

Video Article

Assessment of the Metabolic Profile of Primary Leukemia Cells

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Abstract

The metabolic requirement of cancer cells can negatively influence survival and treatment efficacy. Nowadays, pharmaceutical targeting of metabolic pathways is tested in many types of tumors. Thus, characterization of cancer cell metabolic setup is inevitable in order to target the correct pathway to improve the overall outcome of patients. Unfortunately, in a majority of cancers, the malignant cells are quite difficult to obtain in higher numbers and the tissue biopsy is required. Leukemia is an exception, where a sufficient number of leukemic cells can be isolated from the bone marrow. Here, we provide a detailed protocol for the isolation of leukemic cells from leukemia patients bone marrow and subsequent analysis of their metabolic state using extracellular flux analyzer. Leukemic cells are isolated by the density gradient, which does not affect their viability. The next cultivation step helps them to regenerate, thus the metabolic state measured is the state of cells in optimal conditions. This protocol allows achieving consistent, well-standardized results, which could be used for the personalized therapy.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58426/>

Introduction

The metabolic profile is one of the main characteristics of cells and altered bioenergetics are now considered one of the hallmarks of cancer^{1,2,3}. Moreover, changes in the metabolic setup could be used in the treatment of cancer by targeting signal transduction pathways or enzymatic machinery of cancer cells^{4,5,6}. Knowing the metabolic predisposition of cancer cells is thus an advantage and can help improve the current therapy.

There are a plenty of already established methods which can assess the metabolic activity of cells in culture. Regarding glycolysis, glucose uptake can be measured by the radioactive labeling, using 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) or extracellular lactate levels measured enzymatically^{7,8}. Fatty acid oxidation rate is another metabolic parameter measured by isotopically labeled palmitate^{9,10}. Oxygen consumption rate is a method widely-used for determining mitochondrial activity in cells^{11,12}, together with the mitochondrial membrane potential evaluation^{13,14}. ATP/ADP (adenosine 5'-triphosphate/Adenosine 5'-diphosphate) ratio measurement¹⁵ or total intracellular ATP measurement¹⁶. Signaling pathways known to regulate metabolic processes could be determined by protein quantifications and can improve the understanding of metabolic measurements^{17,18,19}.

However, all these methods measure only one or, in the best scenario, a few metabolic parameters in one sample simultaneously. Importantly, simultaneous measurement of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) can be achieved by the extracellular flux analysis by, for example, Seahorse XFp Analyzer. OCR is an indicator of mitochondrial respiration and ECAR is mainly the result of glycolysis (we cannot ignore CO₂ production possibly elevating ECAR of cells with high oxidative phosphorylation activity)²⁰. So far, various cell types have been studied using these analyzers^{21,22,23}.

Here we describe the protocol for the extracellular flux analysis of primary blasts (leukemia cells derived from the immature hematopoietic stage) from leukemia patients. To the best of our knowledge, a specific protocol for primary blasts is not available yet.

Protocol

All samples were obtained with the informed consent of the children's parents or guardians and approval of Ethical committee of Charles University in Prague, Czech Republic, the study no. NV15-28848A.

1. Preparation of Reagents

1. Prepare 500 mL of PBS by dissolving 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, in ddH₂O. Adjust the pH to 7.4 with HCl. Sterilize by autoclaving.

2. Prepare 100 mL of RPMI medium: RPMI-1640 medium with L-Alanyl-Glutamine supplemented with 10 % fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL).
3. Prepare 50 mL of 0.1 M NaHCO₃ in distilled water. Adjust the pH to 8.1, filter sterilize (0.22 µm) and store at 4 °C.
NOTE: For two 8-well extracellular flux analyzer plates, prepare 250 µL of 0.1 M NaHCO₃ pH 8.1.
4. Prepare 1 mL of 2 M D-glucose in distilled water. Filter sterilize (0.22 µm) and store at -20 °C.
5. Prepare 100 µL of 1 mM Oligomycin A in ethanol. Filter sterilize (0.22 µm) and store at -20 °C.
6. Prepare 250 µL of 1 M 2-deoxy-D-glucose (2-DG) in Minimal DMEM (Dulbecco's Modified Eagle's medium). Warm to 37 °C and adjust the pH to 7.4 (carry out pH measurement at 37 °C). Filter sterilize (0.22 µm) and store at -20 °C.
7. Prepare 100 µL of 1 mM FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) in DMSO. Filter sterilize (0.22 µm) and store at -20 °C.
8. Prepare 100 µL of 1 mM Rotenone in ethanol. Filter sterilize (0.22 µm) and store at -20 °C.
9. Prepare 100 µL of 1 mg/mL Antimycin A in ethanol. Filter sterilize (0.22 µm) and store at -20 °C.
10. Just before the use, prepare 10 mL of Glycolysis stress test medium. Warm Minimal DMEM to 37 °C in a water bath and adjust the pH to 7.4 (carry out pH measurement at 37 °C).
11. Prior to the use, prepare 10 mL of Cell Mito stress test medium with BSA. Supplement Minimal DMEM with 2 mM L-glutamine, 10 mM D-glucose, 1 mM HEPES (pH 7.4), 1 mM pyruvate and 0.1 % BSA (bovine serum albumin). Warm to 37 °C in a water bath and adjust the pH to 7.4 (carry out pH measurement at 37 °C).
12. Prior to the use, prepare 10 mL of Cell Mito stress test medium without BSA. Supplement Minimal DMEM with 2 mM L-glutamine, 10 mM D-glucose, 1 mM HEPES (pH 7.4) and 1 mM pyruvate. Warm Cell Mito stress test medium without BSA to 37 °C in a water bath and adjust the pH to 7.4 (carry out pH measurement at 37 °C).
NOTE: BSA is added to the Cell Mito stress test medium because the cells respond better to FCCP when the medium is supplemented with BSA (different conditions were tested). Cell Mito stress test medium without BSA is used for loading the ports as the manufacturer does not recommend using BSA in the medium.

2. Isolation of Mononuclear Cells from Bone Marrow

NOTE: Ideally, measurement of metabolic state of primary leukemia cells should start immediately after bone marrow collection and cell isolation. Nevertheless, relevant data could be also obtained from cells isolated after transportation from other hematology centers in the Czech Republic. Perform all sub-steps in a sterile tissue culture hood.

1. Warm up PBS and the density gradient medium to room temperature.
2. Dilute the bone marrow sample of leukemia patient with PBS, in a ratio of 1:1. Make sure that the sample contains at least 80% of leukemic blasts.
3. Determine the percentage of blasts by flow cytometry using specific CD markers for immunophenotype characterization of cell types. After density gradient separation, detect nucleic acid positive events with a nuclear dye in order to establish the overall cell count.
4. Then, determine leukemia cells using specific CD markers for each type of leukemia: B-ALL (CD19, CD45), T-ALL (CD3, CD4, CD8, CD5, CD7, CD99) and AML (CD45, CD33, and specific myeloid markers)²⁴. Divide the number of leukemia cells positive for specific CD markers by all nuclear cells to determine the percentage of leukemia blast cells.
NOTE: The bone marrow has to be collected into the tubes with anticoagulants.
5. Carefully layer 6 mL of diluted bone marrow sample over 6 mL of the density gradient medium in a 15 mL conical tube. Centrifuge at 400 x g for 35 min at 4 °C in a swinging-bucket rotor without the brake.
NOTE: Larger volume of the sample could be divided into more aliquots or 50 mL conical tube can be used with the larger amount of density gradient medium.
6. Using a Pasteur pipette, carefully transfer the interphase layer which consists of mononuclear cells (**Figure 1**) to a new 50 mL conical tube with 5 mL of PBS. Centrifuge at 400 x g for 10 min at 4 °C in a swinging-bucket rotor.
NOTE: Transfer all mononuclear cell layers into a single 50 mL conical tube with 5 mL of PBS.
7. Aspirate the supernatant and resuspend the cell pellet in 2 mL of sterile PBS. Count the cells using a hemocytometer.
NOTE: The number of leukemia cells in one mL of aspirated bone marrow differs significantly among patients²⁵.

3. Overnight Cultivation of Mononuclear Cells

NOTE: Perform all sub-steps in a sterile tissue culture hood.

1. Prepare two T75 flasks with 20 mL of RPMI. To each flask, add 30 x 10⁶ isolated mononuclear cells. Incubate the cells with the flask standing up for 16–24 h at 5% CO₂ and 37 °C.

4. Preparation of Cell Adhesive-coated Plates

1. Coat two 8-well extracellular flux analyzer plates.
NOTE: Perform the coating in a sterile tissue culture hood.
2. Add 2.2 µL of cell adhesive (density: 2.54 mg/mL) to 250 µL of 0.1 M NaHCO₃, pH 8.1, and pipette immediately 12.5 µL of the solution into each well.
NOTE: Cell adhesive stock solutions can differ in their density, adjust the volume added to NaHCO₃ accordingly.
3. Let the plates sit in the hood for about 20 min, then aspirate the cell adhesive and wash each well twice using 200 µL of sterile water. Let it sit in the hood with the lid open until wells are dry.
4. Use the plates right away or save up to 1 week at 4 °C with the rim wrapped in paraffin film to avoid condensation. Ensure that the plates are warmed up to room temperature (for about 20 min) in the hood before seeding the cells.

5. Hydration of Sensor Cartridge

NOTE: Hydrate two 8-well extracellular flux analyzer cartridges.

1. Separate the utility plate and the sensor cartridge. Place the sensor cartridge upside down on the lab bench.
2. Fill each well of the utility plate with 200 μ L calibrant. Fill each moat around the outside of the wells with 400 μ L of calibrant.
3. Return the sensor cartridge to the utility plate that now contains the calibrant.
4. Place the cartridge assembly in a humidified, non-CO₂, 37 °C incubator overnight.
5. Turn on the extracellular flux analyzer and let it warm to 37 °C overnight.

6. Seeding Cells in Cell Adhesive-coated Plates

NOTE: For Glycolysis stress test, use Glycolysis stress test medium. For Cell Mito stress test, use Cell Mito stress test medium with BSA.

1. Seed cells for the Glycolysis stress test and the Cell Mito stress test in separate plates. For each test, use cells from one flask with overnight culture.
2. Centrifuge cells at 200 x g for 5 min at room temperature. Resuspend cells in 1 mL of the appropriate medium and count them.
3. Add 4×10^6 of live cells to the final volume of 400 μ L (use appropriate medium).
4. Plate 50 μ L of the cell suspension into wells B-G. Ensure 500,000 cells are seeded in one well.
NOTE: It is crucial to seed exactly 500,000 cells per well as no other normalization is performed. That way, the results from different patients can be compared. The optimal number of replicates is six, as is described here. Using less replicates is not recommended since primary cells could sometimes behave erroneously.
5. Add 180 μ L of the appropriate medium into wells A and H (these wells will serve as a background correction).
6. Centrifuge the plate at 400 x g for 5 min at room temperature with brake set to 1.
7. Add 130 μ L of appropriate medium to wells B-G in two 8-well extracellular flux analyzer plates slowly and carefully. Visually confirm that the cells are stably adhered to the bottom of the wells by viewing under the microscope.
8. Place the plate into a humidified, non-CO₂, 37 °C incubator for 30 min.

7. Loading the Sensor Cartridge

1. For the Glycolysis stress test, prepare 250 μ L each of 100 mM glucose, 20 μ M Oligomycin A and 1 M 2-DG, all in Glycolysis stress test medium.
2. For the Cell Mito stress test, prepare 250 μ L each of 20 μ M Oligomycin A, 15 μ M FCCP, 30 μ M FCCP and a mixture of 10 μ M Rotenone and 10 μ g/ml Antimycin A, all in Cell Mito stress test medium without BSA.
NOTE: Injecting two concentrations of FCCP in one assay is recommended since there is not enough patients' material for the FCCP titration. Nevertheless, the concentrations should be determined by the researcher.
3. Load the compounds into the appropriate injector ports of the cartridge as follows (**Table 1**):

8. Setting Up the Program

1. For the Glycolysis stress test, set up the program as described in **Table 2**.
2. For the Cell Mito stress test, set up the program as described in **Table 3**.
3. Start the program. Replace the calibrant plate with the assay plate (when prompted).

9. Evaluation and Interpretation of the Results

1. In the Glycolysis stress test results, subtract the lowest ECAR value after 2-DG injection from all other ECAR values.
NOTE: This lowest value represents the non-glycolytic acidification. Usually, the lowest value is from the 4th measurement.
2. Calculate Basal acidification, Glycolysis, Maximal glycolysis and Glycolytic reserve parameters from glycolytic function (**Figure 2A**). After subtracting the lowest ECAR value, calculate Basal acidification as a mean of ECAR from the first three measurement points (omit the first ECAR value if it significantly differs from the other two), calculate Glycolysis as a mean of ECAR from three measurement points after glucose injection and calculate Maximal glycolysis as a mean of ECAR from three measurement points after Oligomycin A injection. Calculate Glycolytic reserve as Maximal glycolysis minus Glycolysis.
NOTE: Alternatively, for Maximal glycolysis calculation, use the highest ECAR value from the three points measured.
3. In the Cell Mito stress test results, subtract the lowest OCR value after Rotenone/Antimycin A injection from all other OCR values.
4. Calculate Basal respiration, ATP production, Maximal respiration and Spare capacity parameters from mitochondrial function (**Figure 2B**). After subtracting the lowest OCR value, calculate Basal respiration as a mean of OCR from the first three measurement points.
NOTE: Maximal respiration is the highest OCR value after FCCP injection.
5. For ATP production calculation, subtract the mean of the three OCR measurement points after Oligomycin A injection from Basal respiration. Calculate Spare capacity as the Maximal respiration minus the Basal respiration.
NOTE: Always calculate the Maximal respiration from the highest OCR value, regardless of the FCCP concentration used.

Representative Results

Figure 3 shows the curves after Glycolysis stress test and Cell Mito stress test measurements of leukemic blasts from the BCP-ALL (B-cell precursor acute lymphoblastic leukemia) and AML (acute myeloid leukemia) patients. The calculation of metabolic parameters from these measurements is also indicated. 500,000 cells per well were seeded and all measurements were done in hexaplicates.

In the Glycolysis stress test, the only basal medium is used, so that the cells are deprived of nutrients. The first parameter obtained is the Basal acidification, which should reflect the amount of glucose stored in cells. After the first injection, ECAR is increased since cells utilize glucose and can ferment it to lactate. Oligomycin A in the second injection inhibits ATP-synthase and thus directs the cells to produce ATP mainly via glycolysis. This should cause further elevation of ECAR. Injection of 2-DG completely inhibits glycolysis and ECAR drops.

In the Cell Mito stress test, a medium supplemented with glutamine and glucose are used, so that the cells are not deprived of all nutrients and the Basal respiration parameter reflects their basal metabolic state. After first injection with Oligomycin A, cells inhibit mitochondrial respiration and switch to glycolysis which is represented as a decrease of OCR. FCCP (the second and third injection), on the other hand, uncouples ATP production from respiration, so that the cells now consume oxygen at a maximal rate and OCR rise to its highest value. The last injection of Rotenone and Antimycin A mixture completely inhibits mitochondrial respiration and OCR is decreased close to zero.

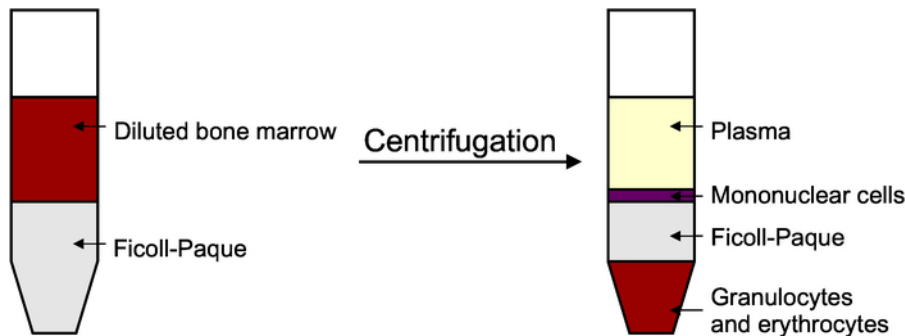


Figure 1: Density gradient centrifugation of the bone marrow sample. Mononuclear cells enriched for leukemic cells are separated by density gradient medium. [Please click here to view a larger version of this figure.](#)

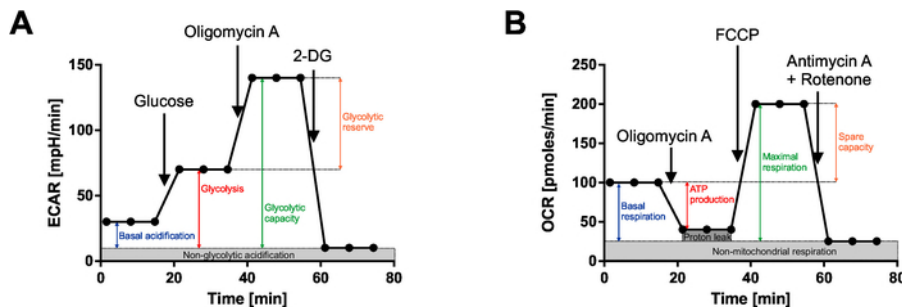


Figure 2: Outline of Glycolysis stress test and Cell Mito stress test. (A) Exemplary result of Glycolysis stress test. (B) Exemplary result of Cell Mito stress test. Parameters are indicated within the curves. [Please click here to view a larger version of this figure.](#)

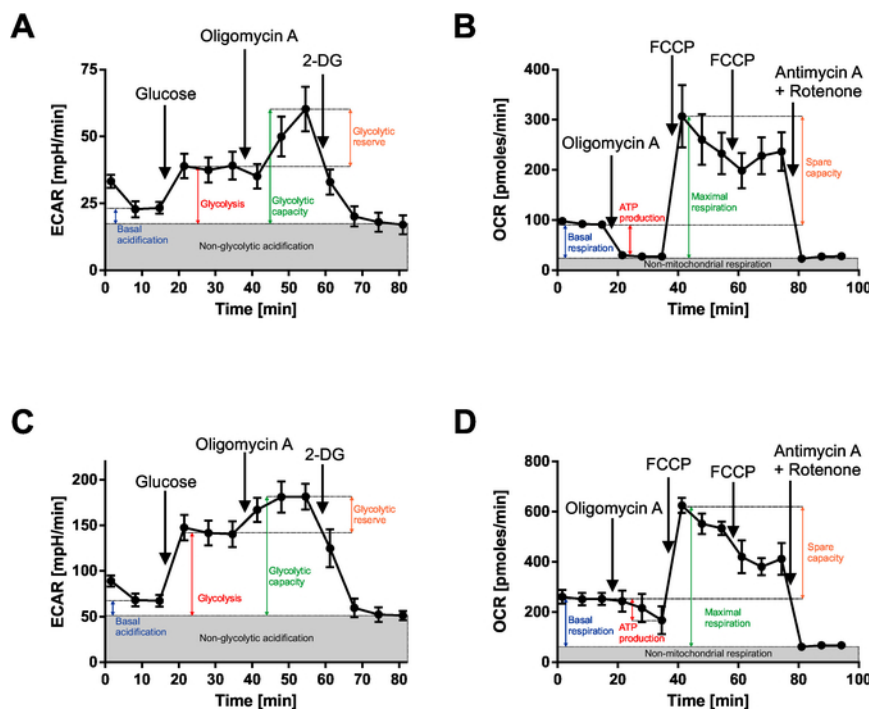


Figure 3: Extracellular flux analysis of leukemic blasts from BCP-ALL (A, B) and AML (B, C) patient. (A and C) Glycolysis stress test results. Please note that the first measurement point can significantly differ from the rest and should be excluded from the analysis in that case. (B and D) Cell Mito stress test results. Parameters are indicated within the curves. [Please click here to view a larger version of this figure.](#)

Port	Glycolysis stress test		Cell mito stress test	
	Load to the port	Final concentration in the wells	Load to the port	Final concentration in the wells
A	20 μ L of 100 mM glucose	10 mM glucose	20 μ L of 20 μ M Oligomycin A	2 μ M Oligomycin A
B	22 μ L of 20 μ M Oligomycin A	2 μ M Oligomycin A	22 μ L of 15 μ M FCCP	1.5 μ M FCCP
C	25 μ L of 1 M 2-DG	100 mM 2-DG	25 μ L of 30 μ M FCCP	4.5 μ M FCCP
D	X		25 μ L of 10 μ M Rotenone and 10 μ g/ml Antimycin A	1 μ M Rotenone and 1 μ g/ml Antimycin A

Table 1: Compound volumes.

Step	Settings
Calibration	Automatic
Equilibration	Automatic
Baseline measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port A	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port B	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port C	Injection
Measurement	Four times: Mix – 3 min, Wait – 0 min, Measure – 3 min

Table 2: Program for Glycolysis stress test.

Step	Settings
Calibration	Automatic
Equilibration	Automatic
Baseline measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port A	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port B	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port C	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port D	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min

Table 3: Program for Cell Mito stress test.

Discussion

The above-described protocol allows for the measurement of the metabolic activity assessed by OCR and ECAR values in primary leukemic blasts derived from patients with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML). The advantage of measurement using an extracellular flux analyzer is that it enables the detection of metabolic profile in the real time in the live cells. Essentially, every step in the provided protocol could be adjusted depending on the cell type one plans to study. Here, we will discuss the most important parameters which could affect the results and could provide less than optimal values.

The first step towards optimization was a comparison of data obtained from fresh material vs frozen material. The ability to measure the metabolic activity from the frozen material would allow for retrospective studies of patients' samples stored in liquid nitrogen bank. In case of ALL samples, we were able to detect consistent metabolic activity only from the fresh material whereas AML cells were measured also after de-freezing with optimal results.

The second step towards optimization is the cultivation of primary leukemic blasts. We have tested the metabolic activity of the cells straight after the density gradient separation (without culturing) or after culturing overnight. Even if the cells after the density gradient separation looked viable and vital under a microscope, their metabolic activity was impaired. Overall ECAR and OCR values were lower and also, after injection, OCR or ECAR values did not respond optimally as they did in cultivated cells.

Cultivation under different conditions can also influence the results. Using insulin transferrin sodium selenite supplement (ITS) is considered a good practice when cultivating primary blasts⁸, but this supplement interferes with the metabolic activity of the cells. During Cell Mito stress test, leukemic blasts cultivated with ITS did not respond to Oligomycin A (OCR should decrease in order to calculate ATP-linked respiration). We also tried to co-cultivate the cells with mesenchymal stem cells (MSC), but in this case, OCR and ECAR values have been lower compared to the cells cultivated without MSCs. In summary, cultivating primary blasts from leukemia patients in RPMI medium with 10 % FBS is the best option.

Patients suitable for the characterization of their metabolic profile must meet certain criteria which on one hand limit the number of tested samples but on the other will yield relevant results. We measured the metabolic function of patients with high cellularity (for one measurement we seeded 500,000 cells/per wells in hexaplicate) and only samples with 80 and a higher percentage of leukemic blasts could be measured to avoid detection of unspecific metabolic activity of other cell types present in the suspension.

One of the crucial steps in data analysis is the normalization, so that metabolic parameters between different leukemic samples could be compared. According to our previous experiments performed with leukemic cell lines, we found that normalization to the number of the cells gives the best results. The specific number of cells per well needs to be determined by the researcher and depends on the size and metabolic activity of tested cells.

Disclosures

The authors have nothing to disclose.

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