Project: TopSpec - 829157

WP5 - Development of pI-Trap-ESI Combination

Deliverable: D5.2 - Two tested, optimized pI-Trap-ESI Installed and tested

Summary: The pI trap system has shown good capability and reproducibility for separating monoclonal antibodies based on isoelectric points (pI). Two automated pI trap systems are successfully installed at Karolinska Institutet and Institut Pasteur. In the most updated pI trap system, the pI cell is assembled with electrodes, and is in pair with two peristaltic pumps. The system is connected to orbitrap-omnitrap platform to achieve fully automated sample injection, capillary isoelectric focusing (cIEF) and subsequent MS and MS/MS analysis. Two intact antibodies were successfully separated by pI trap at a baseline level.

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Description of the action and performance

1.1 Updated pI cell design, and the installation of peristaltic pumps

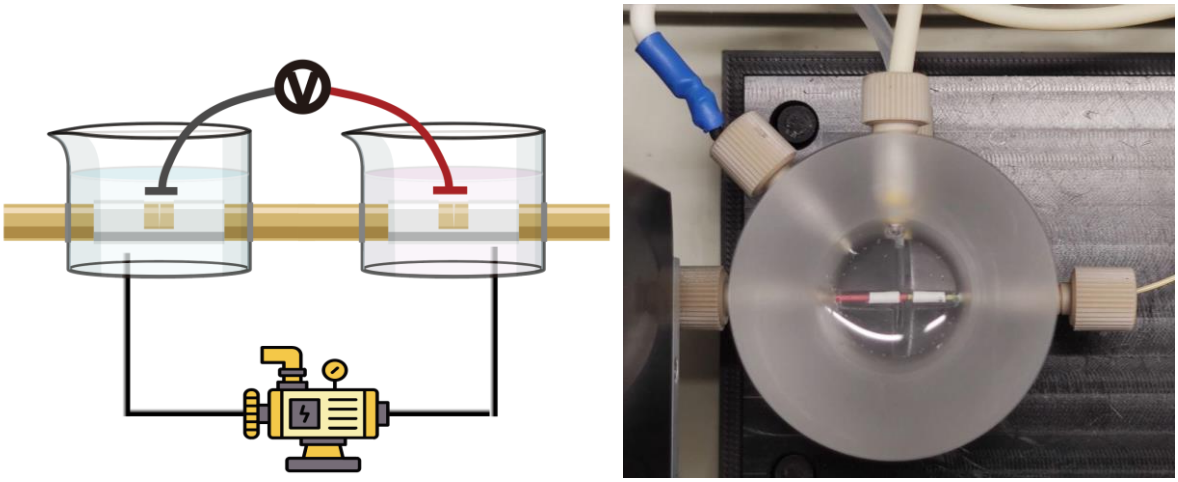

Figure 1. Upgraded pI cell. Nafion membrane and PEEK tube are connected by heat-shrink tubing. Electrode and tubes from and to the peristaltic pump are attached to the cell.

The pI trap is upgraded with new user-friendly and automation features (**Figure 1**). In the past, Nafion tubing is glued to PEEK tubing. The integrated pI cell is hard to be fixed under any leakage condition. The updated version is a modular design, in which the tubing can be installed and removed easily from the machine cell. Inside the heat-shrink tubing sleeve, Nafion membrane wraps the cut on PPEK tubing that allows potential applied to the protein inside the tube. The electrode reaches the buffer through a fingertight connector, while the other two connectors hold the inlet and outlet hose of the peristaltic pump. The introduction of peristaltic pumps is for the automation of cathode and anode buffer replacement. The change buffer session can be added freely to the pI trap method.

1.2 The interface between pI trap and MS

A successful pI trap-MS interface is based on the transformed cable among pI trap, autosampler, and the contact closure wire for Exploris 480 (**Figure 2**). The communication between pI trap and the autosampler is achieved by the signal 1 and 2 sent by pI trap and the output signal sent by the autosampler, via a pair of serial ports and the cable. Among the 9 pins in the port, 2 are connected to pI trap signal leads. We spare one of these two pins to be used for sending a contact closure signal, therefore, having it attached to the MS's triggering wire.

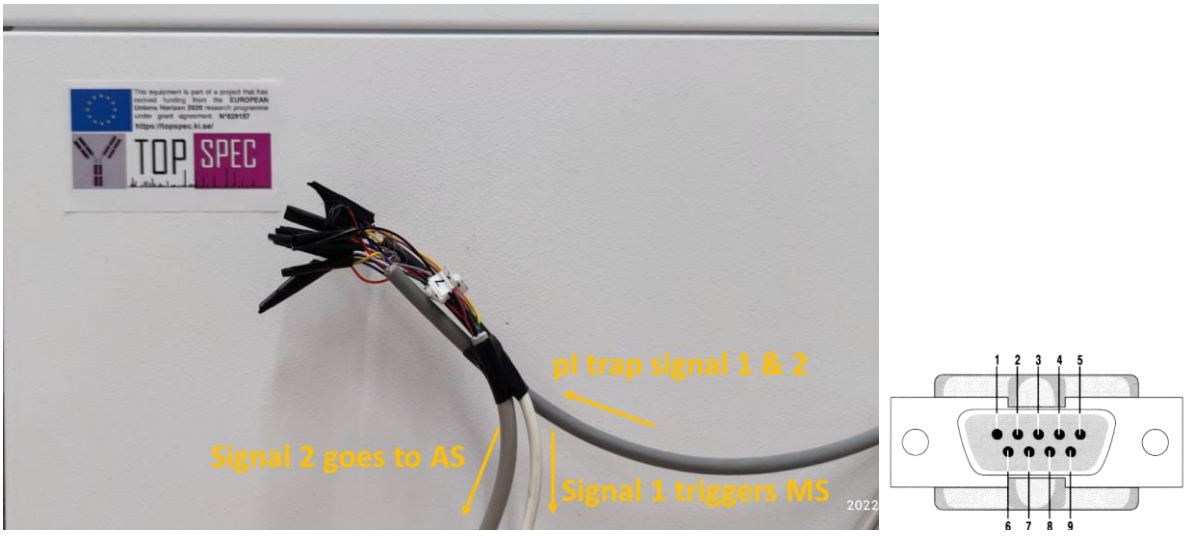

Figure 2. Leads connection among autosampler, pI trap and MS. Two of the 9 leads from the pI trap that transmit signal 1 and ground are connected to MS triggering cable. All the leads except for signal 1 are connected to the autosampler corresponding leads.

Samples are mobilized out of the pI cell after focusing at 0.5 µl/min, subsequently, it is mixed with 4× volume modifier, consisting of 60% acetonitrile and 0.1% formic acid. The modifier not only facilitates ionization but also reduces clogging and expands the selection of emitters (**Figure 3**). The fusion is accomplished in a tee connecter.

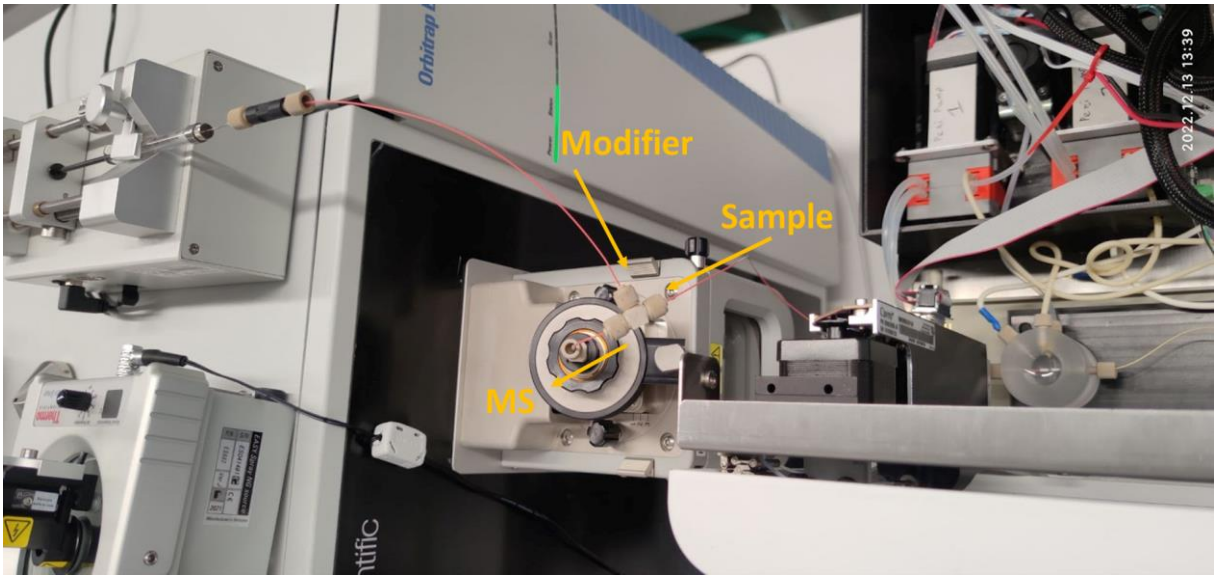

Figure 3. The introduction of modifier to the sample after cIEF. A tee connector is employed to facilitate the mixing.

1.3 Quality control shows high reproducibility and good linear correlation

In order to test the cIEF separation quality and reproducibility on the pI trap system, three small proteins with known isoelectric points are selected (**Table 1**). The pI range of these three proteins covers the range of two monoclonal antibodies' pI values. After 30 min focusing, three proteins were eluted at 0.5 µl/min. β-lactoglobulin which has the smallest pI, was eluted firstly from the pI cell, followed by carbonic anhydrase isozyme I and trypsinogen. One of the most abundant m/s states is extracted for each protein, claiming the good cIEF performed by the pI cell (**Figure 4**). Moreover, we investigated the linear correlation between the retention time and pI. When separating small proteins < 30 kDa, the pI trap is highly reproducible and capable of distributing proteins with great linearity.

Table 1. The pI values and m/z extracted of β-lactoglobulin, carbonic anhydrase isozyme I and trypsinogen.

Name	pI value	Molecular Weight	Ion mass and charge state
β-Lactoglobulin	5.1	~18,400 Da	m/z = 1531.2, charge state 12
Carbonic Anhydrase Isozyme I	6.6	~30 kDa	m/z = 1371.3, charge state 21
Trypsinogen	9.3	23981 Da	m/z = 1602.1, charge state 15

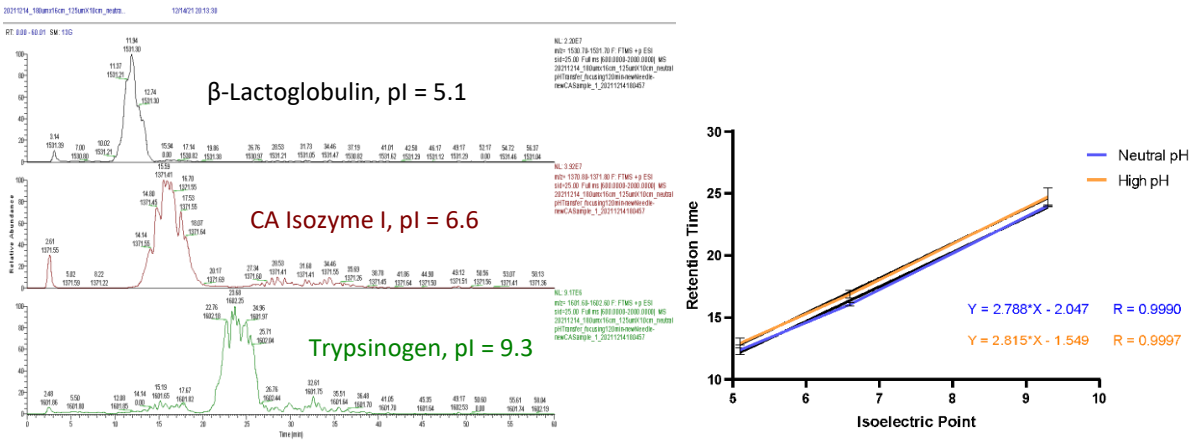

Figure 4. Left: The retention time and intensity of three proteins after 30-minute isoelectric focusing. No evident dispersion or asymmetric peaks was observed. Right: The linear correlation of proteins' retention time vs isoelectric points, among 3 replicates. R values are both >= 0.999 with neutral or basic transfer buffer pushing the sample.

1.4 Optional optimizations for pI trap

A couple of different settings were tested for optimizing pI trap performance. We changed the transfer buffer that pushes sample to MS to basic buffer to reduce dispersion. Figure of Merit (FoM) (**Figure 5**) is calculated and employed as the variable for cIEF quality evaluation. Among the three replicates (**Table 2**), more variation was recorded in the FoM of basic buffer

mobilizing two mAbs. However, the average separation of the basic buffer is larger than that of the neutral buffer.

Table 2. Options for the transfer buffer and FoM of two monoclonal antibodies separation

pH	**Solution**	**Used also as**			
pH = 7	5% IPA	transfer buffer for autosampler			
pH = 10	20 mM ammonium in MQ	pI cell catholyte			
Separation time (min)	**Transfer buffer**	**Figure of Merit (FoM)**			
120	pH = 7	Rep 1	Rep 2	Rep 3	Mean
		3.90	4.57	4.20	**4.22**
	pH = 10	Rep 1	Rep 2	Rep 3	Mean
		5.79	4.04	3.44	**4.42**

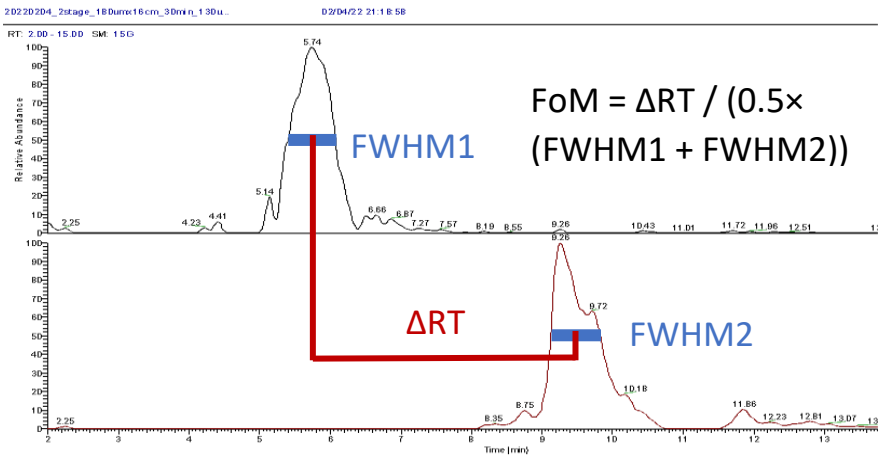

Figure 5. Formula for calculating FoM. The full width at half maximum (FWHM) is measured separately for two peaks. FoM is calculated lettnig the difference in retention time to be divided by the average of two FWHM. The larger the FoM is, the better the two proteins are separated.

Besides the transfer buffer formula, we also compared one- vs two-stage pI cells. By using the two-stage pI cell, sample is first transferred to the first cell, where a relative shorter cIEF is applied, followed by the mobilization of sample to the second cell where potential is applied for a longer time (**Figure 6**). The objective for the 1st cell is to let proteins quickly distribute following their pI in the wide tube, while at the second stage the proteins will sophisticatedly move further away from each other. After the same time of cIEF (60 min vs 30+30 min, and 120 min vs 60+60 or 30+90 min, **Table 3**), two-stage cIEF reached significant better resolution in all groups. The design "Tandem high resolution pI Trap" has been registered as an international patent No. PCT/EP2021/064686. This two-stage upgrade can be optionally installed into the current pI trap system.

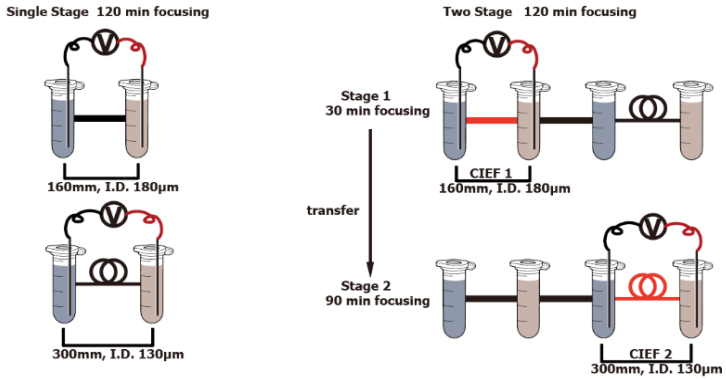

Figure 6. Design of a one- and two-stage pI cell, respectively. For the two-stage cell, it is critical to assure both cells have the same volume. Potential is applied to the two cells sequentially.

Table 3. FoM of two antibodies after the same cIEF duration, using one-or two-stage pI cell.

Focusing time	60 min			120 min		
	pI cell size	Separation time (min)	FoM	pI cell size	Separation time (min)	FoM
Single Stage	16cm x 180µm	60	1.39	16cm x 180µm	120	1.26
	30cm x 130µm	60	1.67	30cm x 130µm	120	2.03
Two Stage	16cm x 180µm + 30cm x 130µm	30 + 30	3.92	16cm x 180µm + 30cm x 130µm	60 + 60	4.89
					30 + 90	4.87

1.5 Installation of pI trap at Institut Pasteur

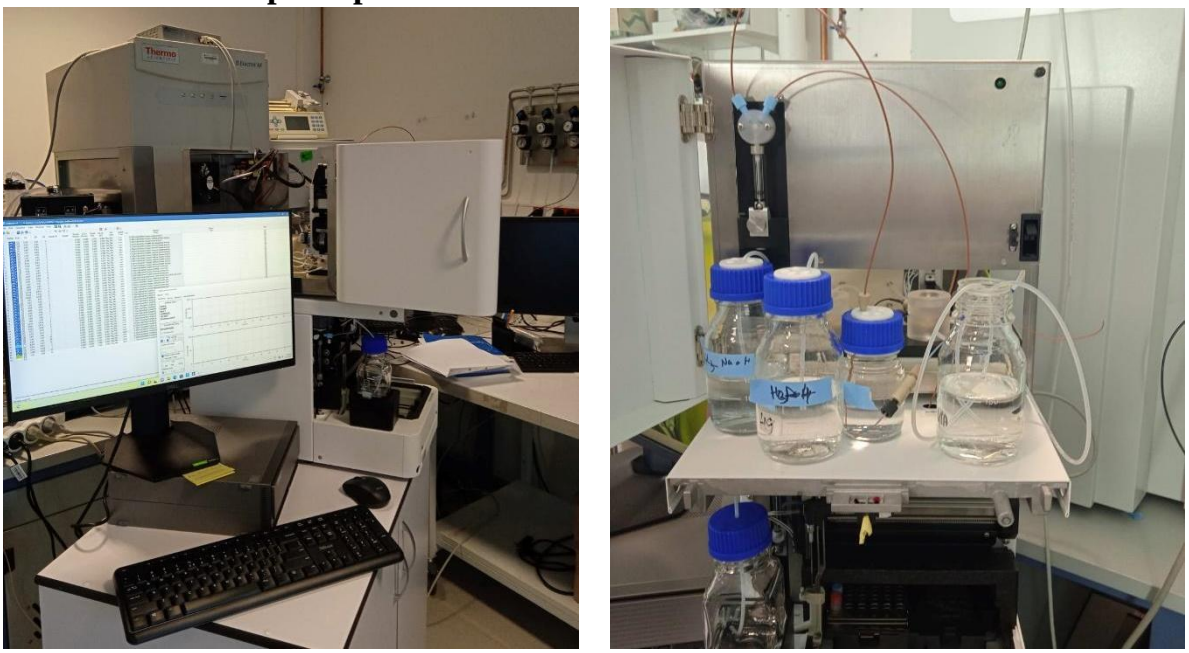

Figure 7. The pI trap at Institut Pasteur.

An intact pI trap system has been installed at Institut Pasteur, France. The communication between pI trap and computer, as well as between pI trap and MS (Q Exactive) has been attained (**Figure 7**).

1.6 pI trap separation of Infliximab and NIST mAb

Two monoclonal antibodies were selected to perform cIEF using the upgraded pI trap system (**Figure 8** and **Table 4**). The antibodies were infliximab, with reported pI 7.1, and NIST mAb with reported pI 8.8. Both mAbs molecular weights (MW) are around 150 kDa. Due to their much larger MW and sizes, they need longer time to mobilize within the pI cell. Hence, a 90-minute potential is applied on the pI cell. The FoM of the two mAbs with the upgraded one-stage pI cell is 2.55, thus, the two antibodies were successfully separated by pI Trap at a baseline level. They are also analyzed by MS and MS/MS performed in the Omnitrap-Orbitrap combination connected online to the pI Trap.

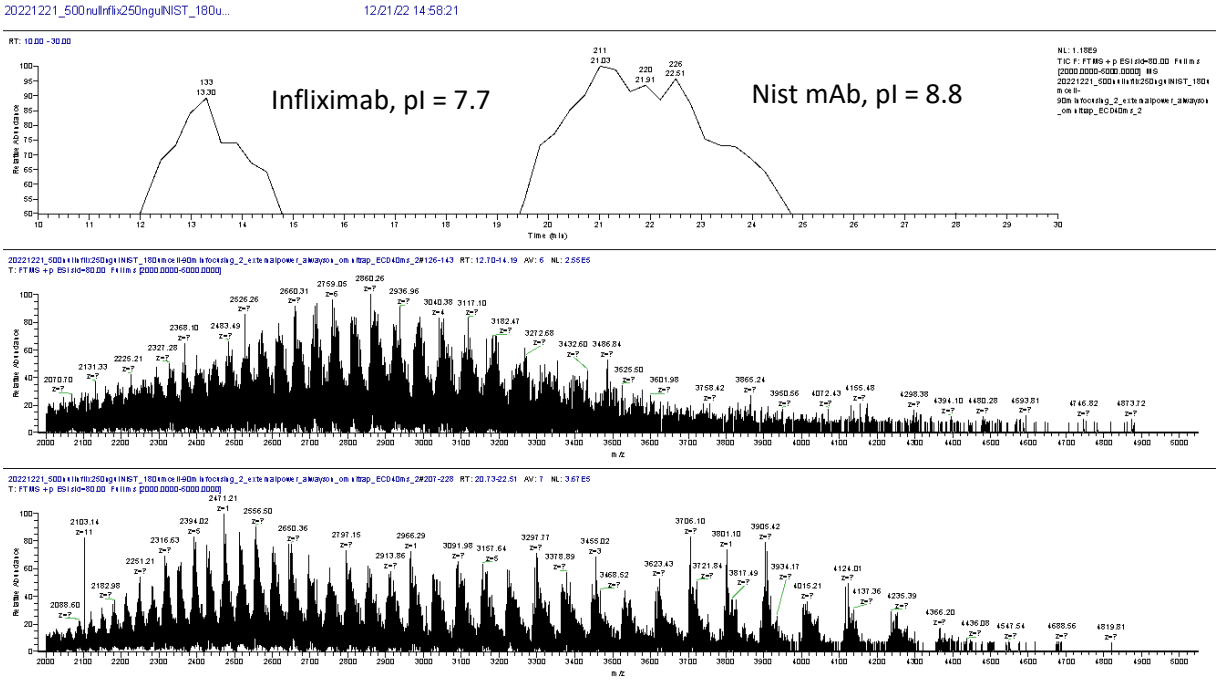

Figure 8. The separation of infliximab and NIST mAb after 90-min cIEF in pI cell, and their full MS spectrum.

Table 4. FoM of two antibodies cIEF separation using new machine pI cell, connected to ORBI-OMNI platform

pI cell size	Separation time (min)	Transfer buffer	Figure of Merit (FoM)			
			Rep 1	Rep 2	Rep 3	Mean
16cm x 180µm machine cell	90	pH = 7	2.56	2.85	2.25	2.55

1.7 Protocols

The Åcelus pI trap system consists of two parts - autosampler and pI trap. The pI cell can be easily assembled, installed, and removed from the pI trap (**Figure 9**). To assemble a new pI cell, PEEK tube with a selected inner diameter (180 µm is used at KI), semi-permeable tubing made from Nafion membrane, and heat-shrink tubes are necessary. The smaller the I.D. is, the better separation will be achieved, however, it requires longer focusing time and causes a higher possibility of clogging. The Nafion tubing should have a smaller I.D. than the O.D. of the PEEK tubing.

It is vital to cut the PEEK tube and cover the cut with Nafion tubing to enable protein mobilization under potential. Due to the feature of Nafion which increases in size by up to 88% when exposed to alcohol, the Nafion tubing can be quickly put over the cut after a soak in ethanol. The heat-shrink tube is employed to wrap up the edge of Nafion tubing, as it is immersed in the cathode or anode buffer over the whole experiment. The tightness and quality of the connection can be evaluated via a leakage test. Subsequently, the tube can be installed in the machine cell, where the cathode buffer (20 nM ammonium in MilliQ water, pH = 10) and anode buffer (0.14% formic acid in MilliQ water, pH = 3) are added.

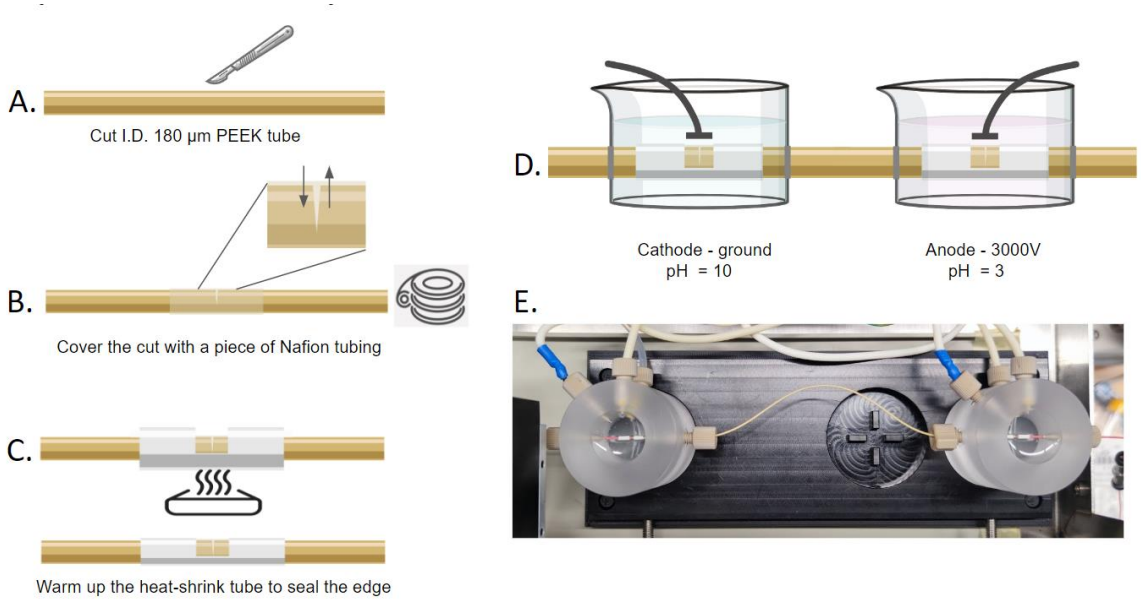

Figure 9. Assembly of a new machine pI cell. A-C: step-by-step procedure of preparing one side of the tube. D-E: let the tube partially go through the machine cell followed by installing the electrode and peristaltic tubings to accomplish the assembly.

The autosampler and pI trap are controlled by two software, with fine real-time communication. The alteration of the buffer is recommended between every 3-4 samples to refrain from proton or hydroxide depletion. The buffer change can be executed by two peristaltic pumps according to the program. The mobilization buffer for samples is 5% IPA in MilliQ water. Samples are

prepared in 0.5% ampholyte (Cytiva) and 5% IPA. A high percentage of IPA should be avoided because it will make the Nafion tubing expand.

To attain good performance and reproducibility of pI cell cIEF separation, inevitably, flowrate tests and emitter spray stability evaluation are supposed to be conducted regularly. Then the flow rate and volume in the method can be changed accordingly. Hereby, we recommend preparing small proteins with known isoelectric points as quality control samples, as it takes a much shorter focusing period to separate them. In addition, blank and wash buffers (50% acetonitrile, 50% water) need to be injected among the protein samples.

The current setting of tested, optimized pI-Trap-ESI interfaced with MS is shown in **Figure 10**.

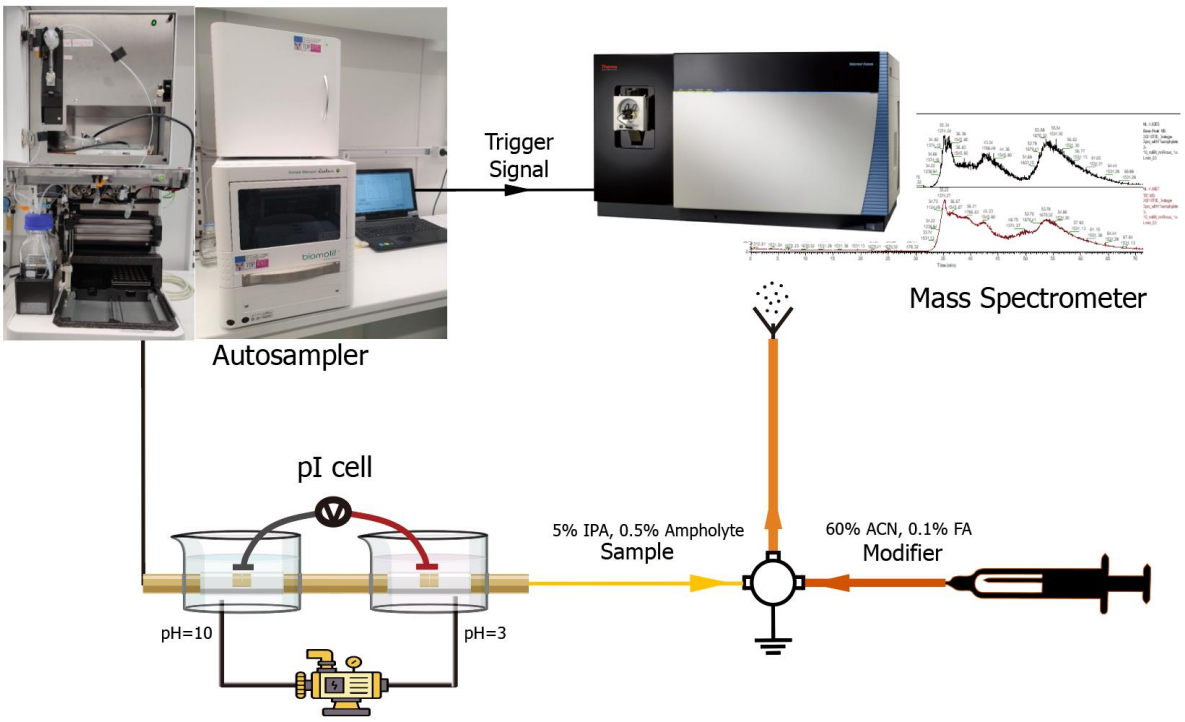

Figure 10. The current setting of tested, optimized pI-Trap-ESI interfaced with MS.