Common procedure for *in gel* protein analysis with 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX)

1. *Wash and destain*. Samples are destained and washed with ammonium bicarbonate and acetonitrile.

2. *Tryptic digestion*. Samples are incubated with porcine modified trypsin that cleaves at specific sites of the aminoacid sequence of the sample protein, generating several peptides that have different molecular weights that are, mostly often, exclusive to a given protein. These peptides constitute the *peptide mass fingerprint* (PMF) of the protein.

3. *Extraction*. The peptides are extracted from the gel and mixed with the matrix, alpha-cyano-4-hydroxy cinnamic acid (CHCA).

4. *Analysis*. Approximately 1 µl of the matrix/peptide solution is spotted on a specific plate. Peptides are analyzed by matrix-assisted laser desorption ionization (MALDI). The mass analyzer is a time-of-flight (TOF) with reflector capabilities. In reflector mode, ions from m/z~700 to m/z~4000 are detected easily at high resolution for a mass accuracy typically less than 20 ppm.

5. *Protein Identification*. Protein identification is determined by comparing the peptide mass fingerprint (PMF) of the unknown protein with the theoretical molecular weights of peptides that are generated by mathematical digestion of each of the proteins from databases like UniProt or NCBI. PMF is the method-of-choice for protein identification in simple protein mixtures because it is a fast, simple, and sensitive technique (femtomole amounts) that can identify proteins with high levels of confidence.

6. *Data analysis*. The results are a ranking list of candidate proteins based on their calculated probability. The protein identity is checked out for plausibility considering trypsin autolysis and cleavage faults, as well as eventual contaminations.

7. *MS/MS analysis and Peptide de novo sequenciation*. Although the PMF approach is useful to identify proteins in simple mixtures, peptide sequence information obtained by MS/MS is required to identify individual proteins in more complex or less abundant samples. Peptide MS/MS data is identified by correlating the experimental MS/MS spectra with simulated product ion spectra derived from peptides of the same mass contained in the databases. For proteins not contained within sequence databases, it is necessary to determine complete or partial amino acid sequences using *de novo* peptide sequence analysis methods.

8. *Protein characterization*. Protein characterization includes the identification of post-translationally modified peptides and localization of modification sites on the peptide. Due to their low abundance, only a few, highly-modified, peptides can be identified while performing routine protein identifications. In most cases, the identification of modified peptides requires additional sample preparation and manual data interpretation. Localization of modification sites often additionally requires *de novo* sequencing of the modified peptide.