**A streamlined mass spectrometry-based proteomics workflow for**

**large scale FFPE tissue analysis**

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**Supplementary materials and methods**

Reference numbers refer to the main text list

**Disclaimer: This protocol includes hazardous chemicals and any use of it is strictly at your own risk (!) Read safety information**

**Detailed sample preparation protocol**

**REAGENTS**

• (!) 2,2,2-Trifluoroethanol (TFE; Sigma-Aldrich, 2860 Søborg, Denmark, cat. no. 96924-250ML-F)

• (!) 1,4-Dithiothreitol (DTT; Sigma-Aldrich, cat. no. 10197777001)

• (!) 2-Chloroacetamide (CAA; Sigma-Aldrich, cat. no. C0267)

• TRIS (Tris(hydroxymethyl)aminomethane, Sigma-Aldrich, cat no. T6066)

• Endoproteinase Lys-C (Wako Chemicals, Richmond, VA, USA, cat. no. 129-02541)

• Proteomics grade modified trypsin (Sigma-Aldrich, cat. no. T6567).

• (!) Acetonitrile (ACN; Merck, Darmstadt, Germany, cat no. 75-05-8)

• (!) Isopropanol (ISO; Sigma-Aldrich, cat. no. 1070222511)

• (!) Trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. 808260)

• (!) Ammonia solution, 25% (NH4OH; Merck, cat. no. 5330030050)

**EQUIPMENT**

• Eppendorf ThermoMixer C (Eppendorf, 2970 Hørsholm, Denmark, cat. no. 5382000015) with 96-well adapter (SmartBlock PCR 96, Eppendorf, cat no. 9.776 915) for PCR tubes.

• 8-channel 200 µl pipette and 8-channel 10 µl pipette.

• 0.2 ml 24-well PCR plate (Thermo Scientific, cat. no. AB-0624).

• Flat 8 Cap Strips (Thermo Scientific, cat. no. AB-0784).

• In-house made 96-well ‘swimming’ adapter for Bioruptor based sonication of PCR tubes. Note, plastic inlets from 200 µl tip boxes can be used for this purpose.

• Solid-phase extraction disks for SDB-RPS StageTips: Empore SDB-RPS (Sigma, cat. no. 66886-U). We use in-house made StageTips [51].

• StageTip Centrifuge STC-V2 (https://www.sonation.com/en/products/STZentrifuge/index.html). Alternatively, if many samples are prepared, we recommend a 3D printed device capable of holding 96 StageTips. A 3D printer employing fused deposition modelling such as the Zortax M200 or Ultimaker 3 instruments enable the rapid (~12 h) and inexpensive fabrication of these devices. If such a device is not available, a pipette-tip box may serve as a suitable StageTip holder.

• Evaporative concentrator: Eppendorf Vacuum Concentrator Plus with 96-well plate rotor.

**REAGENT SETUP**

**Stock solutions:**

1M Tris/HCl pH8 in ddH2O

500 mM 2-chloroacetamide (CAA) in ddH2O

100 mM 1,4-dithiothreitol (DTT) in ddH2O

0.5 μg/μl trypsin protease

0.5 μg/μl LysC protease

These buffers can be aliquoted and stored at -20 °C.

*CAUTION: CAA and DTT are toxic. Prepare this solution in a fume hood and handle with gloves.*

• **Lysis buffer**

Prepare TFE lysis buffer containing 50% (v/v) 2,2,2-trifluoroethanol (TFE), 300 mM Tris-HCl (pH 8.0).   
*CRITICAL: This buffer should be prepared fresh. TFE is highly volatile, close caps directly after pipetting.   
CAUTION: TFE is toxic. Prepare this solution in a fume hood and handle with gloves.*

*NOTE: The high Tris/HCl concentration (300 mM) in the lysis buffer assists in formaldehyde de-crosslinking* [2]

*NOTE: 50% TFE in the lysis buffer can be replaced by 50% acetonitrile for comparable results.*

• **Digestion buffer**

10% (v/v) TFE in ddH20. This buffer should be prepared fresh. Enzymes (trypsin and LysC) are added at a protein:enzyme ratio of 50:1.

• **SDB-RPS StageTip wash buffer 1**

1% (vol/vol) TFA in isopropanol. *CAUTION: TFA solutions are corrosive. Prepare the solutions in a fume hood and handle with gloves. This buffer is stable for >3 months at RT.*

• **SDB-RPS StageTip wash buffer 2**

0.2% (vol/vol) TFA. *CAUTION: TFA solutions are corrosive. Prepare the solutions in a fume hood and handle with gloves. This buffer is stable for >3 months at RT.*

• **SDB-RPS StageTip elution buffer**

1% ammonia, 80% ACN.

• **MS loading buffer**

0.2% TFA/2% (vol/vol) ACN. This buffer is stable for >6 months at RT.

**Tissue preparation**

Prior to tissue collection by macroscopic dissection or laser-capture microdissection (LCM), all FFPE samples were deparaffinized and hematoxylin-eosin (H&E) stained as described below.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Deparaffinization** | |  |  | **H & E staining** |  |
| **Step** | **Time** | |  | **Step** | **Time** |
| Oven at 60 °C | 10 min | |  | Mayer’s Hematoxylin | 1 min |
| Xylene | 10 min | |  | Water | 10 min |
| Xylene | 5 min | |  | Eosin | 30 s |
| 2 x 99 % ethanol | 5 min each | | | Water | 10 dips |
| 2 x 96 % ethanol | 5 min each | | | 70 % ethanol | 10 dips |
| 70% ethanol | 5 min | |  | 96 % ethanol | 10 dips |
| Water | 10 min | |  | 96 % ethanol | 10 dips |
|  |  | |  | 99 % ethanol | 10 dips |
|  |  | |  | 99 % ethanol | 10 dips |

Areas of interest were determined via microscopic inspection by pathologists. For macro-dissected samples, an area of roughly 5 mm x 5 mm was collected by scraping with a razor blade (5 µm or 10 µm thick section). Note, tissue collection into PCR tubes is enhanced with a small droplet (i.e. 3µl) of ddH20 on the scraped area and subsequent transfer by pipetting (supplementary material, Figure S1A). For LCM, FFPE tissues were mounted on PEN membrane slides to enable efficient cutting and collection.An area of approximately 1.5 mm x 1.5 mm (10 µm thick section, approx. 10,000 cells as calculated from dissected area × slide thickness / average mammalian cell volume of 2,250 µm3, BioNumber ID 100434)was collected into adhesive caps and tissue transferred in lysis buffer into PCR tubes for direct in-solution protein extraction and digestion.

**PROTOCOL**

***1. Tissue homogenization and formalin de-crosslinking (day 1,*** ~***2.5 h)***

* Add 100 µl lysis buffer (LB) to FFPE tissue collected into PCR tubes. Close PCR tubes with cap strips (see equipment).   
  *CRITICAL: Make sure that caps are tightly closed.*

*NOTE: We highly recommend working in PCR tubes. If samples were collected in different tubes or adhesive caps, transfer them into PCR tubes with lysis buffer. Make sure to collect all tissue pieces so that sample loss from transfer can be minimized.*

* Sonicate tissue (15 cycles in Bioruptor, high intensity, 30 s on/off cycle).   
  *NOTE: Efficient sonication will lead to a homogeneous tissue powder. For some samples this will only be achieved in a second sonication step after 90 min heating.*
* Centrifuge any condensation down
* Heat tissue at 90 °C for 90 min, no shaking.   
  *CAUTION: TFE is toxic, work under fume hood.   
  CAUTION: TFE is highly volatile and long heating times will result in overpressure during incubation. To minimize cap opening from overpressure during heating, heat-resistant material (i.e. metal plate) can be placed on top. After heating, a short 10 min cooling period to ~60* °C *will reduce high pressure to safely remove samples from the heating block.  
  NOTE: We found that overnight de-crosslinking at 65 °C offers a good alternative to avoid overpressure from high temperatures. Alternatively, a PCR machine can be used for controlled heating.*

*NOTE: 50% TFE in the lysis buffer can be replaced by 50% acetonitrile for comparable results (see supplementary material, Figure S1F–H).*

* Centrifuge down any condensation

***2. Protein reduction, alkylation and tryptic overnight digestion (day1/2, ~16 h)***

* Add DTT (5 mM final) and incubate 20 min at RT, 1500 rpm.

*CRITICAL: We noticed that during the long heating phase, buffer evaporation (if not avoided) can result in slightly different sample volumes after heating. If necessary, adjust sample volumes with ddH20 to the original 100 µl and then add DTT.*

* Add CAA (25 mM final) and incubate 20 min at RT, 1500 rpm.
* Vacuum-dry to a remaining volume of about 20 µl completely (~45 min at 60 °C).   
  *NOTE: Freeze or continue.*
* Add 80 µl freshly prepared digestion buffer including trypsin and LysC at an enzyme/protein ratio of 1:50. Incubate at 37 °C 1500 rpm, overnight.   
  *NOTE: Depending on tissue type and amount, enzyme amounts need to be determined empirically. We found that a tumor area of ~5 mm x 5 mm (10 µm thick section) resulted in 5-15 µg of total protein.*

*NOTE:* Sequential LysC (2-4h) and trypsin (overnight) digestion can be performed.

***3. Peptide clean-up (day 2,*** ~***1 h)***

* Add TFA to a 1% final concentration to acidify the solution and inactivate trypsin and LysC. Mix by pipetting and spin down 5 min to pellet any debris.

*NOTE: Freeze or continue.*

*CRITICAL: A low pH is required for peptide clean-up by SDB-RPS StageTips (see below)*

* ***Peptide purification via StageTips***
* Prepare SDB-RPS StageTip with two layers:
* Load sample directly on SDB-RPS StageTips
* Wash with 200 µl wash buffer 1
* Wash with 200 µl wash buffer 2
* Change collection plate and add 50 µl elution buffer
* Vacuum-dry completely (*~* 30 min at 45 °C),
* Reconstitute peptides in 10 µl MS loading buffer. Measure A280 nm on a Nanodrop spectrophotometer. Store peptides at -20 °C until LC-MS analysis and inject 250-500 ng.  
  *NOTE: A distinct 280 nm peak is indicative of a pure peptide sample.*   
  *NOTE:* Tissue staining such as H&E are largely retained in the filter tip and are not eluted into the sample.

**Sample preparation for proteomics by FASP and RapiGest**

FFPE glioma tissue was used for protocol comparison (Figure 1C–F). Three identical areas from three consecutive sections of the same tissue were collected by macro-dissection into 1.5 ml. Deparaffinization was performed as described above.

FASP method

FASP sample preparation was adapted to the experimental layout of the TFE workflow and performed as described earlier [27]. In brief, proteins were extracted using 100 µl of lysis buffer (1.5% SDS, 50 mM DTT, 100 mM Tris-HCl, pH 7.6), sonication for 15 cycles and heating at 99 °C for 60 min. Subsequent to repeated sonication for 15 cycles, centrifugation at high-speed enabled clearance of the lysate and the supernatant was transferred to a new tube. The following steps of centrifugation comprised 25 min at 10 000 g and 20 °C if not stated otherwise. Samples were diluted by the factor of 2.5 using wash buffer (8 M Urea, 100 mM Tris-HCl, 1 mM DTT, pH 8.5) in a Microcon® 30 centrifugal filter (Merck Millipore). The step was repeated six times using 250 µl wash buffer to remove SDS from the sample. Protein alkylation was achieved by incubation for 20 min in alkylation buffer (50 mM IAA, 8 M Urea, 100 mM Tris-HCl, 1 mM DTT, pH 8.5) and subsequent centrifugation for 10 min. The samples were further purified using wash buffer and digestion buffer (50 mM Tris, 1 mM DTT, pH 8.5) three times by centrifugation, respectively. Proteins were digested using 100 µl of digestion buffer supplemented with an enzyme:protein ratio of 1:50 in a wet chamber at 37 °C overnight. Elution of peptides was achieved using centrifugation and two further washing steps of elution buffer for 10 min, respectively. The enzymatic reaction was quenched by TFA in a final concentration of 1% and MS purification was performed as described for the TFE protocol.

RapiGest method

The previously described RapiGest tissue lysis [9] was adapted to the TFE workflow and accomplished by the commercially available surfactant (RapiGestTM SF Surfactant, Waters, Milford, MA, USA). Tissue lysis employed 100 µl of lysis buffer (0.2% RapiGest, 50 mM ammonium bicarbonate [ABC]) and two heating steps at 95 °C for 30 min and 80 °C for 120 min interrupted by sonication for 15 cycles. Reduction and alkylation was achieved using 5 mM DTT and 25 mM CAA with an incubation at room temperature for 20 min, respectively. The lysate was diluted two-fold with ABC and enzyme was added in an enzyme:protein ratio of 1:50. After overnight digestion at 37 °C, the reaction was quenched with 1% TFA (final concentration) for 45 min at 37 °C. The samples were centrifuged at high-speed and supernatants transferred to a new tube followed by peptide clean-up as described in the detailed TFE protocol.

**LC-MS/MS analysis**

Nanoflow LC–MS/MS analysis of tryptic peptides was conducted on a quadrupole Orbitrap mass spectrometer (Q Exactive HF-X, ThermoFisher Scientific, Bremen, Germany) [5] coupled to an EASY nLC 1200 ultra-high-pressure system (Thermo Fisher Scientific) via a nano-electrospray ion source. Three hundred ng of peptides were loaded on a 50-cm HPLC-column (75 μm inner diameter, New Objective, Woburn, MA, USA; in-house packed using ReproSil-Pur C18-AQ 1.9-µm silica beads; Dr Maisch GmbH, Ammerbuch, Germany).

Peptides were separated using a linear gradient from 2 to 20% B in 55 min and stepped up to 40% in 40 min followed by a 5 min wash at 98% B at 350 nl per min where solvent A was 0.1% formic acid in water and solvent B was 80% ACN and 0.1% formic acid in water. The total duration of the run was 100  min. Column temperature was kept at 60 °C using an in-house-developed oven.

For DDA analysis, the mass spectrometer was operated in ‘top‐15’ data‐dependent mode, collecting MS spectra in the Orbitrap mass analyzer (60,000 resolution, 300–1,650 m/z range) with an automatic gain control (AGC) target of 3E6 and a maximum ion injection time of 25 ms. The most intense ions from the full scan were isolated with an isolation width of 1.4 m/z. Following higher‐energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 27, MS/MS spectra were collected in the Orbitrap (15,000 resolution) with an AGC target of 1E5 and a maximum ion injection time of 28 ms. Precursor dynamic exclusion was enabled with a duration of 30 s. For BoxCar acquisition [35], the mass spectrometer was operated with MaxQuant.Live [52].

The DIA method consisted of one MS1 scan (350 or 300 to 1,650m/z, resolution 60,000 or 120,000, maximum injection time 60 ms, AGC target 3E6) and 32 segments at varying isolation windows from 14,4 m/z to 562,8 m/z (resolution 30,000, maximum injection time 54 ms, AGC target 3E6). Stepped normalized collision energy was 25, 27.5 and 30. The default charge state for MS2 was set to 2.

**MS data analysis**

DIA raw files were analyzed using Spectronaut Pulsar X software (Biognosys, version 12.0.20491.17) and default settings for targeted DIA analysis with the ‘mutated’ as decoy method. We used a project-specific spectral library encompassing 197,622 precursors, corresponding to 10,707 protein groups. The matching library was prepared using the same protocol and was comprised of FFPE tissue of OvCa, adenoma and glioma, as well as AML and fractionated using the high pH reversed-phase fractionator as previously described [37]. In brief, we fractionated a total of about 30 µg of peptides that was automatically concatenated into 8 or 24 fractions using a rotating valve that switches the elution flow every 90 sec. For adenoma tissue analysis (Figure 3 and 4), we used an adenoma tissue specific library, encompassing 7,725 protein groups (77,275 precursors). Data export was filtered by ‘No Decoy’ and ‘Quantification Data Filtering’ for peptide and protein quantifications.

DDA raw files were processed in the MaxQuant environment [22] (version 1.5.0.38 or 1.6.7.0). The integrated Andromeda search engine [53] was used for peptide and protein identification at an FDR of less than 1%. The human UniProtKB database (October 2017) was used as forward database and the automatically generated reverse database for the decoy search. ‘Trypsin’ was set as the enzyme specificity. Search criteria included carbamidomethylation of cysteine as a fixed modification, oxidation of methionine, acetyl (protein N-terminus) and lysine methylation (as stated) as variable modifications. We required a minimum of 7 amino acids for peptide identification. Proteins that could not be discriminated by unique peptides were assigned to the same protein group. Label-free protein quantification was performed using the MaxLFQ [29] algorithm and ‘match-between-runs’ was enabled. We used a minimum ratio count of one peptide and filtered out proteins with only one razor or unique peptide. Proteins, which were found as reverse hits or only identified by site-modification, were filtered out.

**Immunohistochemistry**

Sections freshly cut from FFPE blocks at 3 µm thickness were dewaxed in xylene and rehydrated through a graded series of ethanol. Antigen retrieval was carried out by boiling the sections for 10 min in a conventional microwave oven in target retrieval solution (pH 6), followed by a 30 min cool-down period at room temperature. To block endogenous peroxidase activity, sections were treated with 1% hydrogen peroxide for 10 min. Sections were incubated with primary antibody overnight at 4 °C. Mouse anti-CD44 (144M-94, Cell Marque, Rocklin, CA, USA) and rabbit anti-CDX2 (235R-15, Cell Marque) antibodies were used at 1:1500 and 1:2000 dilution, respectively. Bound primary antibody immune complexes were detected with HiDef Detection HRP Polymer System (Cell Marque), and then visualized using DAB+ substrate-chromogen system (Agilent Technologies, Glostrup, Capital Region, Denmark) for 5 min. Between incubations, the sections were washed thrice with TBS buffer containing 0.5% Triton X-100. The sections were counterstained with Mayer’s hematoxylin. Both positive and negative control tissues were included. Tissue with verified expression of CDX2 and CD44 was used as a positive control, and tissue with low or no expression as a negative control, as stated in the figure legends (supplementary material, Figure S3). As an additional negative control to test for unspecific binding with the HiDef kit, a set of all control tissues were stained as described above, but with diluent instead of primary antibody.