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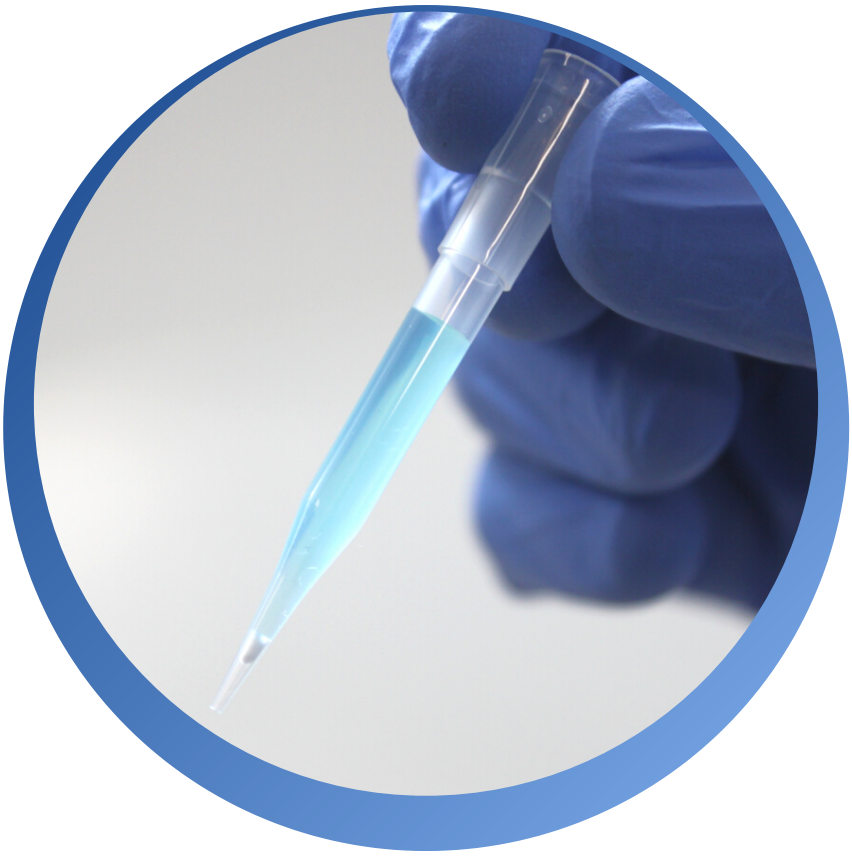
Application Note



Desalting & protein purification

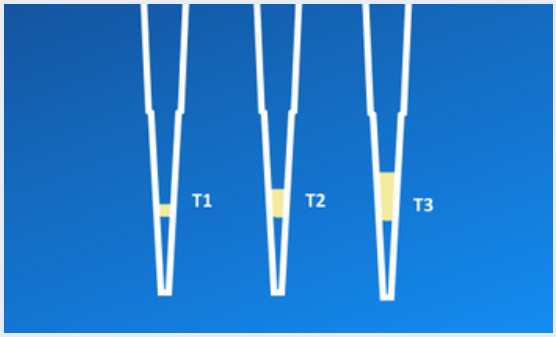
Identification of more than 97% proteins after purification by AttractSPE®Disks SPE Tips / Stage tips - C18

This work was carried out at Institut Curie Centre de Recherche, PSL Research University: Mass Spectrometry and Proteomics facility (LSMP), Paris (France) by Florent Dingli and Damarys Loew.

Introduction :

In this application note, several tips based on C18 were tested for the desalting of 100ng of HeLa digest. 5 replicates of each assay were carried out and peptide MS analysis were compared. The results were compared to a not-purified loading solution (assay 1).

AttractSPE®Disks Tips (AFFINISEP) are Stage Tips based on small particles densely packed and embedded in a soft membrane. These tips are widely used for protein and peptide purifications, fractionation/desalting, or drug analysis. They are available with several capacities T1 (for 15µg), T2 (for 30µg), T3 (for 45µg).



For this application note, 200µL AttractSPE®Disks Tips C18 were tested with capacities T1 (assay 2), T2 (assay 3). For assay 4, 2mg loose powder AttractSPE® C18 was added on top of AttractSPE®Disks Tips C18 – T1 as done by several proteomic labs (assay 4).

AttractSPE®Disks Tips are particularly suitable for centrifugation testings or positive pressure ones. In this application note, the general protocol is based on centrifugation (called general protocol).

These tips were compared to a major competitor product (C18 tips 100µL bed). Competitor tips were tested with the general protocol as for AttractSPE®Disks tips for assay 5 and with the protocol recommended by the supplier (assay 6). For this protocol, pipetting is used instead centrifugation.

Table 1 - brief description of assays

ASSAY NUMBER FOR ALL GRAPHS	TIPS	PROTOCOL
1	NO PURIFICATION	GENERAL PROTOCOL
2	ATTRACTSPE®DISKS TIPS C18 – T1 – 200ML	GENERAL PROTOCOL
3	ATTRACTSPE®DISKS TIPS C18 - T2 – 200ML	GENERAL PROTOCOL
4	ATTRACTSPE®DISKS TIPS C18 - T1– 200ML +	GENERAL PROTOCOL
5	2MG POWDER ATTRACTSPE® C18 COMPETITOR C18 TIPS 100ML BED	GENERAL PROTOCOL
6	COMPETITOR C18 TIPS 100ML BED	SUPPLIER PROTOCOL

Methods

Preparation of the loading solution of HeLa protein digest – 100ng

HeLa Digest was obtained from Fisher scientific (Cat : 88329). One vial of 20µg was resuspended in 1mL of a mixture of 1M Urea and 100mM Tris HCl pH 8.5 to give a solution of 20ng/µL. 5.5µL of this resuspended solution (110ng) and 100µl 0.1% FA) were loaded on each tip.

Protocols used for the tips

Abbreviation: ACN : Acetonitrile ; FA : Formic Acid

Table 2 – Description of general protocol for assay 1 to 5. All assays were carried out manually.

PROCESSING STEP	OPERATION	CENTRIFUGE – TIME AND SPEED
1 - CONDITIONING	100ML 70% ACN ; 0.1% FA	2MIN – 3000 RPM (0.8 RCF)
2 – EQUILIBRATION	100ML 0.1% FA	2MIN – 3000 RPM (0.8 RCF)
3 – LOADING OF THE SAMPLE	110NG HELA DIGEST IN 100ML 0.1% FA	4MIN – 2000 RPM (0.8 RCF)
4 - WASHING	100ML 0.1% FA	2MIN – 3000 RPM (0.8 RCF)
5 - ELUTION	100ML 40% ACN 0.1% FA	2MIN – 3000 RPM (0.8 RCF)
6 – EVAPORATION	SPEED VACUUM DRIED	
7 - RECONSTITUTION	SAMPLE RESUSPENDED IN 5.5ML (WITH IRT) AND 5ML (100NG) INJECTED IN LC-MS/MS	

Table 3 – Description of the competitor protocol for assay 6

PROCESSING STEP	OPERATION
1 - CONDITIONING	100ML 70% ACN ; 0.1% FA
2 – EQUILIBRATION	100ML 0.1% FA
3 – LOADING OF THE SAMPLE	110NG HELA DIGEST IN 100ML 0.1% FA
4 - WASHING	100ML 0.1% FA
5 - ELUTION	100ML 40% ACN 0.1% FA
6 – EVAPORATION	SPEED VACUUM DRIED
7 - RECONSTITUTION	SAMPLE RESUSPENDED IN 5.5ML (WITH IRT) AND 5ML (100NG) INJECTED IN LC-MS/MS

LC-MS/MS Method

Chromatography was performed with an RSLCnano system (Ultimate 3000) coupled online to an Orbitrap Exploris 480 mass spectrometer. Peptides were trapped on a C18 column (75 µm inner diameter × 2 cm; nanoViper Acclaim PepMapTM 100) with buffer A (2/98 MeCN/H2O in 0.1% formic acid) at a flow rate of 3.0 µL/min over 4 min. Separation was performed on a 50 cm x 75 µm C18 column (nanoViper Acclaim PepMapTM RSLC, 2 µm, 100Å) regulated to a temperature of 40°C with a linear gradient of 3% to 29% buffer B (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 91 min. MS full scans were performed in the ultrahigh-field Orbitrap mass analyzer in ranges m/z 375–1500 with a resolution of 120 000 at m/z 200. The top 20 most intense ions were isolated (isolation width of 1.6 m/z) and fragmented (collision energy: 30%) by higher energy collisional dissociation with a maximum injection time of 60 ms, normalized automatic gain control target set to 100%, and 15000 resolution. Charge state from 2+ to 6+ were selected, and dynamic exclusion was set to 40s.

Results and discussion

Data analysis and proteins identification were searched against the Homo sapiens (UP000005640) UniProt database using Sequest HT through Proteome Discoverer (version 2.4) and resulting files were further processes using myProMS (PMID: 17610305, Institut Curie homemade web server).

Peptides comparison

Figure 1 and table 4 show the identified peptides for each assay. In addition, table 4 shows the loss percentage of peptides in comparison to the reference sample (assay 1, not-purified samples). For all assays involving AttractSPE®Disks Tips, the number of identified peptides is very closed to the reference sample (> 32 000 peptides vs ~34 400 for reference).

Assays were carried out with 100ng which is a low concentration. This represents the ideal condition to observe the loss of peptides due to unwanted retention on the sorbent during elution. Indeed, the lower the concentration of peptides, the more visible is the loss due to the sorbent. It is worth noting that the 3 assays with AttractSPE®Disks Tips show similar loss percentage (between 5.1 and 6.1) independently of the quantity of sorbent used. Indeed T1, T2 (twice the capacity of T1) and T1 + loose powder involves different quantities of sorbent. This difference does not impact the loss percentage. That means that main loss observed is not due to the retention on the sorbent but to the overall assays. The loss percentage observed for AttractSPE®Disks Tips is more than acceptable on manual assays.

Competitor tips with the general protocol and with supplier protocol show a loss percentage higher than 22%. Difference between both protocols does not affect this result. In spite of the low concentration of peptides, the loss is very high.

Figure 1 – number of identified peptides - Peptide spectrum matches (PSM)

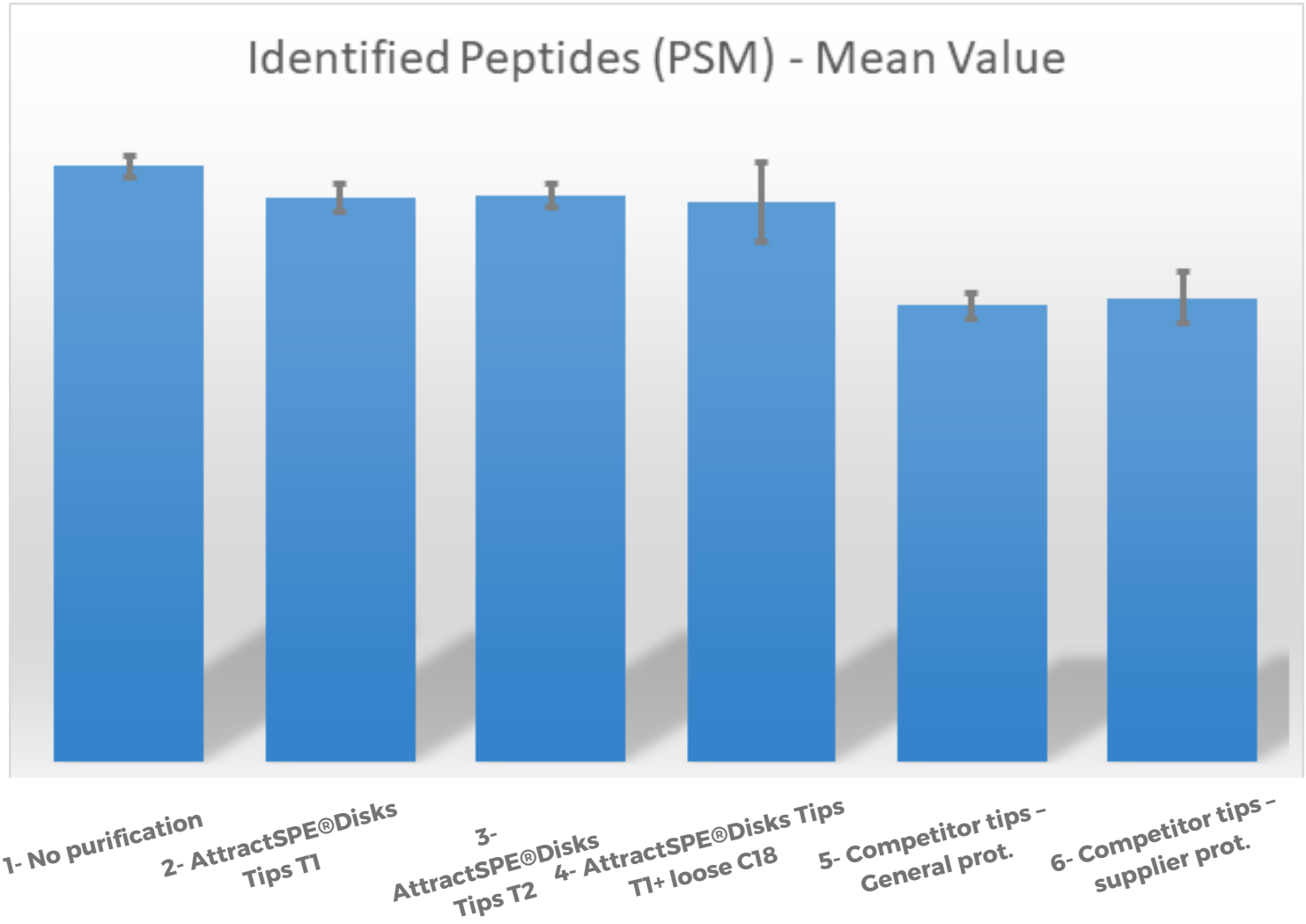


Table 4 – number of identified peptides and loss percentage versus the not-purified sample

ASSAY	IDENTIFIED PEPTIDES (PSM) – MEAN VALUES	LOSS PERCENTAGE (ON MEAN VALUES)
1-NO PURIFICATION	34398	0
2-ATTRACTSPE®DISKS TIPS C18 – T1 – 200ML	32538	5.4
3-ATTRACTSPE®DISKS TIPS C18 - T2 – 200ML	32654	5.1
4-ATTRACTSPE®DISKS TIPS C18 - T1+ LOOSE C18	32300	6.1
5-COMPETITOR C18 TIPS – GENERAL PROTOCOL	26317	23.5
6-COMPETITOR C18 TIPS – SUPPLIER PROTOCOL	26787	22.1

Protein comparison

Figure 2 and table 5 show the identified proteins for each assay. In addition, table 5 shows the loss percentage of proteins in comparison to the reference sample (assay 1, not-purified samples). For all assays involving AttractSPE®Disks Tips, the number of identified proteins is very closed to the reference sample (> 4 000 proteins vs ~4 200 for reference). Less than 3% of proteins were not identified which is a very good performance.

Competitor tips with the general protocol and with supplier protocol show a loss percentage up to 12%. Difference between both protocols does not affect this result. In spite of the low concentration of peptides, the loss remains high.

Figure 2 – number of identified proteins

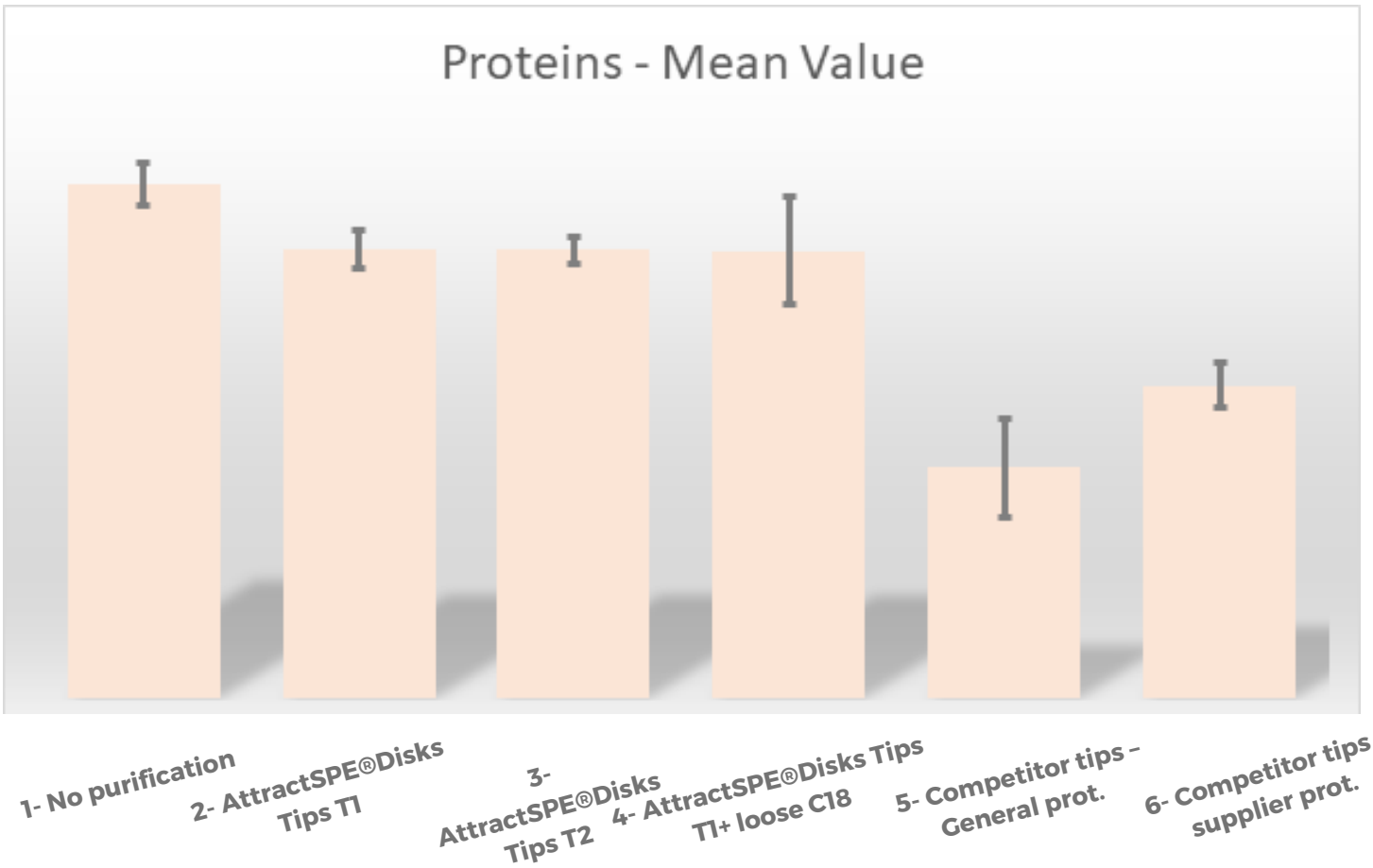


Table 5 – number of identified proteins and loss percentage versus the not-purified sample

ASSAY	IDENTIFIED PEPTIDES - MEAN VALUES	LOSS PERCENTAGE (ON MEAN VALUES)
1-NO PURIFICATION	4205	0
2-ATTRACTSPE®DISKS TIPS C18 – T1 – 200ML	4091	2.7
3-ATTRACTSPE®DISKS TIPS C18 - T2 – 200ML	4089	2.8
4-ATTRACTSPE®DISKS TIPS C18 - T1+ LOOSE C18	4087	2.8
5-COMPETITOR C18 TIPS – GENERAL PROTOCOL	3707	11.9
6-COMPETITOR C18 TIPS – SUPPLIER PROTOCOL	3851	8.4

Analysis of the results by using Proteome Discoverer (Thermo's Software version 2.4)

Figure 3 and figure 4 show respectively the cumulative count of PSM and the count according to the retention time

The retention time is bound to the hydrophicity of the peptides. The shorter, the more hydrophilic the peptides are. The analysis of both figures indicates that as expected according to the PSM, the whole peptides were homogeneously retained on the AttractSPE®Disks Tips independently of the hydrophilicity.
On the same way, for Competitor tips, the loss of peptides on the whole range is observed.

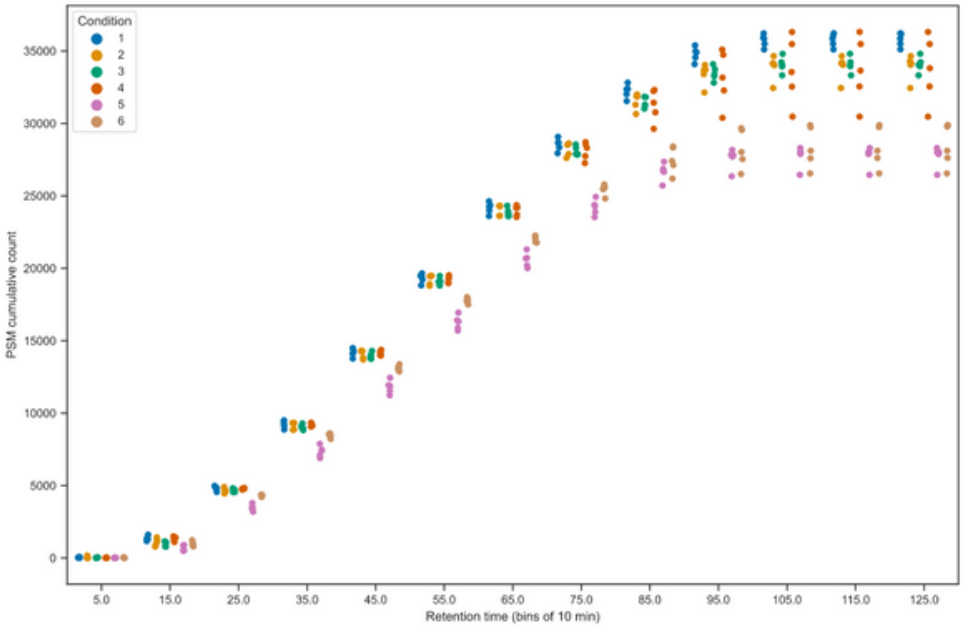


Figure 3 - PSMs according to the Retention Time (RT) - PSM Cumulative count (assays numbers described in table 1)

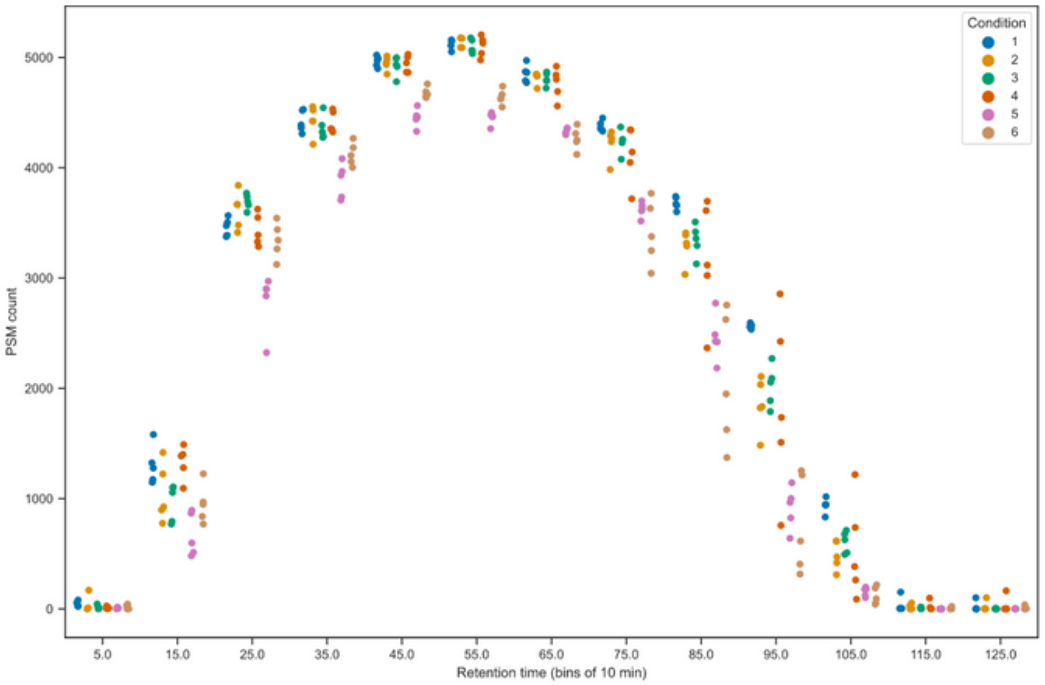


Figure 4 - PSMs according to the Retention Time (RT) - PSM count (Assays numbers described in table 1)

Figure 5 shows the summed peptides group abundances for each tip. Low variation is observed for each 5 replicates showing an excellent repeatability of each experiment and expressing a good reliability of the obtained results.

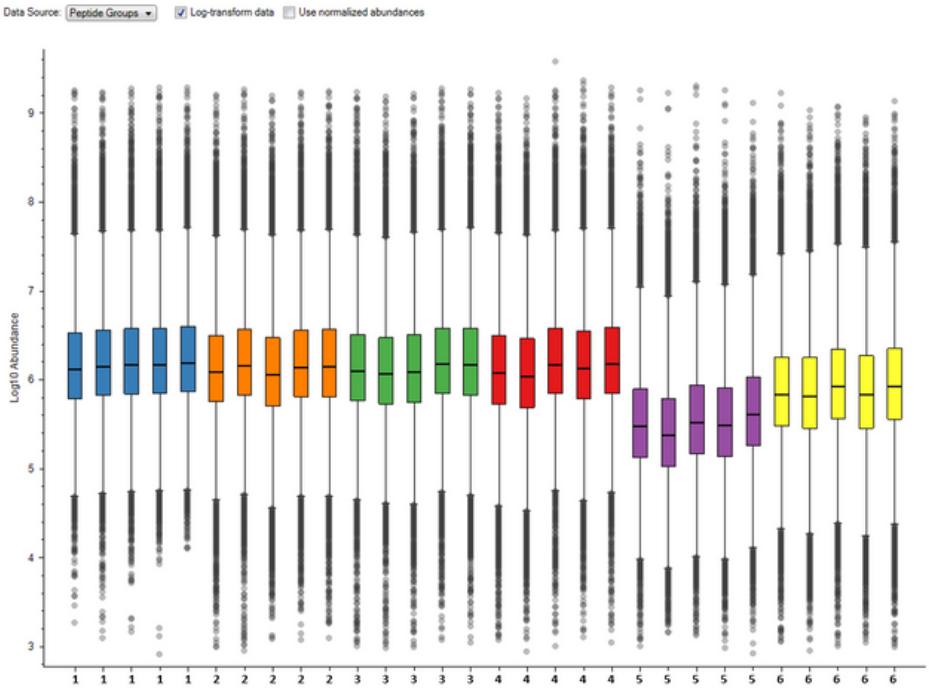


Figure 5 - Peptides group abundances – Total signal (Assays number described in table 1)

Conclusion

This application note compares the desalting performances of several tips with 100ng of HeLa proteins digest. Several Stage Tips AttractSPE®Disks Tips C18 with different capacities T1 (15µg), T2 (30µg) and T1+loose C18 were compared to the reference product (not-purified sample), as well as with competitor Tips C18. Experiments were carried out by centrifugation except for one assay using pipetting for the competitor tips as described on their protocol.

The amount of peptides to be treated is 100ng, the ideal amount to estimate accurately the unwanted retention of peptides on the sorbent.
Analyses of identified peptides show a minimum loss (around 5%) for all AttractSPE®Disks Tips C18 independently of their capacity. The comparison between T1 and T2 shows that by increasing the amount of C18 and so the capacity of tips, the percentages of identified peptides and proteins are similar. Thisshows that peptides loss is not affected by the sorbent and no unwanted retention is observed.

Assays involving competitor tips indicate a loss of peptides higher than 20% for 100ng of peptides. This was observed for the tips having similar protocols than AttractSPE®Disks Tips based on centrifugation as well as for the protocol by pipetting recommended for the competitor. A loss of peptides was observed for the whole range of the retention time analyzed.

With all these AttractSPE®Disks Tips C18, more than 97% of proteins were identified on a reliable way. Very low variability is observed for intra and inter AttractSPE®Disks tips assays.

To conclude, 3 different combinations of AttractSPE®Disks Tips C18 were evaluated for the desalting of peptides. They all performed on an excellent way with a minimum loss of peptides and an identification of 97% of proteins.

Peptides for the full range of RT were efficiently purified. In addition, these tips have a very high capacities making them suitable for very challenging peptides purifications.

Products used in this application note:

AttractSPE®Tips - C18, 200µL, 96/pk

- Tips-C18.T1.200.96
- Tips-C18.T2.200.96

AttractSPE® C18 bulk powder for biological application

- C18-Bio-BP

Related Products

AttractSPE®Disks Spin - C18 – 96/pk

- µSpin-C18.T1.96
- Spin-C18.T1.96

AttractSPE®Disks 96 plate - C18 – 1mL – 1 unit

- 96W-C18.T1.1

AttractSPE®Disks 96 plate - C18 for microelution – 1 unit

- µ96W-C18.T1.1

AttractSPE®Tips SDB – RPS – 200µL, 96/pk

- Tips-RPS-M.T1.200.96