

Metabolomic Applications to the Characterization of the Mode-of-Action of CDK Inhibitors

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Abstract

Cyclin-dependent kinases (CDKs) regulate cell cycle progression, and some of them are also involved in the control of cellular transcription. Dysregulation of these critical cellular processes, due to the aberrant expression of some of these proteins, is common in many neoplastic malignancies. Consequently, the development of chemical compounds capable of inhibiting the biological activity of CDKs represents an attractive strategy in the anticancer area. CDK inhibition can trigger apoptosis and could be particularly useful in hematological malignancies, which are more sensitive to inhibition of cell cycle and apoptosis induction. Over the last few years, a number of pharmacological inhibitors of CDKs (CDKIs) belonging to different chemical families have been developed, and some of them have been tested in clinical trials. Given the complexity of the role of CDKs in cell functioning, it would be desirable to develop new tools that could facilitate a better understanding of the new insights into CDK functions and the mode-of-actions of CDKIs. In this context, this chapter describes an experimental approach to evaluate the metabolic consequences of CDKIs at the cellular level based on metabolomics by NMR. More specifically, a description of a strategy to characterize the biochemical effects of CDKIs acting on mammalian cells is provided, including protocols for the extraction of hydrophilic and lipophilic metabolites, the acquisition of 1D and 2D metabolomic Nuclear Magnetic Resonance (NMR) experiments, the identification and quantification of metabolites, and the annotation of the results in the context of biochemical pathways.

Key words Cancer, CDK inhibitor, Metabolomics, Mode-of-action, Pathway analysis

1 Introduction

Cyclins are important regulators of cell cycle in both normal and transformed eukaryotic cells. Cyclins regulate the cyclin-dependent kinases (CDKs), which have a variety of functions within the cell including the regulation of cell cycle [1, 2]. Because control of cell cycle is an attractive target in cancer therapy [3, 4], inhibitors of CDKs (CDKIs) are being explored as a novel therapeutic class. Although regulation of cell cycle was the original motivation for the identification of CDK inhibitors [5], new insights into the mechanism of CDK inhibitor-mediated cytotoxicity have revealed roles of CDKs unrelated to cell cycle but important for the maintenance

of the cancer cell [6]. The complex and multifaceted role of CDKs in cell functioning would benefit from the introduction of new experimental approaches capable of providing a holistic view of the interplay between the different biological activities associated to CDKs. Furthermore, the progressive development of new CDKIs requires a much better understanding of the mode-of-action of these compounds [7]. In this context, it becomes necessary to introduce new tools capable of providing an in-depth insight about the different biochemical pathways involved in the response to this new class of chemotherapeutic agents.

Metabolomics, a comprehensive tool for monitoring biological systems, impacts on a number of important areas including drug discovery and development [8]. Metabolomics involves the analysis of small molecules present in biological samples and can lead to an improved understanding of small molecule's actions and to a better selection of pharmaceutical targets. This experimental approach has the possibility of transforming our knowledge of inhibitors' action through the examination of chemical-induced metabolic pathways associated to both the efficacy and the development of adverse drug reactions. The application of metabolomic approaches is particularly relevant in oncology [9]. The reason for that is that metabolic requirements of cancer cells are different from those of most normal differentiated cells. Because the metabolome captures the state of a cell, being a direct measure of protein activity, any observed changes in the metabolome as a result of the treatment with inhibitors would provide information on the inhibitor's activity and selectivity [10, 11].

An experimental approach for evaluating the cell metabolome of mammalian cells using metabolomics by NMR is described in this chapter. A description is provided of the procedures involved in the extraction of hydrophilic and lipophilic metabolites from cell cultures in the absence/presence of CDKIs, the acquisition of 1D ^1H -NMR metabolomic experiments leading to the measurement of the metabolic content of mammalian cells, and the chemical characterization of these metabolites. Furthermore, bioinformatic approaches for annotating and interpreting the results obtained in the absence/presence of CDKIs are also described.

2 Materials

2.1 Reagents

1. Sterile cell culture plates.
2. Sterile culture media for mammalian cell culture with filtered inactivated FBS.
3. Mammalian cell line.
4. CDKI stock solution in DMSO (aliquots stored at $-20\text{ }^\circ\text{C}$).
5. Sterile cell scraper (BD Falcon).

6. Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, filtered.
7. Methanol, LC-MS grade.
8. Liquid nitrogen.
9. Chloroform, LC-MS grade.
10. Water, LC-MS grade.
11. 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid or TSP-d₄ (Eurisotop).
12. D₂O 99 % D (Eurisotop).
13. NMR phosphate buffer: 100 mM NaH₂PO₄, 0.1 mM TSP-d₄, pH = 7.4, solution in D₂O.
14. CDCl₃ 99 % D, with 0.0.3 % (v/v) tetramethylsilane (TMS), in capsules (Eurisotop).
15. NMR tubes, 5 mm, quality ≥100 MHz (Norell).

2.2 Equipment

1. Standard equipment for human cell culture.
2. Multiscan reader for 96-well plates.
3. Centrifuge for 15 ml tubes up to 13,000 × *g*.
4. Centrifuge for 1.5 ml tubes up to 13,000 × *g*.
5. Vortex.
6. Lyophilizer.
7. Speed vacuum concentrator.
8. Glass pipettes.
9. NMR spectrometer 400–600 MHz (Bruker).
10. NMR probe with inverse detection, nuclei: ¹H and ¹³C (Bruker).

3 Methods

The analysis of the metabolic changes experienced by a mammalian cell culture in the presence of CDKIs requires a precise combination of cell biology approaches, advanced analytical methods and bioinformatics tools [12]. Cells will be exposed to an effective dose of the CDKI, and the growth and harvest conditions have to be optimized for the metabolite quantification by NMR [13], as first described (*see* Subheading 3.1) (Fig. 1). Afterwards, hydrophilic and lipophilic metabolites are extracted from the harvested pellet using a combination of different solvents (*see* Subheading 3.2). Frozen extracts are then prepared for NMR analysis and optimized 1D and 2D experiments are acquired and processed [14] (*see* Subheading 3.3). Finally, efforts are directed to the assignment of all relevant metabolites [15] and the identification of the metabolic pathways associated to the metabolic changes (*see* Subheading 3.4).

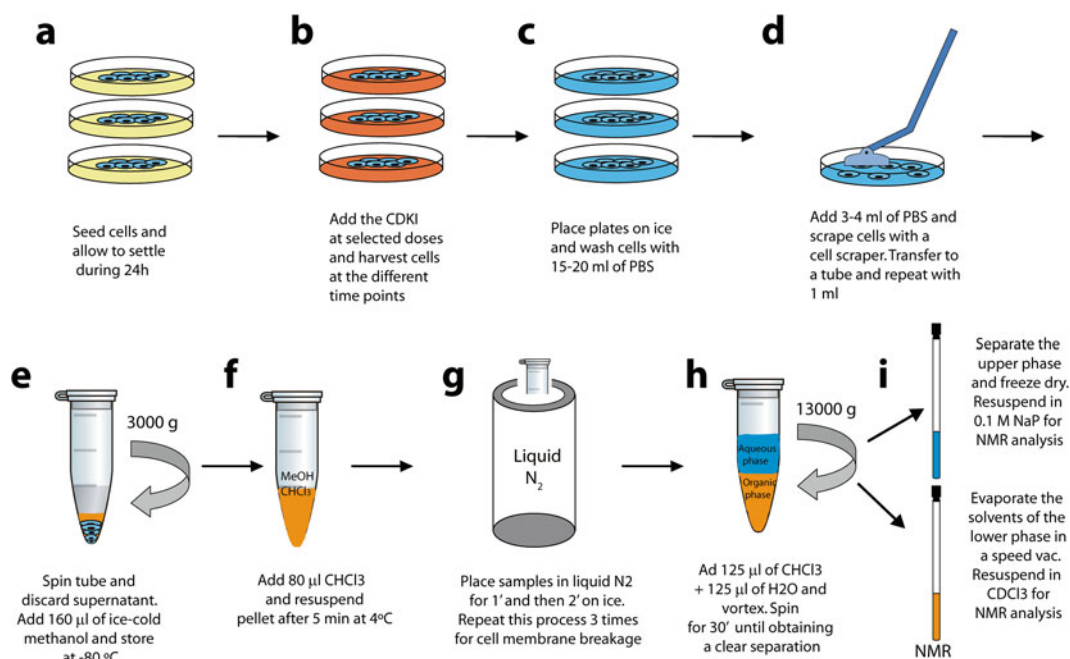


Fig. 1 Summary of the main steps that have to be carried out to obtain a metabolic extract in the presence of a CDKI including: cell culture (a–b), cell harvesting (c–e), metabolite extraction (f–h), and NMR sample preparation (i)

3.1 Cell Culture in the Presence of CDKIs

There are two key factors that have to be taken into account when studying the effect of a CDKI on a cell culture, the dose and the time point. Therefore, the cytotoxic effect of the CDKI should be first evaluated using a dose–response curve at different time points (*see Note 1*). An optimal way to perform the study is to choose at least two different time points and two different doses. This will facilitate the analysis of the effect of the inhibitor over time and the evaluation of the potential differences between doses (i.e., high and low cytotoxicity).

Depending on the cell type, cells may be either grown as a suspension or as adherent cell culture, with a slightly different harvesting protocol, as stated below. The number of cells that are finally harvested for each NMR sample should be between 1 and 20 million cells (*see Note 2*). The reliability of the final results required at least three independent cell cultures, each of them with 2–3 replicates, for each condition.

3.1.1 Cell Growth and Treatment

1. Seed cells in sterile p150 plates (*see Note 3*) at a concentration that has been optimized to have a 95–100 % confluence at the final time point.

2. Allow cells to settle for 24 h. In the case of adherent cells, it should be checked that cells are now well attached to the plate. This will be the initial time point.
3. Treat cells at the time points and doses previously defined with the CDKI of interest, previously filtered with a 0.2 μm filter. Control cells should be treated with the filtered vehicle at the same time points. Particular attention must be paid to avoid contamination of the cells during the treatment with the CDKI.

3.1.2 Cell Harvesting of Adherent Cells

1. Place plates on ice, remove the culture medium (*see Note 4*) and wash cells quickly with 15–20 ml of PBS.
2. Add small amount (3–4 ml) of PBS to the plates and scrape cells from the bottom of the plate with a cell scraper. Transfer the resulting cell suspension to a 15 ml tube. To collect the remaining cells, add again 1 ml of PBS to the plates, resuspend the remaining cells and add this suspension to the tube.
3. Spin the cell suspension at $3000 \times g$ for 5 min at 15 °C and remove the PBS.
4. Add 160 μl of ice-cold methanol to the pellet to ensure that the metabolism is completely quenched [16].
5. Snap-freeze tubes in liquid nitrogen and store them at –80 °C (*see Note 5*).

3.1.3 Cell Harvesting of Cells in Suspension

1. Transfer the cell suspension to plastic tubes, centrifuge at $3000 \times g$ and 15 °C, and remove the medium (*see Note 4*).
2. Add 15 ml of PBS to the pellets and vortex the tubes for 10 s. Spin tubes at $3000 \times g$ for 5 min at 15 °C, and remove the PBS.
3. Add 160 μl of ice-cold methanol to the pellet to ensure that the metabolism is completely quenched.
4. Snap-freeze tubes in liquid nitrogen and store them at –80 °C (*see Note 5*).

3.2 Cell Extraction

Once cells have been collected, an extraction of the intracellular metabolites from the frozen pellets is performed. The optimal method for this procedure is the so-called chloroform-methanol extraction [17], based on the combination of polar and nonpolar solvents to extract hydrophilic metabolites (e.g., sugars, amino acids, organic acid, nucleotides) as well as lipophilic compounds (e.g., fatty acids, phospholipids, steroids). It is critical to follow the time periods described in the procedure and to ensure that cells are kept at a temperature ≤ 4 °C during the whole extraction procedure to achieve reproducible results.

1. Place frozen pellets corresponding to ten million cells (*see Note 6*) on ice and allow them to thaw for 5 min.
2. Add 80 μl chloroform previously cooled to 4 °C.

3. After 30 min, homogenize samples with a vortex, resuspend the pellet with a pipette and transfer the suspension to an eppendorf tube.
4. For uniform cell breakage, place the samples in liquid nitrogen for 1 min and then allow them to thaw on ice for 2 min. Repeat this step two more times (*see Note 7*).
5. Add 125 μl of distilled water and 125 μl of chloroform (both at 4 °C) and vortex the samples.
6. Spin samples at 13,000 $\times g$ for 20 min at 4 °C to obtain two phases (*see Note 8*):
 - An upper methanol/water phase (with hydrophilic metabolites, aqueous phase).
 - A lower chloroform phase (with lipophilic compounds, organic phase).
7. Transfer each phase into a separate tube:
 - Aqueous phase (top) is collected with a micropipette of 200 μl .
 - Organic phase (bottom) is collected with a glass pipette.
8. Spin each phase separately at 13,000 $\times g$ for 2 min at 4 °C and discard the remaining solvent from the other phase with a pipette.
9. Freeze the aqueous phase with liquid nitrogen and lyophilize it overnight to remove water and methanol.
10. Remove the solvents from the organic phase using a speed vacuum concentrator at room temperature (1 h).
11. Store sample extracts at -80 °C until sample preparation for NMR experiments.

3.3 NMR Experiments

To perform the NMR analysis of the metabolic profile of the cell extracts obtained in the presence of the CDKI, the cell extracts have to be dissolved in suitable NMR solvents. Most of the time, these solvents are deuterated to avoid the presence of extra signals in the ^1H NMR spectra. Aqueous solutions are usually buffered at a physiological pH value (by default 7.4) to avoid pH differences between samples, which may result in signal shifts in the spectra. Once NMR samples have been prepared, 1D spectra are acquired for all samples. It is critical to acquire all NMR experiments with the same parameters to facilitate a reliable comparison of the metabolites in different conditions.

After acquisition, NMR spectra have to be processed (*see Note 9*). A fundamental step is the referencing of all spectra to a specific signal (TSP and TMS at 0 ppm are normally used, but also other signals such as specific metabolites that are present in all samples may be used) to ensure that spectra are perfectly aligned before they are integrated.

3.3.1 Preparation of NMR Samples

1. Place samples on ice and allow them to thaw for 5 min.
2. Add 600 μl of phosphate NMR buffer to the aqueous phase and vortex the samples. Spin at $12,000 \times g$ for 5 min. Transfer 550 μl of the supernatant into a 5 mm NMR tube (*see Note 10*).
3. Dissolve the organic extract in 600 μl of deuterated chloroform with 0.03 % TMS. Vortex the sample and then centrifuge at $12,000 \times g$ for 5 min. Transfer 550 μl of the supernatant into a 5 mm NMR tube with a glass pipette.
4. NMR samples should be prepared the same day they are analyzed, and stored at 4 °C before the spectrum acquisition.

3.3.2 Acquisition of NMR Spectra

1. Place the sample inside the magnet and adjust the temperature to 27 °C. Allow the sample to equilibrate for 5 min.
2. Tune and match the probe for ^1H (and ^{13}C in the case of acquiring also 2D heteronuclear experiments).
3. Lock the sample for D_2O (aqueous phase) or CDCl_3 (organic phase).
4. Adjust the field homogeneity until obtaining a linewidth $<1\text{ Hz}$ for the TSP (aqueous samples) or the TMS (organic samples) signal.
5. Determine the ^1H 90° pulse.
6. For aqueous samples, acquire a 1D ^1H -NMR spectrum with presaturation over a region of 25 Hz and optimize the value of the center of the spectrum to minimize the water signal.
7. Acquire 1D NOESY spectra for all samples (*see Note 11*). Record an appropriate number of free induction decays (FIDs) providing a good signal to noise (S/N) ratio in minimal experimental time. Total experiment time should not exceed 30–60 min.
8. Select 1–2 representative samples from each sample group and acquire a 2D Total Correlation Spectroscopy (TOCSY) experiment and 2D Heteronuclear Single Quantum Correlation (HSQC) experiment (*see Note 12*).

3.3.3 Processing of NMR Spectra

1. Transform all spectra applying an exponential function with a 0.5 Hz line broadening factor (*see Note 13*).
2. Correct the phase of each spectrum with the manual phase option.
3. Reference the spectra to the TSP (aqueous samples) or the TMS (organic samples) signal at 0 ppm.

3.4 Analysis of Metabolomic Data

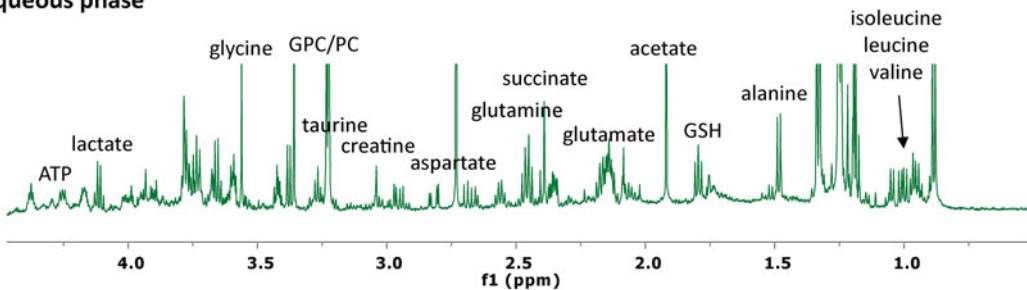
A comprehensive understanding of the metabolic consequences of the addition of CDKIs to a cell culture requires a thorough analysis of all the metabolites present in the aqueous and organic phases

obtained after the extraction procedure. This information is also required for performing statistical and quantitative analysis of the data. Therefore, the analysis of the metabolomic data generated in previous steps involves the assignment, quantification and characterization, in terms of their involvement in different biological pathways, of all the metabolites identified in the absence/presence of CDKIs.

3.4.1 Assignment of Relevant Metabolites

Metabolite assignment is usually performed using a combination of different approaches, including Analysis of Mixtures (AMIX 3.9.7; Bruker BioSpin, Karlsruhe, Germany), the SBASE compound library (BBIORFCODE 2.0.0 database), the standard spectra database in HMDB 3.0 (<http://www.hmdb.ca>), as well as other existing public databases and literature reports. Ambiguities in the assignment of metabolites associated to severe overlapping of signals in the 1D ^1H -NOESY experiments are usually resolved using 2D ^1H - ^1H -TOCSY and ^1H - ^{13}C -HSQC. The assignment of key metabolites can be confirmed by a spiking procedure, based on the addition of the candidate metabolite to a sample and the acquisition of an NMR spectrum to confirm that the signals match the proposed assignment. Figure 2 shows as an example the assignment of MCF7 cells.

Aqueous phase



Organic phase

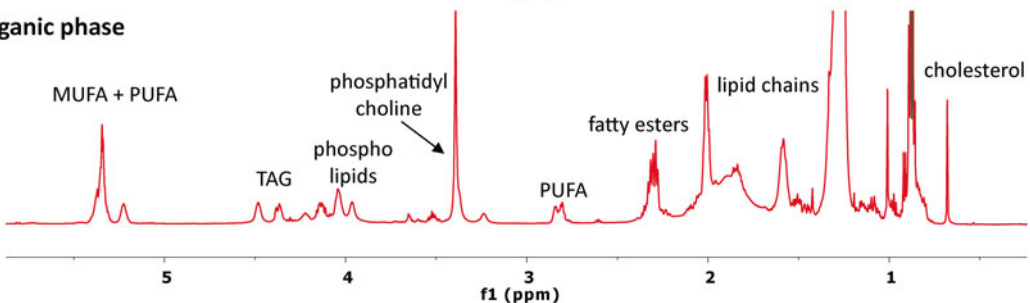


Fig. 2 1D ^1H -NMR spectrum obtained from the aqueous and organic extracts of MCF7 cells acquired with a 600 MHz NMR spectrometer with a TCI cryoprobe at 27 °C in the absence of a CDKI. NMR assignment for some of the metabolites detectable in both phases is indicated. *GPC* glycerophosphocholine, *PC* phosphocholine, *GSH* reduced glutathione, *ATP* adenosine triphosphate, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

3.4.2 Statistical and Quantitative Analysis

Once the metabolites detectable in the different extracts have been assigned, spectral regions corresponding to the different metabolites are automatically integrated. The statistical and quantitative analysis comprises the following steps:

1. Use the variable size bucketing module in AMIX 3.9.7 (*see Note 14*) to obtain the exact integral value corresponding to the NMR signal of each selected metabolite in each NMR spectrum (*see Note 15*).
2. Calculate an average fold change for each metabolite included in the analysis. This value is calculated as the ratio of average value in the cell culture exposed to the CDKI to that in the absence of the compound, indicating an overall change direction of the metabolite in both situations.
3. Perform an analysis of the statistical significance of the differences between the means of the two groups using the nonparametric Mann–Whitney *t* test. A *p*-value <0.05 (confidence level 95 %) is considered statistically significant.

3.4.3 Identification of Relevant Metabolic Pathways

Based on the results obtained in the previous steps, a molecular pathway and network analysis is performed to identify the key biological pathways that could likely explain the observed differences of the metabolic profiles in the presence of CDKIs. The analysis is carried out using Ingenuity Pathway Analysis (IPA; <http://www.ingenuity.com>), a web-based software application that identifies biological pathways and functions relevant to biomolecules of interest. This process consists of the:

1. Evaluation of the systematic influence of the treatment with CDKIs. This step is performed using MetaMapp [18] and the Cytoscape plug-in Metscape [19], and by uploading onto an IPA server the metabolites identified as statistically significant, as well as their change directions.
2. Evaluation of canonical pathways and molecular interaction networks based on the knowledge sorted in the Ingenuity Pathway Knowledge Base.
3. Display the ratio of the number of metabolites that map to the canonical pathway divided by the total number of molecules that map to the pathway.
4. Calculation of a *p*-value based on Fischer's exact test to determine the probability of the association between the metabolites and the canonical pathways.
5. Calculation of network scores with the right-tailed Fischer's exact test. The higher a score is, the more relevant the submitted metabolite is to the network.

This analysis should facilitate the identification of the most relevant molecular pathways associated to the response to CDKIs.

4 Notes

1. A typical cell viability assay can be performed with MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide). To carry out this experiment, cells are seeded into sterile 96-well microtiter plates at 25,000 cells/cm². Cells are allowed to settle for 24 h before the inhibitor, previously filtered with 0.2 µm filter, is added. Cells are treated with at least ten different concentrations of inhibitor within a range of 1:100. After 72 h of incubation, 10 µl MTS–PMS (20:1) are added to each well, and the cells are incubated for 2 further hours. Then, plates are read spectrophotometrically at 490 nm using a plate reader. Data are represented as the percentage of viable cells after the treatment, taken as 100 % of cell viability the untreated cells.
2. The number of cells that are required for each NMR sample depends on the cells size and has therefore to be optimized in each case. A factor that has to be taken into account is the kind of NMR spectrometer used for the analysis. If a strong field (600–800 MHz) equipment or cryoprobe is available, samples from 1 million or even 0.5 million cells will provide high quality spectra. However, lower fields (300–500 MHz) NMR spectrometers would require at least five million cells to obtain an adequate signal to noise ratio in a reasonable acquisition time.
3. In general, P150 plates are an optimal choice for metabolomic analysis by NMR. The amounts of PBS indicated at the cell harvesting step are adjusted for these plates. In the case of cells grown in suspension, these steps are adjusted to a T75 dish with 15 ml of medium. When smaller or bigger plates are used, the PBS volume should be slightly increased/decreased in order to achieve an efficient washing.
4. In some studies, the analysis of the metabolic profile of the culture medium can provide valuable information about the metabolites that are generated/consumed by the cells [20]. Therefore, it is advisable to store 1 ml of culture medium from each plate/flask at –80 °C, in case it may be needed later. Medium samples can be directly measured by NMR after mixing 300 µl of medium with 300 µl of PBS NMR buffer in an NMR tube.

For adherent cell cultures, the culture medium should be always checked for the presence of cells in suspension resulting from detachment due to the treatment. If this is the case, then the culture medium should be treated following the procedure described in Subheading 3.1.3 and put together with the pellet of the adhered cells.
5. Cell pellets can be stored at –80 °C up to 6 months before performing the metabolite extraction. If several batches of cells

are grown, it is advisable to freeze the pellets, and extract and analyze all at the same time to reduce the variability associated to different extraction or NMR acquisition days.

6. The solvent volumes described in the extraction procedure are optimized for a pellet of ten million cells. When working with a significantly different number of cells, solvent volumes should be recalculated. This is important for two reasons; if an insufficient solvent volume is used, not all metabolites may be extracted, and if too much solvent volume is used, more cell proteins may be solubilized.
7. For a complete extraction of the metabolites, cell and organelle walls have to be completely broken during the extraction procedure. This breakage may already have taken place when cells are thawed after storage at -80°C . Still it is important to carry out at least 1–2 freeze–thaw cycles to ensure a homogeneous cell breakage and higher reproducibility in the final data.
8. If no clear separation is observed, samples may be centrifuged again, but ensure that the same centrifugation time is applied for all samples.
9. If spectra are acquired using Bruker NMR spectrometers, then the software used for spectra acquisition and processing is TopSpin. Spectra integration can then be carried out with the complementary software AMIX, also from Bruker. Another suitable option for processing and integration is the program MestReNova, which reads raw data from different kind of NMR spectrometers.
10. It is advisable to use NMR tubes with a quality of at least 100–200 MHz to ensure that good field homogeneity and water suppression can be obtained for the NMR experiments.
11. Although the relaxation time filtered CPMG (Carr–Purcell–Meiboom–Gill) experiment is widely used for metabolomic samples, in the case of cell extracts, that do not contain many protein signals, a 1D NOESY spectrum is often sufficient for capturing the metabolic profile. Suitable parameters for this experiment are:
 - A NOESY mixing time of 10 ms.
 - A calibrated ^1H 90° pulse.
 - A relaxation delay of 3–4 s in order to ensure full relaxation and thus increase the reliability of the quantification of the metabolites.
 - An acquisition time of 2 s corresponding to a digitalization of 64 k data points and a spectral width of 30 ppm.
 - The same receiver gain (rg) value should be applied for all spectra, optimal values are between 64 and 256. Higher rg

values provide a better signal to noise ratio, but may cause baseline and phase problems when working with concentrated samples.

12. Suitable parameters for 2D experiments of cell extract samples are 256–512 increments in the indirect dimension and 32–96 transients in the direct dimension. In the case of the HSQC experiment, the sensibility enhanced variant with adiabatic pulses in ^{13}C provides an improved signal to noise ratio. TOCSY spectra can be recorded using standard MLEV or DIPSI pulse sequences with mixing times (spin-lock) of 60–80 ms.
13. When working with more diluted samples, also higher line-broadening factors (1–2 Hz) can be applied to achieve a better signal to noise ratio in the final spectrum. However, it should be taken into account that spectra transformed with higher line-broadening factors may suffer from a loss in resolution.
14. The variable size option of AMIX requires a pattern file with the regions of all assigned metabolites to be previously created.
15. The integration modes in AMIX allows the normalization of the signal integrals to total spectrum intensity (this option is useful to avoid the influence of dilution effects when samples of different cell concentration are analyzed) or to the intensity of a specific reference signal, for example the TSP signal (this option allows the calculation of the exact concentration of each metabolite in the sample).

References

1. Hirama T, Koeffler H (1995) Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* 86:841–854
2. Murray AW (2004) Recycling the cell cycle: cyclins revisited. *Cell* 116:221–234
3. Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 9:153–166
4. Shapiro GI (2006) Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol* 24:1770–1783
5. Fischer PM, Gianella-Borradori A (2005) Recent progress in the discovery and development of cyclin-dependent kinase inhibitors. *Expert Opin Investig Drugs* 14:457–477
6. Bose P, Simmons GL, Grant S (2013) Cyclin-dependent kinase inhibitor therapy for hematologic malignancies. *Expert Opin Investig Drugs* 22:723–738
7. Blachly JS, Byrd JC (2013) Emerging drug profile: cyclin-dependent kinase inhibitors. *Leuk Lymphoma* 54:2133–2143
8. Nicholson JK, Lindon JC (2008) Systems biology: metabolomics. *Nature* 455:1054–1056
9. Spratlin JL, Serkova NJ, Eckhardt SG (2009) Clinical applications of metabolomics in oncology: a review. *Clin Cancer Res* 15:431–440
10. Wei R (2011) Metabolomics and its practical value in pharmaceutical industry. *Curr Drug Metab* 12:345–358
11. Powers R (2014) The current state of drug discovery and a potential role for NMR metabolomics. *J Med Chem* 57:5860–5870
12. Zhang A, Sun H, Xu H et al (2013) Cell metabolomics. *OMICS* 17:495–501
13. Aranibar N, Borys M, Mackin NA et al (2011) NMR-based metabolomics of mammalian cell and tissue cultures. *J Biomol NMR* 49:195–206

14. Barding GA Jr, Salditos R, Larive CK (2012) Quantitative NMR for bioanalysis and metabolomics. *Anal Bioanal Chem* 404:1165–1179
15. Wishart DS, Jewison T, Guo AC et al (2013) HMDB 3.0 – the human metabolome database in 2013. *Nucleic Acids Res* 41:D801–D807
16. Teng Q, Huang W, Collette TW et al (2009) A direct cell quenching method for cell-culture based metabolomics. *Metabolomics* 5: 199–208
17. Beckonert O, Keun HC, Ebbels TM et al (2007) Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2:2692–2703
18. Barupal DK, Haidiya PK, Wohlgemuth G et al (2012) MetaMapp: mapping and visualizing metabolomic data by integrating information from biochemical pathways and chemical and mass spectral similarity. *BMC Bioinformatics* 13:99
19. Gao J, Tarcea VG, Karnovsky A et al (2010) Metscape: a cytoscape plug-in for visualizing and interpreting metabolomic data in the context of human metabolic networks. *Bioinformatics* 26:971–973
20. Duarte TM, Carinhas N, Silva AC et al (2014) ¹H-NMR protocol for exometabolome analysis of cultured mammalian cells. *Methods Mol Biol* 1104:237–247