



SOP Title:

Extraction and quantitative analysis of proteins from FFPE tissue

The STORE processing methods were shown to be fit-for purpose for DNA, RNA and protein extraction from FFPE material. The STORE characterisation methods were shown to be fit for-purpose for Quality Control of the extracted DNA, RNA and proteins.

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**Standard Operating Procedure for
Extraction and quantitative analysis of proteins from FFPE tissue**

Created

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Summary

The following protocol describes the procedure of FFPE label-free quantitative proteomics analysis.

The quality of the FFPE tissue samples is examined by conventional histology and immunoblotting analysis. This is followed by protein extraction, using detergent containing buffer and accurate protein concentration measurement. The extracted proteins are subjected to be resolved on 1D SDS-PAGE before in-gel digestion and LC/MS-MS analysis. The obtained peptide spectra are quantified using the Progenesis software (Nonlinear).

Safety considerations

Gloves and protective clothing should be worn throughout.

Reagents and Equipments

Reagents needed:

SDS and ammonium bicarbonate (Sigma, St. Luis, MO); RapiGest from (Waters, USA); acetone, acetonitrile, formic acid, trifluoroacetic acid (TFA) (Roth, Karlsruhe, Germany); dithiothreitol (DTT), iodoacetamide, urea, thiourea, tris-(hydroxymethyl) aminomethane (Tris), CHAPS and IPG buffer 3-10 (GE Healthcare, Freiburg, Germany). Sequencing grade trypsin (Promega, Madison, WI); cyano-4-hydroxycinnamic acid (Bruker Daltonik, Bremen, Germany). All solutions are prepared using HPLC grade water (Roth, Karlsruhe, Germany).

Equipment needed:

Staining dishes for deparaffinization of FFPE sections, microcentrifuge (with rotor for 1.5 ml tubes), vortexer, water bath or heating block capable of reaching 100°C, thermomixer (e.g. Eppendorf, Hamburg, Germany).

Histology

To examine the quality of the FFPE samples, tissue morphology and the tissue structure of the samples are controlled by histology.

3 µm paraffin sections are cut from the paraffin embedded tissue blocks for HE staining. Deparaffinisation of the microscope slides is performed according to the following protocol: xylene (4 changes), 96 % (v/v) ethanol (3 changes), 70 % (v/v)

ethanol (3 changes) followed by 2 washes by de-ionized water. Each step is performed for 3 minutes.

For HE staining, slides are placed in Mayer's acid heamalum for 3 min, rinsed by running tap water 10 min, followed by incubating the slides in Eosin for 2 min. Slides are dehydrated in increasing ethanol series and mounted with Eukitt® (Sigma-Aldrich, Germany) and cover slips from Menzel-Glas (Menzel, Germany).

Images are taken with the Olympus Dotslide2.0® Slide scanning system.

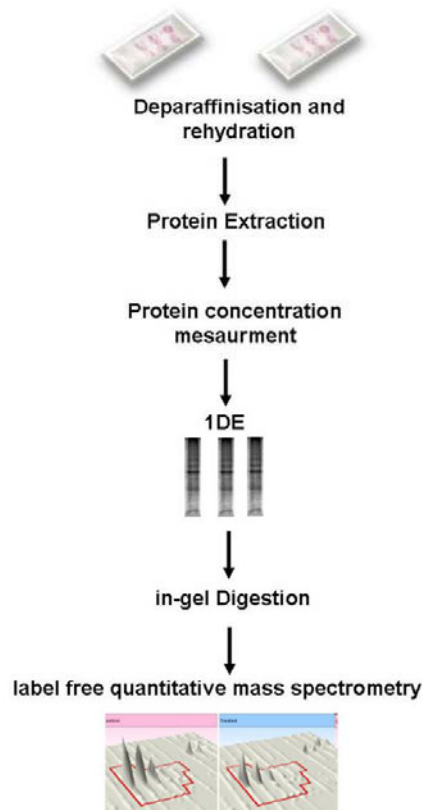
Immunoblotting Analysis

To examine the quality of the extracted proteins from FFPE samples, the samples are controlled by western blot and antibody detection as follow:

- Solubilise 20 µg samples of the protein extract from FFPE tissue in SDS-PAGE sample buffer, and separate by 12% SDS-PAGE under reducing conditions. ²
- Transfer proteins to nitrocellulose membranes (GE Healthcare) using a TE 77 semidry blotting system (GE Healthcare) at 1 mA/cm for 1h.
- Block the membranes using 3 % BSA in PBS, pH 7.4, for 1 h at room temperature wash three times in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl for 5 min
- Incubate overnight at 4°C with antibodies against candidate proteins using dilutions recommended by the manufacturer.
- Wash the membrane 3 times as described above.
- Incubate the blots with secondary antibody and developed using the ECL system (GE Healthcare) following standard procedures.

Workflow of FFPE tissue label free quantitative proteomics

Extraction and Quantitative Analysis of Proteins from FFPE Tissues using LC ESI /MS-MS



Protein extraction

The protein extraction and digestion would be performed as described before.¹

- Place FFPE tissue sections (20 μm thick, 80 mm^2) on microscope slides.
- Deparaffinise the slides by incubating twice with xylene for 10 min at room temperature before rehydration in a graded series of ethanol (100%, 95% and 70%) for 10 min each.
- Scrap the tissue sections from the slides and collect in 1.5 ml tubes.
- Wash the scrapped tissue with 0.5% beta-octylglucoside at RT for 10 min.
- Centrifuge the tubes at for 5 min at 14,000 g at 4°C.
- Resuspend the tissue in a extraction buffer containing 20 mM Tris-HCl, pH 8.8, 2% SDS, 1% beta-octylglucoside, 200 mM DTT, 200 mM glycine and mixture of protease inhibitor cocktail (Complete, Roche Diagnostics).
- Incubate samples in the buffer at 100°C for 20 min, and at 80°C for 2 h with shaking.
- Centrifuge the extract for 30 min at 14,000 g at 4°C.

- Precipitate protein extract with the 2D clean up kit (GE Healthcare) following the manufacturer's instructions.
- Quantify the protein amount from the pellet resuspended in Tris buffer in triplicate using both Bradford method and 2D Quant Kit (GE Healthcare) following the manufacturer's instructions.

Protein separation

1D SDS PAGE

- Solubilise 60 µg samples of the protein extract from FFPE tissue in SDS-PAGE sample buffer, and separate by 12% SDS-PAGE under reducing conditions.²
- Stain the gel with Brilliant blue G-250.³

In-gel Digestion

- Cut 1D stained gel slices from polyacrylamide gel and transfer into an Eppendorf tube.
- Wash 1x 10 minutes with 200 µl 60 % acetonitrile in 50 mM NH₄HCO₃.
- Wash 1x 10 minutes with 200 µl H₂O.
- Add 100 µl 5 mM DTT, incubate 15 minutes 60 °C.
- Remove DTT solution from gel pieces.
- Add 100 µl freshly prepared 25 mM iodacetamide (0.046 g / 10 ml), incubate 15 minutes at room temperature in the dark.
- Remove iodacetamid from gel pieces.
- Wash 1 x 5 minutes with 100 µl H₂O.
- Wash 1 x 10 minutes with 100 µl 100 % acetonitrile.
- Wash 1 x 10 minutes with 100 µl 50 mM ammoniumbicarbonate.
- Wash 1 x 10 minutes with 100 µl 60 % acetonitrile.
- Wash 1 x 10 minutes with 100 µl 100 % acetonitrile.

Prepare the trypsin stock with 1 mM HCl as described in the manual.

- Trypsin digestion: Overlay the gel pieces with 0,01 µg / µl trypsin diluted in 50 mM ammoniumbicarbonat, and digest the samples overnight at 37 °C.

- Peptide elution: Add 1-2 μl 0.5 % TFA, shake 15 minutes to stop the trypsin activity.
- Transfer supernatant to a tube and combine it with the solutions from the next two elution steps.
- Add enough volume of 60 % acetonitrile / 0.1 % TFA in order to cover the gel pieces completely and shake for 15 minutes.
- Add 100 % acetonitrile / 0.1 % TFA and shake again for 15 minutes and combine with the other fractions. As a result the gel pieces should shrink and become white.
- Dry samples in the speed vac to complete dryness.
- Re-dissolve in 2 % ACN / 0.5 % TFA.

Mass Spectrometry

LC-MSMS analysis is performed on an Ultimate3000 nano HPLC system (Dionex, Sunnyvale, CA) online coupled to a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific) by a nano spray ion source.

Peptides generated from in-gel digests of FFPE are acidified with TFA and automatically loaded to the HPLC system equipped with a nano trap column (100 μm i.d. \times 2 cm, packed with Acclaim PepMap100 C18, 5 μm , 100 \AA , Dionex) at a flow rate of 30 $\mu\text{l}/\text{minute}$ in 5% buffer B (80% acetonitrile, 0.1% formic acid (FA) in HPLC-grade water) and 95% buffer A (5% acetonitrile, 0.1% FA in HPLC-grade water). After 5 min, the peptides are eluted and separated on the analytical column (75 μm i.d. \times 15 cm, Acclaim PepMap100 C18, 3 μm , 100 \AA , Dionex) by a gradient from 5% to 50% of buffer B at 300 nL/minute flow over 140 min. Remaining peptides are eluted by a short gradient from 50% to 100% buffer B in 5 min. The eluting peptides are analysed by the LTQ OrbitrapXL. From the high resolution MS pre-scan, the five most intense peptide ions are selected for fragment analysis in the linear ion trap if they exceed an intensity of at least 200 counts and if they are at least doubly charged. The normalised collision energy for CID is set to a value of 35 and the resulting fragments are detected with normal resolution in the linear ion trap. The lock mass option is activated, and the background signal with the mass of 445.120020 is used as lock mass. Every ion selected for fragmentation is excluded for 30s by dynamic exclusion.

Label-Free Peptide Quantification

The label-free quantitative approach is performed using Progenesis software (version 2.5, Nonlinear).⁴ Profile data of the MS scans are transformed to peak lists with Progenesis LC-MS using a proprietary algorithm. This method uses wavelet-based filtering to smooth the peak envelopes and identify noisy areas, *i.e.* areas deemed to contain no ion peaks. Peaks are then modelled in non-noisy areas to record their peak m/z value, intensity, abundance (area under the peak) and m/z width. MS/MS spectra are transformed similarly and then stored in peak lists comprising m/z and abundance. After selecting one sample as a reference, the retention times of all other samples within the experiment are aligned by manually creating three to five landmarks followed by automatic alignment of all retention times to maximal overlay of the 2D feature maps. Features with only one charge or more than seven charges are masked at this point and excluded from further analyses. After alignment and feature exclusion, samples are divided into the appropriate groups (healthy and ERU), and raw abundances of all features are normalized. Normalization corrects for factors resulting from experimental variation and is automatically calculated over all features in all samples. It results in a unique factor for each sample that corrects all features in the sample in a similar way for experimental variation. After normalization, statistical analysis is performed using transformed (“log-like” arcsinh(.)function) normalized abundances for one-way analysis of variance (ANOVA) calculations of all detected features. No minimal thresholds are set neither for the method of peak picking nor selection of data to use for quantification. Co-detection across all runs ensures that abundance data used for relative quantification is obtained for every peptide ion in every run resulting in data variance that is representative of the full dataset. For quantification, all peptides (with Mascot score ≥ 30 and $p < 0.01$) of an identified protein are included and the total cumulative abundance is calculated by summing the abundances of all peptides allocated to the respective protein. Calculations of the protein p value (one-way ANOVA) are then performed on the sum of the normalised abundances across all runs. ANOVA values of $p \leq 0.05$ and additionally regulation of ≥ 1.5 -fold or ≤ 0.7 -fold are regarded as significant for all further results.

After quantification of peptides, those features not having an MS/MS scan from the initial samples run are exported to Excel (Microsoft) and used as an inclusion list for a replicate run of samples on the Orbitrap. Resulting raw data files are aligned to the

experiments in Progenesis and additional MS/MS scans resulting from these measurements are added to the previous ones; however, the original quantifications and statistics are not changed.

Database Search and Protein Identification

All MS/MS spectra are exported from the Progenesis software as Mascot generic file (mgf) and used for peptide identification with Mascot (version 2.2) in the Ensembl database for *Mus musculus*. Search parameters used are: 10 ppm peptide mass tolerance and 1-Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation is set as fixed modification and methionine oxidation, Phosphorylation of tyrosine, serine, and threonine are allowed as variable modifications. A Mascot-integrated decoy database search calculates a false discovery rate. Searching is performed on the concatenated mgf files with an ion score cut-off of 30 and a significance threshold of $p \leq 0.01$. Only peptides with ion scores of 30 and above and only proteins with at least one unique peptide ranked as top candidate (bold red in Mascot) are considered and re-imported into Progenesis software.

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