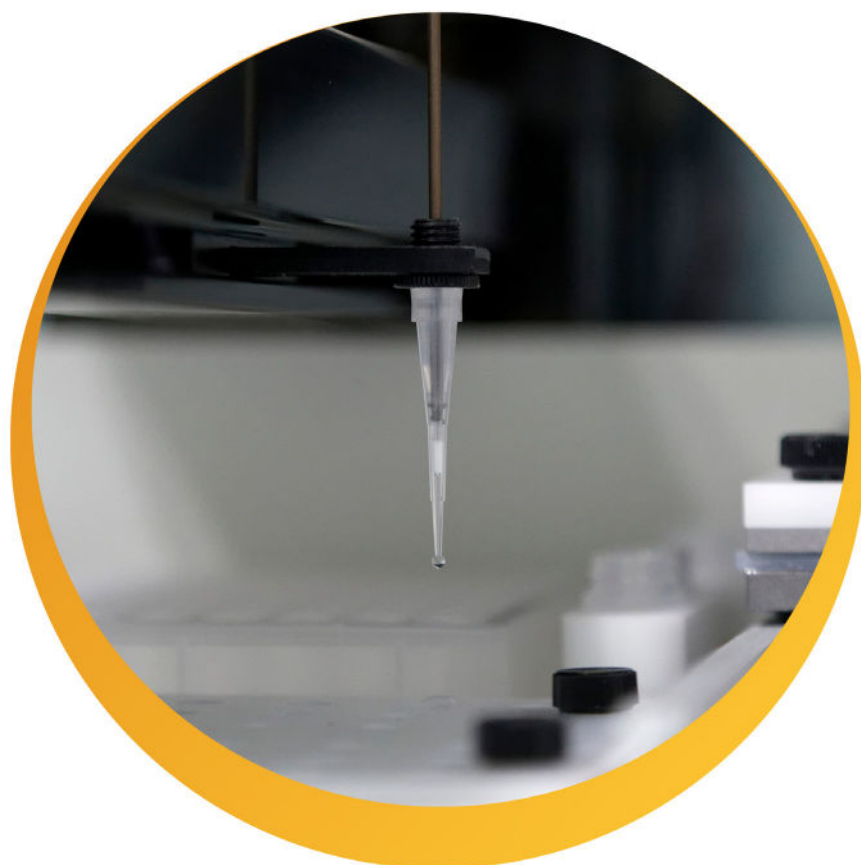


# Application Note



**Development and optimization of a method for automated peptide desalting on the DigestPro MSi robot using**

**AttractSPE® Disks Tips C18**

This work was carried out at Sorbonne Université, INSERM, UMS PASS, Plateforme Post-génomique de la Pitié Salpêtrière (P3S), Paris (France) by Soumia Hamada, Célia Jardin, Cédric Pionneau, Viviane de Almeida Bastos and Solenne Chardonnet, and in collaboration with Martin Technau from CEM GmbH (Kamp-Lintfort, Germany).

Peptide desalting is a crucial step of sample preparation to perform bottom-up approach in proteomics analyses by LC-MS/MS. However, salts in the digestion buffer can generate ion suppression effects that limit the detection of peptides and greatly impact protein identification.

In this application note, we describe the creation and optimization of an automated method for peptide desalting on the DigestPro MSi robot (CEM). Throughout this study, AttractSPE® Disks Tips C18, packed with a membrane made of small sorbent particles tightly embedded in a monolithic disk to combine high capacity and small dead volume, were evaluated and compared to homemade stagetips (made with Empore™ C18 SPE disks). Two different amounts of starting material were used (100ng and 2µg) to evaluate the performance of the desalting process with middle and low amounts of protein digest.

AttractSPE® Disks Tips are packed using fully automated and highly repeatable methods, thus preventing the tip-to-tip variability often observed for homemade stagetips. Plus, AttractSPE® Disks Tips are available with different binding capacities (referred as T0, T1, T2 and T3), ranging from a few ng and up to 100µg, to perfectly adapt to the quantity of peptides to be purified.

# 1 Methods

Sample name	Content	Source	Quantity injected	LC gradient	Acquisition mode
Low amount	100ng HeLa digest	Thermo Fisher Scientific, ref 88329	10ng	30min 5-22% B	DDA-PASEF
Middle amount	2µg rat brain digest	Prepared at P3S core facility: reduction, alkylation and tryptic digestion	200ng	70min 2-30% B	DDA-PASEF
High amount	10µg mouse serum digest	Prepared at P3S core facility: reduction, alkylation and tryptic digestion	100ng	30min 5-25% B	DDA-PASEF

Samples were desalted on the DigestPro MSi robot (CEM former Intavis) using non-skirted 96-well plates for samples and eluates, glass vials for the solutions, and a specific needle\* for SPE Tips desalting. The SPE Tips were either 10µL AttractSPE® Disks Tips C18 provided from Affinisep (AFF) or 10µL homemade stagetips packed with 1 or 2 layers of Empore™ C18 SPE disks (HM-T1 or HM-T2). The desalted peptides were dried out in a vacuum centrifuge and resuspended in 20 µL of acetonitrile 2% / formic acid 0.1%. LC-MS/MS analysis was performed on a nanoElite – timsTOF Pro system (Bruker) with a 25 cm Aurora2 column (75 µm i.d., 120 Å, 1.6 µm) from IonOpticks with no pre-column. The ion mobility range was from 0.7 to 1.2 1/K0, the mass range was set from 100 to 1700 m/z, and 100 msec accumulation time was selected.

Protein identification was performed with X!tandem (Alanine 2017.2.1.4) using i2MassChroQ (0.4.68) from TDF files with 20 ppm mass tolerance in MS and MS<sup>2</sup> and FDR 1%.

Protein quantification was performed using MaxQuant (2.1.3.0) with min ratio 1, 20 ppm mass tolerance, no MBR, no normalization, and FDR 1%.

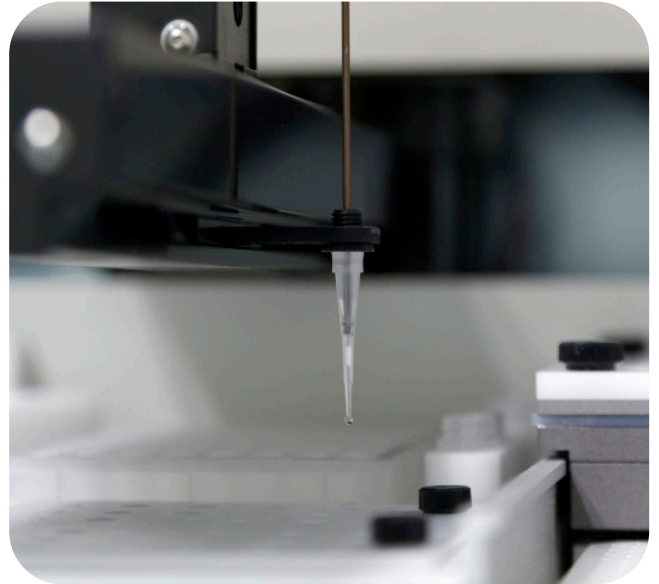
For statistical analysis, a t-test was used (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001, \*\*\*\*p < 0.0001).

\*part number of the specific needle indicated in the related products section at the end of the application note

### 1) Creation and optimization of a SPE Tips desalting method on the DigestPro MSi robot

The DigestPro MSi robot is a dedicated sample preparation system for MS-based proteomics that ensures minimal sample handling and a high or medium throughput under controlled and reproducible conditions. This robot has been designed for in-gel digestion, liquid digestion and SPE Tips desalting.

In order to implement a new application for SPE Tips desalting, the shape of the tip needle has been adapted to create a new needle dedicated to 10 $\mu$ L AttractSPE® Disks Tips (Picture 1).



**Picture 1.** Tip needle specifically designed by CEM to adapt to 10 $\mu$ L SPE Tips

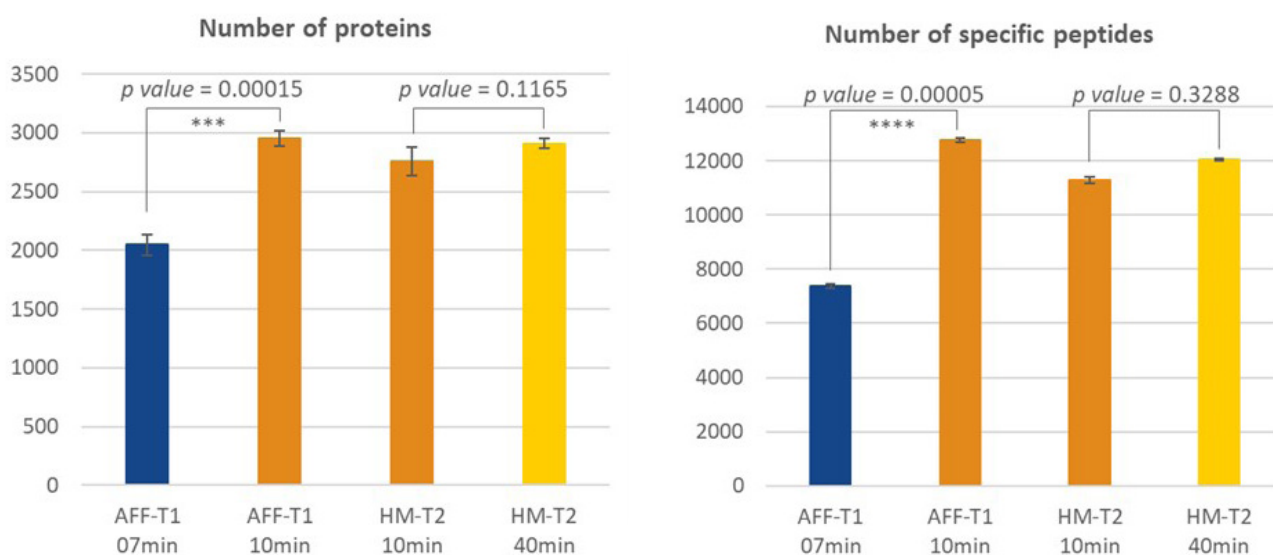
The DigestPro MSi robot offers complete access to all configuration parameters and the capacity for users to create new methods. P3S core facility has developed a first method based on their homemade stagetips protocol (adapted from Rappsilber et al. [1]). The method consisted in the following steps: 1) Sample dilution in 0.5% acetic acid; 2) Tip conditioning; 3) Sample loading on the tip; 4) Tip wash; 5) First elution; 6) Short needle wash; 7) Second elution. The homemade stagetips were prepared in 10 $\mu$ L Gilson tips packed with 1 or 2 layers of Empore™ C18 SPE disk.

The duration of the initial method was 40 min. To improve the throughput, we tested different flow rates and volumes for each step (**Table 1**). To ensure optimal peptide interaction with the C18 membrane during loading and elution, a lower flow rate was used for those two steps. These programs were tested with 100 ng HeLa to evaluate peptide recovery from low amounts of protein digest (**Figure 1**).

[1] Rappsilber J., Mann M. and Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc. 2007;2(8):1896-906. doi: 10.1038/nprot.2007.261.

Step	Solutions	40min program		10min program		7min program	
		Volume (µL)	Flow rate (mL/min)	Volume (µL)	Flow rate (mL/min)	Volume (µL)	Flow rate (mL/min)
Tip conditioning	1) Methanol 2) 50% ACN/0.5% acetic acid 3) 0.5% acetic acid	1) 200 2) 100 3) 200	0.1	1) 200 2) 100 3) 200	0.2	1) 20 2) 20 3) 20	0.2
Sample loading	0.5% acetic acid	68	0.01	68	0.05	68	0.05
Sample wash	0.5% acetic acid	100	0.1	100	0.2	50	0.2
Elution n°1	80% ACN/0.5% acetic acid	100	0.01	60	0.1	20	0.1
Elution n°2	80% ACN/0.5% acetic acid	60	0.01	60	0.1	20	0.1

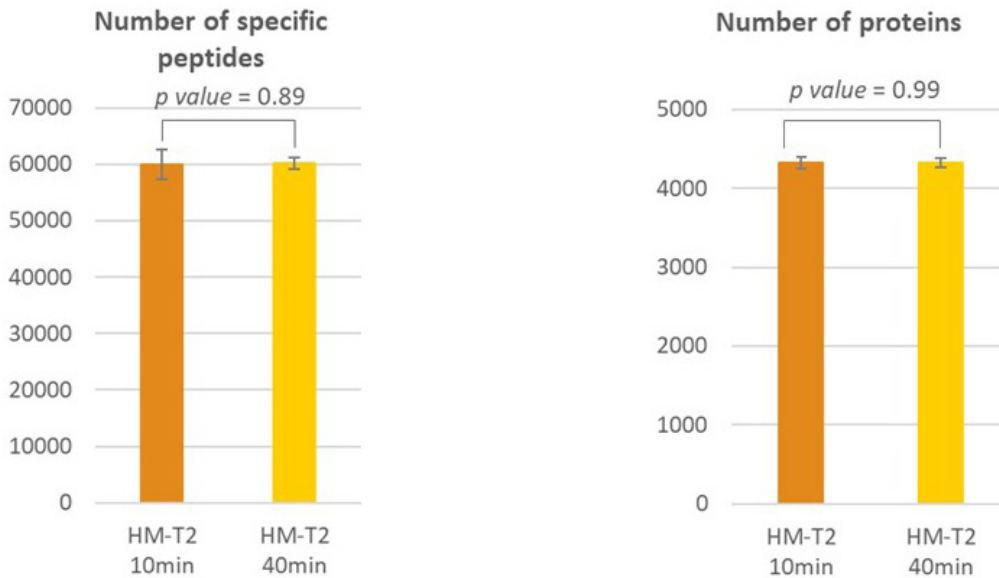
**Table 1.** Description of the solutions, volumes, and flow rates for the three programs with respective duration of 40min, 10min, and 7min. ACN = acetonitrile



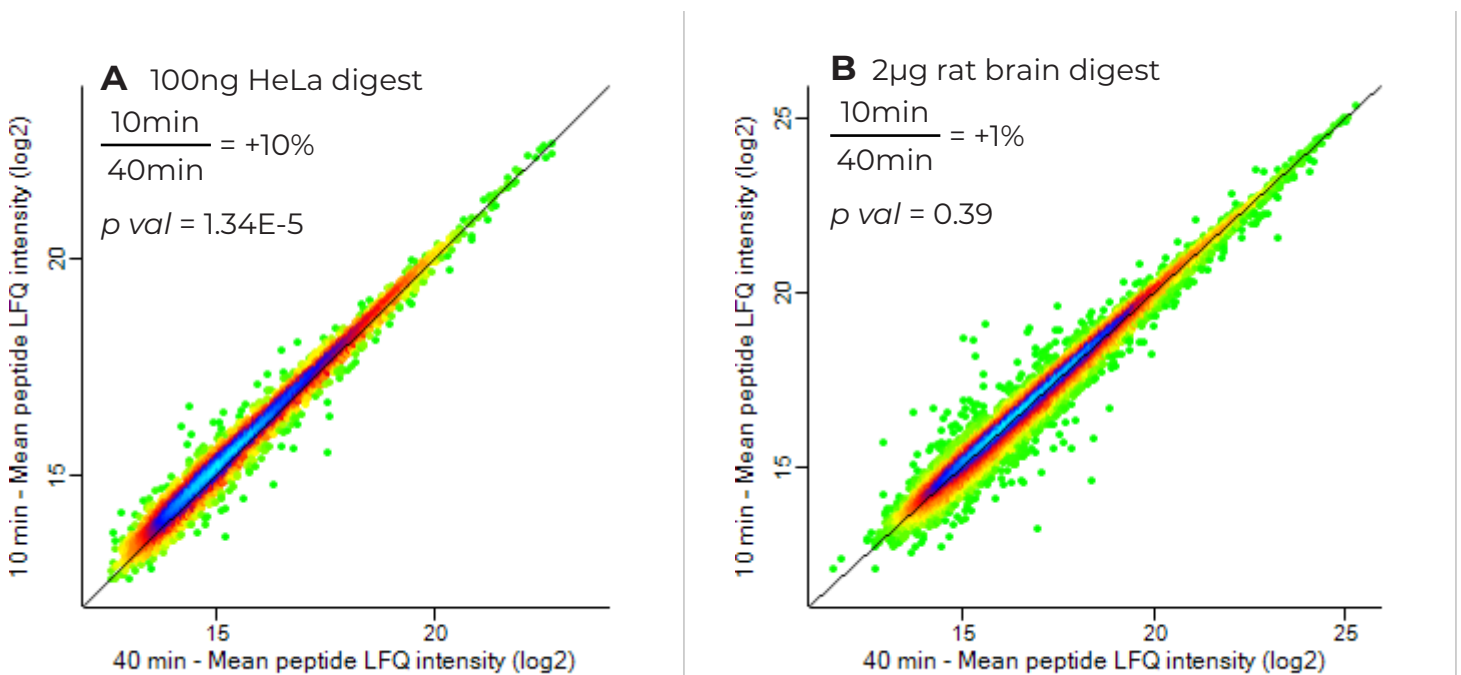
**Figure 1.** Comparison of the 7min, 10min, and 40min programs for the desalting of 100ng of HeLa digest in terms of protein identifications and specific peptides (injection of 10% of the volume of desalted peptides). The comparison of the 7min and 10min programs was performed with AttractSPE® Disks Tips C18 T1 capacity (AFF-T1, binding capacity up to 7µg of peptide) in triplicate, and the comparison of the 10min and 40min programs was carried out with homemade 2-layer C18 Stage-tips (HM-T2) in triplicate.

Both the 40min and 10min programs proved to be very efficient, with no significant difference between the two methods. Thus, increasing the flow rates had no substantial effect on peptide recovery in terms of protein and peptide identification. However, the use of lower volumes in the 7min program induced a great loss in both the number of proteins (-31%) and peptides (-42%) identified compared to the 10min program. Consequently, the 7min program was excluded from further investigations.

The comparison of the 10min and 40min programs with an intermediate amount of peptides (2 $\mu$ g of rat brain digest) showed very similar results in terms of protein and peptide identifications (**Figure 2**). Regarding peptide abundance, a comparison of the two programs is given in **Figure 3**.



**Figure 2.** Comparison of the 10 min and 40 min programs for the desalting of 2 $\mu$ g rat brain digest in terms of numbers of proteins and specific peptides (injection of 10% of the volume of desalted peptides). These experiments were performed with homemade 2-layer C18 stagetips (HM-T2) in triplicate.



**Figure 3.** Comparison of the 10min and 40min programs for both 100ng of HeLa digest (A) and 2 $\mu$ g of rat brain digest (B) in terms of peptide intensity (mean peptide LRFQ intensity from triplicates). The mean peptide LRFQ intensity is given for the peptides quantified in all experiments (no missing value).

When desalting 2µg of peptides, no difference was observed between the two programs in terms of peptide intensity (p value = 0.39). A small increase was observed when desalting a small quantity of protein digest, with a 10% increase in peptide intensity (p value = 1.34E-5).

In conclusion, the 10min program gave similar results than the 40min program in terms of peptide identification and quantification when using both low amounts and intermediate amounts of starting material. Oppositely, the 7min program, based on reduced volumes, induced great loss.

► The 10min program was the best compromise for high peptide recovery with limited time consumption. A detailed description of this program is given in **Table 2**.

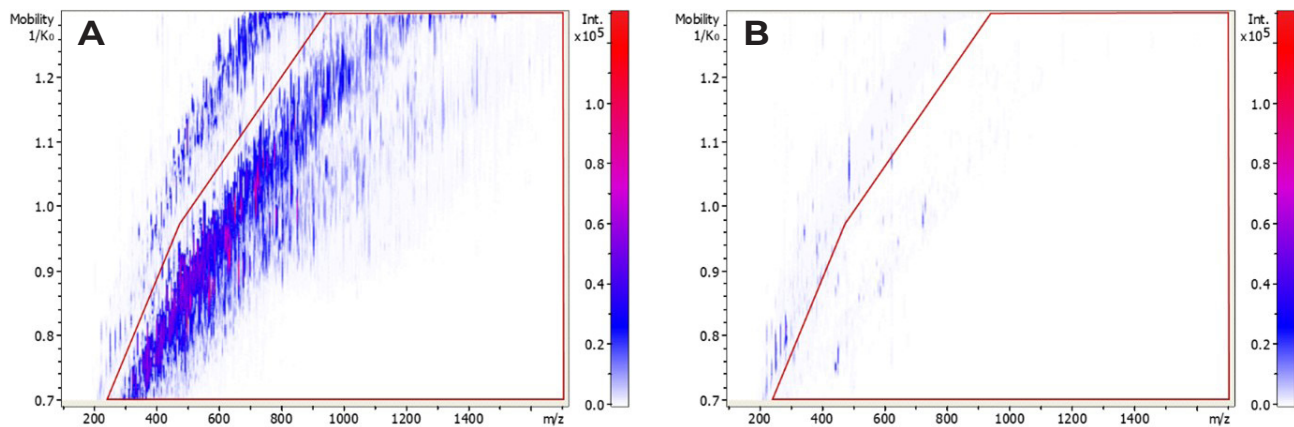
#	Step	Description	Program task	Dispense speed
1	Needle wash	TFA 0.1%: 500µL inside / 1500µL outside	RinseNeedle	10 mL/min
2	Sample dilution	To a final volume of 70µL with acetic acid 0,5% The pH must be around 2	IncubateAdd	8 mL/min
3	Dry Needle Tip	DryTip	MoveToZone	
4	Loop	Will be repeated cyclically for each sample		
4.1	Tip conditioning	200µL methanol 100µL 50% acetonitrile / 0.5% acetic acid 200µL 0.5% acetic acid	TipWash	0.2 mL/min
4.2	Sample loading	68µL uptake (2µL dead volume out of the 70µL)	TipLoadEx	0.05 mL/min
	Sample wash	Wash with 100µL 0.5% acetic acid		0.2 mL/min
4.3	Elution n°1	60µL 80% acetonitrile / 0.5% acetic acid	TipElute	0.1 mL/min
4.4	Needle wash	TFA 0.1%: 50µL inside / 250µL outside	RinseNeedle	5 mL/min
4.5	Elution n°2	60µL 80% acetonitrile / 0.5% acetic acid	TipElute	0.1 mL/min
4.6	Needle wash	TFA 0.1%: 50µL inside / 250µL outside	RinseNeedle	5 mL/min
5	Needle wash	TFA 0.1%: 100µL inside / 500µL outside	RinseNeedle	8 mL/min

**Table 2.** Description of the optimized method developed on the DigestPro MSi robot. The duration of the method is 10 min/sample. Samples were manually distributed in a non-skirted 96-well plate with the same starting volume for each sample, typically 10 or 20 µL.

Of note, the recovery rate with either one or two elution steps (120µL once versus twice 60µL) was evaluated. The elution with twice 60µL gave the highest recovery (data not shown).

### 3 Evaluation of cross-contamination effects in automated desalting

In order to fully validate the automated method, and considering the fact that the robot uses the same needle to process all samples, cross-contamination tests were performed. 10µg of mouse serum digest were used for these tests, since abundant serum proteins are known to induce abundant carryover. In the sample plate in the robot, 10µg of mouse serum was placed in alternate wells with a solution of acetic acid 0.5% (v/v) in 4 replicates. All samples were desalted using 10µL AttractSPE® Disks Tips C18 with T2 capacity (binding capacity up to 15µg of peptides) and analyzed by LC-MS/MS. An illustration of the heatmaps (ion mobility against m/z) is given in **Figure 4**.



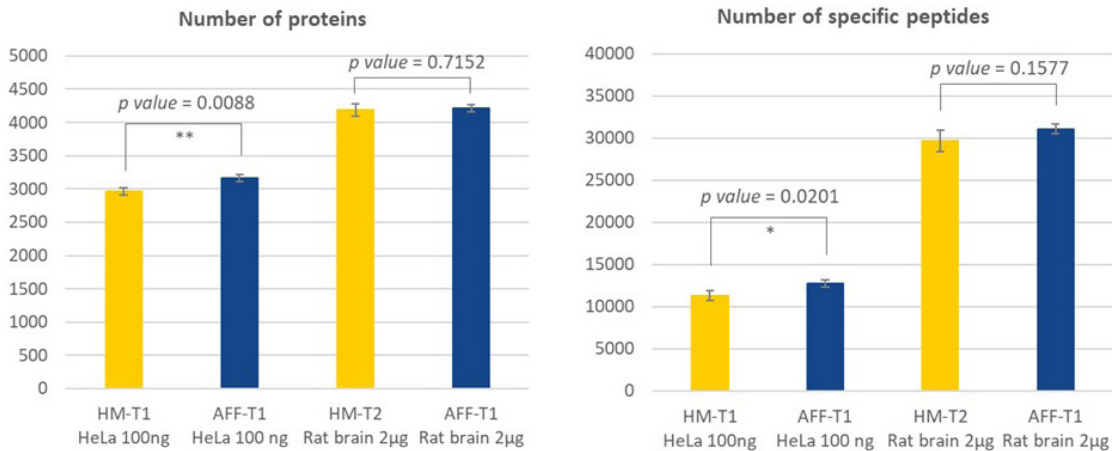
**Figure 4.** Heatmap of ion mobility against m/z of (A) mouse serum sample and (B) blank sample run on the DigestPro MSi robot following a mouse serum sample (these heatmaps are representative of the 4 replicates). The area framed in red represents the ion-mobility zone of multicharged ions, selected for acquisition. The signal detected outside this area corresponds to single-charged ions that are excluded from MS/MS analyses

Protein quantification of the serum and blank samples was performed with MaxQuant. The results showed a mean of 17 proteins identified in blank samples, while more than 570 proteins were identified in the neat serum samples. As expected, Albumin induced the highest carry-over. In terms of protein intensity, Albumin was found 850 times less intense in blank samples (0.12%) compared to serum samples. The other proteins had 3400 times less intense signal (0.03%) in blank samples on average.

➤ These results demonstrate that the program used on the DigestPro MSi robot ensures very clean sample preparation with no cross-contamination between samples.

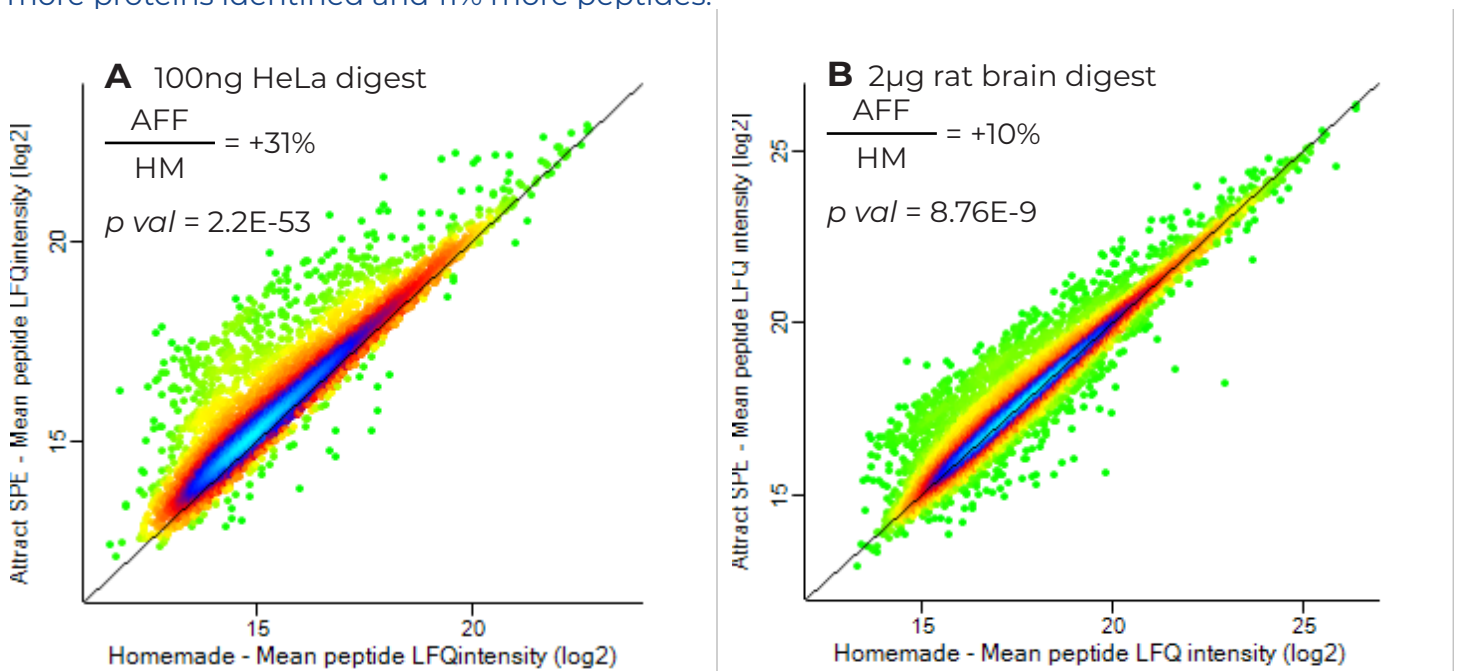
## 4 Comparison between homemade stagetips and AttractSPE® Disks Tips C18

In order to evaluate the efficiency of Affinisep AttractSPE® Disks Tips C18 on the DigestPro MSI robot, homemade stagetips (packed with Empore™ C18 phase) were compared to AttractSPE® Disks Tips C18 using the 10 min program (**Figures 5 and 6**).



**Figure 5.** Comparison of homemade stagetips (HM) and AttractSPE® Disks Tips C18 (AFF) for the desalting of 100ng of HeLa digest and 2µg of rat brain digest in terms of numbers of proteins and specific peptides (injection of 10% of the volume of desalted peptides). These experiments were performed in triplicate.

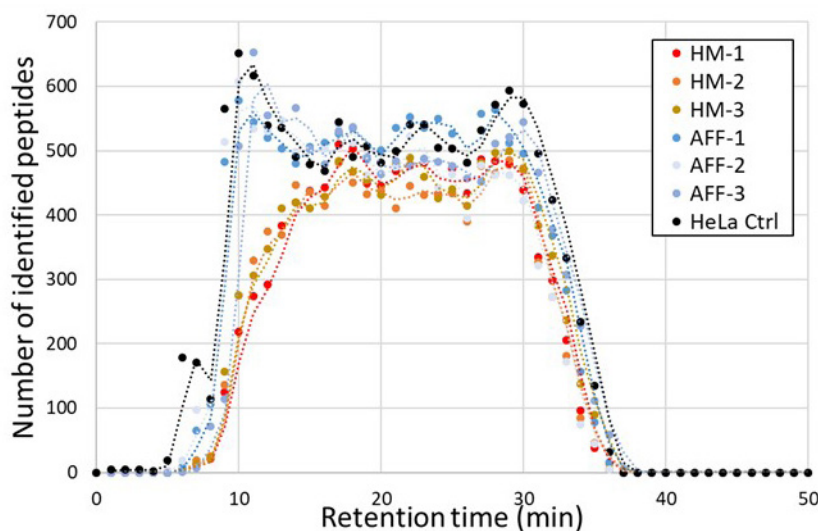
No significant difference was observed between homemade stagetips and AttractSPE® Disks Tips C18 with 2µg of starting material regarding the number of peptides and proteins. When working with a lower amount of starting material, AttractSPE® Disks Tips C18 gave better results with 6% more proteins identified and 11% more peptides.



**Figure 6.** Comparison of homemade (HM) stagetips and AttractSPE® Disks Tips C18 (AFF) for the desalting of 100ng of HeLa digest and 2µg of rat brain digest in terms of mean peptides intensity (injection of 10% of the volume of desalted peptides). These experiments were performed in triplicate. The mean peptide LFC intensity is given for the peptides quantified in all experiments (no missing value).

▶ Regarding peptide intensity, AttractSPE® Disks Tips C18 showed an increase in peptide intensity by 10% for middle amounts of starting material and by 31% for low amounts. Both variations were deemed significant.

To further investigate the advantage of using AttractSPE® Disks Tips C18, the hydrophobicity of the peptide eluted from homemade stagetips and AttractSPE® Disks Tips C18 was evaluated and compared (**Figure 7**).



**Figure 7.** Number of peptides identified according to the retention time for homemade (HM) stagetips and AttractSPE® Disks Tips C18 (AFF) for the desalting of 100ng of HeLa digest. Non-desalted HeLa digest is given as a reference (dark blue dots).

▶ AttractSPE® Disks Tips C18 show much better recovery of hydrophilic peptides compared to homemade stagetips. The Retention Time distribution of the peptides desalted with AttractSPE® Disks Tips C18 is highly similar to the non-desalted HeLa peptides distribution, meaning these SPE tips allow very complete peptide recovery.

## CONCLUSION

This study showed that SPE Tips could be used for automated peptide desalting on the Digest Pro MSi robot. Several programs were created on the robot with different flow rates and volumes. The best compromise was found to be a 10min program. The comparison of AttractSPE® Disks Tips C18 with homemade stagetips prepared with Empore™ C18 phase showed that AttractSPE® Disks Tips C18 allowed higher peptide recovery both in terms of number of peptides and peptide intensity. The gain was higher when working with low amounts of starting material (100ng of HeLa digest). Moreover, AttractSPE® Disks Tips C18 offered very high recovery for hydrophilic peptides.

In conclusion, automated desalting with AttractSPE® Disks Tips C18 on the Digest Pro MSi robot offers a clean, efficient, and complete peptide recovery with a throughput of 36 samples per day.

### Products used in this application note



**AttractSPE® Disks Tips**  
**C18, 10µL, 96/pk**  
**Tips-C18.T1.10.96**  
**Tips-C18.T2.10.96**

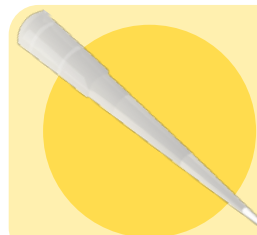
The CEM part number for the specific needle to handle the 10µL AttractSPE® Disks Tips is **# 34101 - PROBE, TEFLON COATED**

Any DigestPro user who has the TipHandling module just needs this special needle to handle the 10µL AttractSPE® Disks Tips.

### Related products



**AttractSPE® Disks 96 plate**  
**for microelution - C18, 1/pk**  
**µ96W-C18.T0.1**  
**µ96W-C18.T1.1**



**AttractSPE® Disks Tips**  
**C18, 200µL, 96/pk**  
**Tips-C18.T1.200.96**  
**Tips-C18.T2.200.96**  
**Tips-C18.T3.200.96**