Protocol MSU_MSMC_012
MSMC Glutathione cycle metabolite analysis: sample preparation

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Based on methods from: J of Pharm and Biomed Anal 160 (2018) 289-296.

Objective: quantitative measurement of reduced (GSH) and oxidized (GSSG) glutathione in biological matrices and covalently bound to proteins via disulfide bonds.

- Issues to consider
    - Oxidation of reduced glutathione (GSH) during extraction
        - Alkylation of reduced glutathione with *N*-ethylmaleimide (NEM) in the extraction solvent to prevent oxidation during processing or storage
    - Purity of reduced glutathione standards (labeled and unlabeled) should be verified before use in calibration standards.
    - A significant amount of glutathione may be bound to proteins through disulfide bonds and knowing the amount of free glutathione (both reduced and oxidized forms) as well as the amount of protein bound glutathione is much more informative for understanding the actual state of oxidative stress in a sample.
    - Use of organic solvents to denature proteins has been observed to release substances that oxidize GSH during extraction of whole blood owing to denaturation of oxyhemoglobin and concomitant release of H_2O_2 which oxidizes GSH (Rossi et al. Clin. Chem. 2002, 52: 1406). This may be minimized if NEM is present in the extraction solvent.
    - Acidification before NEM derivatization can shift GSH/GSSG equilibria and should be avoided. Acidification after derivatization can stabilize NEM derivatives from hydrolysis
    - Should also consider measuring all metabolites in the glutathione cycle
        - 5-oxoproline, Cys-Gly, Glu, Cys, gamma-Glu-Cys, Gly, GSH, GSSG
        - The more polar compounds (Glu, Gly, 5-oxoproline) will likely require a separate chromatography method for their analysis

Materials:

Internal standards

- Available from Toronto Research Chemicals
    - **G597952 – [$^{13}C_2$,^{15}N]-GSH (MSMC stock IS_0037)**
    - G597953 – [$^{2}H_5$]-GSH (MSMC stock IS_0001)
    - **G597957 – [$^{2}H_{10}$]-GSSG (MSMC stock IS_0038)**
    - G597972 – [$^{13}C_4$,$^{15}N_2$]-GSSG (MSMC stock IS_0002)
    - *The bold standards are the preferred combination of internal standards for GSH/GSSG (see note below)*
- If measuring other compounds in the same analysis, try to include the labeled internal standards when available (ie. $^{13}C_3$,^{15}N-cysteine)

External standards

- Reduced GSH – Sigma G4251 (Note: reduced GSH slowly oxidizes to GSSG in the presence of air). Verification of GSH content can be achieved by spectrophotometric (Ellman's) assay
- Oxidized GSSG – Sigma G4376

Methanol (HPLC grade or better)
0.1% formic acid in Milli-Q water
Milli-Q water
N-ethylmaleimide (Sigma 04260 or 04259)
TCEP (tris(2-carboxyethyl)phosphine) – Sigma 646547 (10 x 1 ml aliquots of 0.5 M) or C4706
LC vials, caps and inserts (available in BMB stores)

Sample collection:

- If possible, NEM should be added directly to fresh whole blood immediately after collection from the animal to prevent oxidation of GSH (and other thiols) during sample handling (~ 50 mM final concentration of NEM). If NEM is not added during blood collection, it is better to use plasma rather than serum due to the longer time needed for clotting prior to freezing. Oxidation of GSH can occur quickly during this time. We recommend the use of heparin blood collection tubes for preparation of plasma (separate plasma by centrifugation at 1100 x *g* for 10 minutes (4˚C).
    - When adding NEM to blood (or plasma, serum), make a fresh working stock of NEM in water and use within 6 hours. It's best not to store NEM long term in water due to its reactivity toward water (hydrolysis).
- Tissue samples should be snap frozen in liquid nitrogen immediately after collection

Extraction solvents:

- Prepare fresh within 12 hours before extraction to limit oxidation of GSH
- ***Plasma/serum/tissue extraction solvents***
    - Solvent A: Methanol containing 100 mM NEM
        - 12.5 mg/ml = 100 mM NEM
    - Solvent B: Methanol containing 0.50 μM **[$^{13}C_2$,^{15}N]-GSH** and 0.050 μM **[$^{2}H_{10}$]-GSSG**
- ***Whole blood extraction solvents***
    - Solvent A: Milli-Q water containing 100 mM NEM
        - 12.5 mg/ml = 100 mM NEM
    - Solvent B: Milli-Q water containing 0.50 μM **[$^{13}C_2$,^{15}N]-GSH** and 0.050 μM **[$^{2}H_{10}$]-GSSG**
    - Solvent C: Methanol (HPLC grade); kept on ice
- ***Protein thiol extraction solvents***
    - Solvent A: Milli-Q water containing 1.0 μM **[$^{13}C_2$,^{15}N]-GSH**

Extraction of free GSH and GSSG:

Whole blood protocol (freshly collected)

1. Process whole blood as quickly as possible after collection. Blood may be kept on ice briefly while samples await processing.
2. Prepare a labeled 1.7-mL polypropylene microcentrifuge tube by adding 50 µL of Whole Blood Extraction Solvent B (containing labeled internal standards) to a labeled 1.7-mL polypropylene microcentrifuge tube. Store the tube on ice. Prepare a second tube labeled with the same specimen identifier.
3. Immediately before adding freshly-collected whole blood, transfer 50 µL of Whole Blood Extraction Solvent A (NEM) to the tube containing extraction solvent B
4. Transfer 10 µL of whole blood into the tube with extraction solvents A & B
5. Vortex to mix thoroughly then incubate for 45 min at room temp to allow for derivatization of thiols with NEM
6. Add 300 µL of ice-cold Whole Blood Extraction Solvent C (HPLC-grade methanol) and vortex briefly; return the tube to ice for 10 minutes
7. Centrifuge samples at 4°C at top speed in a microcentrifuge for 10 min to pellet insoluble material
    a. *Save pelleted material for measurement of protein bound thiols if desired*
8. Transfer supernatant to the other labeled polypropylene microcentrifuge tube
9. Evaporate solvent to dryness using a speed-vac (preferred) without application of heat (or under a stream of nitrogen gas, without heat). Dried samples may be stored in the freezer (-20˚C or colder) if necessary.
10. Reconstitute samples in 100 µL of 0.1% aqueous formic acid (final IS concentration should be 0.25 µM **[$^{13}C_2$,^{15}N]-GSH** and 0.025 µM **[$^{2}H_{10}$]-GSSG**) with vortexing, and transfer to LC autosampler vial with a low-volume glass insert. The low pH minimizes basic hydrolysis of the maleimide group while the extracts wait to be analyzed.
    a. May need to dilute samples in water further for GSH-NEM analysis if levels are too high

Blood plasma or serum samples

1. Thaw plasma or serum on ice, briefly vortex once the specimen has thawed
2. While the specimen is thawing, transfer 50 µL of Plasma/serum/tissue Extraction Solvent B (labeled internal standards) to a labeled 1.7-mL polypropylene microcentrifuge tube. Prepare a second tube labeled with the same specimen identifier.
3. Immediately before adding plasma/serum, transfer 50 µL of Plasma/serum/tissue Extraction Solvent A (NEM) to the tube containing extraction solvent B
4. Transfer 10 µL of serum/plasma into the tube containing the extraction solvents
5. Vortex to mix thoroughly then incubate for 45 min at room temp to allow for derivatization of thiols with NEM
6. Centrifuge samples at 4°C at top speed in a microcentrifuge for 10 min to pellet insoluble material
    a. *Save pelleted material for measurement of protein bound thiols if desired*
7. Transfer supernatant to the other labeled polypropylene microcentrifuge tube

8. Evaporate solvent to dryness using a speed-vac (preferred) without application of heat (or under a stream of nitrogen gas, without heat). Dried samples may be stored in the freezer (-20˚C or colder) if necessary.
9. Reconstitute samples in 100 μL of 0.1% aqueous formic acid (final IS concentration should be 0.25 μM **[$^{13}C_2$,^{15}N]-GSH** and 0.025 μM **[$^{2}H_{10}$]-GSSG**) with vortexing and transfer to LC autosampler vial with a low-volume glass insert. The low pH minimizes basic hydrolysis of the maleimide group while the extracts wait to be analyzed.
    a. May need to dilute samples in water further for GSH-NEM analysis if levels are too high

Tissue specimens

1. Frozen tissue specimens may be cut with a clean razor blade or scalpel, with the goal of obtaining 20 mg of frozen tissue. Use forceps to transfer to a tared 1.7-mL polypropylene microcentrifuge tube, and determine the weight the tissue on an analytical balance (to 0.1 mg). Prepare a second microcentrifuge tube labeled with the same sample identifier.
2. Add a single clean 3-mm stainless steel ball bearing to the tube to aid with tissue maceration.
3. Add 200 μL of Plasma/serum/tissue Extraction Solvent A (NEM in methanol)
4. Add 200 μL of Plasma/serum/tissue Extraction Solvent B (internal standards in methanol) and cap the tube
5. Vortex at maximum speed for 5 minutes to allow the ball bearing to macerate the tissue, then incubate for an additional 45 min at room temp to allow for derivatization of thiols with NEM.
6. Centrifuge samples at 4°C at top speed in a microcentrifuge for 10 min to pellet insoluble material
    a. *Save pelleted material for protein analysis for normalization of data*
    b. *Protein pellet can also be used for measuring protein bound thiols if desired*
7. Transfer 200 μL supernatant to a new microcentrifuge tube (Tube A), save the remaining supernatant in another tube for archiving in the freezer (Tube B); also freeze the tube with the pellet unless it is processed immediately.
8. Evaporate solvent to dryness from 200 μL of supernatant (Tube A) using a speed-vac (recommended) or stream of nitrogen gas without application of heat. Dried samples may be stored in the freezer (-20˚C or colder) if necessary.
9. Reconstitute in 200 μL of 0.1% aqueous formic acid (final IS concentration is 0.25 μM **[$^{13}C_2$,^{15}N]-GSH** and 0.025 μM **[$^{2}H_{10}$]-GSSG**) with vortexing, and transfer to LC autosampler vial with a glass insert
    a. May need to dilute samples in water further for GSH-NEM analysis if levels are too high

Optional: Extraction of protein bound thiols

1. Uses saved protein pellet from extraction of free GSH
2. Rinse pellet with Milli-Q water containing 1 mM *S*-methylglutathione (Toronto Research Chemicals #M303750) to remove any residual non-covalently bound GSH
    a. Add 0.5 mL, vortex, centrifuge at top speed in a microcentrifuge for 5 min
    b. Repeat 3 times
3. Discard supernatant from washes

4. Reconstitute the protein pellet in 200 μL of an aqueous solution containing 10 mM TCEP and 1 mM tributylphosphine by gently drawing up the suspension using a 1000-μL pipetter multiple times until the pellet is suspended.
5. Withdraw a 50-μL aliquot for subsequent protein assay and store on ice
6. Incubate the remaining solution at room temp for 15 min
7. Add 75 μL of Whole Blood Extraction Solvent A (50 mM NEM, aqueous)
8. Add 75 μL of Protein thiol Extraction Solvent A 1 μM **[$^{13}C_2$,^{15}N]-GSH** (final IS concentration is 0.25 μM **[$^{13}C_2$,^{15}N]-GSH**)
9. Incubate 45 min at room temp
10. Centrifuge sample at top speed in a microcentrifuge for 10 min.
11. Transfer supernatant to LC autosampler vial.

**** Note on internal standards:**

- Standards with different labeling patterns enables you to distinguish how much oxidation of GSH occurs during sample prep.
- [$^{13}C_2$,^{15}N]GSH and [$^{2}H_{10}$]GSSG are the preferred combination of internal standards. [$^{13}C_2$,^{15}N]GSH will oxidize to [$^{13}C_2$,^{15}N]GSSG or [$^{13}C_4$,$^{15}N_2$]GSSG. Neither or these oxidation products have isotope patterns that will overlap with the [$^{2}H_{10}$]GSSG internal standard.
- [$^{2}H_5$]GSH oxidizes to either [$^{2}H_5$]GSSG or [$^{2}H_{10}$]GSSG, both of which have a different MW than GSSG or [$^{13}C_4$,$^{15}N_2$]GSSG. However, the issue with this combination of internal standards is the overlap of the +1 isotope of [$^{2}H_5$]GSSG with the [$^{13}C_4$,$^{15}N_2$]GSSG internal standard.

Standard Curve preparation

1. Prepare a mix of unlabeled standards at 40 μM in water (4X of the highest concentration for the standard curve, 10 μM)
2. Add an equal volume of freshly prepared water containing 50 mM NEM (concentration of standards is now 2X or 20 μM)
3. Incubate for 45 min at room temp
4. Prepare a mix of the labeled internal standards at 4X concentration (1 μM **[$^{13}C_2$,^{15}N]-GSH** and 0.1 μM **[$^{2}H_{10}$]-GSSG**)
5. Add an equal volume of freshly prepared water containing 50 mM NEM (concentration of labeled standards is now 2X)
6. Incubate for 45 min at room temp
7. Make serial dilutions (1:4) of alkylated unlabeled standards (20 μM, 5 μM, 1.25 μM, 0.313 μM, 0.078 μM, 0.0195 μM, 0.0049 μM)
8. Mix 1:1 each of the serial dilutions of the unlabeled standards with the 2X stock of alkylated internal standards
9. Final standard curve is 10 μM, 2.5 μM, 0.625 μM, 0.156 μM, 0.039 μM, 0.0098 μM, 0.0024 μM all containing 0.25 μM **[$^{13}C_2$,^{15}N]-GSH** and 0.025 μM **[$^{2}H_{10}$]-GSSG**

Note: Can include more standards and internal standards for quantitative analysis of other related compounds in the same analysis.

LC/MS/MS Method:

- Use a Waters Acquity UPLC HSS-T3 2.1 x 100 mm column with a water + 0.1% formic acid and methanol mobile phase gradient

Time (min)	Flow rate mL/min	% A	% B	Curve
0.00	0.3	100	0	6
1.00	0.3	100	0	6
5.00	0.3	50	40	6
7.00	0.3	1	99	6
8.00	0.3	1	99	6
8.01	0.3	100	0	6
10.00	0.3	100	0	6

- Analyze in positive ion mode (can be done in an untargeted way using QTof2 or the QE, or could be done in a targeted method using a triple quad (TQD or TQ-XS)

MRM table (Waters Acquity TQD), positive-ion mode

Compound	**Precursor ion (*m/z*)**	**Product ion (*m/z*)**	**Cone voltage (V)**	**Collision voltage (V)**
5-oxoproline	130	84	26	10
[$^{13}C_5$]5-oxoproline	135	88	26	10
Glutamic acid	148	84	18	18
Cystine	241	73	18	34
Cysteine-NEM	247	87	26	26
Homocysteine-NEM	261	56	26	18
GSH	308	179	30	16
GSH-NEM	433	304	30	16
[$^{2}H_5$]GSH-NEM	438	304	30	16
[$^{13}C_2$,^{15}N]GSH-NEM	436	307	30	16
GSSG	613	355	30	22
[$^{13}C_2$,^{15}N]GSSG	616	358	30	22
[$^{2}H_5$]GSSG	618	355	30	22
[$^{13}C_4$,$^{15}N_2$]GSSG	619	361	30	22
[$^{2}H_{10}$]GSSG	623	355	30	22

Accurate mass table

Compound	**$[M+H]^+$**
5-oxoproline	130.0499
$[^{13}C_5]$5-oxoproline	135.0666
Glutamic acid	148.0604
Cystine	241.0311
Cysteine-NEM	247.0747
Homocysteine-NEM	261.0904
GSH	308.0911
GSH-NEM	433.1388
$[^2H_5]$GSH-NEM	438.1701
$[^{13}C_2,^{15}N]$GSH-NEM	436.1425
GSSG	613.1592
$[^{13}C_2,^{15}N]$GSSG	616.1630
$[^2H_5]$GSSG	618.1906
$[^{13}C_4,^{15}N_2]$GSSG	619.1667
$[^2H_{10}]$GSSG	623.2220