Automated protein digestion workflows for MS-based proteomics applications

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Overview

Three reasons for automating workflows in a proteomic laboratory:

- Reduce hands-on time on repetitive work
- Increase consistency
- Increase reproducibility

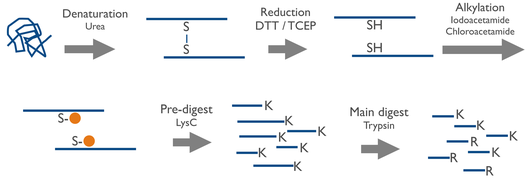
WORKFLOW

- In-solution digest
- In-gel digest

Mass spectrometry (MS) based bottom-up proteomics is built upon large scale identification of peptides, and depends on proteins being efficiently converted to peptides by a protease of known specificity. The most common preparation methods are digestion in solution (ISD) or digestion of proteins separated on an SDS-PAGE gel, in-gel digestion (IGD). Both methods consist of a lengthy sequence of washing and chemical modification steps. To increase throughput and reproducibility, automation of these processes is highly desired. Contrary to other „omic"-applications, proteomics analysis by LC-MS/MS remains time-intensive, making the measurement the rate-determining step in the pipeline. Thus the preparation of samples does not require a high- but rather a medium-throughput setup.

The normal benchtop methods for the IGD and the ISD were adapted to make automation in a robotic setup possible. In addition, we modified a standard CTC-PAL robot setup with a vacuum chamber that can be controlled by the robot's software and facilitates the removal of large volumes of washing solvents required by IGD leading to enhanced sensitivity.

In-solution digest



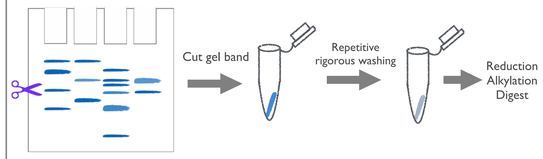
Peptides are generated in a multi-step digestion procedure. The first steps occur at highly denaturing conditions (8 M urea). Proteins are reduced (TCEP) and blocked by alkylation (chloroacetamide). These conditions ensure complete unfolding of the proteins so all parts of polypeptide are accessible for the protease. Since the protease has only limited activity in 8 M urea, the solution is diluted and a second protease, trypsin, is added. An additional incubation at 37°C finalises the digest and allows reproducible production of small peptides.

The PAL robot performs all liquid solvent transfer steps on a 96-well plate based setup.

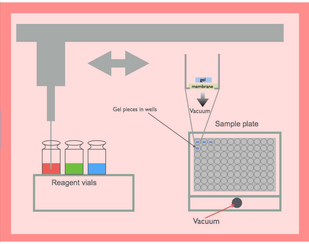
A representation of an RTC-PAL setup. Each module is variable and thus the robotic setup can be extended by introducing new tools.

Transfer of liquids is achieved by a syringe tool. Syringes of different volumes can be automatically exchanged during the process and are washed in a washing station with organic solvent and water.

In-gel digest

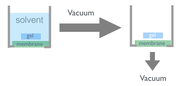


Realisation on the PAL



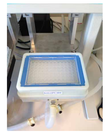
Gel pieces are placed in wells of a 96-well plate equipped with a PTFE membrane that keeps the gel in the well but allows draining liquid by vacuum.

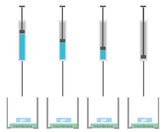
The robot arm transfers - step by step - the washing solutions and reagents to the reagent wells with the gel. After each washing step, vacuum is applied to soak out the washing solvent.



The setup allows extensive washing of the gel bands by transfer of organic and inorganic solvent in alternating steps for up to 20 times, each followed by 10 min of incubation.

Multi-dispense can be realised by the Chronos software and allows speeding-up of the overall process while avoiding cross-contaminations.

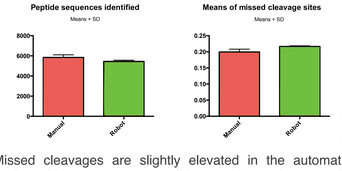




A self-constructed tray holder for a vacuum chamber that is connected to a vacuum pump. The vacuum is regulated by an electric valve controlled by the PAL software.

Quality control

In-solution digest



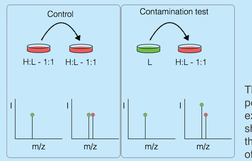
Yeast lysate was digested both manually and using the PAL in five replicates and measured on an orbitrap mass spectrometer.

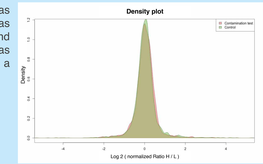
Peptide sequences identified after digestion with the PAL are slightly decreased. However, the overall reproducibility, as reflected by the standard deviations (SD), is increased.

Missed cleavages are slightly elevated in the automatic procedure. This can be explained by a better mixing of the samples in the manual procedure compared to the automated procedure where reactions take place in a 96-well plate.

Cross-contaminations

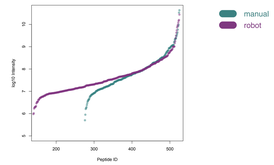
By using SILAC labelling, cross-contamination was evaluated. A yeast lysate from „light"-only cells was digested in wells next to a 1:1 mixture of „heavy" and „light" labelled lysate. As a control, a 1:1 mixture was placed next to another 1:1 mixture. In case of a contamination, an overall shift to „light" is expected.





The density plot shows the distribution of the H/L ratios of peptides for the contamination test and the control experiment. The curves almost overlap completely and no shift of the contaminant test to the left is visible, indicating that cross-contaminations are avoided by efficient washing of the syringe between each transfer step.

In-gel digest



A mixture of proteins was separated by SDS-PAGE and stained with Coomassie. Bands were cut out and digested by hand and by robot, respectively.

As a result, we gain more peptide identifications (412 vs. 270) in the automated procedure, most likely due to increased washing efficiency.

Summary

The PAL system provides an affordable and reliable platform optimised for medium-throughput peptide preparation for shotgun-proteomics based mass spectrometry