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Extraction of aqueous metabolites from cultured adherent cells for metabolomic analysis by capillary electrophoresis-mass spectrometry

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TITLE:

Extraction of Aqueous Metabolites from Cultured Adherent Cells for Metabolomic Analysis by Capillary Electrophoresis-Mass Spectrometry

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KEYWORDS:

Metabolome, cancer cells, capillary electrophoresis-mass spectrometry (CE-MS), Glucose-6-phosphate dehydrogenase (G6PD), oxidative stress, diamide

SUMMARY:

The purpose of this article is to describe a protocol for extraction of aqueous metabolites from cultured adherent cells for metabolomic analysis, particularly, capillary electrophoresis-mass spectrometry.

ABSTRACT:

Metabolomic analysis is a promising omics approach to not only understand the specific metabolic regulation in cancer cells compared to normal cells but also to identify biomarkers for early-stage cancer detection and prediction of chemotherapy response in cancer patients. Preparation of uniform samples for metabolomic analysis is a critical issue that remains to be addressed. Here, we present an easy and reliable protocol for extracting aqueous metabolites from cultured adherent cells for metabolomic analysis using capillary electrophoresis-mass spectrometry (CE-MS). Aqueous metabolites from cultured cells are analyzed by culturing and washing cells, treating cells with methanol, extracting metabolites, and removing proteins and

macromolecules with spin columns for CE-MS analysis. Representative results using lung cancer cell lines treated with diamide, an oxidative reagent, illustrate the clearly observable metabolic shift of cells under oxidative stress. This article would be especially valuable to students and investigators involved in metabolomics research, who are new to harvesting metabolites from cell lines for analysis by CE-MS.

Introduction:

Otto Warburg observed that cancer cells acquire the unusual ability to take up glucose and ferment it to produce lactate in the presence of adequate oxygen—a phenomenon termed as Warburg effect or aerobic glycolysis[1,2]. Mitochondrial respiration defects are speculated as the underlying basis for aerobic glycolysis in cancer cells[3]. Indeed, the Warburg effect is the basis for tumor imaging by fluorodeoxyglucose (FDG)-positron emission tomography (PET), which is widely used in clinical practice[4,5]. A high rate of aerobic glycolysis is considered a key feature of cancer and has been recently adopted as one of the well-known "hallmarks of cancer," as described by D. Hanahan and B. Weinberg[6]. Somatic mutations in oncogenes and tumor suppressor genes—such as *HRAS/KRAS/NRAS*, *EGFR*, *BRAF*, *MYC*, *TP53*, isocitrate dehydrogenase (*IDH*), and fumarate hydratase (*FH*)—have been linked to specific metabolic changes in cancer cells, believed to be a result of the Warburg effect[7].

Metabolomic analysis is a promising approach not only to understand metabolic regulation in cancer cells but also to identify early-stage cancer biomarkers and chemotherapy response prediction. Following treatment of sensitive or resistant cancer cells with anticancer compounds, tracking of their metabolic responses facilitates identification of metabolic biomarkers to predict efficacy of specific anticancer therapies in cancer patients[8-11]. In this article, cancer cell lines derived from a lung adenocarcinoma with an *EGFR* mutation treated with diamide—which causes oxidative stress—were used as models for metabolomic analysis. The advantage of this analytical method using capillary electrophoresis-mass spectrometry (CE-MS) is its comprehensive measurement of charged metabolites with the mass range *m/z* 50-1000[12,13]. The purpose of this article is to provide novices a detailed stepwise visual protocol for preparation of aqueous metabolites from cultured cancer cells and subsequent metabolomic analysis, particularly by CE-MS.

PROTOCOL:

1. Cell culture on day 1

NOTE: Each sample for metabolite extraction should be prepared from a single 100 mm tissue culture dish that is moderately but not fully confluent (containing approximately 2–5 million cells). Calculate the number of dishes needed for the assay and prepare them accordingly.

1.1. Culture HCC827 and PC-9 cells in 5% CO_2 at 37 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

1.2. Aspirate the cell culture media from the 100 mm culture dishes.

1.3. Wash cells on each dish using 2 mL of phosphate buffered saline (PBS) solution without calcium and magnesium. Gently rock each dish so that the PBS solution completely covers the surface of the dish.

1.4. Aspirate the wash buffer from the culture dishes.

1.5. Warm 0.25% trypsin-EDTA solution to 37 °C and add 2 mL of trypsin-EDTA solution with a 5 mL serological pipette. Gently rock each dish so that the trypsin completely covers the surface of the dish.

1.6. Incubate the culture dishes at 37 °C for approximately 5 min.

1.7. Add 4 mL of pre-warmed complete growth medium per dish. Resuspend the cells in medium by gently pipetting several times.

1.8. Transfer each cell suspension to a separate 15 mL conical tube and centrifuge at 800 × *g* for 5 min.

1.9. Resuspend each cell pellet in 2 mL of pre-warmed complete growth medium.

1.10. Determine the total number and percent viability of the cells using the automated cell counter and 0.4% trypan blue solution.

1.10.1. Mix 10 µL of cell suspension and 10 µL of 0.4% trypan blue solution.

1.10.2. Load 10 µL of sample into the cell counting chamber slide through capillary action.

1.10.3. Insert the chamber slide into the automated cell counter. The transmitted light automatically illuminates and the instrument auto focuses on the cell.

1.10.5. Press the **capture** button to capture the image and display the results.

1.10.6. If necessary, add further growth medium to obtain the desired cell concentration.

1.11. Seed approximately 1–2.5 million cells per 100 mm cell culture dish.

NOTE: Metabolite concentrations determined by CE-MS analysis will be normalized based on the number of viable cells. For the purpose of cell counting, it is necessary to prepare at least one extra seeded culture dish for each group.

1.12. Incubate the culture dishes in 5% CO_2 at 37 °C for 18 h.

2. Preparation of reagents

2.1. Dilute a commercial internal standard solution including L-methionine sulfone and *d*-camphor-10-sulfonic acid 1000-fold in ultrapure water.

NOTE: For fewer than 80 samples, simply mix 50 µL of the Internal Standard Solution 1 and 45 mL of ultrapure water in a 50 mL volumetric flask, then bring the solution up to 50 mL with ultrapure water.

2.2. Prepare a 0.05 g/mL mannitol solution in ultrapure water as wash buffer.

NOTE: For fewer than 30 samples, simply dissolve 25 g of mannitol in 500 mL of ultrapure water. Approximately 15 mL of wash buffer is required per 100 mm culture dish, so prepare a sufficient volume of wash buffer according to the number of samples.

3. Pre-washing centrifugal filter units

3.1. Pipette 250 µL of ultrapure water into the filter cup of each centrifugal filter unit (see the **Table of Materials**).

NOTE: Two filter units per sample are required.

3.2. Cap the filter units tightly and centrifuge at 9,100 × *g* at 4 °C for 5 min.

3.3. Check the volume of each filtrate—if significant filtrate has accumulated during the first short spin, the filter unit may be defective. In this case, discard the filter unit and use a new filter unit instead.

3.4. Close the lids of the filter units tightly and centrifuge again at 9,100 × *g* at 4 °C for 30 min.

3.5. Ensure that no ultrapure water remains in any of the filter cups; remove the filtered ultrapure water in each collection tube with a pipette and discard.

NOTE: Do not try to remove residual water in a filter cup with a pipette as it may damage the filter.

3.6. Replace the filter cups into their collection tubes.

NOTE: Use the centrifugal filter units within an hour since the filters may become damaged upon drying.

4. Cell culture on day 2

NOTE: The recommended cell culture duration is 18 h.

4.2. Aspirate the cell culture medium from each 100 mm culture dish.

4.3. Add 10 mL of cell culture medium that includes the appropriate concentrations of compounds or drugs to each dish, taking care to not disturb the cell layer.

NOTE: For demonstration purposes, we added 10 µL of 250 mM diamide dissolved in PBS (final concentration of 250 µM) in this experiment.

4.4. Incubate the culture dishes at 37 °C for 30 min in the presence of diamide or PBS as a control.

4.5. Aspirate the cell culture medium from each 100 mm culture dish.

4.6. Wash cells by gently adding 2 mL of 5% mannitol solution to the edge of each dish, taking care to not to disturb the cell layer, then slightly tilt the dish.

NOTE: PBS or saline solution interferes with CE-MS-based metabolomic analysis and adversely affects measurement results, and thus should **not** be used as wash buffer.

4.7. Aspirate the wash buffer from each culture dish, then wash cells again by gently adding 10 mL of wash buffer per dish and slightly tilting the dish.

4.8. Completely aspirate the wash buffer from the edge of each culture dish.

NOTE: Aspirate as much wash buffer as possible, while paying attention to not aspirate the cells. Residual mannitol may interfere with CE-MS analysis; aspiration of cells will decrease the number of cells and thus become a source of error in data normalization.

5. Extraction of metabolites from cultured cells

5.1. Add 800 µL of 99.7% methanol per culture dish. Gently rock each culture dish back and forth to cover its entire surface. Leave the dishes at room temperature for 30 s.

5.2. Slowly add 550 µL of the diluted internal standard solution per dish by immersing the tip of the pipette into the methanol and gently pipetting up and down several times.

5.5. Gently rock each culture dish back and forth to cover its entire surface.

5.6. Leave the dishes at room temperature for 30 s.

6. Ultrafiltration of cell extracts

6.1. Transfer the extracted solution from each culture dish to a separate 1.5 mL microcentrifuge tube.

6.2. Centrifuge the tubes at 2300 × *g* at 4 °C for 5 min.

6.3. Transfer 350 µL of each supernatant into two centrifugal filter units per sample.

NOTE: From each culture dish, a total of 700 µL of the extracted solution is transferred into two filter tubes (350 µL/tube).

6.4. Centrifuge the filter tubes at 9,100 × *g* at 4 °C for approximately 2 h until no liquid remains in the filter cups.

6.5. Remove the filter cups and tightly close the lids of the collection tubes.

7. Sample evaporation

7.1. Prepare a centrifugal evaporator—typically, this consists of an evaporator, a cold trap, and a vacuum pump.

7.2. Place the collection tubes in the centrifugal evaporator.

NOTE: Leave the lids of the tubes open.

7.3. Evaporate the extracted sample solutions under vacuum conditions at room temperature.

NOTE: Typical configurations for the number of rotations and pressure are 1,500 rpm and 1,000 Pa, respectively, and it usually takes approximately 3 h to completely evaporate the samples.

7.4. Confirm that no liquid remains in any of the collection tubes and close the lids of the tubes tightly.

7.5. Store the collection tubes in an ultra-low temperature (−80 °C) deep freezer until metabolomic analysis.

8. Metabolomic analysis by CE-MS

8.1. Resuspend the filtrate in 50 µL of ultrapure water immediately before CE-MS analysis.

8.2. Perform CE-MS analysis by methods described previously[12,13] using capillary electrophoresis system and time-of-flight mass spectrometer system equipped with an isocratic pump, a CE-MS adapter, and a CE-ESI-MS sprayer.

NOTE: Both systems can be controlled by the software of the system vendors and are connected by a fused silica capillary (50 µm internal diameter × 80 cm total length).

8.2.1. Set up instruments and sample vials, prepare the capillary with a capillary cassette, replenish sheath liquids and appropriate electrophoresis buffers depending on anion or cation analysis mode, and then apply voltages.

NOTE: Instrumentation and analytical conditions are described in detail elsewhere[12,13].

8.2.2. Open the software and prepare a worklist containing data acquisition methods and sample information.

8.2.3. Start a test run and check the data such as the signal intensity and the peak shape of internal standards and the peak resolution of other standard compounds.

8.2.4. Fine-tune the analytical conditions if necessary.

8.2.5. Inject the sample solutions at 50 mbar for 3 s and a voltage of 30 kV.

NOTE: CE-MS was conducted either in the positive or negative ion mode. Set the spectrometer to scan the mass range *m/z* 50–1000. The capillary voltage was set at 4 kV; the flow rate of nitrogen gas (heater temperature 300°C). For positive mode, the fragmentor, skimmer and OCT RFV voltage were set at 75, 50 and 125 V, respectively. For negative ion mode, the fragmentor, skimmer, and OCT RFV voltage was set at 100, 50, and 200 V, respectively.

8.3. Analyze spectrum data.

8.3.1. Extract peaks from the mass spectral data using automatic integration software in order to obtain peak information including *m/z*, peak area, and migration time (MT).

NOTE: The method is described in detail elsewhere[14].

8.3.2. Exclude signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites.

8.3.3. Annotate remaining peaks with information from the HMT metabolite database according to *m/z* values and MTs.

8.3.4. Normalize areas of the annotated peaks to internal standard levels and numbers of cells per sample.

8.3.5. Evaluate the concentration of each metabolite in the cultured cells (pmol/10^6 cells) using standard curves prepared for each metabolite.

8.3.6. Use the quantified metabolite concentrations for subsequent statistical analyses and biological interpretations[14].

REPRESENTATIVE RESULTS:

Since metabolite concentrations in cancer cells (pmol/10^6 cells) are normalized to the number of viable cells, experimental conditions should be set up with care so as to minimize variation in the number of viable cells between conditions. For example, diamide treatment was at a relatively high concentration (250 µM) but for a short time to allow all the cells to grow as equally as possible, thereby equalizing the number of viable cells analyzed. Under these experimental conditions, HCC827 and PC-9 cells grew equally for 3 h (**Figure 1**). CE-MS analysis of diamide-treated cells compared with PBS-treated (control) cells revealed 175 and 150 differential metabolites in HCC827 and PC-9 cells, respectively. Among these, several intermediates in the pentose phosphate pathway (PPP) and in upper glycolysis were significantly higher in the diamide-treated conditions in both cell lines, whereas a few tricarboxylic acid (TCA) cycle intermediates were lower in the treated conditions (**Figure 2** and **Figure 3**).

The PPP generates reducing equivalents in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is used for redox homeostasis maintenance and fatty acid biosynthesis[15]. Following diamide treatment, the level of gluconic acid—an oxidized glucose—increased 12-fold in HCC827 cells and 10-fold in PC-9 cells; similarly, following diamide treatment, the level of glucose 6-phosphate (G6P)—a phosphorylated glucose and the first hexokinase-catalyzed glycolysis product—also increased 6.3- and 3.5-fold in HCC827 and PC-9 cells, respectively (**Figure 4**). In addition, following diamide treatment, the levels of 6-phosphogluconate (6PG)—the first intermediate in PPP—dramatically increased 89-fold in HCC827 cells and 231-fold in PC-9 cells compared to the levels seen in the PBS controls (**Figure 4**). In contrast, levels of other glycolytic intermediates, such as fructose 6-phosphate (F6P) and fructose 1,6-bisphosphate (F1,6P), did not change in the diamide experimental condition (**Figure 4**). Total nicotinamide adenine dinucleotide phosphate ($NADP^+$) levels were nearly equivalent between diamide treatment and PBS control conditions (**Figure 4**), suggesting that glucose was mainly catabolized via the PPP.

FIGURE LEGENDS:

Figure 1. Unchanged cell numbers upon diamide treatment. Cell growth responses to 250 µM of diamide were measured using trypan blue staining. Cell numbers of (**A**) HCC827 and (**B**) PC-9 cells treated with PBS (blue) or diamide (red; 250 µM) for 1 or 3 h are shown. Data are shown as the mean ± SD (*n* = 6).

Figure 2. Representative MS peaks of metabolites. Electropherograms annotated as (**A**) gluconic acid, (**B**) glucose 6-phosphate (G6P), (**C**) 6-phosphogluconate (6PG), and (**D**) nicotinamide

adenine dinucleotide phosphate ($NADP^+$) obtained by CE-MS analysis. Each line indicates the cell line (solid, HCC827; dotted, PC-9) and treatment (blue, PBS; red, diamide) used.

Figure 3. Metabolome profiles of intracellular metabolites. Fold changes of metabolites in (**A**) HCC827 and (**B**) PC-9 cells treated with diamide are shown as $\log_2$(diamide/PBS). In total, 175 and 150 metabolites were annotated in HCC827 and PC-9 cells, respectively.

Figure 4. Up-regulation of PPP upon diamide treatment. Intracellular concentrations (pmol/10^6 cells) of key metabolites involved in glycolysis and the pentose phosphate pathway (PPP) after treatment with diamide are shown. Metabolites were extracted from HCC827 and PC-9 cells treated with PBS (blue) or diamide (red, 250 μM) for 30 min. Representative metabolites such as gluconic acid, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), 6-phosphogluconate (6PG), and nicotinamide adenine dinucleotide phosphate ($NADP^+$) are shown.

DISCUSSION:

Here, we describe a widely accessible methodology to prepare metabolites from cultured cancer cells for CE-MS-based metabolomic analysis. One of the most critical points in this protocol is the proper preparation of cancer cells, because measured metabolite concentrations are normalized to the number of viable cells. For accurate estimation of cell number, it is necessary to prepare at least one additional culture dish per experimental group to count the number of viable cells in parallel with the extraction of metabolites for metabolomic analysis. In addition, the same number of cells should be seeded in each dish for the replicates and in the dish for counting; in the future, this would be aided by a quick and stressor-free (e.g., trypsin-free) cell counting protocol that allows the same dish to be used for both counting viable cells and extracting metabolites. Care should be taken during washes so that cells do not detach from the surface of the dishes. Severe cytotoxicity tests and other experiments that reduce cell adhesion may be unsuitable for this extraction protocol due to potential loss of cells during the washing procedure. It is important to use a 5% mannitol solution as the wash buffer for extracting metabolites from cultured cells for CE-MS-based metabolomic analysis, because salt-based buffers, such as PBS, interfere with metabolomic analysis and adversely affect measurement.

Two or three dishes can be combined as a single sample by individually extracting metabolites from each dish and then pooling samples; however, combining multiple dishes often increases residual mannitol in the extracted metabolite solution. This may also interfere with metabolomic analysis by CE-MS. Hence, it is recommended to not use multiple dishes or wells as a single sample.

This metabolomic analysis method using CE-MS has been developed for comprehensive measurement of charged molecules with molecular weights between 50 and 1000 Da; thus, this protocol is optimized for extraction of aqueous, low molecular weight compounds. Therefore, this protocol is not suitable for extracting hydrophobic metabolites such as lipids or macromolecules such as proteins and nucleic acids. Since there is an increasing demand for comprehensive lipid analyses or lipidomics of cultured cell samples, the development of an easy

and effective protocol for simultaneous extraction of both hydrophilic and hydrophobic metabolites is needed.

The first step of metabolite extraction—aspirating medium and washing cells with mannitol—should be conducted as quickly as possible to minimize changes to the metabolic profile of the cells. Treatment of cells with methanol after washing with mannitol is assumed to denature proteins and thereby prevent enzymes from catalyzing further metabolic reactions. However, even after methanol treatment, non-enzymatic chemical reactions—such as redox reactions, some decarboxylation processes, and thiol linkages—may take place. As such, any concentrations of metabolites involved in these reactions measured by this protocol should be interpreted with caution. In contrast to the genome or transcriptome, the metabolome consists of molecules with a wide variety of chemical properties; hence, no single protocol can extract all metabolites without any loss or disturbance. For more accurate measurements of such highly reactive metabolites, a protocol specifically designed to extract certain groups of metabolites, which requires fractionations and derivatizations, should be consulted. The protocol presented here, however, describes a simple and quick extraction of aqueous metabolites from cultured cell samples for metabolomic analysis by CE-MS. In this paper we could not describe how to set up CE-MS in detail because the focus of the present manuscript is different, however, describing detailed steps to set up CE-MS may require a separate dedicated article.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGMENTS:

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Figure 1

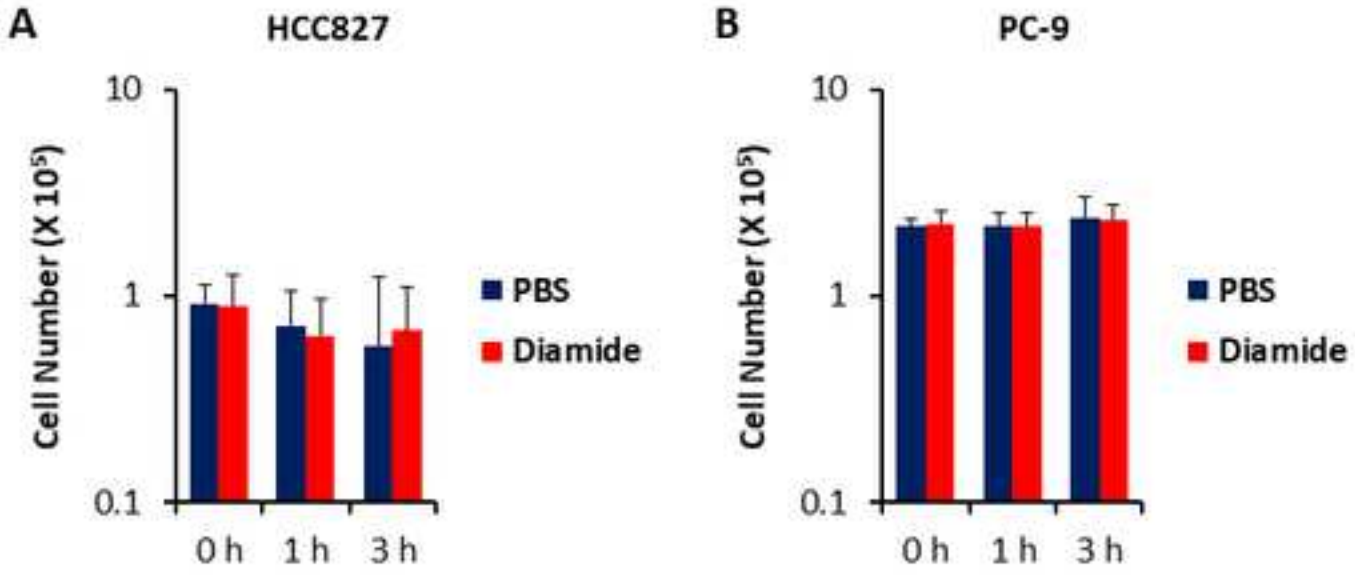

Figure 2

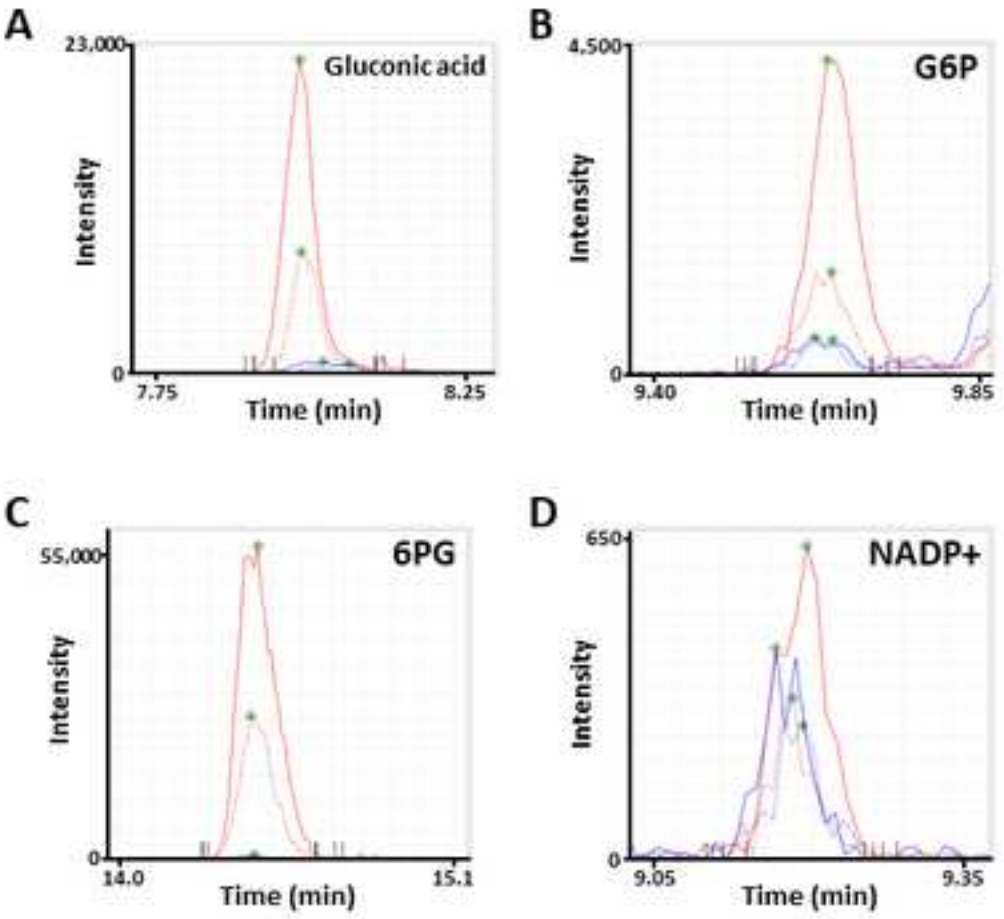

Figure 3

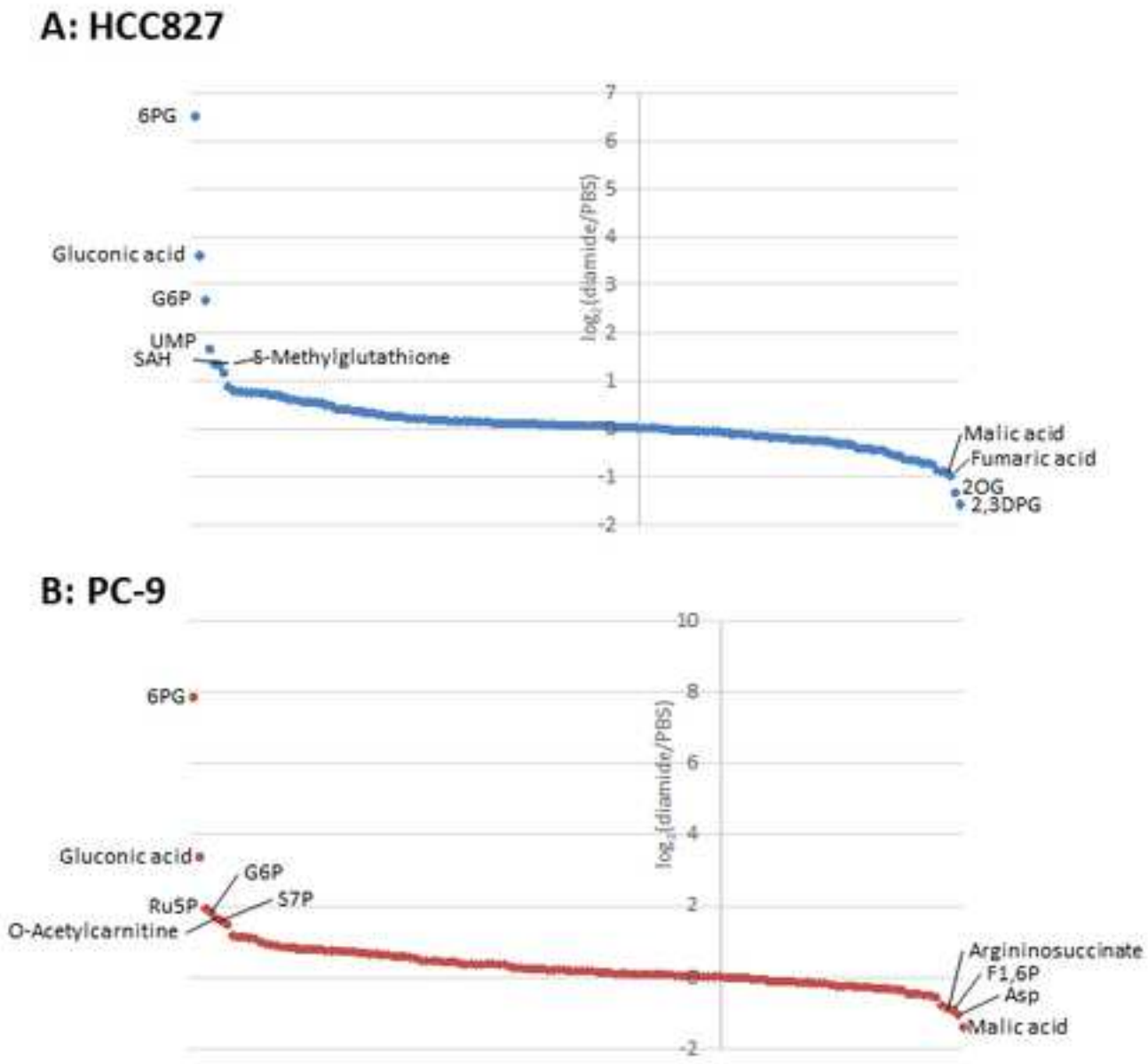

Figure 4

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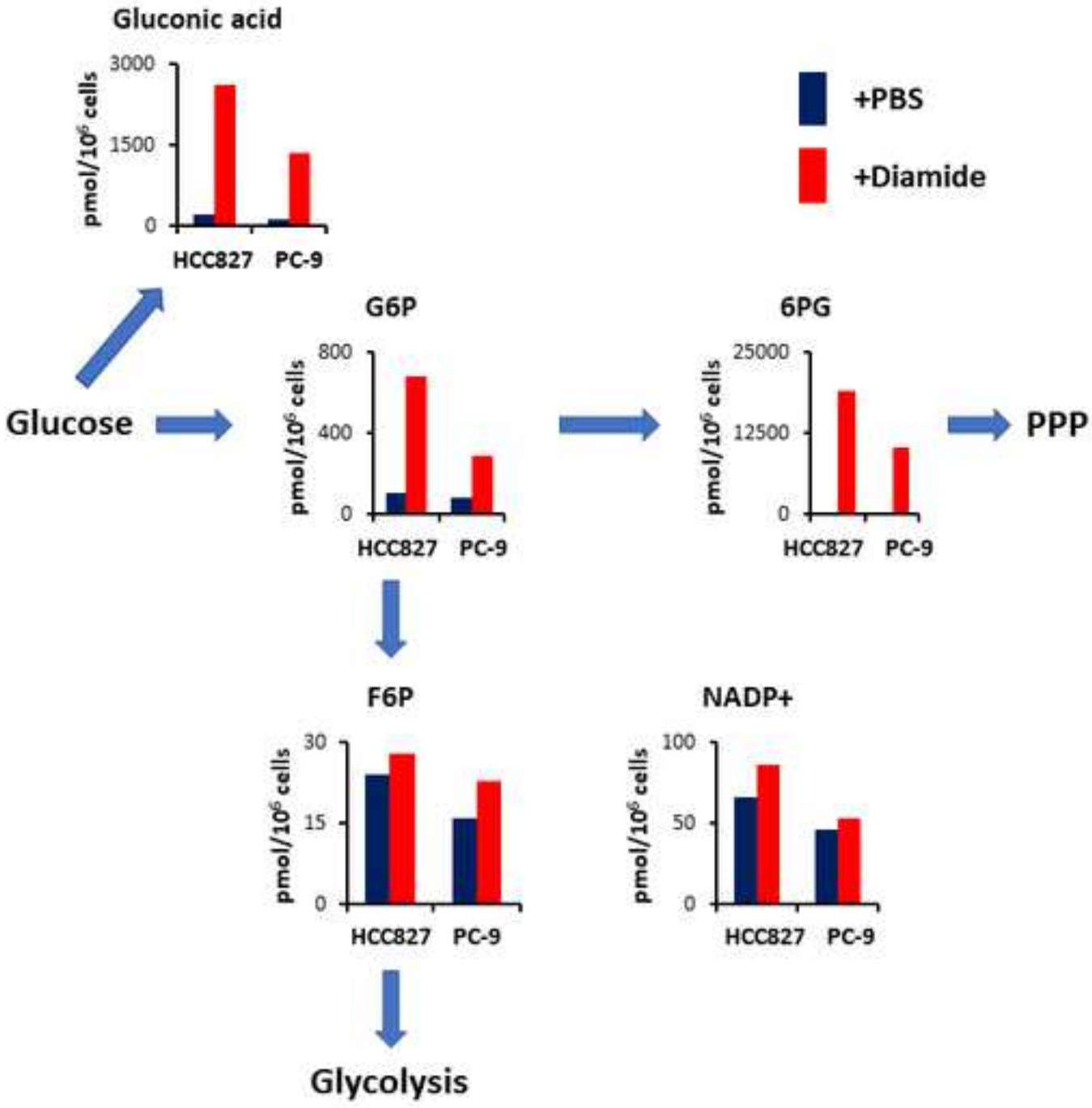

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Automated cell counter	Thermo Scientific	AMQAX1000	Countess II automated cell counter
Automatic integration software	Agilent Technologies	MassHunter G3335-60041	version B.02.00
CE system	Agilent Technologies	Agilent 7100 CE system	
CE/MS adapter kit	Agilent Technologies	G1603A	
CE-ESI-MS Sprayer kit	Agilent Technologies	G1607A	
Cell counting chamber slide	Thermo Scientific	C10282	Countess cell counting chamber slides
Centrifugal filter device, 5 kDa	Human Metabolome Technologies	ULTRAFREE MC PLHCC, UFC3LCCNB-HMT	
Conical sterile polypropylene tube, 15 ml	Thermo Scientific	N339651	
Conical sterile polypropylene tube, 50 ml	Thermo Scientific	N339653	
Costar stripette, 10 ml	Corning	4488	
Costar stripette, 5 ml	Corning	4487	
D (-)-Mannitol	Wako	133-00845	500 g
Dulbecco's phosphate buffered saline (DPBS)	Sigma-Aldrich	D8537-500ML	
Electrophoresis buffer	Human Metabolome Technologies	H3301-1001	for cation analysis
Electrophoresis buffer	Human Metabolome Technologies	H3302-1021	for anion analysis
Fetal bovine serum	Biowest	S1780	
Filter tip, 1000 µl	Watson	124P-1000S	
Filter tip, 20 µl	Watson	124P-20S	
Filter tip, 200 µl	Watson	1252-703CS	
Fused silica capillary	Polymicro Technologies	TSP050375	50 µm i.d. × 80 cm total length
HCC827	American Type Culture Collection	CRL-2868	
Internal standard solution	Human Metabolome Technologies	H3304-1002	
Isocratic pump	Agilent Technologies	Agilent 1100 Series Isocratic Pump	
Methanol	Wako	138-14521	1 L, LC/MS grade
Microtube, 1.5 ml	Watson	131-415C	
Operating Software	Agilent Technologies	ChemStation G2201AA	version B.03.01 for CE
PC-9	RIKEN Bio Resource Center	RCB4455	
RPMI-1640	Sigma-Aldrich	R8758-500ML	
Sterile tissue culture dish, 100 mm	Corning	430167	
Time-of-flight mass spectrometer	Agilent Technologies	Agilent G1969A Time-of-Flight LC/MS	
Trypan blue solution, 0.4%	Thermo Scientific	T10282	
Trypsin-EDTA solution	Sigma-Aldrich	T4049-100ML	
Ultrapure water	Merck	Milli-Q water	18.2 MΩ・cm pure water
Volumetric flask, 50 ml	Iwaki	5640FK50E	TE-32

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Title of Article:	Extraction of aqueous metabolites from cultured adherent cells for metabolomic analysis by capillary electrophoresis-mass spectrometry
Author(s):	Ami Maruyama, Kenjiro Kami, Kazunori Sasaki, Hajime Sato, Yuzo Sato, Katsuya Tsuchihara, Hideki Makinoshima

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Title:	Extraction of aqueous metabolites from cultured adherent cells for metabolomic analysis by capillary electrophoresis-mass spectrometry
Signature:	Hideki Makinoshima
Date:	13. Dec. 2018

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To,
JoVE Senior Review Editor
1 Alewife Center #200
Cambridge, MA 02140
TEL: +1-617-674-1888

Subject: Manuscript-JoVE59551

Dear Dr. Alisha Dsouza,

Thank you very much for your review of our manuscript entitled "Extraction of aqueous metabolites from cultured adherent cells for metabolomic analysis by capillary electrophoresis-mass spectrometry," for publication in *JoVE*.

We have modified the original manuscript according to the editorial members and reviewers' comments and are submitting a revised manuscript for your consideration. We believe that this version of the manuscript has corrected the deficiencies of the original manuscript. We greatly appreciate the editorial members and reviewers for their insightful comments, which have helped us to improve the paper significantly.

The detailed line-by-line responses are provided below. We hope this revised version of our manuscript can be published in JoVE.

Sincerely yours,

Hideki Makinoshima, Ph.D.
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Line-by-line replies to the editorial comments

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: Thank you very much for your comment. We carefully read our manuscript to ensure that there are no spelling or grammatical errors in the revised manuscript.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Response: We thank the editorial members for careful reading of our manuscript.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We thank again the editorial members for reading our manuscript carefully and giving constructive comments on the manuscript. The detailed line-by-line replies are provided below.

1) 1 Note: what is the surface treated with? Please mention reagent and concentration used. Add to the table of materials as well.

Response: We thank the editorial members for raising this concern and we agree with it. Therefore, we have revised this sentence as follows.

NOTE: Each sample for metabolite extraction should be prepared from a single 100 mm tissue culture dish that is moderately but not fully confluent (containing approximately 2–5 million cells). We also added this material in the table.

2) 1.1: mention cell lines used here (or tested by you). Mention culture media and environmental conditions.

Response: We have added this sentence as follows.

1-1. Culture HCC827 and PC-9 cells in 5% CO_2 at 37 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

3) 1.4: Prewarmed to what temperature? Mention pipette tip size to avoid cell damage.

Response: We have revised this sentence as follows.

1-5. Warm 0.25% trypsin-EDTA solution to 37 °C and add 2 mL of trypsin-EDTA solution with 5 ml stripette. Gently rock each dish so that the trypsin completely covers the surface of the dish.

4) 1.9 what is the trypan blue concentration?

Response: We have revised this sentence as follows.

1-10. Determine the total number and percent viability of the cells using the automated cell counter and 0.4% trypan blue solution.

5) 2.1: define the composition of the internal standard solution 1

Response: This product is commercially available. Therefore, we have revised this sentence as follows.

2-1. Dilute the commercial internal standard solution 1000-fold in ultrapure water.

6) 2.2: provide concentration in mol/L or g/mL to avoid ambiguity.

Response: We have rewritten this sentence as follows.

2-2. Prepare a 0.05 g/mL mannitol solution in ultrapure water as wash buffer.

7) 4.3: is diamide diluted in DMSO? What is the DMSO concentration? Please provide examples of drug to be diluted in DMSO or PBS.

Response: Diamide was dissolved in PBS. Thus, we have revised this sentence as follows.

4-3. Add 10 mL of cell culture medium that includes the appropriate concentrations of compounds or drugs to each dish, taking care to not disturb the cell layer.

For demonstration purposes, we added 10 µL of 250 mM diamide dissolved in PBS (final concentration 250 µM) in this experiment.

8) 4.4: mention example of the appropriate time period.

Response: We have rewritten this sentence as follows.

4-4. Incubate the culture dishes at 37 °C for 30 min in the presence of diamide or PBS as a control.

9) 5.1: 100% methanol?

Response: We have revised this sentence as follows.

5-1. Add 800 µL of 99.7% methanol per culture dish.

10) 5.4: internal standard solution 1? Please clarify.

Response: This product is commercially available. The detail information is described in Material Table.

11) 7.3: What is the vacuum strength? For how long is evaporation performed?

Response: We have added this sentence as follows.

Note: Typical configurations for the number of rotations and pressure are 1,500 rpm and 1,000 Pa, respectively, and it usually takes approximately 3 h to completely evaporate the samples.

12) 8.2: Please describe the exact steps. Mention all software actions in detail. How is the analysis performed? Citing a reference is insufficient.

13) 8.3: Mention software button clicks and selections in detail.

Response: We thank the editorial members for raising this concern and we agree with it. However, the main focus of this article is on how to extract aqueous metabolites from cultured adherent cells for metabolomic analysis. Our study is designed to focus specifically on culturing and washing cells, treating cells with methanol, extracting metabolites, removing proteins and macromolecules this time. In addition, this CE-MS part is not filmed this time. Thus, we would like to keep the focus intact, but we have changed three sentences as following your suggestion.

1. We have deleted the sentence “setting up CE-MS metabolomic analysis” from Abstract.

2. We have revised the 8. Metabolomic analysis by CE-MS section as follows.

---

8. Metabolomic analysis by CE-MS

8-1. Resuspend the filtrate in 50 µL of ultrapure water immediately before CE-MS analysis.

8-2. Perform CE-MS analysis by methods described previously[12,13] using capillary electrophoresis system and time-of-flight mass spectrometer system equipped with an isocratic pump, a CE-MS adapter, and a CE-ESI-MS sprayer.

Note: Both systems can be controlled by the software of the system vendors and are connected by a fused silica capillary (50 µm internal diameter × 80 cm total length).

8-2-1. Set up instruments and sample vials, prepare the capillary with a capillary cassette, replenish sheath liquids and appropriate electrophoresis buffers depending on anion or cation analysis mode, and then apply voltages.

Note: Instrumentations and analytical conditions are described in detail elsewhere[12,13].

8-2-2. Open the software and prepare a worklist containing data acquisition methods and sample information.

8-2-3. Start a test run and check the data such as the signal intensity and the peak shape of internal standards and the peak resolution of other standard compounds.

8-2-4. Fine-tune the analytical conditions if necessary.

8-2-5. Inject the sample solutions at 50 mbar for 3 s and a voltage of 30 kV.

Note: CE-MS was conducted either in the positive or negative ion mode. Set the spectrometer to scan the mass range *m/z* 50–1000The capillary voltage was set at 4 kV; the flow rate of nitrogen gas (heater temperature 300°C). For positive mode, the fragmentor, skimmer and OCT RFV voltage were set at 75, 50 and 125 V, respectively. For negative ion mode, the fragmentor, skimmer, and OCT RFV voltage was set at 100, 50, and 200 V, respectively.

8-3. Analyze spectrum data.

8-3-1. Extract peaks from the mass spectral data using automatic integration software in order to obtain peak information including *m/z*, peak area, and migration time (MT).

Note: The method is described in detail elsewhere[14].

8-3-2. Exclude signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites.

8-3-3. Annotate remaining peaks with information from the HMT metabolite database according to *m/z* values and MTs.

8-3-4. Normalize areas of the annotated peaks to internal standard levels and numbers of cells per sample.

8-3-5. Evaluate the concentration of each metabolite in the cultured cells (pmol/10^6 cells) using standard curves prepared for each metabolite.

8-3-6. Use the quantified metabolite concentrations for subsequent statistical analyses and biological interpretations[14].

---

3. We have added the sentence “In this paper we could not describe how to set up CE-MS in detail because the focus of the present manuscript is different, however describing detailed steps to set up CE-MS may require a separate dedicated article.” in Discussion.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

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2) The highlighted steps should form a cohensive naarative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

5) Please bear in mind that theory/calculations cannot be filmed.

Response: I have highlighted protocol step which will be filmed in yellow.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: Thank you very much for this comment.

• **Figures:**

1) Please remove the text "Maruyama et al… from all figures"

Response: We have removed the text from all figures.

2) Fig 1: Change "hrs" to "h"

Response: We have changed "hrs" to "h".

3) Fig 2: Axis ticks and labels are too small to read.

Response: We have re-labeled the axis ticks.

• **References:** Please spell out journal names.

Response: We have corrected it.

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2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Response: We revised the words containing commercial sounding language to generic names.

• **Table of Materials:**

1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as cell lines, filters, software etc

2) Sort items in alphabetical order based on Name of material.

Response: We updated the table and sorted items in alphabetical order.

Line-by-line replies to the Peer-reviewers' comments

Reviewer #1:

Manuscript Summary:

In this study, the authors applied the capillary electrophoresis- mass spectrometry (CE-MS) to analyze the cells subjected to diamide treatment. They used two cell lines, namely HCC827 and PC-9 in their study. They found that a number of metabolites related to pentose phosphate pathway increased in abundance in these cells after

treatment.

Major Concerns:

This manuscript should have focused on the CE-MS analysis which is more eye catching to the scientist of the field. Instead, the authors described the procedures of cell cultivation and methanolic extraction in detail. These procedures can be easily performed by most scientists. It is better to add more details about CE-MS analysis, such as preparation of capillary; preparation of running buffer; preparatory work involved in interfacing between CE and MS; running conditions of CE and MS; peak picking; and data analysis. Such information is more useful to any analytical scientists working in the field.

Response: We agree with the reviewer that it would be interesting to show the details on how to use CE-MS. However, the main focus of this article is on how to extract aqueous metabolites from cultured adherent cells for metabolomic analysis. This study was designed to focus specifically on culturing and washing cells, treating cells with methanol, extracting metabolites, removing proteins and macromolecules this time. Thus, we would like to keep the focus of the paper intact, but we have changed three sentences as following reviewers' suggestion.

1. We have deleted the sentence "setting up CE-MS metabolomic analysis" from Abstract.

2. We have revised the 8. Metabolomic analysis by CE-MS section as follows.

---

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Note: Both systems can be controlled by the software of the system vendors and are connected by a fused silica capillary (50 µm internal diameter × 80 cm total length).

8-2-1. Set up instruments and sample vials, prepare the capillary with a capillary cassette, replenish sheath liquids and appropriate electrophoresis buffers depending on anion or cation analysis mode, and then apply voltages.

Note: Instrumentations and analytical conditions are described in detail elsewhere[12,13].

8-2-2. Open the software and prepare a worklist containing data acquisition methods and sample information.

8-2-3. Start a test run and check the data such as the signal intensity and the peak shape of internal standards and the peak resolution of other standard compounds.

8-2-4. Fine-tune the analytical conditions if necessary.

8-2-5. Inject the sample solutions at 50 mbar for 3 s and a voltage of 30 kV.

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8-3. Analyze spectrum data.

8-3-1. Extract peaks from the mass spectral data using automatic integration software in order to obtain peak information including *m/z*, peak area, and migration time (MT).

Note: The method is described in detail elsewhere[14].

8-3-2. Exclude signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites.

8-3-3. Annotate remaining peaks with information from the HMT metabolite database according to *m/z* values and MTs.

8-3-4. Normalize areas of the annotated peaks to internal standard levels and numbers of cells per sample.

8-3-5. Evaluate the concentration of each metabolite in the cultured cells (pmol/10^6 cells) using standard curves prepared for each metabolite.

8-3-6. Use the quantified metabolite concentrations for subsequent statistical analyses and biological interpretations[14].

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3. We have added the sentence “In this paper we could not describe how to set up CE-MS in detail, because the focus of the present manuscript is different, however describing detailed steps to set up CE-MS may require a separate dedicated article.” in Discussion.

Minor Concerns:

1. A detailed procedure for cell number determination should be given.

Response: We thank the reviewer for raising this concern and we agree with it. In accordance with the reviewer’s comment, we have inserted the following sentences.

1-10. Determine the total number and percent viability of the cells using the automated cell counter and 0.4% trypan blue solution.

1-10-1. Mix 10 µL of cell suspension and 10 µL of 0.4% trypan blue solution.

1-10-2. Load 10 µL of sample into the cell counting chamber slide through capillary action.
1-10-3. Insert chamber slide into the automated cell counter.
1-10-4. The transmitted light automatically illuminates and the instrument auto focuses on the cell.
1-10-5. Press the capture button to capture the image and to display the results.
1-10-6. If necessary, add further growth medium to obtain the desired cell concentration.
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2. The manuscript is well written.
Response: We thank the reviewer for carefully reading of our manuscript and giving constructive comments on the manuscript.

Reviewer #2:
Well written manuscript with detail protocol for extraction of metabolites from cell cultures and their analysis by CE-MS. I have no comments on it and, in my opinion, manuscript may be accepted in its present version.
Response: We are grateful to the reviewer for the recognition of our work.