

## Determining Phosphorylation Sites by LC-MS/MS

Procedure for the identification of precise phosphorylation sites by LC-MS/MS. We use LC-MS/MS to identify the phosphorylation sites of phosphopeptides that are identified from MALDI-TOF experiments. The LC/MS/MS is operated in a unique fashion that MS/MS (or CID) is performed on a single m/z value during the entire LC run. As default, the m/z value is calculated based on the estimated charge state of the phosphopeptide, which typically equals to one plus the total number of basic residues in the phosphopeptide. One should keep in mind that in ESI a peptide often exists in more than one charge state, and the charge state distribution can be changed by many factors.

The remaining sample after phosphopeptide mapping (about 80% of total sample) is dried in a SpeedVac and then re-dissolved in 10% of mobile phase B that is used for the LC experiment. 1-5 ml of the sample is then injected into HPLC for LC-MS/MS analysis. The volume of injection depends on the amount of phosphopeptides available and the total number of unique phosphopeptides identified in the previous mapping step. Since the sensitivity of micro LC-ESI-MS/MS is comparable or better than MALDI-TOF, even 1 ml of sample is often enough to obtain a decent MS/MS spectrum. It is suggested to save some samples for extra LC-MS/MS runs. Some runs may need to be repeated because the estimated charge state is not correct, or electrospray is not stable, or column is blocked during the LC procedure.

Data analysis. If the protein digest is separated with high resolution in the LC/MS/MS procedure, data analysis is often straightforward for a person who is trained in biological mass spectrometry. Since only one precursor is selected for MS/MS during the entire LC run, there are often few peaks in a chromatography of total ion current (TIC). By visual inspection of MS/MS spectrum corresponding to each peak, the selected phosphopeptide may be identified. This is the distinct advantage of this combined MALDI-TOF and LC/MS/MS approach for the identification of phosphorylation sites, which utilizes the best properties of the two different mass spectrometers while circumventing their shortcomings.

Sensitivity of the ion trap in determining phosphorylation sites by the LC-MS/MS procedure. This procedure is very sensitive in determining phosphorylation site. Its ultimate sensitivity will only be limited by the detection limit of the mass spectrometer. With the help of the capillary LC-MS technique, the detection limit of current commercial ion trap mass spectrometer can reach the attomole level. So the limiting factor lies in sample handling, in which sufficient amounts of phosphopeptides need to be present in a small volume so that it can achieve the concentration that the amount of phosphopeptide in 1 to 5 ml volume is above the detection limit of LC/MS/MS.

In principle LC/MS/MS should be capable of analyzing very low stoichiometric phosphorylation that is even difficult for MALDI-TOF based phosphopeptide mapping. Since the phosphopeptide is separated from non-phosphopeptides in LC prior to MS detection, the presence of large excess of other peptides often doesn't interfere with the analysis of this phosphopeptide. As a result, even very low stoichiometric phosphorylated peptide may be analyzed by this procedure if it is present above the detection limit of the mass spectrometer, and if it is well separated from other peptides and if the m/z value is known. Problems in phosphorylation site determination by capillary LC-MS/MS. There are some general difficulties associated with determination of phosphorylation sites by LC-MS/MS. It is often difficult to determine the exact phosphorylation sites when a peptide is multiply phosphorylated, especially when several Ser or Thr residues are adjacent to each other. If peptide bond cleavage is not observed between these adjacent S/T residues, the exact sites of phosphorylation cannot be determined. This is a general problem for any mass spectrometry. The facile loss of H<sub>3</sub>PO<sub>4</sub> in ion trap exacerbates the difficulty, in which multiple losses of H<sub>3</sub>PO<sub>4</sub> are so dominant that peptide bond cleavage along the peptide backbone is negligible. This is

particularly problematic for phosphopeptides that their amounts are limited. Sometimes, large and hydrophobic phosphopeptides are refractory to LC/MS/MS, because they are not eluted from the LC column, although they are readily observed in MALDI-TOF. The size of the peptide is not the only parameter that dictates whether it can be sequenced in LC/MS/MS. We sequenced phosphopeptides with masses over 5000 Da. On the other hand small and hydrophilic phosphopeptides do not bind the column and are eluted in the void volume. Since inorganic salt is often present in the void volume, as a result the quality of MS/MS spectra is compromised, rendering data analysis more difficult. In a few lucky cases, we were able to identify phosphorylation sites for phosphopeptides that elute very early in the gradient or in the void volume. Nevertheless, this is a problem for any method that LC is used or a desalting step is involved. Only MALDI based methods such as MALDI/Ion trap and MALDI/TOF operated in the post source decay mode can circumvent this problem.

Alternative procedures for determining phosphorylation sites using LC-MS/MS. The procedure we described above can only analyze one phosphopeptide at a time. One may argue that it is not the most efficient way to use the sample and time. However, since determination of protein phosphorylation sites is still a challenging task, focus on sensitivity and reliability in this procedure is still well justified. Nevertheless multiple phosphopeptides can be analyzed in a single LC-MS/MS procedure. We provide two alternative procedures here.

One method is to perform data-dependent MS/MS with the parent masses selected from a list of phosphopeptides mapped by MALDI-TOF. The LCQ mass spectrometer can be programmed to input m/z values of selected phosphopeptides into the parent masses list and select "Nth Most Intense from List" instead of "Nth Most Intense Ion" in Scan Event setting for data-dependent MS/MS procedure. Since one can input up to 25 parent masses, it is possible to select several different charge states for each phosphopeptide if total number of phosphopeptides to be sequenced is small. Another alternative method is to determine the retention time of phosphopeptides by a LC-MS procedure. If phosphopeptides elute at different time, it is possible to divide the MS/MS procedure into several time segments, each one is unique to one phosphopeptide. Multiple phosphopeptides can thus be sequenced in one LC run.