**Magnetic Resonance Spectroscopy (MRS)-based methods for examining cancer metabolism in response to oncogenic kinases drug treatment.**

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**Summary:**

Magnetic resonance spectroscopy (MRS) is an analytical technique that has been extensively used to examine reprogrammed metabolism and treatment response in cancer cells and solid tumours both *in vivo* and *ex vivo*. High-resolution MRS (HR-MRS) is one of the best methods for metabolic profiling, as it is highly quantitative, robust, and reproducible. The protocols for dual-phase extraction of cancer cells and tumours and sample preparations for high-resolution 1H and 31P HR-MRS analysis are described here. Descriptions of spectra acquisition and analysis are also included in this chapter.

**Running title:** Study of cancer metabolism and treatment response by HR-MRS.

**Key Words:** Magnetic resonance spectroscopy, nuclear magnetic resonance spectroscopy, dual phase extraction, 1H-MRS, 31P-MRS, cancer metabolism

**1. Introduction**

Magnetic resonance spectroscopy (MRS) or nuclear magnetic resonance spectroscopy (NMR) is an analytical technique that has been widely used to study metabolism and monitor treatment response in cancer [1]. This technique is based on certain nuclei, such as, proton (1H), phosphorus (31P) and carbon (13C), possess the property of magnetic spin, and this spin causes the nuclei to behave like small magnetics. When they are placed in a magnetic field, these nuclei became align (lower energy state) or oppose (higher energy state) to the external magnetic field with slightly more nuclei in the lower energy state. A radiofrequency pulse is then applied, many of the nuclei are flipped into the higher energy state and the differences in the populations between these two magnetic energy states are detected as a radio wave as the system returns to equilibrium. The static magnetic field induces small electronic currents in atoms and molecules, which produce a small local field and the strength of this local field depends on the electronic environment around the nucleus. Therefore, different chemical structures possess different electronic environments and lead to nuclei resonating at slightly different frequencies. These frequencies are presented as the dimensionless units, parts per million (ppm) in the spectrum and represent the metabolite signal in the sample [2]. High-resolution MRS (HR-MRS) can be used to obtain metabolic profiles from body fluids, cell or tissue extracts and cell culture media. Furthermore, intact tissues can be examined by High-Resolution Magic Angle Spinning-MRS and *in vivo* MRS can be also used to obtain metabolic profile in tissue non-invasively.

MRS of various nuclei, e.g., 1H, 31P and 13C, provides information on different metabolites and insights into various aspect of cellular metabolism. 1H is the most natural abundant nuclei and hence with the highest sensitivity to detect low molecular weight metabolites in body fluids, *ex vivo* cells and tissues and *in vivo*. 1H-MRS is also used to analyse culture medium to acquire information on metabolite uptake and secretion by the cells, by comparing the metabolite levels in the starting media with the media that have been incubated with the cells. This information will help to evaluate whether the observed changes in some of the cellular metabolite levels are associated with the increase or decrease rate of metabolite uptake or secretion. 13C-MRS is usually too insensitive to detect signals from the naturally-abundant 13C, but it can be used to follow the flux of 13C-labelled substrates (e.g., glucose, glutamine) through different metabolic pathways in cells and perfused biological systems [3, 4]. 31P-MRS can be used to observe markers for cell and tissue bioenergetics and phospholipid membrane turnover [1].

HR-MRS is one of the best methods for metabolic profiling, as it is highly quantitative, robust, and reproducible. This is because all the detectable molecules in the sample are measured simultaneously without prior assumptions as to the molecular types. HR-MRS can be fully automated with high degree of reproducibility. The metabolite identifications are relatively easy from simple 1D spectrum. It also has the ability to identify unknown metabolites by 2D-NMR methods [5]. A list of metabolites that are routinely measured by HR-MRS of culture media and cell and tumour extracts is illustrated in Table 1.

The investigations of cell or tissue samples by HR-MRS require the samples to be extracted beforehand, which is usually accomplished either by perchloric acid [6] (only for the water-soluble metabolites), or by dual phase method (methanol, chloroform and water), where both water-soluble metabolites (in the water and methanol phase) and lipid metabolites (in the chloroform phase) are extracted [7]. The protocols for dual phase extraction of cancer cells and tumour tissues and for sample preparation of extracted samples and cell culture media will be described in this chapter. Descriptions of 1H- and 31P- HR-MR spectra acquisition and data analysis are also included.

1. **Materials**

All the chemicals and materials are purchased from Sigma-Aldrich Chemical Company (Dorset, UK), unless otherwise stated.

***2.1 Dual phase extraction of cell or tissue:***

1. 100% Chloroform

2. 100% Methanol

3. Saline

4. De-ionised water

Chill chloroform, methanol, saline and de-ionised water in the fridge at 4 oC overnight

5. Chelex 100

6. Universal pH indicator solution, with pH4-11 range

***2.2 Sample preparations for MRS analysis:***

1. 5mm NMR tubes

2. Deuterated water (D2O)

3. 0.75% sodium 3-trimethylsilyl- 2,2,3,3‐tetradeuteropropionate (TSP) in D2O

4. 60 mM Ethylenediaminetetraacetic acid (EDTA) in double-distilled water

5. 10 mM Methylene diphosphonic acid (MDPA) in D2O

6. 0.6% Perchloric acid in double-distilled water

7. 0.1 M Potassium hydroxide in double-distilled water

8. Deuterated chloroform (CDCl3)

9. 0.1% tetramethylsilane (TMS) in CDCl3

1. **Methods**

***3.1* Dual phase extraction of cultured cells:**

1) Plate and treat the cells according to your treatment schedule (see **Note 1**).

2) Remove the culture medium from the cell culture flask using a pipette.

3) Keep 1ml of the medium and store in a -20 oC freezer. Media can also be analysed by 1H HR-MRS to obtain information on the footprint of some metabolites. This method is described later in the chapter (see **Method 3.6**).

4) Wash the cells three times with 10ml ice-cold saline and remove the saline carefully after each wash by using a pipette (see **Note 2**).

5) Add 3ml of ice-cold methanol into each flask (for T75 flasks) to cover the cells (see **Note 3**). Keep the flasks on ice (5-10 mins) to prevent methanol evaporation during manipulation of the flasks.

6) Scrape off the cells from the culture flask surface into the added methanol using a cell scraper.

7) Pipet the cell/methanol mixture into a clean centrifuge tube (orange cap Corning CentriStar tubes).

8) Use a glass pipette to add 3ml of ice-cold chloroform into the tube.

9) Vortex the tube vigorously for 30 sec.

10) Add 3ml of ice-cold de-ionised water into the tube and again vortex the tube vigorously for 30 sec.

11) Keep the extraction mixtures on ice while extracting other samples.

12) Centrifuge the samples for ~5000g at 4oC for 20 mins for phase separation. The final chloroform:methanol:water ratio should be 1:1:1 (v/v/v). The upper methanol-water phase contains the water-soluble cellular metabolites, the middle phase contains the protein pellet and the bottom chloroform phase contains the cellular lipids.

13) Keep and store the cell pellets (the middle phase) at -80oC (for protein concentration determination if required).

*Recovery of the water-soluble metabolites:*

14) Pipette the upper methanol-water phase into a clean centrifuge tube (orange cap Corning CentriStar tubes) and treat with Chelex 100 (~5mg) to remove the divalent ions.

15) Centrifuge the sample for ~5000g at 4oC for 5 mins to separate the beads from the solution.

16) Transfer the clear supernatant to a clean centrifuge tube (orange cap Corning CentriStar tubes) and add 2 drops of pH indicator solution into the supernatant.

17) Store the supernatant in a -80oC freezer until freeze-drying.

*Recovery of the lipid metabolites:*

18) Place the bottom chloroform phase into a glass tube using a glass pipette.

19) Leave the samples to dry in a fume hood overnight or until all the chloroform are evaporated.

20) Store the dried samples in a -20oC freezer until analysis.

***3.2* Dual phase extraction of tumours:**

1) Weigh the freeze-clamped or snapped frozen tumour sample and make note of the tumour weight.

2) Immerse the mortar and pestle in liquid nitrogen.

3) Put some liquid nitrogen in the mortar.

4) Place the tumour samples in the mortar.

5) Grind the tumour into fine powder in liquid nitrogen using a pestle.

6) Put the grounded tumour in a clean centrifuge tube (orange cap Corning CentriStar tubes).

7) Add 3ml of ice-cold methanol into the tube with the grounded tumour (see **Note 4**).

8) Vortex the tube vigorously for 30 sec.

9) Use a glass pipette to add 3ml of ice-cold chloroform into the tube.

10) Vortex the tube vigorously for 30 sec.

11) Stand the tube in ice for about 10 mins.

12) Add 3ml of ice-cold de-ionised water into the tube and again vortex the tube vigorously for 30 sec.

13) Keep the extraction mixtures on ice while extracting other samples.

14) Centrifuge the samples for ~5000g at 4oC for 20 mins for phase separation. The final chloroform:methanol:water ratio should be 1:1:1 (v/v/v). The upper methanol-water phase contains the water-soluble cellular metabolites, the middle phase contains the protein pellet and the bottom chloroform phase contains the cellular lipids.

15) Refer to the protocol in **Method 3.1** for the recovery of the water-soluble and lipid metabolites.

***3.3* Sample preparation of water-soluble metabolites for 1H-MRS analysis:**

1) Lyophilise the methanol and water phase of the cell or tumour extracts in a freeze-drier until the samples are in a powder form.

2) Reconstitute the freeze-dried sample in 650 μl D2O and 50 μl of 0.75% sodium 3-trimethylsilyl-2,2,3,3‐tetradeuteropropionate (TSP) in D2O (see **Note 5**).

3) Centrifuge the sample for ~5000g at 4oC for 5 mins.

4) Place 600 μl of the sample into a 5 mm NMR tube.

5) Adjust the sample to pH7 using 0.1 M potassium hydroxide and 0.6% perchloric acid.

6) Perform 1H-MRS analysis on the samples (see **Section** **3.7**), ideally using a 500 or 600 MHz NMR system.

***3.4* Sample preparation of water-soluble metabolites for 31P-MRS analysis:**

1) After 1H-MRS analysis, add 50 μl of 60 mM ethylenediaminetetraacetic acid (EDTA) and 25 μl of 10 mM methylene diphosphonic acid (MDPA) to the sample solution in the NMR tube (see **Note 6**).

 2) Adjust the sample to pH7 using 0.1 M KOH and 0.6% perchloric acid.

3) Perform 31P-MRS analysis on the samples (see **Section** **3.7**).

***3.5* Sample preparation of lipid metabolites for 1H-MRS analysis:**

1) Leave the chloroform phase of the cell or tumour extracts (containing the lipid metabolites) in a fume hood until all the chloroform is evaporated.

2) Reconstitute the dried lipid metabolites in 450 μl of deuterated chloroform and 150 μl of 0.1% tetramethylsilane (TMS) in deuterated chloroform (see **Note 7**).

3) Place the whole sample into a 5mm NMR tube.

4) Perform 1H-MRS analysis on the samples (see **Section 3.7** and **Note 8**).

***3.6* Sample preparation of culture medium for 1H-MRS analysis (Note 9):**

1) Place 500 μl of the culture medium into a 5 mm NMR tube.

2) Add 50 μl D2O and 50 μl 0.75% TSP in D2O in the sample.

3) Perform 1H-MRS analysis on the samples (see **Section** **3.7**).

***3.7* MR spectral acquisition parameters:**

1) Acquire MR spectra on the samples at 25oC, ideally using a 500 or 600 MHz NMR system.

2) A pulse and collect NMR sequence with water suppression is used to acquire the 1H-MR spectra of water-soluble metabolites from the cell or tumour extracts or culture media (see **Note 10**).

3) A pulse and collect NMR sequence is used to acquire the 1H-MR spectra of lipid metabolites from the cell or tumour extracts.

4) A 1H decoupled 31P NMR sequence is used to acquire the 31P-MR spectra of water-soluble metabolites from the cell or tumour extracts.

5) A list of example parameters for different sample types is shown in Table 2.

***3.8* MR spectral analysis:**

1) MR spectra are analysed using commercially available software packages, such as Bruker Topsin-3.2 or MestRe-C-4.9.9.6.

2) Spectra are first processed by using exponential multiplication with a line broadening of 0.5Hz for 1H-MR spectra and 3Hz for 31P-MR spectra, then followed by Fourier- transform, zero and first order phase correction, baseline correction and spectral peak integration.

3) Spectral assignments are based on literature values of 1H-MRS [8, 9] and 31P-MRS [10] of aqueous extracts or 1H-MRS of lipid extracts [11].

4) Calculation of cellular metabolite concentrations, lipid levels and rates of metabolite uptake and secretion are shown below:

i) Calculation of cellular metabolite concentrations from a 1H-MR spectrum of cell or tumour extract:

[M]sample =

NTSP . Isample . [TSP]

NMet . Wsample . ITSP

 [M]sample  is the cellular concentration of metabolite.

NTSP and NMet are the number of proton giving rise to signal integral of TSP and metabolite of interest, respectively; NTSP = 9.

ITSP and Isample are the signal integral of TSP and metabolite of interest, respectively.

[TSP] is the concentration of TSP.

Wsample is the sample cell number, protein concentration or tumour wet weight.

ii) Calculation of cellular metabolite concentrations from a 31P-MR spectrum of cell or tumour extract:

[M]sample =

NMDPA . Isample . Vsample . [MDPA]

NMet . Wsample . IMDPA . Vtube

 [M]sample  is the cellular concentration of metabolite.

NMDPA and NMet are the number of phosphorus atom giving rise to signal integral of MDPA and metabolite of interest, respectively; NMDPA = 2.

IMDPA and Isample are the signal integral of MDPA and metabolite of interest, respectively.

[MDPA] is the concentration of MDPA.

Wsample is the sample cell number, protein concentration or tumour wet weight.

Vsample is the total reconstituted sample volume (700 µl as described in **Method 3.3**).

Vtube is the reconstituted sample volume that is placed into the NMR tube (600µl as described in **Method 3.3**).

 iii) Calculation of cellular lipid levels from a 1H-MR spectrum of cell or tumour extract:

Lipid/TMS sample =

 Isample

Wsample . ITMS

 Lipid/TMSsample  is the cellular lipid level.

ITMS and Isample are the signal integral of TMS and lipid metabolite of interest, respectively.

Wsample is the sample cell number, protein concentration or tumour wet weight.

iv) The rates of metabolite being taken up from or secreted into the culture media are measured from a 1H-MR spectra of the starting culture media and the media that has been incubated with cells:

KUptake/Secretion =

(ISM-ICM) . NTSP . VTot . [TSP]

NMet . Wsample . ITSP . Vtube . T

KUptake/Secretion is the rate of metabolite uptake from or secreted into the culture media by the cells. A positive value indicates the rate of metabolite being taken up from the media by the cells and a negative value shows the rate of metabolite being secreted into the media by the cells.

ISM is the signal integral of metabolite in the starting media.

ICM is the signal integral of metabolite in the media incubated with the cells.

ITSP is the signal integral of TSP.

NTSP and NMet are the number of proton giving rise to signal integral of TSP and metabolite of interest, respectively; NTSP = 9.

[TSP] is the concentration of TSP.

Wsample is the sample cell number or protein concentration.

VTot is the total volume of media that are incubated with the cells.

Vtube is the volume of media that are placed into the NMR tube (500µl as described in **Method 3.6**).

T is the length of time since the last media change.

5) Example 1H- and 31P-MR spectra of an ovarian carcinoma xenograft extract are illustrated in Figure 1 and 2, respectively.

**4. Notes**

1) Set up an extra flask of cell for cell counting and/or protein concentration estimation, so that the metabolites could be standardised to cell number or protein concentration.

2) It is very important to wash the cells carefully and thoroughly to ensure that all the residue culture media is removed from the flask before extraction, as any residue media that is left in the flask will cause contaminations in the MR spectra of the cell extract.

3) The use of 3 ml methanol, chloroform and de-ionised water described in this protocol are for extracting confluent cells from a T75 flask. 6 ml of methanol, chloroform and de-ionised water should be used to extract confluent cells from a T175 flask.

4) The use of 3 ml methanol, chloroform and de-ionised water described in this protocol are for extracting tumour up to about 1g in weight. For tumours that are very small (200mg or less), 1 ml methanol, chloroform and de-ionised water could be used instead.

5) The addition of TSP into the samples is for 1H-MR spectral chemical shift calibration and metabolite quantification.

6) The additions of EDTA and MDPA into the sample solutions are for chelation of metal ions in the samples and for 31P-MR spectral chemical shift calibration and metabolite quantification, respectively.

7) The addition of TMS into the samples is for 1H-MR spectral chemical shift calibration.

8) Prepare the samples on the same day as the 1H-MRS analysis to avoid evaporation of the solvent, which may affect the subsequent comparison of lipid metabolite levels between the samples.

9) This approach is not valid if the cells are undergoing apoptosis after treatment, as less cells are present at the end of the study than the start and this will result in over-estimation of metabolite uptake and secretion.

10) The water resonance signal from the media and water-soluble cell or tumour extract samples is normally suppressed by a gated irradiation centred on the water frequency at about 4.7ppm, so that other metabolites can be visualised.

11) The number of scans for both 1H- and 31P-MR spectra are depended on the number of extracted cells or size of the extracted tumour in the sample. The number of scans listed in Table 2 is based on an extracted sample from about 20 million cells or 300mg of tumour tissue.

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 **Figure Captions**

**Figure 1**: 1H-MR spectrum of the aqueous phase from an ovarian carcinoma xenograft extract. **(A)** The aliphatic region and **(B)** the aromatic region (expanded 8x vertically) of the spectrum are shown. Peaks assigned  as: TSP (1- internal reference), valine (2), lactate (3), alanine (4), acetate (5), glutamate (6), succinate (7) glutamine (8), glutathione (9), creatine + phosphocreatine (10), phosphocholine (11), glycerophosphocholine (12), scyllo-inositol (13), taurine (14), glycine (15), creatine (16), phosphocreatine (17), myo-inositol (18), β-glucose (19), α-glucose (20), fumarate (21), histidine (22), tyrosine (23), phenylalanine (24), ATP+ADP (25), NAD++NADH (26), formate (27). For detailed peak assignments see References 8 and 9.

**Figure 2:** 31P- MR spectrum of the aqueous phase from an ovarian carcinoma xenograft extract. Peak assignments were: β-nucleotide triphosphate [β-NTP] (1), α-nucleotide triphosphate [α-NTP] (2), α-nucleotide diphosphate [α-NDP] (3), γ-nucleotide triphosphate +β-nucleotide diphosphate [γ-NTP+β-NDP] (4), phosphocreatine (5), glycerophosphocholine (6), glycerophosphoethanolamine (7), inorganic phosphate (8), phosphocholine (9), phosphoethanolamine (10), methylene diphosphonic acid (11 – internal reference).