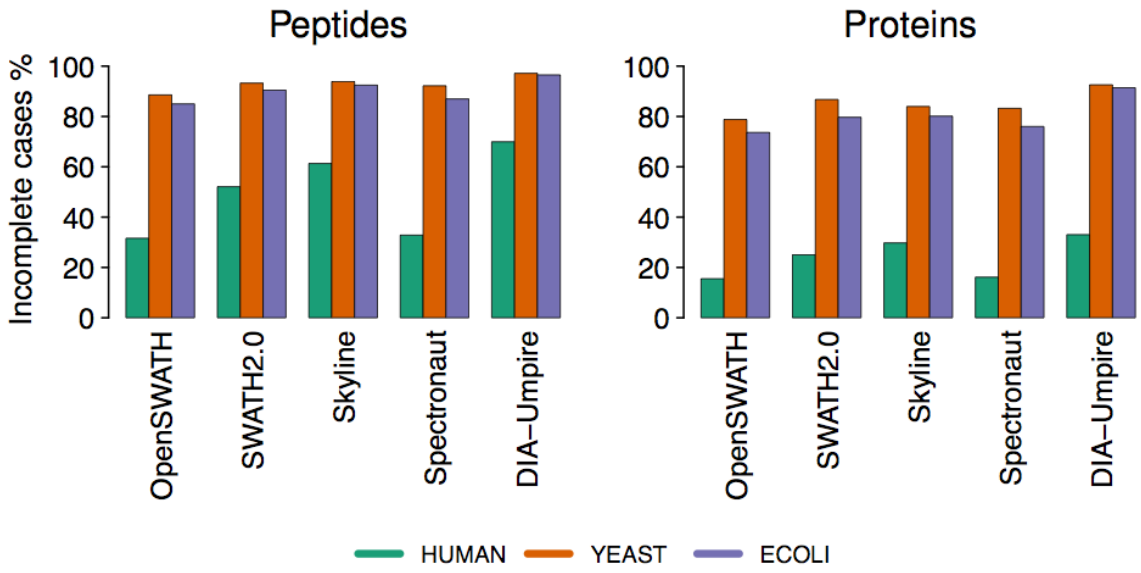
Supplementary Figure 17.D: Number of incomplete cases for HYE 124, TripleTOF 6600, 64var setup.



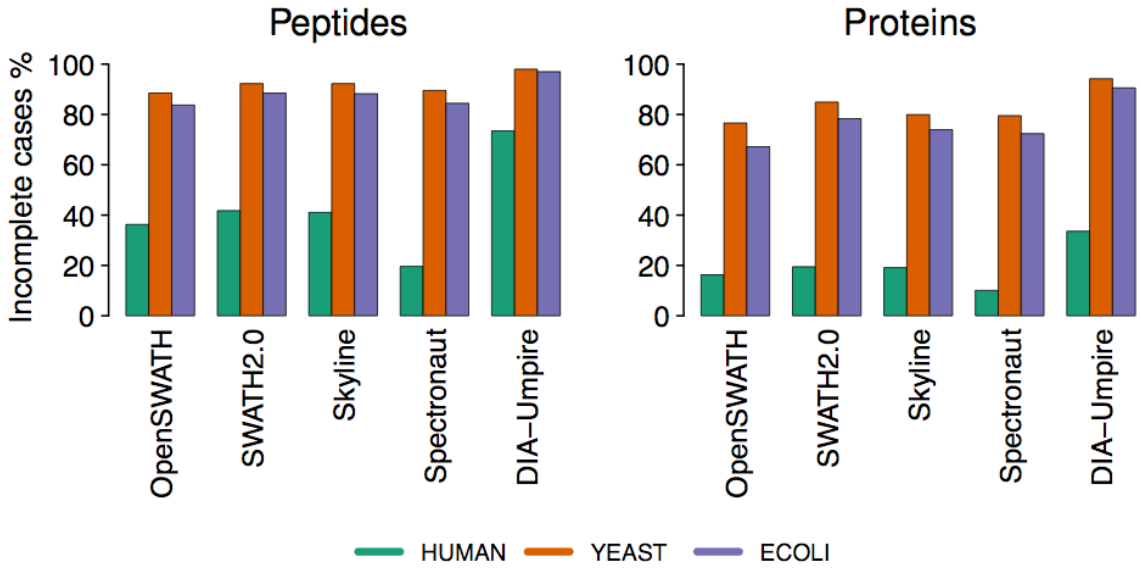
Supplementary Figure 17.E: Number of incomplete cases for HYE 110, TripleTOF 6600, 32fix setup.



Supplementary Figure 17.F: Number of incomplete cases for HYE 110, TripleTOF 6600, 32var setup.



Supplementary Figure 17.G: Number of incomplete cases for HYE 110, TripleTOF 6600, 64fix setup.

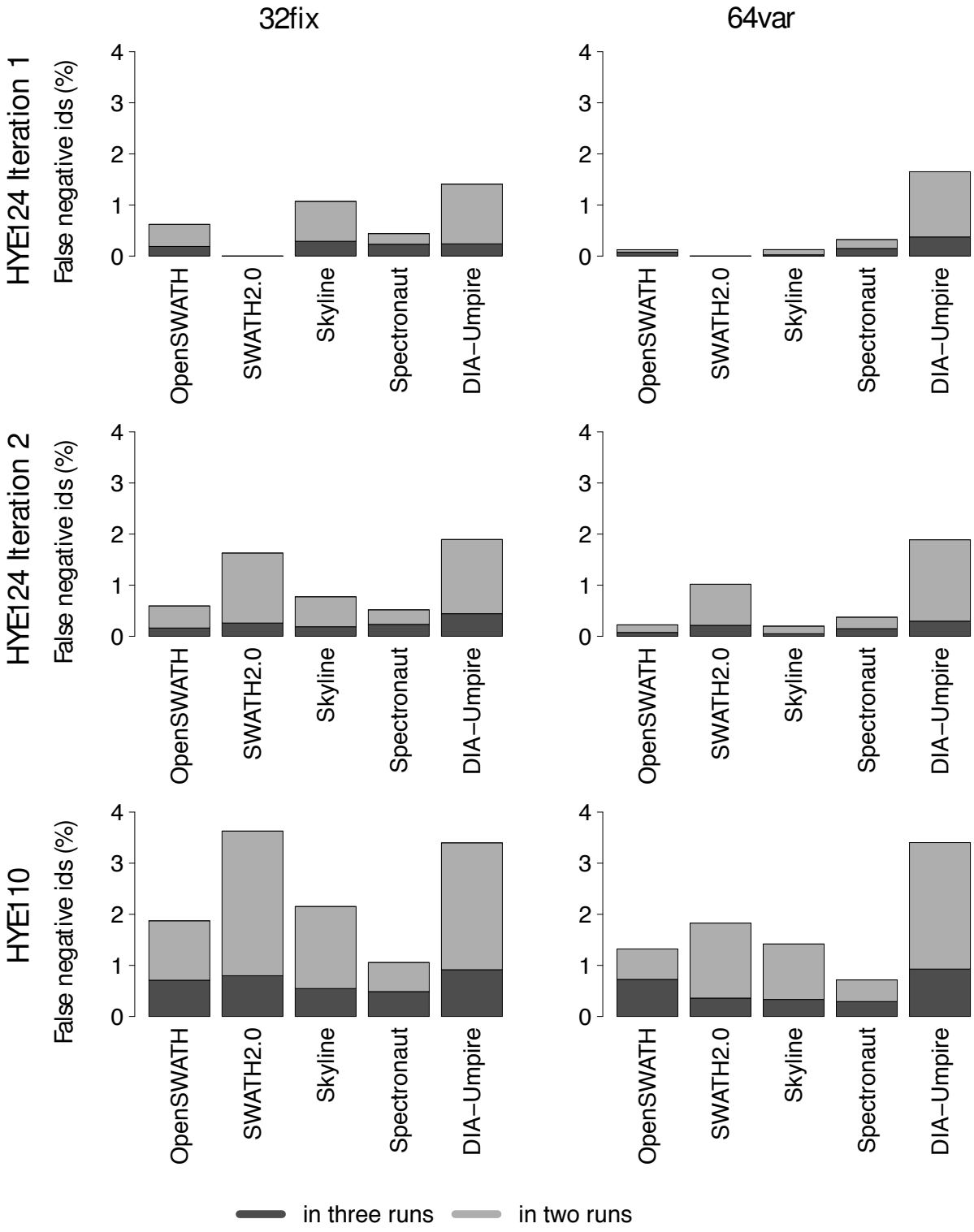


Supplementary Figure 17.H: Number of incomplete cases for HYE 110, TripleTOF 6600, 64var setup.

Supplementary Figure 18. Percentage of false negative human proteins (human proteins detected in only one of the samples) in the second iteration with replication rate filtering.

According to sample composition, human proteins should always be detectable in both samples (A and B). Human proteins that are false-negative in one of the samples (and thus falsely reported as "exclusively detected" in the other samples) are highly problematic from a biological perspective. The data indicate that requiring a protein to be quantified in at least two biological replicates (bar plots group by number of technical replicates – runs – the peptide or protein is detected in the other sample) reduces the false negative rate by up to 70%, depending on instrument, software and SWATH window setup.

The plots show the percentage of false negative identifications after filtering those identifications, which were found in less than two technical replicates in both samples A and B (Supplementary Figure 12 shows unfiltered data).

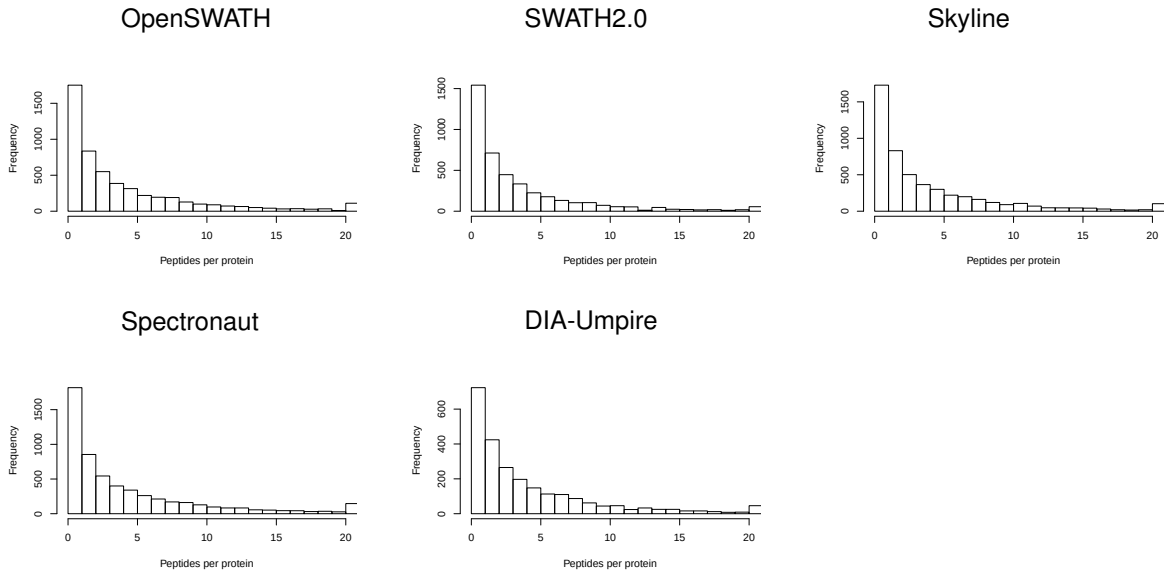


Supplementary Figure 18: Percentage of false negative human proteins (human proteins detected in only one of the samples) in the second iteration with replication rate filtering.

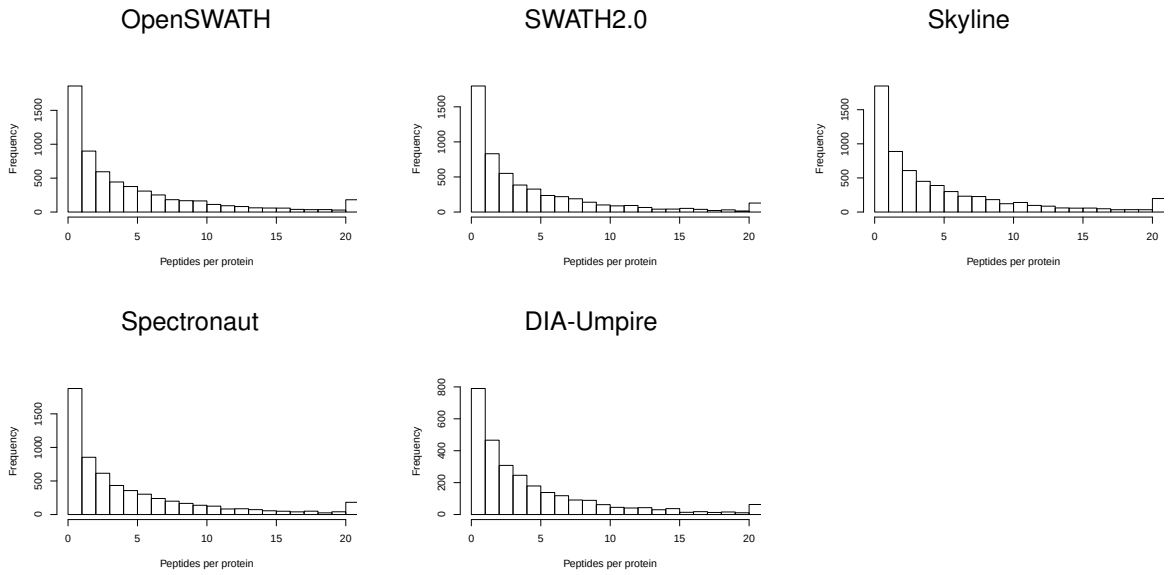
Supplementary Figure 19. Number of quantified peptides per protein.

The following pages show the numbers of quantified peptides per protein for:

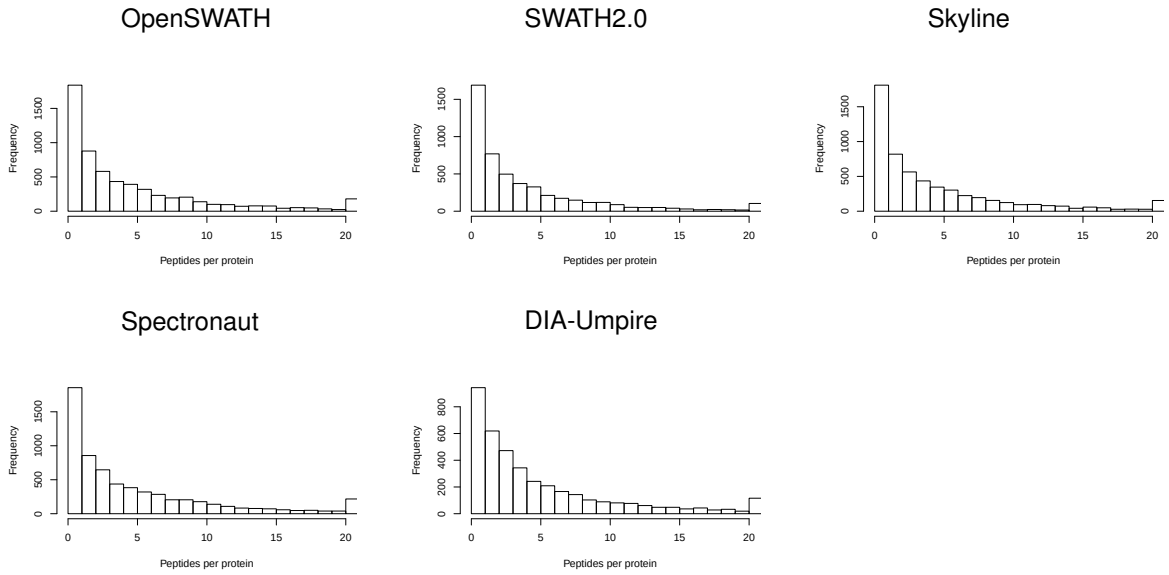
- Supplementary Figure 19.A: HYE 124, TripleTOF 5600, 32fix setup, iteration 1
- Supplementary Figure 19.B: HYE 124, TripleTOF 5600, 64var setup, iteration 1
- Supplementary Figure 19.C: HYE 124, TripleTOF 6600, 32fix setup, iteration 1
- Supplementary Figure 19.D: HYE 124, TripleTOF 6600, 64var setup, iteration 1
- Supplementary Figure 19.E: HYE 124, TripleTOF 5600, 32fix setup, iteration 2
- Supplementary Figure 19.F: HYE 124, TripleTOF 5600, 64var setup, iteration 2
- Supplementary Figure 19.G: HYE 124, TripleTOF 6600, 32fix setup, iteration 2
- Supplementary Figure 19.H: HYE 124, TripleTOF 6600, 64var setup, iteration 2
- Supplementary Figure 19.I: HYE 110, TripleTOF 6600, 32fix setup
- Supplementary Figure 19.J: HYE 110, TripleTOF 6600, 32var setup
- Supplementary Figure 19.K: HYE 110, TripleTOF 6600, 64fix setup
- Supplementary Figure 19.L: HYE 110, TripleTOF 6600, 64var setup



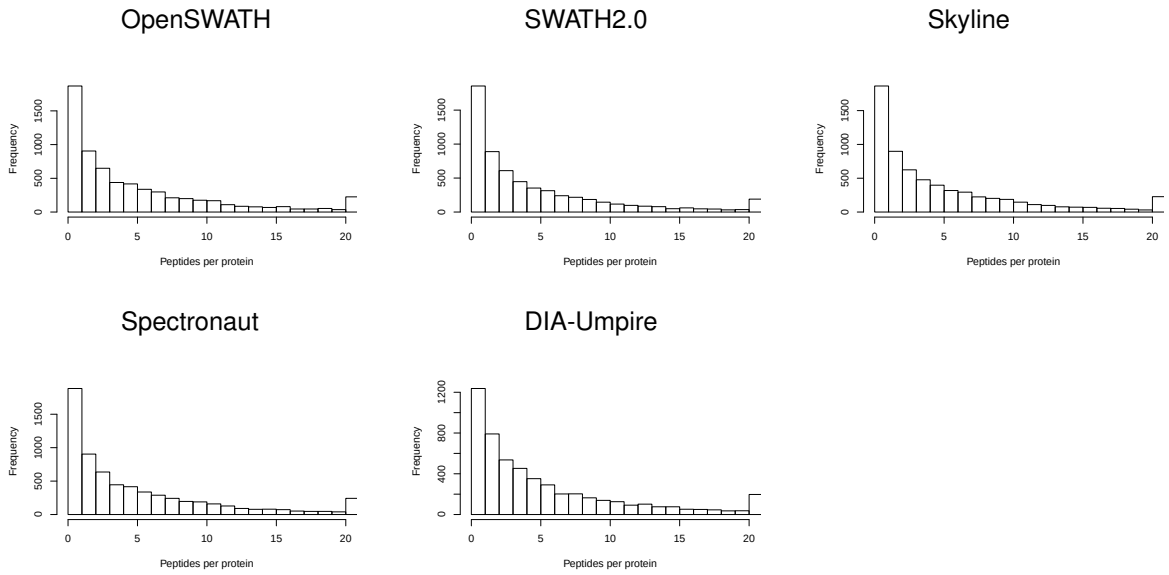
Supplementary Figure 19.A: Number of quantified peptides per protein for HYE 124, TripleTOF 5600, 32fix setup, iteration 1.



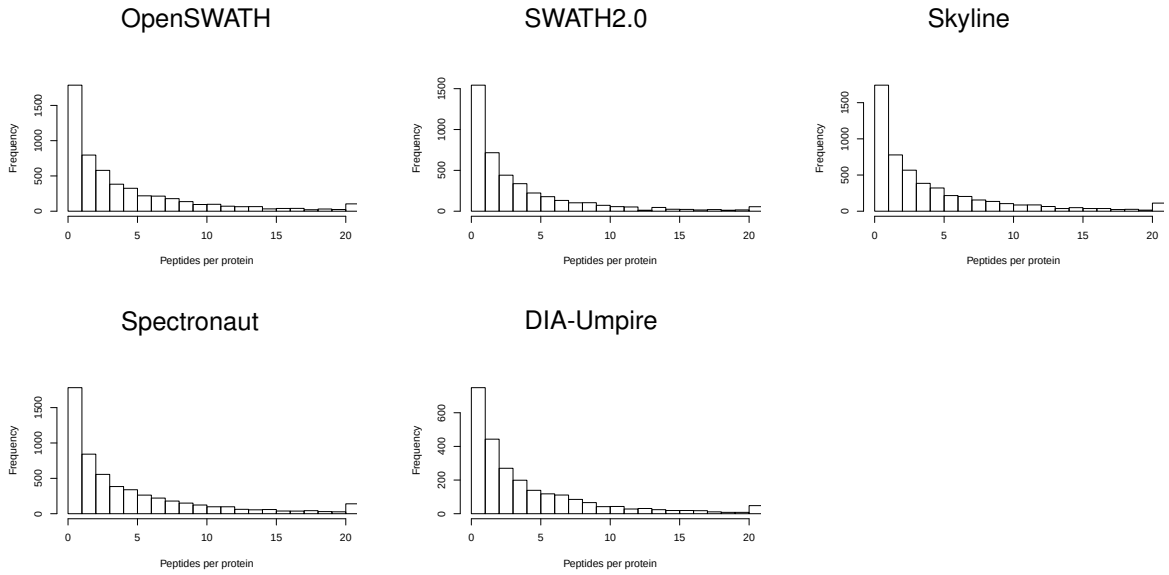
Supplementary Figure 19.B: Number of quantified peptides per protein for HYE 124, TripleTOF 5600, 64var setup, iteration 1.



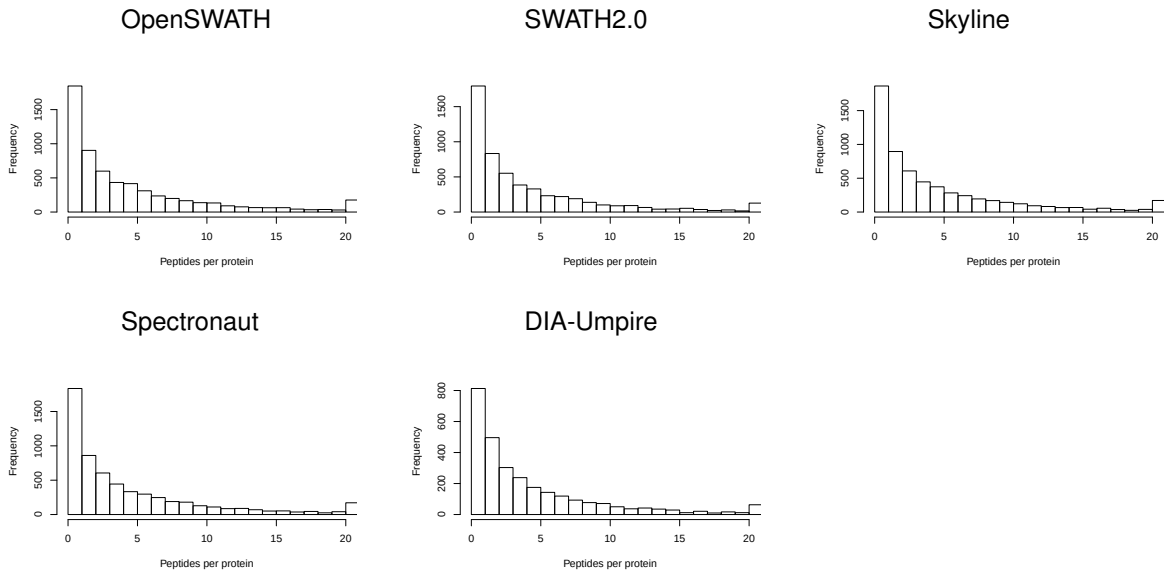
Supplementary Figure 19.C: Number of quantified peptides per protein for HYE 124, TripleTOF 6600, 32fix setup, iteration 1.



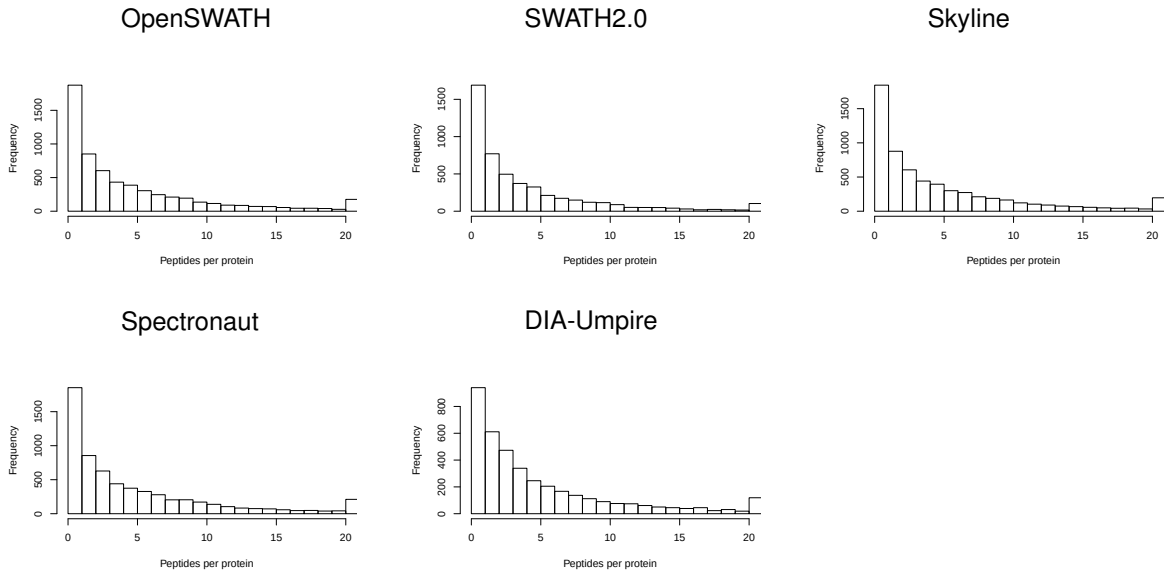
Supplementary Figure 19.D: Number of quantified peptides per protein for HYE 124, TripleTOF 6600, 64var setup, iteration 1.



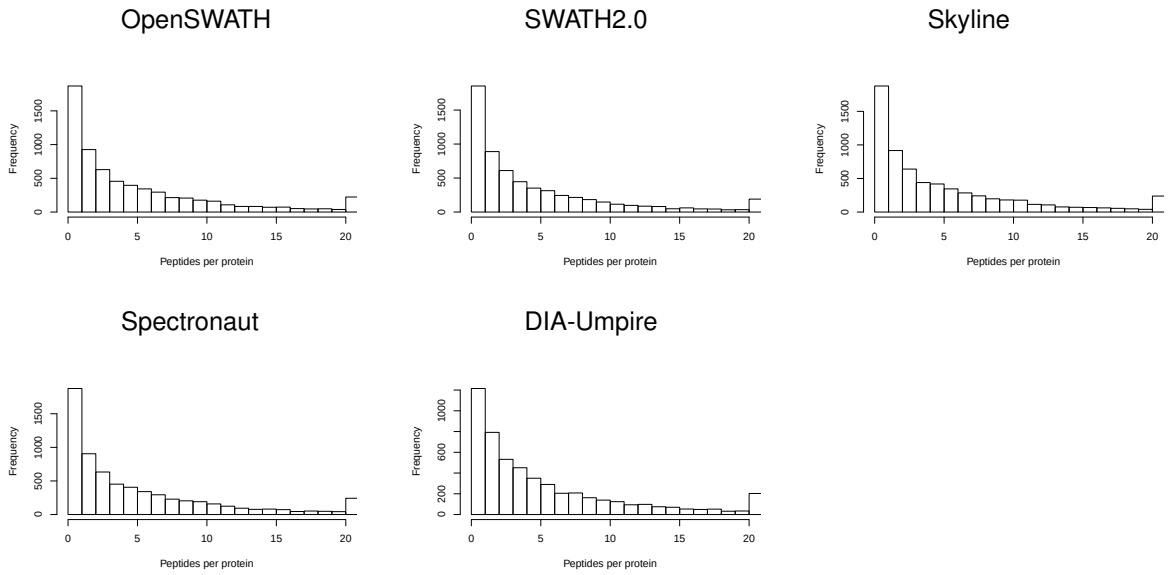
Supplementary Figure 19.E: Number of quantified peptides per protein for HYE 124, TripleTOF 5600, 32fix setup, iteration 1.



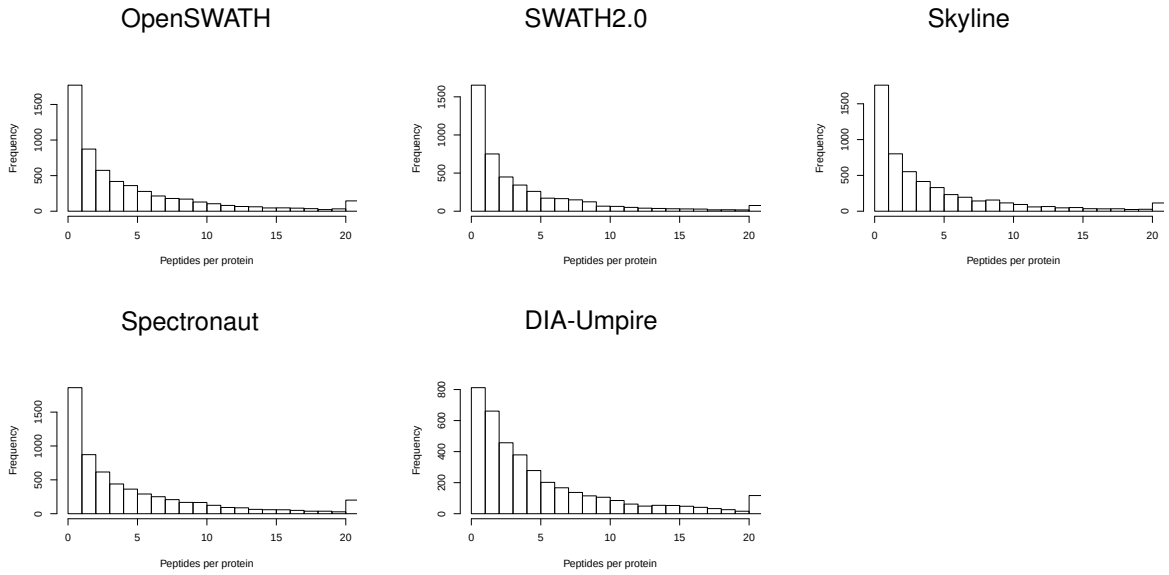
Supplementary Figure 19.F: Number of quantified peptides per protein for HYE 124, TripleTOF 5600, 64var setup, iteration 1.



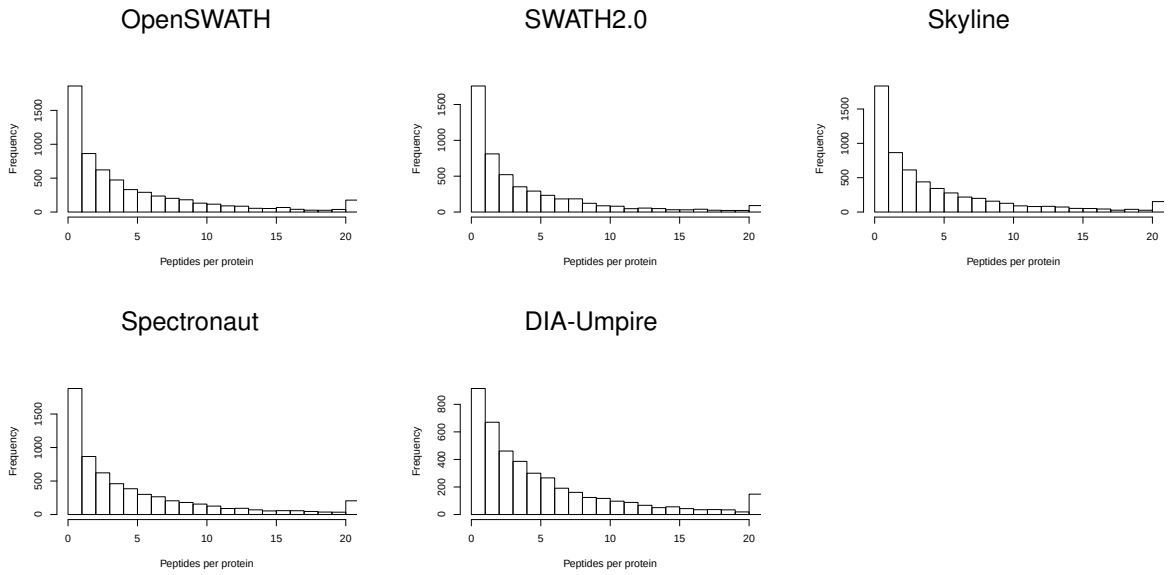
Supplementary Figure 19.G: Number of quantified peptides per protein for HYE 124, TripleTOF 6600, 32fix setup, iteration 1.



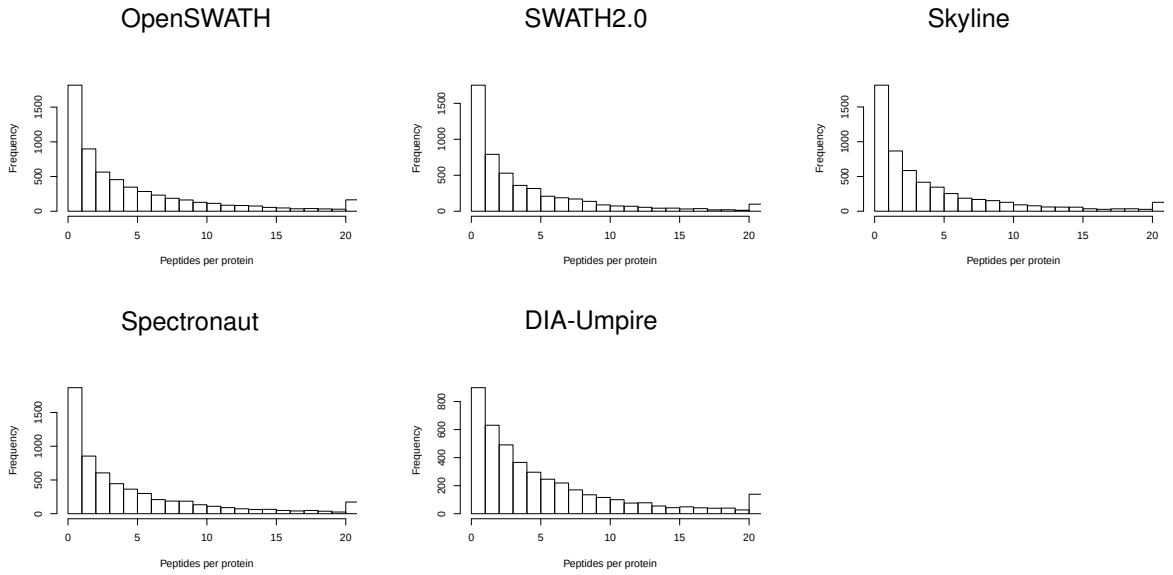
Supplementary Figure 19.H: Number of quantified peptides per protein for HYE 124, TripleTOF 6600, 64var setup, iteration 1.



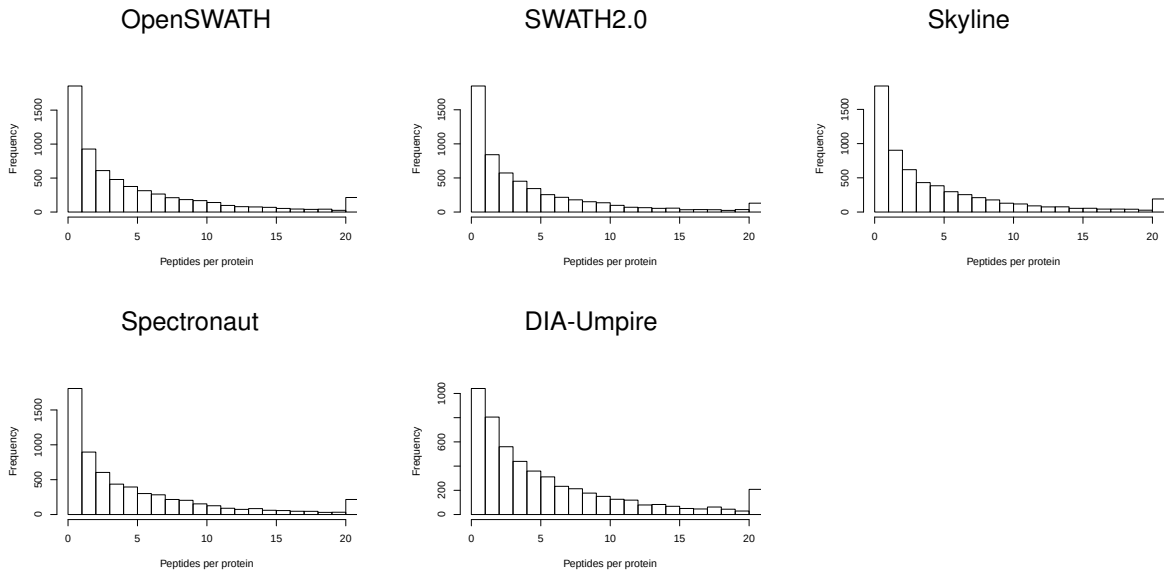
Supplementary Figure 19.I: Number of quantified peptides per protein for HYE 110, TripleTOF 6600, 32fix setup.



Supplementary Figure 19.J: Number of quantified peptides per protein for HYE 110, TripleTOF 6600, 32var setup.



Supplementary Figure 19.K: Number of quantified peptides per protein for HYE 110, TripleTOF 6600, 64fix setup.

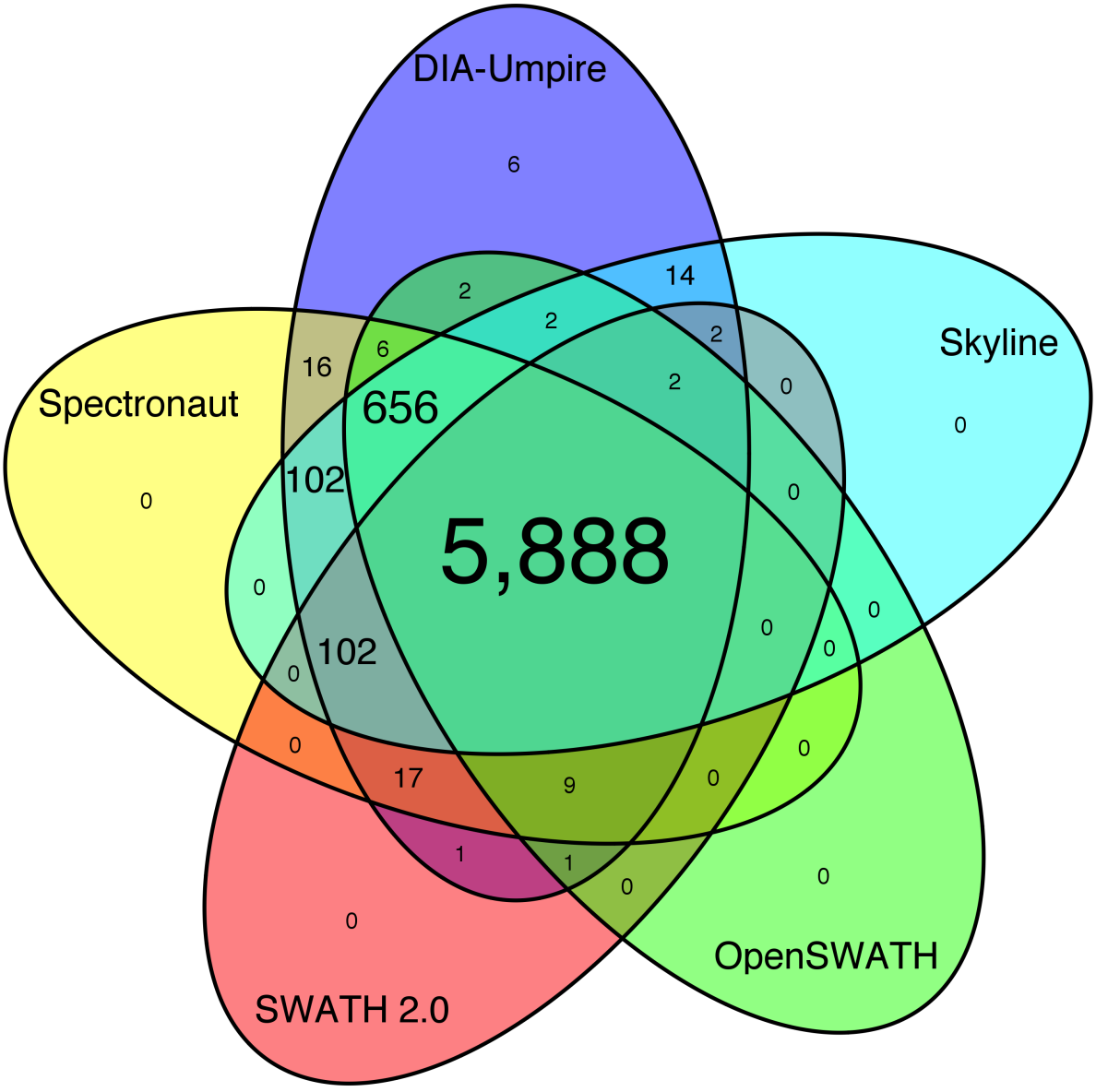


Supplementary Figure 19.L: Number of quantified peptides per protein for HYE 110, TripleTOF 6600, 64var setup.

Supplementary Figure 20. Peptide overlap between the five software tools using a dedicated library with peptides identified initially only by DIA-Umpire.

A dedicated library (built with Orbitrap/Fusion assays) containing peptides exclusively detected by DIA-Umpire was interrogated using the four library-based software tools. Only unmodified peptides were considered to estimate the overlap with DIA-Umpire's exclusively identified peptides.

Notably, more than 99% of the 6,826 peptides in this library were detectable by at least two library-based tools.

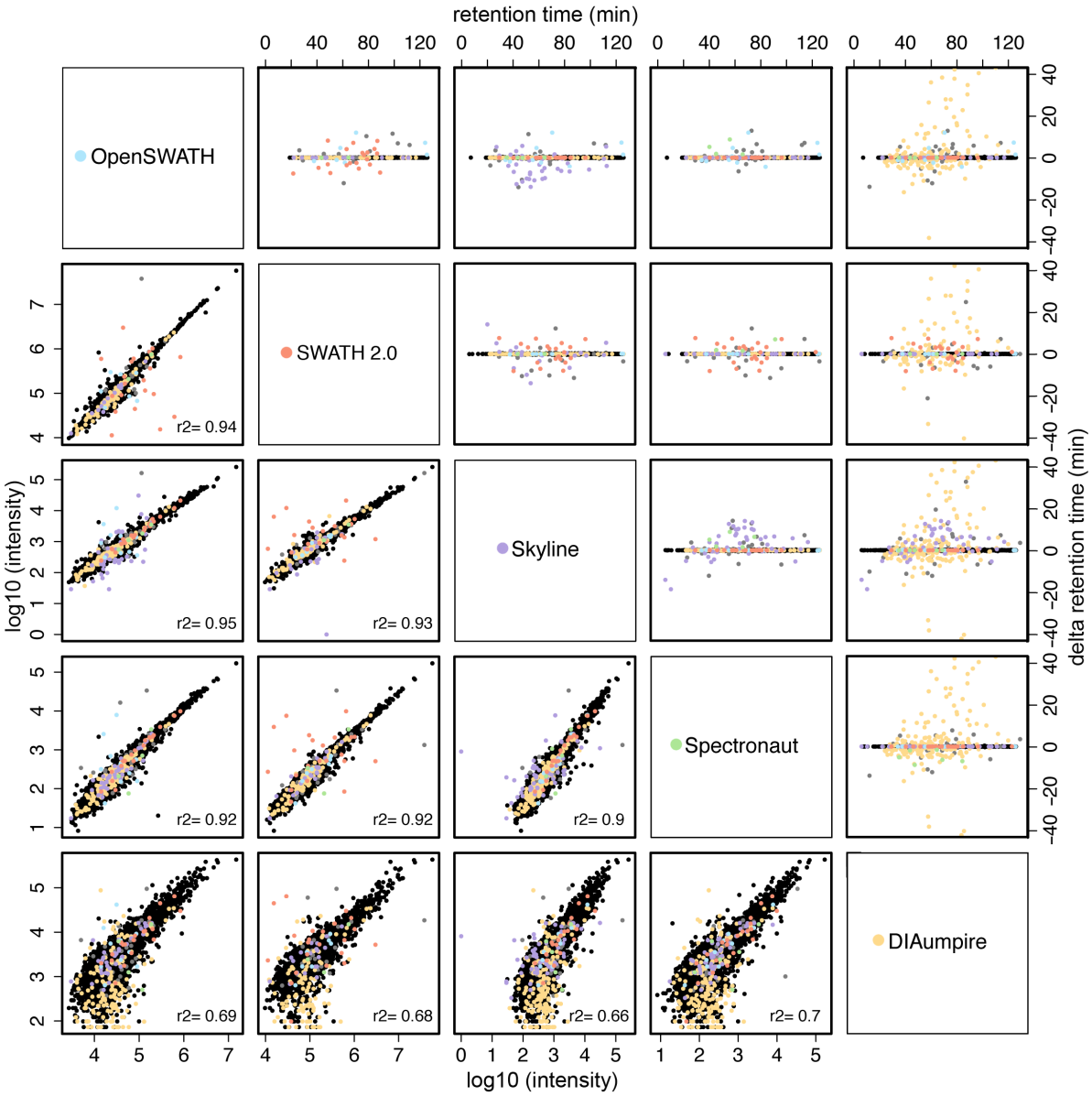


Supplementary Figure 20: Peptide overlap between the five software tools using a dedicated library with peptides identified initially only by DIA-Umpire.

Supplementary Figure 21. Retention time differences and correlation of reported peak intensities between all software tools for extractions of a dedicated library with peptides identified only by DIA-Umpire.

The present figure shows the retention time differences (upper right panels) and correlation of reported peak intensities (lower left panels) between all software tools for extractions of a dedicated library with peptides identified only by DIA-Umpire. A dedicated library (built with Orbitrap/Fusion assays) containing peptides exclusively detected by DIA-Umpire was interrogated by using the four library-based software tools. Only unmodified peptides were considered to compare the peak retention times and intensities of all five software tools.

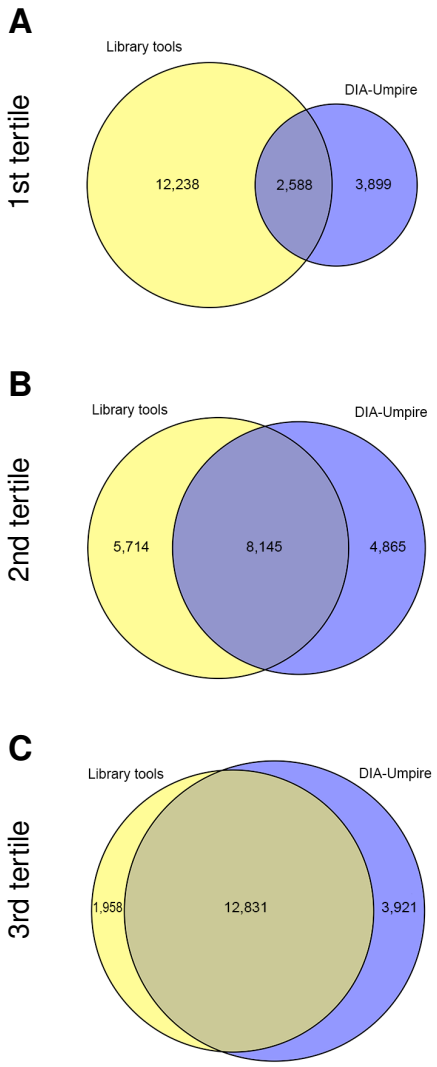
Retention time differences (upper right panels) and correlation of reported peak intensities (lower left panels) between all software tools for the respective matching precursors. Outliers are plotted in the color of the outlier software tool (see color legend in the diagonal panels). Diagonal panels show the total number and percentage (to the total number of common detected peptides) of outliers of each respective software tool. Outliers have been defined as producing a standard deviation of the peak retention time greater than 0.2 minutes relative to all other software tools detecting that precursor, after removing ambiguous cases, in which more than one software tool produce a greater standard deviation in the peak retention time.



Supplementary Figure 21: Retention time differences (upper right panels) and correlation of reported peak intensities (lower left panels) between all software tools for extractions of a dedicated library with peptides identified only by DIA-Umpire.

Supplementary Figure 22. Overlap of quantified peptides between DIA-Umpire and the library based tools.

The present figure shows the overlap of quantified peptides between DIA-Umpire and the library based tools in the lowest (A) middle (B) and highest (C) intensity tertiles. Peptide identifications were grouped in tertiles of total signal intensities (average of the intensities detected by all software tools for each peptide). The first tertile (lowest intensities) is the most dissimilar group and shows that both library-based tools and DIA-Umpire identify different peptides at the lower intensity range. The second and third tertiles show increasingly better agreement between DIA-Umpire and library-based software tools.



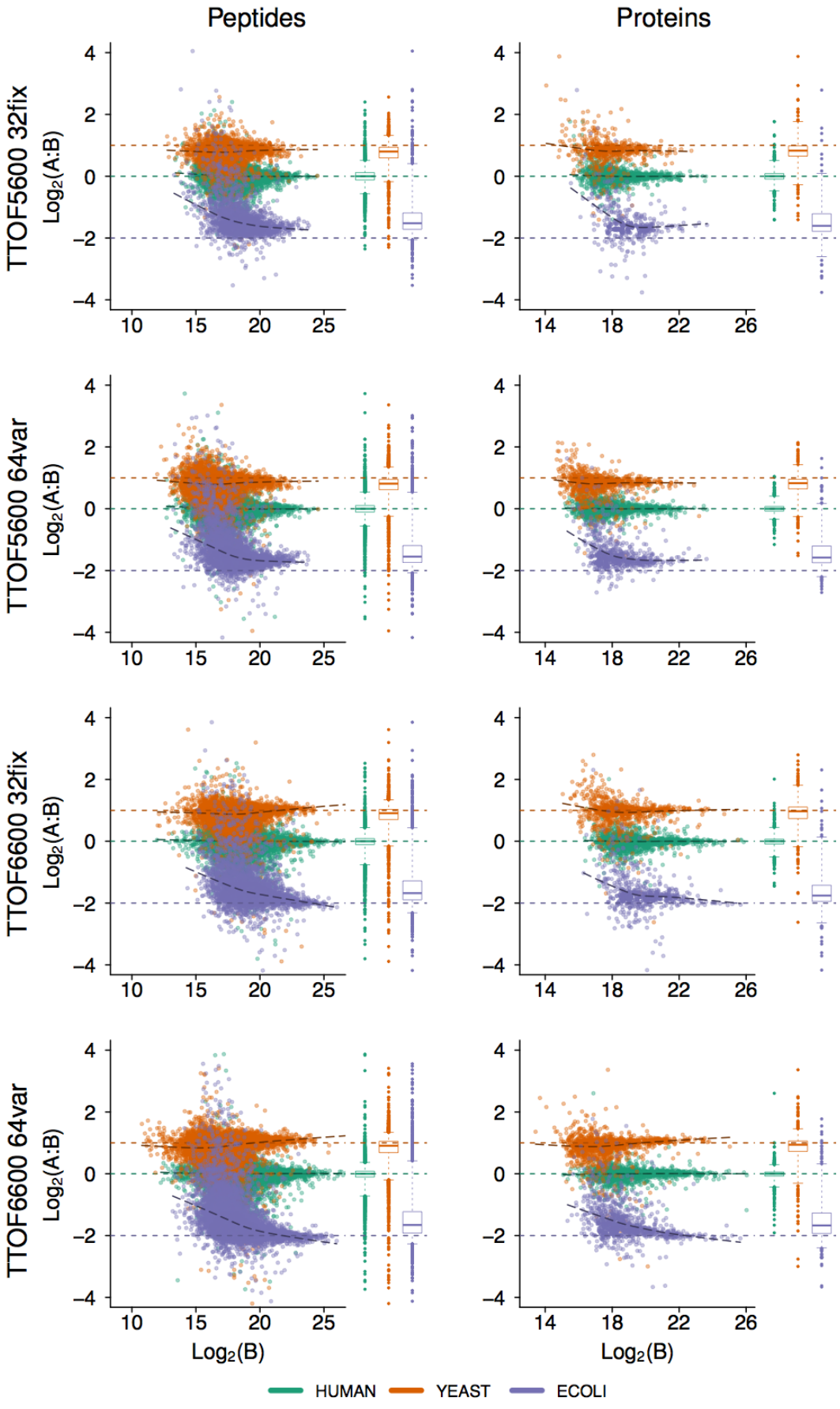
Supplementary Figure 22: Overlap of quantified peptides between DIA-Umpire and the library based tools in the lowest (A) middle (B) and highest (C) intensity tertile.

Supplementary Figure 23. LFQbench peptide and protein level benchmarks using Skyline´s user recommended values.

Skyline's web page suggests to users a configuration parameter setting, which has also been evaluated in this study (LFQbench results shown in this figure), and afterwards optimized for the iteration 1.

Log-transformed ratios (log_2(A/B)) of proteins were plotted for each benchmarked software tool over the log-transformed scaled intensity of sample B. Dashed colored lines represent the expected log_2(A/B) values for human, yeast, and E.coli peptides. Black dashed lines represent the local trend along the x-axis of experimental log-transformed ratios of each population (human, yeast, and E.coli). In case of sample HYE124, panel A shows both iterations for all software tools, and panel B shows the log_2(A/B) of the averages between technical replicates of A and B for E.coli peptides in the lowest intensity tertile. Boxes represent 25% and 75% percentiles, whiskers cover data points between 1% and 99% percentiles.

Intensities of multiply charged precursors were summed up, and averaged between technical replicates of each sample. Protein quantities were estimated in each technical replicate by the average of the most 3 intense peptides reported for each protein. Single hit proteins (a single peptide detected in a protein) and proteins detected in less than two injections in both samples A and B were discarded.



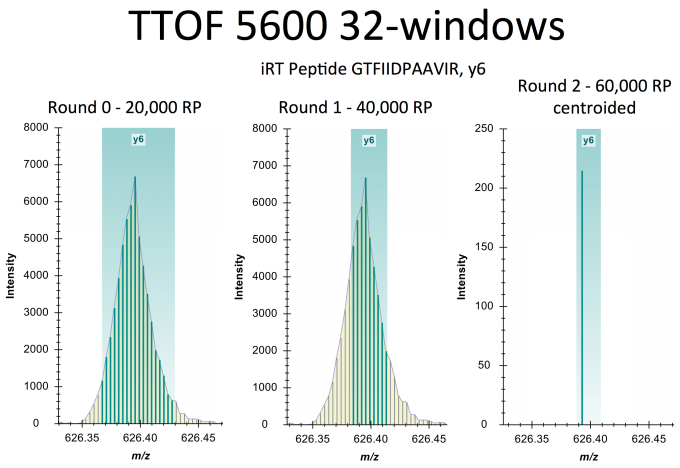
Supplementary Figure 23: LFQbench peptide and protein level benchmarks using Skyline´s user recommended values.

Supplementary Figure 24. Isolation ranges used in Skyline.

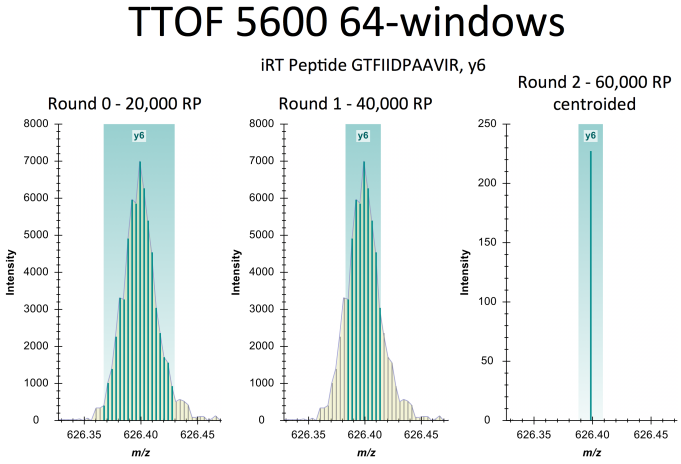
Isolation ranges used in Skyline (shaded in blue) shown over the extracted ion chromatogram of the y6 (z = 1) fragment ion of the peptide GTFIIDPAAVIR (choosen as an example).

The following pages show:

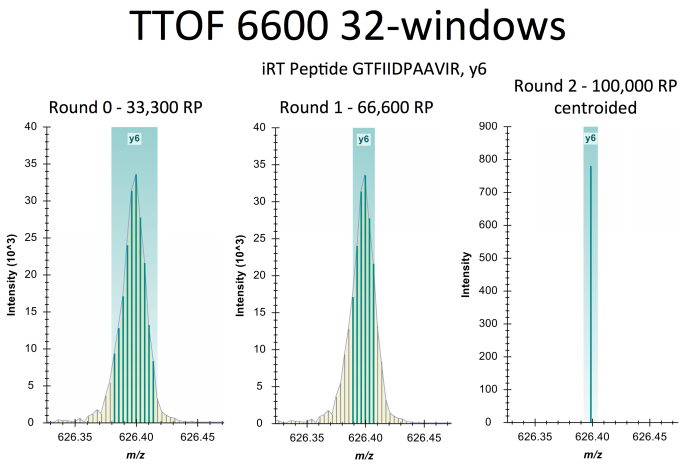
- Supplementary Figure 24.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 24.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 24.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 24.D: HYE 124, TripleTOF 6600, 64var setup



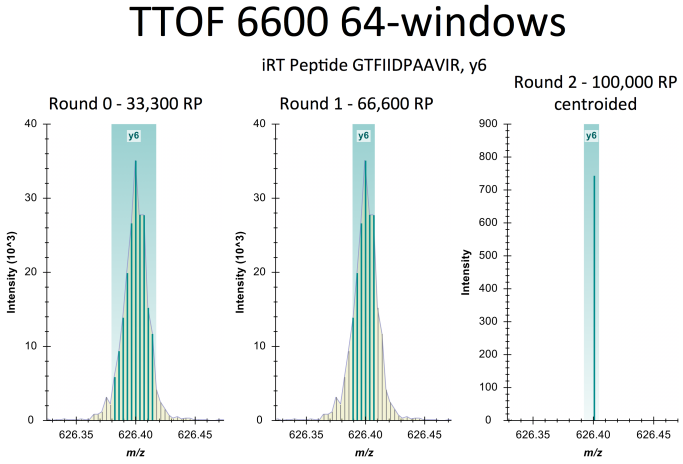
Supplementary Figure 24.A: Isolation ranges used in Skyline for HYE 124, TripleTOF 5600, 32fix setup.



Supplementary Figure 24.B: Isolation ranges used in Skyline for HYE 124, TripleTOF 5600, 64var setup.



Supplementary Figure 24.C: Isolation ranges used in Skyline for HYE 124, TripleTOF 6600, 32fix setup.



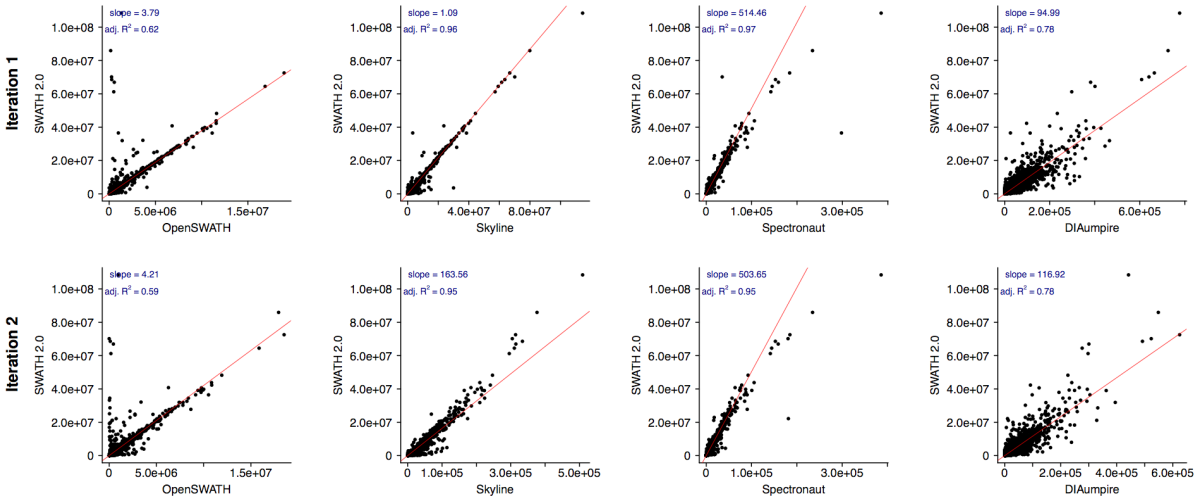
Supplementary Figure 24.D: Isolation ranges used in Skyline for HYE 124, TripleTOF 6600, 64var setup.

Supplementary Figure 25. Linear models of intensity scales (CIS).

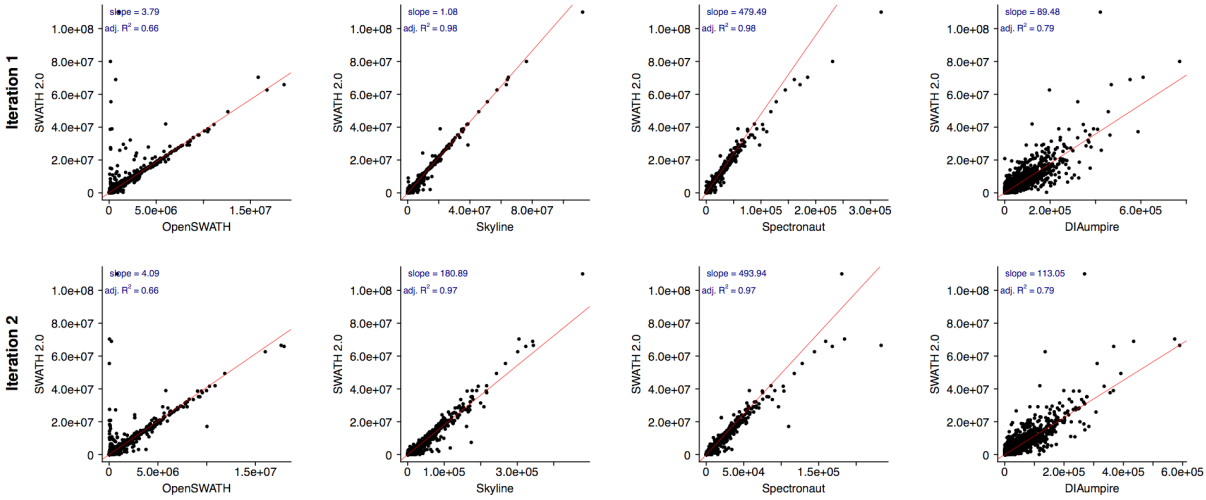
Intensity values reported by each software tool were scaled to an intensity reference (SWATH 2.0 reports were used as reference). For each software tool peptide report, precursor intensities were paired with precursor intensities of the reference, and the 98th lowest percentile of the paired intensities was used to calculate a linear model (origin was set to 0) to estimate the intensity factor scale. This factor scale was then used to scale both peptide and protein reports of each evaluated software tool.

The following pages show linear models of intensity scales for:

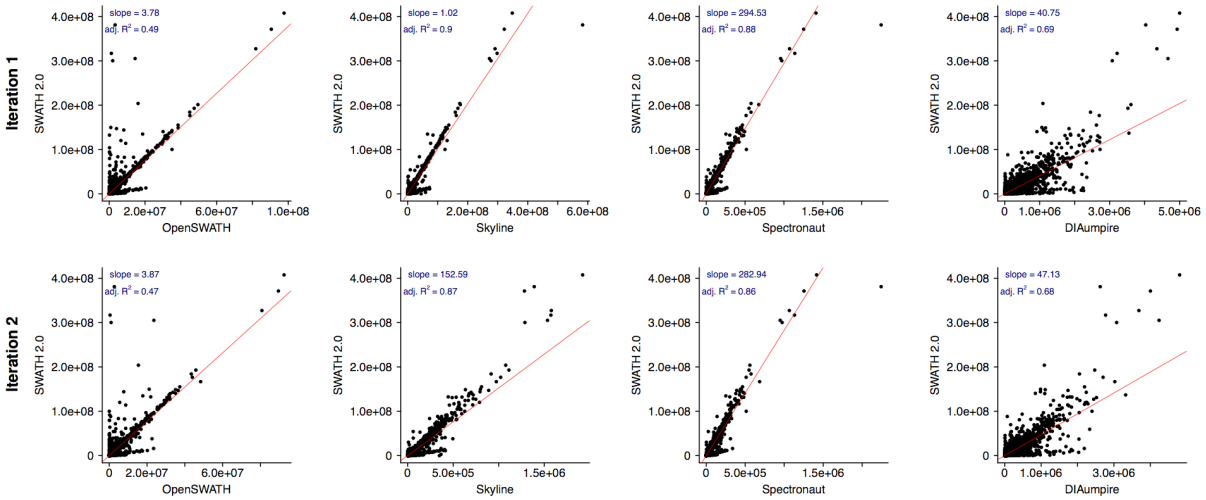
- Supplementary Figure 25.A: HYE 124, TripleTOF 5600, 32fix setup
- Supplementary Figure 25.B: HYE 124, TripleTOF 5600, 64var setup
- Supplementary Figure 25.C: HYE 124, TripleTOF 6600, 32fix setup
- Supplementary Figure 25.D: HYE 124, TripleTOF 6600, 64var setup
- Supplementary Figure 25.E: HYE 110, TripleTOF 6600, 32fix setup
- Supplementary Figure 25.F: HYE 110, TripleTOF 6600, 32var setup
- Supplementary Figure 25.G: HYE 110, TripleTOF 6600, 64fix setup
- Supplementary Figure 25.H: HYE 110, TripleTOF 6600, 64var setup



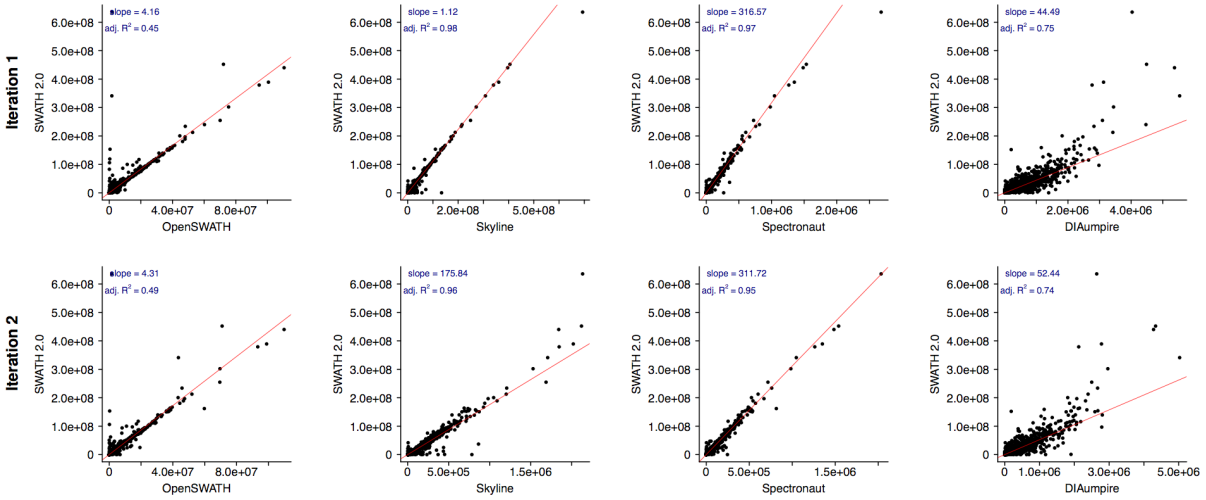
Supplementary Figure 25.A: Linear models of intensity scales for HYE 124, TripleTOF 5600, 32fix setup.



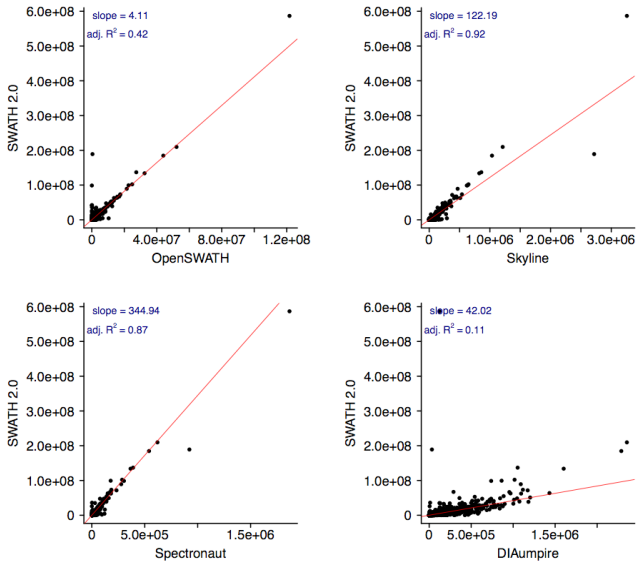
Supplementary Figure 25.B: Linear models of intensity scales for HYE 124, TripleTOF 5600, 64var setup.



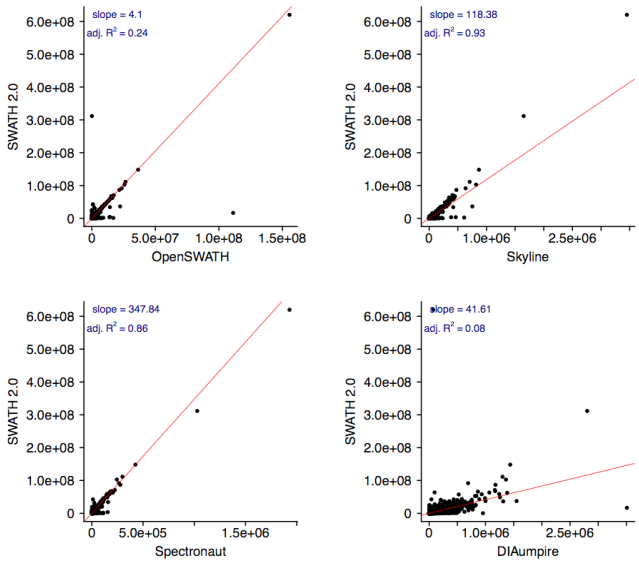
Supplementary Figure 25.C: Linear models of intensity scales for HYE 124, TripleTOF 6600, 32fix setup.



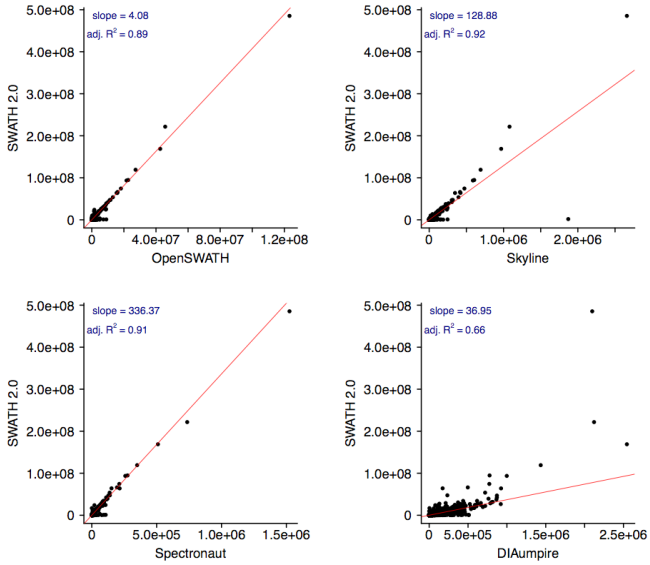
Supplementary Figure 25.D: Linear models of intensity scales for HYE 124, TripleTOF 6600, 64var setup.



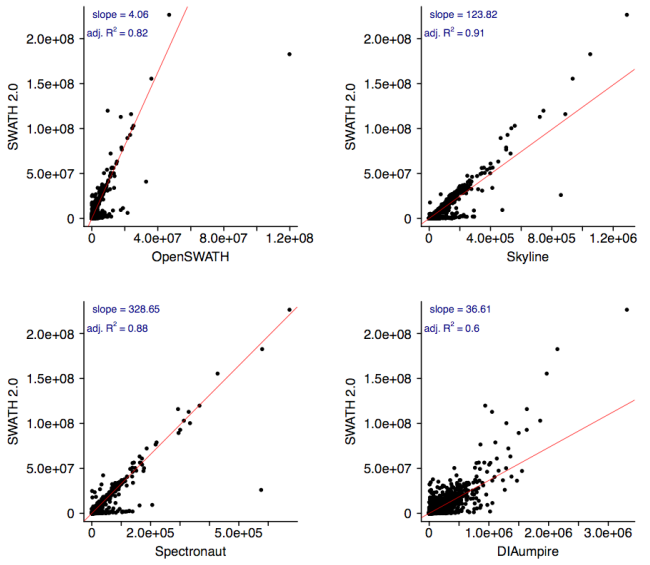
Supplementary Figure 25.E: Linear models of intensity scales for HYE 110, TripleTOF 6600, 32fix setup.



Supplementary Figure 25.F: Linear models of intensity scales for HYE 110, TripleTOF 6600, 32var setup.



Supplementary Figure 25.G: Linear models of intensity scales for HYE 110, TripleTOF 6600, 64fix setup.



Supplementary Figure 25.H: Linear models of intensity scales for HYE 110, TripleTOF 6600, 64var setup.

Step-by-Step Analyses

1. OpenSWATH

This step-by-step describes how to analyse a dataset of SWATH files with OpenSWATH. It exemplarises how to process one dataset containing 6 files with generic names (filename_001, filename_002,..., filename_006). For a more detailed information about the different parameters of OpenSWATH, the user may be interested on reading an article posted in bioRxiv: http://dx.doi.org/10.1101/044552

1.1 Convert the provided csv file to TraML format for OpenSWATH

```
ConvertTSVToTraML \
    -in ecolihumanyeast_concat_mayu_IRR_cons_openswath_32sw-var_curated.csv \
    -out ecolihumanyeast_concat_mayu_IRR_cons_openswath_32sw-var_curated.TraML
```

1.2 Append decoy assays to the TraML file for OpenSWATH

```
OpenSwathDecoyGenerator \
    -in ecolihumanyeast_concat_mayu_IRR_cons_openswath_32sw-var_curated.TraML \
    -out ecolihumanyeast_concat_mayu_IRR_cons_openswath_32sw-var_curated_decoy.TraML \
    -method pseudo-reverse \
    -append \
    -exclude_similar
```

1.3 Execute OpenSWATH workflow (iRT re-alignment, fragment ion chromatogram extraction, scoring)

```
OpenSwathWorkflow \
    -in /PATH/filename_001.mzXML.gz \
    -tr /PATH/ecolihumanyeast_concat_mayu_IRR_cons_openswath_32sw-var_curated_decoy.TraML.csv \
    -out_tsv /PATH/filename_001.tsv \
    -tempDirectory /PATH/ \
    -readOptions cacheWorkingInMemory \
    -batchSize 1000  \
    -Scoring:TransitionGroupPicker:background_subtraction original \
    -Scoring:stop_report_after_feature -1 \
    -min_upper_edge_dist 1 \
    -tr_irt /PATH/hroest_DIA_iRT.TraML \
    -extra_rt_extraction_window 100 \
    -min_rsq 0.95 \
    -min_coverage 0.6 \
    -Scoring:Scores:use_dia_scores true \
    -rt_extraction_window 600 \
    -mz_extraction_window 30 \
    -threads 4 \
    -ppm
```

(Repeat this step for every file of the dataset – in this example, 6 files)

1.4 Prepare the scores to be used for mProphet

```
mProphetScoreSelector.sh \
    /PATH/filename_001.tsv \
    xx_swath_prelim_score \
    bseries_score \
    intensity_score \
    isotope_correlation_score \
    isotope_overlap_score \
    library_corr \
    library_rmsd \
```

```
    log_sn_score \
    massdev_score \
    massdev_score_weighted \
    norm_rt_score \
    xcorr_coelution \
    xcorr_coelution_weighted \
    xcorr_shape \
    xcorr_shape_weighted \
    yseries_score
```

(Repeat this step for every file of the dataset – in this example, 6 files)

1.5 Run the python implementation of mProphet

```
pyprophet \
    --ignore.invalid_score_columns \
    --target.dir=/PATH/PyProphet/  \
    --xeval.num_iter=10 \
    --d_score.cutoff=1 \
    /PATH/OpenSwathWorkflow/filename_001.tsv
```

(Repeat this step for every file of the dataset – in this example, 6 files)

1.6 Run the cross-run alignment procedure

```
feature_alignment.py \
    --file_format openswath \
    --in \
        /PATH/filename_001_with_dscore_filtered.csv \
        /PATH/filename_002_with_dscore_filtered.csv \
        /PATH/filename_003_with_dscore_filtered.csv \
        /PATH/filename_004_with_dscore_filtered.csv \
        /PATH/filename_005_with_dscore_filtered.csv \
        /PATH/filename_006_with_dscore_filtered.csv \
    --out /PATH/feature_alignment.tsv \
    --out_meta /PATH/feature_alignment.yaml \
    --tmpdir /PATH.tmpdir/ \
    --mst:useRTCorrection False \
    --method global_best_overall \
    --max_rt_diff auto_3medianstdev \
    --frac_selected 0 \
    --target_fdr 0.01 \
    --realign_method lowess_cython \
    --disable_isotopic_grouping
```

2. SWATH 2.0

This step-by-step manual describes how to analyse SWATH-MS data with the SWATH 2.0 micro-app integrated in the software PeakView. SWATH 2.0 supports several input format files for the libraries; this step-by-step describes only the workflow used in this manuscript.

2.1 Library preparation

- Prepare an ion library in a plain text file, formatted as tab separated values. Each ion fragment of the library must be reported in a different row.

The following column values are necessary:

Column name	value
Q1	Precursor m/z
Q3	Fragment m/z
RT_detected	retention time in which peptide was detected in library generation
protein_name	any protein identificator
isotype	light or heavy (may be left in blank)
relative_intensity	normalized intensity of the fragment
stripped_sequence	peptide sequence without modifications
modification_sequence	peptide sequence including modifications in ProteinPilot format.
prec_z	charge state of the precursor ion
frg_type	fragment ion type (y, b, ...)
frg_z	charge state of the fragment ion
frg_nr	number of amino acids of the fragment ion sequence
iRT	iRT values
uniprot_id	UniProt ID
decoy	is decoy? TRUE/FALSE
N	peptide ranking. Set all to 1
shared	Peptides shared in several proteins. Set to FALSE to use all peptides (LFQbench filter them)

- Retention time calibration peptides must be labeled as [RT-Cal protein] at the *protein_name*

2.2 Analysis in SWATH 2.0 micro-app

- Open the program *PeakView*.
- Choose: Quantitation –> SWATH Processing –> Import Ion Library
  - Browse for the ion library (remember to select at the bottom the filter for _Ion Library Text Files (*.txt)_)
  - *Import settings*: untick the option *Do not import shared peptides*. For label-free quantification, leave *Sample Type* as *Unlabeled*.
  - *Select Samples*: choose the directory containing .wiff files at the *Source*

    * You can drag and drop the samples you want to analyse from the *Available* frame to the *Selected* frame. You may also drag and drop file folders.

- At the main panel, there are four sub-panels, from top to bottom and from left to right: Proteins in library, Peptides in protein, composed extracted ion chromatograms of the fragment ions of the selected peptide, composed MS2 scan of the best peak selected by the algorithm (top, in blue), and relative intensity and m/z of the library fragment ions of the selected peptide.

- Click on *Processing Settings* at the top of the *Proteins in library* panel. Use the following parameters:

- Peptide filter

Parameter	value
Number of Peptides per Protein	2000
Number of Transitions per Peptide	6
Peptide Confidence Threshold % (0-99)	99
False Discovery Rate Threshold % (0-100)	1.0
Exclude Modified Peptides	unticked
Exclude Shared Peptides	unticked
Fix Rank	unticked

- XIC Options

Parameter	value
XIC Extraction Window (min)	10.0
XIC width (ppm)	50 for TripleTOF 5600, 30 for TripleTOF 6600
XIC width (Da)	unticked

- Once you have configured processing settings, click the *Process* button at the right side of the *Processing Settings* button.
  - choose a name for saving the SWATH 2.0 session at the displayed dialog.
- **Report results**: when SWATH 2.0 finishes to process, export results by choosing at the main menu: Quantitation –> Export –> All
  - choose a name for saving the report at the displayed dialog.

3. Skyline

There are many Skyline tutorials available at the Skyline's tutorial web page. This short step-by-step tutorial deals exclusively with the kind of DIA (SWATH-MS) analysis performed in this article, and it exemplarises how to analyse with Skyline a dataset acquired in a TripleTOF 6600 with the settings proposed for the second iteration.

The whole analysis is summarised in 9 steps:

1. Create a new blank document
2. Import transitions list
   - File –> Import –> Transition List... (select Skyline designed transition list: both openswath and peakview formats work fine)
3. Configure settings
   - Settings are descripbed at the Supplementary Information Table 6. For convinience, they are also described at the end of this document.
4. Add decoys
   - Edit –> Refine –> Add Decoy peptides... –> Decoy generation method: Reverse sequence
5. Integrate All (for quantification)
   - Settings –> Integrate All
6. Remove precursors with less than 3 transitions
   - Edit –> Refine –> Advanced –> Min transitions per precursor: 3
7. Import wiff files into the Skyline document: File –> Import –> Results... –> Add single-injection replicates in files (Optimizing: None)
   - After selecting wiff files: do not remove common prefix.
8. Use mProphet model
   - Edit –> Refine –> Reintegrate (here: train the model and use all enabled features). Apply to all precursors, and tick the "report Q-value" and "Overwrite manual integration" options.
9. Save a report file
   - File –> Export –> Report... (use the designed report: SWATHbenchmark_long.skr)

3.1 Settings

Document Settings

- check "annotation_QValue"

Transition Settings

Full-Scan

MS1 filtering

- Isotope peaks included: None

MS/MS filtering

- Acquisition method: DIA
- Product mass analyzer: TOF
- Resolving power: 100,000
- Isolation scheme:
  - manually designed: use the mass ranges defined in Supplementary Table 8.

Retention time filtering

- Use only scans within "10" minutes of predicted RT.

Instrument

- min m/z : 50 m/z
- max m/z : 2000 m/z
- Method match tolerance m/z: 0.01 m/z
- Other parameters: left in blank or not ticked

Library

- Ion match tolerance: 0.1 m/z
- If a library spectrum is available, pick its most intense ions
- Pick: 6 product ions
  - from filtered ion charges and types

Filter

- Precursor charges: 2, 3
- Ion charges: 1, 2
- Ion types: y, b
- Product ions: from ion "3" to "last ion -1"
  - Special ions: none checked
  - Use DIA precursor window for exclusion: checked
- Auto-select all matching transitions: checked

Prediction

These parameters are less important in our case, since we are not doing using any transition prediction (we use a DDA library).

- Precursor mass: Monoisotopic
- Product ion mass: Monoisotopic
- Collision energy: ABI 5500 QTrap
- Declustering potential: None

- Optimization library: None

Peptide Settings

Digestion

- Enzyme: Trypsin [KR|P]
- Max missed cleavages: 0

- Background proteome: None

Prediction

- Retention time predictor:
  - Use the following standard peptides and iRT values:

Modified Sequence	iRT Value
ADVTPADFSEWSK	54.97
DGLDAASYYAPVR	43.28
GAGSSEPVTGLDAK	0.23
GTFIIDPAAVIR	86.72
GTFIIDPGGVIR	71.38
LFLQFGAQGSPFLK	98.09
LGGNEQVTR	-28.31
TPVISGGPYEYR	29.00
TPVITGAPYEYR	33.63
VEATFGVDESNAK	13.11
YILAGVENSK	22.38

- Measured peptides:
  - Add... -> A named spectral library : (select the spectral library you have previously generated)
- Time window: 3 min
- Drift time predictor: None

Filter

- Min length: 7
- Max length: 36
- Exclude N-terminal AAs: 36
- Auto-select all matching peptides: checked

Library

- built from corresponding file

- Pick peptides matching: Library

Modifications

- Structural modifications:
  - Carbamidomethyl (C) *Fixed*
  - Oxidation (M) *Variable*
- Max variable mods: 3
- Max neutral losses: 1
- Isotope label type: heavy
- Isotope modifications:
  - Label: 13C(6)15N(2)(C-term K)
  - Label: 13C(6)15N(4)(C-term R)
- Internal standard type: heavy

4. Spectronaut

A complete manual of Spectronaut is available on-line at the Bignosys web page:.

This step-by-step manual is focused exclusively on how to analyse SWATH-MS data with Spectronaut when you have already a library (in text format), and how to get a report for LFQbench.

4.1 Convert .wiff files to Spectronaut's raw format (.htrms)

Spectronaut can read .wiff files directly, but in order to reduce the analysis time, they may be converted to a Spectronaut's internal format (.htrms). This is strongly recommended if you need to perform several analyses over the same dataset.

1. Organise all .wiff files you want to convert into a single folder (Example: \wiff_files).
2. Start the program *"Wiff to HTRMS Converter"*.
3. Select the tab *Auto Conversion*.
4. Select as *Input Directory* the folder containing the .wiff files (\wiff_files).
5. Select as *Output Directory* any folder of your choice (Example: \htrms_files).
6. Press the button at the bottom right corner: *Convert*

4.2 Analysis in Spectronaut

Once all .wiff files are converted to .htrms, you can start the analysis in Spectronaut.

1. Start the main program *"Spectronaut"*.
2. Select the tab: *Review*.
3. Press the link: *Load Raw Data...*
   - Select the folder containing the .htrms files (\htrms_files), and choose all files you want to analyse.
   - If Spectronaut detects a common prefix at the file names, it will display an option to remove this prefix. Do not remove the prefix if you want to analyse files with LFQbench (otherwise you need to take note of the new file names).
   - Write an Experiment name (Example: Spectronaut_HYE124_TTOF6600_64var).

Configure the spectral library

- Select the tab *From File*
  - Browse your spectral library. You may use any spectral library formatted in plain text. OpenSWATH's and PeakView's (SWATH2.0's) formats work fine.
    * If this is the first time you use a spectral library type (i.e. OpenSWATH format), you need to relate column headers to Spectronaut's internal values. Spectronaut has a dictionary for most of these headers, but some of them may be missing. As an example, the following table contains the relationships between OpenSWATH's headers and Spectronaut's internal variables:

OpenSWATH header	Spectronaut variable
Q1	PrecursorMz

OpenSWATH header	Spectronaut variable
Q3	FragmentMz
iRT	iRT
relative_intensity	RelativeFragmentIntensity
stripped_sequence	StrippedSequence
prec_z	PrecursorCharge
frg_type	FragmentType
frg_nr	FragmentNumber
frg_z	FragmentCharge
protein_name	ProteinId
modification_sequence	ModifiedSequence
uniprot_id	UniProtId
isotype	IsotopicLabel
RT_detected	EmpiricalRT (not used if iRT variable is present)
confidence	blank (not registered)
decoy	blank (not registered)
N	blank (not registered)
shared	blank (not registered)

- After relating headers and internal variables, press the bottom right button *Load*.

Configure Conditions

- At the Experiment Setup: Raw Data, press the buttom *Configure Conditions...*
  - Write at the *Condition* column a condition name for each file.
    - Replicate numbers are automatically assigned for same conditions.
    - Labels also change with Conditions.
    - Choose a reference condition at the *IsReference* column. Usually, your control condition.
  - Press the button *Apply*.

Configure Analysis Settings

- At the Experiment setup, you may choose for different analysis schemas. If you didn't save yet any schema in Spectronaut, you have just the default option. You may save a set of parameters as an analysis schema at the *Settings* tab.
  - In this manuscript we use the following parameters for the analysis. You may configure them each time you run Spectronaut, or you may save a schema at the *Settings* tab.
- XIC Extraction:

Parameter	Value
XIC RT Extraction Window	Dynamic

- Calibration:

Parameter	Value
iRT Calibration Strategy	Non-linear iRT calibration

- Identification:

Parameter	Value
Pvalue Estimator	Normal distribution estimator
Qvalue Cutoff	0.01

- Quantitation:

Parameter	Value
Interference Correction	checked
Cross Run Normalization	checked

- Workflow:

Parameter	Value
Profiling Strategy	iRT Profiling
Profiling Row Selection	Minimum Qvalue Row Selection
Qvalue Threshold	0.01
Profiling Target Selection	Automatic Selection
Default Labeling Type Assumed	LABEL

- Protein Inference:

Parameter	Value
Protein Inference Workflow	Automatic

- Post Analysis:

Parameter	Value
Differential Abundance Testing	Student's t-test
Quantity	MS2 peak area
Data Filtering	Qvalue
Differential Abundance Grouping	By protein group
Smallest Quantitative Unit	Precursor ion (summed fragment ions)
Include MS1	checked

- Reporting:

If you have already loaded the SWATHbenchmark schema, tick it at the *Pipeline Report Schema*. Otherwise, you can load it after analysis at the *Report* tab.

Parameter	Value
Pipeline Reporting Unit	Experiment

Parameter	Value
Scoring Histograms	checked
Generate SNE File	checked

Report results

- Select the *Report* tab.
  - If you didn't load yet the SWATHbenchmark report schema, you may do it here at the Schemas section:
    - Press the *Import Schema* button at the bottom of the *Schemas* section.
    - Browse for the SWATHbenchmark report schema, and load it.
  - Select the SWATHbenchmark report schema at the *Schemas* section (in *Normal Report* subsection).
  - Press the button *Export Report...* at the bottom left.
    - Choose a name for the report, and save it.

5. DIA-Umpire

This document describes the steps to analyze a dataset of DIA files with the DIA-Umpire pipeline. We show the pipeline using a dataset with 6 files. In this example, injection files are generically named: injection_001, injection_002, ..., injection_006, and the database: mydatabase.fasta. Detailed information about the parameters for each of the used tools in the pipelines may be found on their respective websites.

5.1 Convert WIFF raw files

Convert WIFF raw files into mzXML format (centroid spectra)

- use the peakpicking option in msconvert to generate centroid spectra

```
msconvert.exe --mzXML --filter "peakPicking true 1-2"  ~/workdir/injection_001.wiff
msconvert.exe --mzXML --filter "peakPicking true 1-2"  ~/workdir/injection_002.wiff
msconvert.exe --mzXML --filter "peakPicking true 1-2"  ~/workdir/injection_003.wiff
msconvert.exe --mzXML --filter "peakPicking true 1-2"  ~/workdir/injection_004.wiff
msconvert.exe --mzXML --filter "peakPicking true 1-2"  ~/workdir/injection_005.wiff
msconvert.exe --mzXML --filter "peakPicking true 1-2"  ~/workdir/injection_006.wiff
```

Run DIA-Umpire Signal Extraction (SE) for each mzXML file to generate pseudo MS/MS spectra (MGF format)

```
java -jar -Xmx15G ~/DIA-Umpire/DIA_Umpire_SE.jar \
    ~/workdir/injection_001.mzXML ~/workdir/diaumpire_se.params
```

(execute the same command for the other 5 injections too)

Convert the resulting MGF files into mzXML format using msconvert.exe

```
msconvert.exe --mzXML ~/workdir/injection_001_Q1.mgf
msconvert.exe --mzXML ~/workdir/injection_001_Q2.mgf
msconvert.exe --mzXML ~/workdir/injection_001_Q3.mgf
```

(execute the same commands for the other 5 injections too)

5.2 Database searches

X! Tandem MS/MS database search

To run X! Tandem for each pseudo MS/MS spectra file, you will need to have an X! Tandem parameter file for each pseudo MS/MS mzXML file. The definitions of most of X! Tandem parameters are available at http://www.thegpm.org/TANDEM/api/index.html. For each pseudo MS/MS mzXML file, an X! Tandem parameter file is generated and the input spectrum files and output tandem output files are specified in the parameter file.

Run X! Tandem for each mzXML file

```
tandem ~/workdir/tandem_param_injection_001_Q1.xml
tandem ~/workdir/tandem_param_injection_001_Q2.xml
tandem ~/workdir/tandem_param_injection_001_Q3.xml
```

(execute the same commands for the other 5 injections too)

Convert tandem output files to pepXML files using Tandem2XML

```
tandem2XML ~/workdir/injection_001_Q1.tandem ~/workdir/injection_001_Q1.tandem.pep.xml
tandem2XML ~/workdir/injection_001_Q2.tandem ~/workdir/injection_001_Q2.tandem.pep.xml
tandem2XML ~/workdir/injection_001_Q3.tandem ~/workdir/injection_001_Q3.tandem.pep.xml
```

(execute the same commands for the other 5 injections too)

Execute xinteract (from TPP package) on each X! Tandem pepXML file

```
xinteract -OpdEAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q1.tandem.pep.xml \
        ~/workdir/injection_001_Q1.tandem.pep.xml
xinteract -OpdEAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q2.tandem.pep.xml \
        ~/workdir/injection_001_Q2.tandem.pep.xml
xinteract -OpdEAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q3.tandem.pep.xml \
        ~/workdir/injection_001_Q3.tandem.pep.xml
```

(execute the same commands for the other 5 injections too)

Comet MS/MS database search

To run Comet, you will need to have an Comet parameter file. The definitions Comet parameters are available at http://comet-ms.sourceforge.net/parameters/parameters_201601/

```
comet.2015025.linux.exe -Pcomet.params -N~/workdir/injection_001_Q1.comet \
        ~/workdir/injection_001_Q1.mzXML
comet.2015025.linux.exe -Pcomet.params -N~/workdir/injection_001_Q2.comet \
        ~/workdir/injection_001_Q2.mzXML
comet.2015025.linux.exe -Pcomet.params -N~/workdir/injection_001_Q3.comet \
        ~/workdir/injection_001_Q3.mzXML
```

(execute the same commands for the other 5 injections too)

Execute xinteract (from TPP package) for each Comet pepXML file:

```
xinteract -OpdAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q1.comet.pep.xml \
        ~/workdir/injection_001_Q1.comet.pep.xml
xinteract -OpdAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q2.comet.pep.xml \
        ~/workdir/injection_001_Q2.comet.pep.xml
xinteract -OpdAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q3.comet.pep.xml \
        ~/workdir/injection_001_Q3.comet.pep.xml
```

(execute the same commands for the other 5 injections too)

MS-GF+ search engine

The format of command for MS-GF+ search engine can be found at https://omics.pnl.gov/software/ms-gf

Run MS-GF+

```
java -Xmx3500m \
    -jar ~/MSGFPlus.20140716/MSGFPlus.jar \
    -s ~/workdir/injection_001_Q1.mzXML \
    -d ~/mydatabase.fasta \
    -o ~/workdir/injection_001_Q1.msgf.mzid
```

```
    \-t 30ppm \
    -thread 1 \
    -tda 0 \
    -m 0 \
    -ti 0,0 \
    -inst 2 \
    -e 1 \
    -mod ~/msgf.mod
java -Xmx3500m \
    -jar ~/MSGFPlus.20140716/MSGFPlus.jar \
    -s ~/workdir/injection_001_Q2.mzXML \
    -d ~/mydatabase.fasta \
    -o ~/workdir/injection_001_Q2.msgf.mzid \
    -t 30ppm \
    -thread 1 \
    -tda 0 \
    -m 0 \
    -ti 0,0 \
    -inst 2 \
    -e 1 \
    -mod ~/msgf.mod
java -Xmx3500m \
    -jar ~/MSGFPlus.20140716/MSGFPlus.jar \
    -s ~/workdir/injection_001_Q3.mzXML \
    -d ~/mydatabase.fasta \
    -o ~/workdir/injection_001_Q3.msgf.mzid \
    -t 30ppm \
    -thread 1 \
    -tda 0 \
    -m 0 \
    -ti 0,0 \
    -inst 2 \
    -e 1 \
    -mod ~/msgf.mod
```

(execute the same commands for the other 5 injections too)

Convert mzid files to pepXML using idconvert.exe

```
idconvert ~/workdir/injection_001_Q1.msgf.mzid --pepXML
idconvert ~/workdir/injection_001_Q2.msgf.mzid --pepXML
idconvert ~/workdir/injection_001_Q3.msgf.mzid --pepXML
```

(execute the same commands for the other 5 injections too)

Execute xinteract (from TPP package) for each MS-GF+ pepXML file:

```
xinteract -OpdEAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q1.msgf.pep.xml \
        ~/workdir/injection_001_Q1.pepXML
xinteract -OpdEAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q2.msgf.pep.xml \
        ~/workdir/injection_001_Q2.pepXML
xinteract -OpdEAP -PPM -p0.1 -dreverse -Ninteract-injection_001_Q3.msgf.pep.xml \
        ~/workdir/injection_001_Q3.pepXML
```

(execute the same commands for the other 5 injections too)

5.3 Perform iProphet analysis to combine the results of the three search engines

```
InterProphetParser NONSP ~/workdir/interact-injection_001_Q1.tandem.pep.xml \
        ~/workdir/interact-injection_001_Q1.comet.pep.xml \
        ~/workdir/interact-injection_001_Q1.msgf.pep.xml \
        ~/workdir/interact-injection_001_Q1.iproph.pep.xml
InterProphetParser NONSP ~/workdir/interact-injection_001_Q2.tandem.pep.xml \
        ~/workdir/interact-injection_001_Q2.comet.pep.xml \
        ~/workdir/interact-injection_001_Q2.msgf.pep.xml \
        ~/workdir/interact-injection_001_Q2.iproph.pep.xml
```

```
InterProphetParser NONSP ~/workdir/interact-injection_001_Q3.tandem.pep.xml \
        ~/workdir/interact-injection_001_Q3.comet.pep.xml \
        ~/workdir/interact-injection_001_Q3.msgf.pep.xml \
        ~/workdir/interact-injection_001_Q3.iproph.pep.xml
```

(execute the same commands for the other 5 injections too)

5.4 Perform ProteinProphet analysis

```
ProteinProphet IPROPHET MINPROB0.5 ~/workdir/*.iproph.pep.xml \
        ~/workdir/interact.iproph.prot.xml
```

5.5 Perform DIA-Umpire quantification

```
java -jar --Xmx20G ~/DIA-Umpire/DIA_Umpire_Quant.jar \
        ~/workdir/diaumpire_quant.params
```