FSP Title: # NMR Metabolomics

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1.0 BACKGROUND

When the NMR-based metabonomics approaches are employed for metabolite composition analysis, the demands for extraction method will include optimal concentrations and consistent pH values for the resultant extracts so as to maximise signal-tonoise ratio (SNR) and minimise the inter-sample chemical shift variations.

This procedure should be used as a guide for sample preparation. Any required adjustments should be worked out during method development stage.

2.0 SCOPE

This facility specific protocol (FSP) describes the tissue, serum, cecal content, feces, urine and cell sample preparation steps for analysis of metabolites via NMR.

3.0 MATERIALS

3.1 Equipment

Ice	Homogenization tubes	Acrylic Homogenization Beads
Homogenizer	NMR tubes	Vortexer
Centrifuge		

3.2 Reagents

2:1 Methanol:H_2O	PBS (0.045M/0.1M/1.5M, K_2HPO_4:NaH_2PO_4)
D_2O (deuterium oxide)	TSP-d4 (sodium3-trimethylsilyl [2,2,3,3-d4] propionate
NaN_3	EDTA-d12
KF	KOH

4.0 PROCEDURE

This procedure assumes there is adequate amount or volume of sample available to perform all the described analysis. The minimum volume of all the study samples should be checked ahead of time. If there are samples with low volume, then the volumes described in this protocol can be adjusted as long as the ratios of the reagents are kept constant.

Important: Prepare a blank. Follow all steps for blank along with samples.

4.1 Tissues extraction for NMR metabolomics

1. Preparation of 0.1 M Na+/K+ PO_4 Buffer (100 ml, pH = 7.4)

K_2HPO_4: 0.8*100 (ml)*0.1(M)*174.18/1000/0.99 = 1.408 g

NaH_2PO_4: 0.2*100 (ml)*0.1(M)*119.98/1000/0.99 = 0.242 g

H_2O: 100 ml*0.5 = 50 ml

D_2O: 100 ml*0.5 = 50 ml

TSP: 0.005% (w/v)*100 = 0.005 g

NaN_3: 0.01 g (Preservative)

2. Tissue sample extraction

1) Weigh ~50 mg tissues and make a record; mark and save on ice;
2) Add 8-10 beads and 1 ml of pre-cooled methanol:H_2O (2:1) to homogenization tubes;
3) Homogenization with 6500 – 1x20 – 005 program for 2-3 times;
4) Incubate 5 min at room temperature;
5) Centrifuge at 4°C, 11180 g for 10 min;
6) Transfer the supernatants to 2 ml EP tubes;
7) Add 0.6 ml solution (methanol: H2O (2:1)) to the pellets, repeat the above procedure (4-6 steps).
8) Combine the supernatants;
9) Dry down and then save it at -80°C.
10) Resuspend in 0.6 ml 0.1 M PBS, centrifuge at 4°C for 10 min, then transfer 0.55 ml supernatants to NMR tubes.

4.2 Serum or plasma for NMR metabolomics

4.2.1 Non-protein precipitation method

1. Preparation of 0.045 M Na+/K+ PO_4 Buffer (100 ml, pH = 7.4)

K_2HPO_4: 0.8*100(ml)*0.045(M)*174.18/1000/0.99 = 0.633 g

NaH_2PO_4: 0.2*100(ml)*0.045(M)*119.98/1000/0.99 = 0.110 g

NaCl: 0.9 g

H_2O: 50 ml

D_2O: 50 ml

2. Serum or plasma sample extraction

If the volume of serum is enough:

1) 200 µl samples mixed with 400 µl PBS (0.045M) containing 50% D_2O;
2) Vortex samples for 10 s;
3) Centrifuge at 18100 g for 10 minutes, 4°C;
4) Transfer 550 µl supernatants into 5.0 mm NMR tubes.

If the volume of serum is not enough (≤ 50 µl):

1) 20 µl samples mixed with 40 µl PBS (0.045M) containing 100% D_2O;
2) Vortex samples for 10 s;
3) Centrifuge at 18100 g for 10 minutes, 4°C;
4) Transfer 60 µl supernatants into 1.7 mm NMR tubes. (NS = 256 when acquire ^1H NMR spectra)

4.2.2 Protein precipitation method

1. Preparation of 0.1 M Na+/K+ PO_4 Buffer (100 ml, pH = 7.4)

K_2HPO_4: 0.8*100 (ml)*0.1(M)*174.18/1000/0.99 = 1.408 g

NaH_2PO_4: 0.2*100 (ml)*0.1(M)*119.98/1000/0.99 = 0.242 g

H_2O: 100 ml*0.5 = 50 ml

D_2O: 100 ml*0.5 = 50 ml

TSP: 0.005% (w/v)*100 = 0.005 g

NaN_3: 0.01 g (Preservative)

2. Serum or plasma sample extraction

1) Add 80-300 µl blood and 3 volume of pre-cooled methanol to tubes and vortex;
2) Incubate 20 min at -20°C;
3) Centrifuge at 4°C, 11180 g for 10 min;
4) Dry down and then save it at -80°C.
5) Resuspend in 0.6 ml 0.1 M PBS, centrifuge at 4°C for 10 min, then transfer 0.55 ml supernatants to NMR tubes.

4.3 Cecal content or feces for NMR metabolomics

1. Preparation of 0.1 M Na+/K+ PO_4 Buffer (100 ml, pH = 7.4)

K_2HPO_4: 0.8*100 (ml)*0.1(M)*174.18/1000/0.99 = 1.408 g

NaH_2PO_4: 0.2*100 (ml)*0.1(M)*119.98/1000/0.99 = 0.242 g

H_2O: 100 ml*0.5 = 50 ml

D_2O: 100 ml*0.5 = 50 ml

TSP: 0.005% (w/v)*100 = 0.005 g

NaN_3: 0.0195 g (Preservative)

2. Cecal content or feces sample extraction

1) Weigh 50~60 mg samples and make a record; mark and save it on ice;
2) Add 8-10 beads and 0.6 ml PBS (0.1M) solution containing 50% D_2O to homogenization tubes, Vortex 30s;
3) Homogenization with 6500 – 1x20 – 005 program for 2-3 times;
4) Freeze-thawing two times with Liquid nitrogen;
5) Centrifuge at 4°C, 11180 g for 10 mins;
6) Transfer the supernatants to 2 ml new EP tubes;
7) Add 0.6 ml PBS solution to the pellets followed with Vortex 30s and Centrifuge at 4°C, 11180g for 10 mins.
8) Combine the supernatants, centrifuge at 4°C and 16099g for 10 mins; Transfer the supernatants (0.55 ml) to NMR tubes.

4.4 Urine for NMR metabolomics

1. Preparation of 1.5 M Na+/K+ PO_4 Buffer (100 ml, pH = 7.4)

K_2HPO_4: 0.8*100(ml)*1.5(M)*174.18/1000/0.99 = 21.113 g

NaH_2PO_4: 0.2*100(ml)*1.5(M)*119.98/1000/0.99 = 3.636 g

H_2O: 100ml*0.5 = 50 ml

D_2O: 100ml*0.5 = 50 ml

TSP: 0.005% (w/v)*100 = 0.005 g

NaN_3: 0.01 g (Preservative)

2 . Preparation of 5M KF solution (**25 ml,** save in a plastic container)

$KF{\cdot}2H_2O$: 25(ml)*5(M)*94.13/1000/0.99 = 11.885g

H_2O: 25 ml

3. Preparation of 0.12M EDTA-d12 solution (**25 ml**)

EDTA-d12: 25(ml)*0.12(M)*304.34/1000 = 0.913g

KOH: 0.593g

H_2O: 25 ml

4. Urine sample extraction

For mice urine

1) 60 µl urine mixed with 4.8 µl KF (5M) and 440 µl D_2O (20%);
2) Vortex samples for 10 s;
3) Centrifuge at 11800 g for 10 minutes, 4°C;
4) Add 5 µl EDTA-d12 (0.12M) into NMR tubes;
5) Transfer 450 µl supernatants into NMR tubes, blending;

6) Add 45 µl PBS (1.5M) into NMR tubes.

For human urine

1) 500 µl urine mixed with 14 µl KF (5M);
2) Vortex samples for 10 s;
3) Centrifuge at 11800 g for 10 minutes, 4°C;
4) Add 8.3 µl EDTA-d12 (0.12M) into NMR tubes;
5) Transfer 450 µl supernatants into NMR tubes, blending;
6) Add 45 µl PBS (1.5M) into NMR tubes.

4.5 Cell extraction for NMR metabolomics

1. Preparation of 0.1 M Na+/K+ PO_4 Buffer (100 ml, pH = 7.4)

K_2HPO_4: 0.8*100 (ml)*0.1(M)*174.18/1000/0.99 = 1.408 g

NaH_2PO_4: 0.2*100 (ml)*0.1(M)*119.98/1000/0.99 = 0.242 g

H_2O: 100 ml*0.5 = 50 ml

D_2O: 100 ml*0.5 = 50 ml

TSP: 0.005% (w/v)*100 = 0.005 g

NaN_3: 0.01 g (Preservative)

2 . Cell sample extraction

1) Add 1 ml of pre-cooled methanol:H2O (2:1);
2) Vortex samples for 10 s;
3) Freeze-thawing three times with Liquid nitrogen;
4) Centrifuge at 11800 g for 10 minutes, 4°C;
5) Transfer the supernatants to 2 ml new EP tubes;
6) Add 0.6 ml solution (methanol: H2O (2:1)) to the pellets, repeat the above procedure (2-5 steps).
7) Combine the supernatants;
8) Dry down and then save it at -80°C.
9) Resuspend in 0.6 ml 0.1 M PBS (D_2O), centrifuge at 4°C for 10 min, then transfer 0.55 ml supernatants to NMR tubes.

5.0 REFERENCES

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