DELIVERABLE D3 – SOP

Grant Agreement number	15HLT02 ReMIND
Project short name	ReMind
Project full title	**Role of metals and metal containing biomolecules in neurodegenerative diseases such as Alzheimer's disease**
Version numbers of latest contracted Annex 1 and Annex 2 against which the assessment will be made	Annex 1: V# Annex 2: V#
Period covered (dates)	From 01July 2016 To 28 February 2019

For JRPs:

Project start date and duration:	1 July 2016, 36 months	
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Internal Funded Partners:	External Funded Partners:	Unfunded Partners:
1 PTB, Germany 2 BAM, Germany 3 LGC, UK 4 TUBITAK, Turkey	5 Charité, Germany 6 UGent, Belgium 7 UNIABDN, UK 8 UNIVIE, Austria	9 10 11 12

HTL02 ReMiND WP3

Deliverable 3 (D3). Standard Operating Procedure for the Quantification of SOD1 in body fluids

1 Terms and abbreviations

- Ar: argon
- CAS: Chemical Abstracts Service
- $CHCl_3$: chloroform
- CSF: cerebrospinal fluid
- Cu: copper
- DAD: diode array detector
- EtOH: ethanol
- ESI-MS: electrospray ionisation mass spectrometry
- HAc: acetic acid
- HGB: haemoglobin
- HNO_3: nitric acid
- HPLC: high performance liquid chromatography
- ICP-MS: inductively coupled plasma mass spectrometry
- IDMS: isotope dilution mass spectrometry
- ME: measurement equipment
- MWCO: molecular-weight-cut-off
- NH_4Ac: ammonium acetate
- PP: polypropylene
- Sample spike blend bx: mix of erythrocyte sample and SOD1 spike material
- Reference spike blend bz: mix of SOD1 reference and spike material
- r.t.: room temperature
- SOD1: Cu,Zn-superoxide dismutase
- SOP: standard operating procedure
- Spike material: SOD1 containing Cu enriched in ^{65}Cu and Zn enriched in ^{67}Zn
- T: temperature
- TRA: time resolved analysis
- Tris: Tris(hydroxymethyl)aminomethane
- Zn: zinc

2 Description

Cu,Zn-superoxide dismutase (SOD1) is an homodimeric enzyme which contains a closely spaced non-covalently bound Cu and Zn ion pair per subunit and has a molecular mass of about 31800 g mol^{-1} for the human species. It catalyses the disproportionation of the superoxide anion (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The Cu ion is responsible for the enzymatic activity as the disproportionation of O_2^- is driven by the [Cu(II)/Cu(I)] ion redox cycle. The protein plays a role in medical diagnostics. SOD1 activity is known to decrease in malignant lymphoma, chronic alcoholism and medullar carcinoma; its activity is increased in leukemia. It can be used as a prenatal marker for Down's syndrome and is involved in neurodegenerative diseases such as Alzheimer's disease. Furthermore, it is a potential marker for gastric cancer. When results from the quantitative determination of SOD1 are used for clinical diagnostics, it is of great importance that the results of the protein quantification are comparable and reliable (inter- and intra-laboratory). In routine or clinical laboratories, immunoassays such as enzyme-linked immunosorbent assays (ELISA) or spectrophotometric assays are used to quantify SOD1, but there are no reference methods or materials available yet. Comparison of some of these methods shows large differences in the results, the limits of quantification (LODs) and the reproducibility. None of these clinical assays is based on metrological principles and results are not traceable to the International System of Units (SI) as demanded by EU regulations such as 2017/745 and 2017/746. Furthermore, no uncertainty budget according to the Guide to the Expression of Uncertainty in Measurement (GUM) has been estimated for the quantification of SOD1 for these methods.

Species-specific double IDMS is used in this reference measurement procedure for the quantification of SOD1 via its Cu content. By measuring the Cu containing part of the SOD1, only the amount of active protein is quantified, which is the important quantity. Thus, the method can serve as a reference method of higher order for routine measurements. The basic principle of IDMS is the change of the natural isotopic composition of the interesting element in the sample by the addition of a substance enriched in a minor isotope of this element, called spike. For the double IDMS approach, a blend of a reference material with the spike, the reference spike blend, is prepared and both, sample spike and reference spike blend, are measured using ICP-MS. In the reference material, the analyte is well characterised and acts as a back spike. In species-specific IDMS, the species-specific spike material (here isotopically labelled SOD1) is added to the sample as soon as possible before any sample preparation and separation, which has the advantage that any losses or changes of the analyte during the sample preparation process can be compensated. Commercially available SOD1 was characterised for use as a reference material and the species-specific spike material was prepared in-house from this. To avoid interferences from HGB in erythrocytes, HGB has to be precipitated before measuring the samples. Both, sample spike and reference spike blend, share the same fate during sample preparation and measurement.

3 Chemicals and materials

3.1 Warning and safety precautions

When handling proteins and erythrocytes of human origin suitable protective gloves have to be worn (e.g. nitrile gloves) and disinfection solution (e.g. Meliseptol®, B. Braun, Melsungen, Germany) has to be available at all times. Contaminated surfaces have to be disinfected immediately and contaminated clothes and gloves have to be removed. Contaminated waste has to be discarded in containers designated for this kind of waste. After finishing the work all surfaces have to be cleaned and disinfected as well as the hands.

3.2 Article of daily use

- Low protein binding tubes (1.5 mL and 2.0 mL, Eppendorf, Hamburg, Germany)
- Aluminium boats 0.12 mL (L12/W4/H2,7 mm) (e.g. IVA Analysentechnik, Meerbusch, Germany)
- Antistatic gun
- MWCO filter with 3000 g mol^{-1} cut-off (e.g. Amicon Ultra Centrifugal Filter von Merck Millipore, Darmstadt, Germany)
- HPLC-Vials (2.0 mL) with 250 µL Inlets (e.g. Agilent, Waldbronn, Germany)
- Various pipettes (e.g. Eppendorf, Hamburg, Germany)
- Duran bottles (1 L, 2 L) (e.g. Schott, Mainz, Germany)

3.3 Instrumentation

- Vortex Mixer, e.g. ZX4 (VELP Scientifica, Usmate, Italy)
- pH Meter, e.g. pH 3110 (WTW, Weilheim, Germany)
- Magnetic stirrer, e.g. RCT basic (IKA, Staufen, Germany)
- Microbalance with a precision of ≤ 0.05 mg, e.g. MC210S (Sartorius, Göttingen, Germany)
- Top-loading balance (e.g. BP2100S, Sartorius, Göttingen, Germany or ME4002, Mettler-Toledo, Gießen, Germany)
- Multimeter, e.g. testo 650 (Testo GmbH und Co. KG, Lenzkirch, Germany)
- Centrifuge, temperature controlled, e.g. Sigma 3-16PK (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany)
- Freeze dryer, e.g. Alpha 1-4 LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Deutschland)
- HPLC equipped with degasser, binary pump, temperature controlled autosampler and oven, UV/Vis detector (e.g. Agilent 1200, Agilent Technologies, Santa Clara, USA)
- Quadrupole ICP-MS equipped with temperature controlled Scott spraying chamber, microconcentric nebulizer, shielded torch with a 1.0 mm injector, lens system fit for high matrix content, collision/reaction cell (e.g. Agilent 7700x, Agilent Technologies, Santa Clara, USA)

3.4 Reagents

Reagent	Purity	Storage conditions	Molar mass / g mol^{-1}	CAS	Hazards	Precautions
human SOD1	≥ 80 % (Biuret)	-20 °C	31872	9054-89-1	-	-
human SOD1 enriched in ^{65}Cu and ^{67}Zn	produced from SOD1 named above	-20 °C	31878		-	-
Tris	ultra, ≥ 99.0 %	r.t.	121.44	77-86-1	-	-
NH_4Ac	ultra, ≥ 99.0 %	sufficient ventilation	77.08	631-61-8	-	-
HAc	100 %, suprapure	sufficient ventilation	60.05	64-19-7	H226-H314	P210-P260-P280-P303 + P361 + P353-P305 + P351 + P338-P370 + P378
HNO_3	65 %, p.a, subboiled	sufficient ventilation	63.01	7697-37-2	H272-H290-H314	P210-P220-P260-P280-P305 + P351 + P338-P370 + P378
Ar	5.0 (99.9990 %)	pressurised gas	39.948	7440-37-1	H280	P403
$CHCl_3$	HPLC grade	sufficient ventilation	119.38	67-66-3	H302-H315-H319-H331-H351-H361d-H372	P302 + P352- P304 + P340-P305 + P351 + P338- P308 + P310
EtOH,	HPLC grade	sufficient ventilation	46.07	64-17-5	H225-H319	P210-P240-305 + P351 + P338-P403 +

						P233

Comment: Details of the Hazard and precaution codes can be found at e.g. the MSDS pages of Sigma

4 Required solutions

- 12.5 mM Tris (1.51 g) in ultrapure water (1000.00 g), pH 7.2
- 12.5 mM Tris (1.51 g) + 125 mM NH_4Ac (9.64 g) in ultrapure water (1000.00 g), pH 7.2
- If necessary precipitation reagent for HGB precipitation: $CHCl_3$ (500 µL) and EtOH (1500 µL) ratio 1:3

5 Sample preparation

5.1 Storage of body fluids

For the determination of SOD1, CSF samples should be stored cooled in PP tubes (protein lobind). For short-term storage (up to two weeks) a storage temperature of 4 °C is sufficient, whereas for longer periods of time (up to six weeks) the samples should be stored at -20 °C. For long-term storage (several months) a storage temperature of -80 °C is recommended. Freeze/thaw cycles should be avoided.

Brain tissue samples should be stored at -80 °C.

5.2 Preparation of required solutions

Glassware and PFA bottles have to undergo a special cleaning procedure to minimise Cu and Zn contamination. This includes washing in the dishwasher followed by cleaning with 10% HNO_3 for several hours (at least 3 h). Finally, the bottles and glassware are rinsed with ultrapure water three times and dried in the drying cabinet. All vessels have to be discharged using the antistatic gun before weighing.

All solutions are prepared gravimetrically and the weights are corrected for air buoyancy. Therefore, humidity, temperature and pressure are noted at each weighing step. All vessels, solutions and substances are stored for at least 1 h in the balance room for acclimatisation before weighing.

Preparation of protein reference solution

→ Stock solution (using the microbalance)

- Weighing of 1.5 mL tube
- Addition of lyophilised SOD1, weighing
- Addition of 1000 µL 12.5 mM Tris, pH 7.2 using a pipette, weighing
- Shaking for 5 min at 800 rpm

- Storage at 4 °C

→ Dilutions (using the microbalance)

Dilute the stock solution with 12.5 mM Tris buffer (pH 7.2) to a concentration of about 0.1 mg/g SOD1:

- Weighing of empty 1.5 mL tube
- Addition of 12.5 mM Tris, pH 7.2, weighing
- Addition of SOD1 stock solution, weighing
- Storage at 4 °C

Preparation of protein spike solution

→ Apo-SOD1: commercially available SOD1 is dissolved in 10 mM NH_4Ac + 5 mM EDTA buffer (pH 3.8). The protein solution is transferred into a dialysis membrane with a MWCO of 5000 g mol^{-1}. The dialysis is performed for 24 h at r.t. with 300 mL of 10 mM NH_4Ac + 5 mM EDTA buffer (pH 3.8). The buffer is changed every hour. The second (dialysis against 10 mM NH_4Ac + 100 mM NaCl (pH 3.8)) and the third dialysis step (dialysis against 10 mM NH_4Ac (pH 3.8)) are performed following the same conditions and the buffer is also changed every hour. Alternatively, the last two dialysis steps can be replaced by desalting the produced apo-SOD1 with PD-10 desalting columns. The dialysed proteins were freeze-dried (e.g. using the Freeze dryer Alpha 1-4 LSCplus) and stored at -20 °C prior to further use.

→ Remetallation: the freeze-dried apo-SOD1 is dissolved in 10 mM NH_4Ac buffer at pH 6.8. Firstly, ^{67}Zn in threefold surplus is added and the mixture is incubated for 12 h at r.t. Secondly, ^{65}Cu in threefold surplus is added to the solution and again the mixture is incubated for 24 h at r.t. Finally, the excess metals are removed by centrifugal filtration using a spin-filter with MWCO of 3000 g mol^{-1}. The concentrated remetallated protein was washed with buffer three times, freeze-dried and stored at -20 °C. Alternatively, the surplus metal ions can be separated by desalting the prepared protein spike with the help of PD-10 desalting columns.

→ Spike stock solution (using the microbalance)

- Weighing of the 1.5 mL cup with lyophilised protein spike material
- Addition of 1000 µL 12.5 mM Tris, pH 7.2, weighing
- Shaking of the solution for about 1 min at 800 rpm
- Weighing of empty 1.5 mL tube
- Transfer of the spike solution into the empty tube, weighing
- Cleaning, drying and weighing of the tube in which the spike material was stored and calculation of the weight of the spike material by differentiation

→ Dilutions (using the microbalance)

- Dilute the stock solution with 12.5 mM Tris buffer (pH 7.2) to a concentration of (0.1 – 0.33) mg g^{-1} SOD1 spike:
- Weighing of empty 1.5 mL tube
- Addition of 12.5 mM Tris, pH 7.2, weighing
- Addition of SOD1 spike stock solution, weighing
- Storage at 4 °C

5.3 Preparation of sample spike and reference spike blends

All solutions necessary to prepare the required blends have to be shaken at 500 rpm for 1 h for homogenisation in the balance room for temperature adjustment. When preparing the blends, it is important that the ratio of reference and spike isotope in the sample is as close to 1 as possible. The blend bz has to be similar to the blend bx in both concentration and isotope ratio. In case of an unknown sample, the mass fraction of SOD1 (w_x) should be estimated previously by for example external calibration. At least three blends bx and three blends bz have to be prepared independently using the microbalance.
Preparation of sample spike blend bx (using the microbalance)

- Weighing of empty 1.5 mL tube
- Addition of body fluid (about (100 – 200) µL) and weighing
- Addition of SOD1 spike to achieve the intended isotope ratio and weighing
- Shaking for 1 h at 800 rpm using the vortex mixer

Preparation of reference spike blend bz (using the microbalance)

- Weighing of empty 1.5 mL tube
- Addition of SOD1 reference to achieve a SOD1 concentration similar to the one expected in the erythrocytes and weighing
- Addition of SOD1 spike to achieve the intended isotope ratio and weighing
- Shaking for 1 h at 800 rpm using the vortex mixer

Further sample preparation when erythrocytes are used as sample

- After homogenisation the solutions are centrifuged at 12000 rpm for 15 min at 4 °C
- Transfer of supernatant into another tube and discarding of solid which consists of cell fragments
- Addition of precipitation reagent to remove HGB at 1:1 ratio solution:reagent and shaking for 20 min at 800 rpm using the vortex mixer
- Centrifugation at 12000 rpm for 15 min at 4 °C
- Transfer of supernatant into another tube and discarding of the solid which consists mainly of HGB

- Removal of solvent by centrifugation of solutions for 45 min at 4500 rpm and 4 °C using MWCO filter with a cut-off of 3000 g mol^{-1} which has been conditioned according to filter manual
- Discarding of filtrate and cleaning of the residue with 1 mL ultrapure water and additional centrifugation for 45 min at 4500 rpm and 4 °C
- Discarding of filtrate, transfer of the residue into a tube and addition of ultrapure water to achieve the original blend volume

5.4 Measurement

To prepare the hyphenated HPLC-ICP-MS system for measurement and for optimisation of the system refer to the according manuals.
Transfer the blend solutions into HPLC vials with 250 µL inlets and quantify them using double IDMS. For the determination of the mass discrimination of the system, inject a protein solution without spike at least three times for and at the end of each sequence. Every blend solution has to be measured at least three times. A so called bracketing sequence should be used for measuring SOD1: bz_1, bx_1, bz_2, bx_2, bz_3, bx_3, bz_4, bx_4, bz_5, bx_5, bz_6, and so on. The peak of SOD1 is expected at a retention time of (15.55 ± 0.5) min.
Conditions:

ICP-MS	
RF power	1500 W
Sampling depth	(7.0 - 8.0) mm
Spraying chamber temp	2 °C
Isotopes detected	63, 65, 66, 67
Collision gas	(4.5 – 6.0) mL/min He
Cones	Ni
Cell entrance	-30 V
Cell exit	-50 V
Mode	TRA
Integration time	0.1 s
Measurement time	1500 s
Repetitions	1
HPLC	
Autosampler temp	4 °C
Injection volume	50 µL
Flow rate	0.5 mL/min
Column	MonoQ® 5/50 GL

Column temp	30 °C
Eluent	A: 12.5 mM Tris, pH 7.2
	B: A + 125 mM NH4Ac, pH 7.2
Gradient	(0 – 5) min, 7% B
	(5 – 10) min, (7 – 50)% B
	(10 – 12) min, 50% B
	(12 – 17) min, (50 – 86)% B
	(17 – 19) min, 86% B
	(19 – 20) min, (86 – 100)% B
	(20 – 25) min, 100% B

Quality Assurance

Prior to first-time use a function test measuring a protein-mix containing conalbumin, α-lactalbumin and soybean trypsin inhibitor applying an appropriate measurement method should be performed. The resulting chromatogram is used as reference chromatogram for future function tests. The performance of the separation of the column has to be checked regularly by injecting the performance check solution named above prior to every measurement sequence. When a decreasing separation performance or an increasing column back pressure is observed, the column has to be cleaned following the instructions in the column handbook. If necessary, the column needs to be replaced. The analyte peak shape has to be similar in both reference spike and sample spike blend and the retention time must not vary by more than 0.5 min. Otherwise the column has to be cleaned or replaced.

All relevant information about the performance of the instruments has to be recorded in the instrument's logbooks of HPLC and ICP-MS as well as in the scientist notebook and included in the report.

5.5 Data evaluation

For the evaluation the equations for the double IDMS approach.

To calculate the isotope ratios in bx and bz, the peak areas in the according chromatograms of the various isotopes are integrated using a proper software recommended by the manufacturer of the ICP-MS and the ratio is calculated according to:

$$R_{\mathrm{bx}} = \frac{A_{\mathrm{yx}}}{A_{\mathrm{x}}};\ R_{\mathrm{bz}} = \frac{A_{\mathrm{yz}}}{A_{\mathrm{z}}};$$

R_{bx}, R_{bz},	isotope ratio in the blends bx and bz, resp.
A_{yx}, A_{yz}	peak area of the spike isotope (m/z = 65) in the blends
A_{x}, A_{z},	peak area of the reference isotope (m/z = 63) in the blends

Double IDMS is used because in this case the reference serves as a kind of back spike to characterise the isotopically enriched spike material and, therefore, it is not necessary to know the exact mass fraction of the protein in the spike. The concentration of SOD1 in the sample is calculated according to:

$$w_x = w_z * \frac{m_{yx}}{m_x} * \frac{m_z}{m_{yz}} * \frac{R_y - R_{bx}}{R_{bx} - R_x} * \frac{R_{bz} - R_z}{R_y - R_{bz}}$$

w_x	mass fraction of SOD1 in sample x
w_z	mass fraction of SOD1 in reference z
m_z	mass of reference z in the blend bz
m_{yz}	mass of spike y in the blend bz
m_x	mass of sample x in the blend bx
m_{yx}	mass of spike y in the blend bx
R_z	isotope ratio of SOD1 in reference z
R_{bz}	isotope ratio of SOD1 in blend bz
R_x	isotope ratio of SOD1 in sample x
R_{bx}	isotope ratio of SOD1 in blend bx
R_y	isotope ratio of SOD1 in spike y

To calculate the mass fraction of SOD1 in reference w_z, the purity of the protein used as reference has to be determined using various methods such as amino acid analysis, ESI-MS as well as total metal quantification and post-column IDMS.
For the isotope ratios R_x and R_z the isotope abundances listed in the IUPAC table can be used as the natural variation of Cu is too small to be detected by quadrupole ICP-MS. R_y has to be measured beforehand.

A full uncertainty budget for w_x has to be estimated according to the Guide of the Expression of Uncertainty in Measurement (GUM).

5.6 Reporting

The report should include:

- Identification of source and type of sample,
- Date of sampling and date of measurement,
- Reference to measurement method and/or measurement procedure employed,
- Results with measured quantity name, numerical value, and measurement unit,
- Statement of measurement uncertainty,
- Observations of unusual properties of sample which might influence the measurement,

- Observations as regards unusual features of the measurement procedure or use of modifications, physiological and clinical information, if relevant.

6 Literature

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