Sample Preparation and Protocols

MIDAS

Protein constructs: MIDAS can be performed on any soluble protein, domain, or complex with a MW ≥ 15 kDa. MIDAS is permissive to a variety of epitope- (e.g. His, FLAG, etc.) and protein/domain-tags (GST, MBP, etc.) commonly used for protein purification. Analysis of proteins with protein/domain-tags will require parallel and independent analysis of the protein/domain-tag to subtract off-target interactions.

Protein sample preparation: Proteins for MIDAS analysis require purification to ≥ 95% purity to prevent false positive discovery from off-target interactions. Protein samples should be provided in LC-MS grade 150 mM ammonium acetate pH 7.4 (titrated with NH_4OH). Freeze-thaw solubility of the protein in this solution should be determined by the user prior to sample shipment. Cryoprotectants, reducing agents, non-volatile salts, additives, etc. should be avoided. Please contact the Core director to discuss necessary protein sample buffer amendments. The sensitivity of the MIDAS platform is directly proportional to the concentration of the protein sample. For optimal discovery, we suggest a protein sample concentration of **500 µM**. If high protein concentration is not possible, we recommend a protein sample concentration between 100 – 500 µM. Protein concentrations below 100 µM must be discussed with the Core director. For MIDAS analysis (triplicate dialysis across all for metabolite pools), we require **150 µL** of snap-frozen protein sample shipped on dry ice.

Protein sample buffer: Proteins for MIDAS analysis must be provided in LC-MS grade 150 mM ammonium acetate pH 7.4 (pH titrated with NH_4OH). Freeze-thaw solubility of the protein in this solution should be determined by the user prior to sample shipment. Cryoprotectants, reducing agents, non-volatile salts, additives, etc. should be avoided. Please contact the Core director to discuss necessary protein sample buffer amendments.

MIDAS protein-metabolite screening: The day of MIDAS screening, a number of MIDAS metabolite library, 384-well small volume working stock plates, corresponding to the number of proteins to be screened (eight proteins per plate), are defrosted at 30°C for 5 minutes and metabolites were combined de novo to generate four predetermined MIDAS screening pools. The MIDAS screening pools are prepared in LC-MS grade 150 mM ammonium acetate pH 7.4 and pH-adjusted with ammonium hydroxide. The majority of metabolites are prepared to a final screening concentration of 50 µM in the metabolites pools, with a subset at higher or lower concentration dependent on their FIA-MS ionization properties. For each metabolite pool, 8 µL of target protein is arrayed in a minimum of a triplicate across a 10 kDa MWCO 96-well microdialysis plate and sealed with aluminum foil seals to create the protein chambers. To the reverse side, 300 µL of metabolite pool is aliquoted per target protein replicate and sealed with aluminum foil seals to create the metabolite chambers. Where necessary and just prior to screening, proteins provided in alternative buffer systems were in situ, sequentially exchanged into 150 mM ammonium acetate pH 7.4 on the 96-well microdialysis screening plate. Loaded dialysis plates are placed in the dark at 4°C on a rotating shaker (120 rpm) and incubated for 40 hours. Post-dialysis, protein and metabolite chamber dialysates are retrieved, sample volume normalized and diluted 1:10 in 80% methanol to precipitate protein, incubated 30 mins on ice, and centrifuged at 3200 x g for 15 mins to sequester precipitated protein. Processed protein and metabolite chamber dialysates are retrieved and arrayed across a 384-well microvolume plate, sealed with a silicon slit septum cap mat, and placed at 4°C for FIA-MS analysis.

MIDAS flow injection analysis mass spectrometry analysis: All MIDAS metabolite pool FIA-MS is performed on a Shimadzu Nexera HPLC system equipped with binary LC-20ADXR pumps and a SIL-20ACXR autosampler coupled to a SCIEX X500R ESI-QTOF MS. Briefly, 2 µL of each processed protein and metabolite chamber dialysate (~10 pmoles per metabolite, depending on metabolite) is injected in technical triplicate with blanks injections interspersed between technical replicates. Mobile phase flow rate is 0.2 mL/min. The following mobile phases were used according to the MIDAS metabolite pool being analyzed: pool 1, 5 mM ammonium acetate pH 5, 50% methanol; pools 2 and 4, 5 mM ammonium acetate pH 6.8, 50% methanol; pool 3, 10 mM formic acid pH 3, 50% methanol. Pools 1 and 2 are analyzed in positive mode and pools 3 and 4 are analyzed in negative mode. Source conditions consisted of 40 psi for ion source gas 1 and 2, 30 psi curtain gas, 600°C source temperature, and +5500 V or −4500 V spray. Method duration was 1 min. All target proteins for a given metabolite pool and MS method are analyzed together before switching FIA-MS methods. Between FIA-MS methods, the Shimadzu Nexera HPLC system and SCIEX X500R ESI-QTOF MS are equilibrated for 40 min to the next FIA-MS method. Auto-calibration of positive or negative mode was performed approximately every 45 mins at the beginning of a target protein-metabolite pool batch to control detector drift. Non-dialyzed MIDAS metabolite pools were assayed at the beginning, middle, and end of each metabolite pool method batch to control detector sensitivity.

Differential Scanning Fluorimetry

Thermal differential scanning fluorimetry (DSF) is performed similar to Niesen et al. Briefly, DSF thermal shift assays are performed to assess protein melting point (Tm) and thermal stability in the presence of putative small molecule ligands. Where appropriate, DSF experiments are performed using either the standard SYPRO orange fluorescent system or PROTEOSTAT® Thermal shift stability assay kit. A final concentration reaction mixture of 10 µL containing 25mM HEPES pH 7.4, 50mM NaCl, 0.1 mg/mL (SYPRO system) or 0.75 mg/mL (PROTEOSTAT system) target protein, 7.5X SYPRO orange or 1x PROTEOSTAT® reagent, and a concentration titration of putative ligand are arrayed across a MicroAmp™ optical 384-Well reaction plate and sealed with MicroAmp™ optical adhesive film. Protein denaturation is measured in sextuplicate technical replicates for SYPRO orange and PROTEOSTAT experiments with an excitation of 470 nm and emission of 580 nm on an Applied Biosystems Quantstudio 7 Flex from 25°C to 95°C at a ramp rate of 0.05°C/second. DSF experiments are performed in triplicate. Protein Thermal Shift software 1.4 (Applied Biosystems) is used to interpret and determine protein Tm from the first derivative of the fluorescence emission as a function of temperature (dF/dT). A change in ligand-induced protein melting point (ΔTm) is determined from the difference of the ligand induced Tm and no-ligand control Tm. Apparent binding affinity (Kdapp) is determined by fitting the specific binding and Hill slope equation to ΔTm as a function of ligand concentration in GraphPad Prism 9 software.

Shipping Address

Kevin Hicks

University of Utah

Department of Biochemistry

15 N Medical Dr. East

Rm 4720

Salt Lake City, UT 84112

Tel. (801) 581-8718

Please include reference # 00221-9900-21888 on the shipping label for internal accounting.

All protein samples must be shipped on dry ice.

Contact

Hours of Operation

Monday-Friday 9am-5pm

Location

University of Utah

Department of Biochemistry

Emma Eccles Jones Medical Research Building (EEJ) Room 5520

15 N Medical Dr East

Salt Lake City, UT 84112

Staff

Kevin Hicks, Ph.D., Director

Phone: (801) 581-8718

kevin.hicks@biochem.utah.edu

Samantha Tilley, Technician

samantha.tilley@biochem.utah.edu

Andrea Guzman, Technician

Andrea.Paredes@biochem.utah.edu

Referencing Us

Protein-metabolite interactomics was performed at the Protein-Metabolite Discovery Core at the University of Utah, supported in part by a grant from the NIH National Institute of Diabetes and Digestive and Kidney Diseases, Grant number U54DK110858."

Data Analysis

MIDAS data processing and analysis: MIDAS FIA-MS spectra are processed in SCIEX OS software using a targeted method to determine metabolite abundances in the protein chamber and metabolite chamber by integrating the mean area under the curve for each extracted ion chromatogram. If necessary, up to one dialysis replicate per pool per protein is removed if processing or autosampling abnormalities are identified. For each dialysis replicate, log2(fold change) for each metabolite is calculated as the difference between the log2 abundance in the protein chamber and metabolite chamber. Log2(fold change) for metabolite isomers (e.g. L-Leu/L-Ile/L-Allo-Ile) within the same screening pool are collapsed to a single entry prior to further data processing leading to 333 unique metabolite isomer analytes. Using the replicate protein-metabolite log2(fold change) values as input, a processing method developed in R (https://github.com/KevinGHicks/MIDAS) is used to capture and remove extreme outliers and non-specific systematic variation and to determine significant protein-metabolite interactions. Briefly, for each dialysis replicate set, up to one outlier is removed using a z-score cutoff of five (<0.2% of observations). Technical replicates are then averaged yielding one fold-change summary per protein-metabolite pair. To remove fold-change variation that is not specific to a given protein-metabolite pair, the first three principal components of the total screening dataset are removed on a per metabolite pool basis by subtracting the projection of the first three principal components, creating log2(corrected fold change). Protein-metabolite z-scores are determined by comparing the target protein-metabolite log2(corrected fold change) to a no-signal model for that metabolite using measures of the central tendency (median) and standard deviation (extrapolated from the inter quartile), which are robust to the signals in the tails of a metabolite's fold-change distribution. Z-scores are false-discovery rate controlled using Storey's q-value (30) and protein-metabolite interactions with q-values < 0.01 were considered significant. Since correcting for non-specific binding, and estimating metabolite-specific standard deviation both benefit from the inclusion of additional proteins, MIDAS data from anonymized proteins is analyzed alongside the users target protein. Users are provided a list of metabolites that significantly interact with their target protein and publication-quality volcano plots visualizing the screening results. Additional data visualizations are available on request.

Electrum: MIDAS protein-metabolite interaction data can be visualized and explored using Electrum (v0.0.0; https://github.com/Electrum-app/Electrum).