

Cellular Analysis Application Note No. 2

Gene Expression Analysis of Paclitaxel-Treated HT29 Cells

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1 Introduction

The analysis of gene expression profiles provides a more detailed understanding of the genetic basis of disease helping to guide diagnosis and therapeutic treatment at the molecular level.

With gene expression profiling, the process of rating candidate compound activity and efficiency, as well as the subsequent optimization of identified lead compounds, is enhanced. Equally important, suitable biomarkers can be identified using gene expression profiling. With gene expression analysis the safety of compounds can be evaluated early in the developmental process and used to avoid the risks of severe side effects.

Sensitive and specific gene expression assays require workflows using reliable robust techniques that are synergistically linked to each other. Here we describe a workflow, using the xCELLigence System, to monitor cellular responses after treatment with the anti-cancer agent paclitaxel *in vitro*, combined with qRT-PCR using the LightCycler® 480 Real-Time PCR Instrument. Continuous monitoring of cellular behavior after treatment with paclitaxel pinpointed the key times for collecting sample material for subsequent analysis using qRT-PCR.

Human colon carcinoma HT29 cells were treated with paclitaxel or its solvent DMSO as a control. Cellular responses to paclitaxel were continuously monitored during the course of the experiment using the Real-Time Cell Analyzer (RTCA) SP Instrument. Based on the Cell Index profile recorded with the xCELLigence system, time points were selected for the collection of sample material for RNA isolation and cell viability tests. High quality RNA was purified using the High Pure RNA Isolation Kit and cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit. The expression levels of

84 different human apoptosis related genes and 84 different human cell cycle related genes were compared for all cDNAs using the LightCycler® 480 Instrument with the RealTime ready Human Apoptosis Panel, 96 and the RealTime ready Human Cell Cycle Panel, 96.

Continuous monitoring of the growth behavior of a cell line after treatment with paclitaxel for the first time provides a means for defining the optimal time points for the collection of sample material for subsequent analysis by RT-qPCR.

2 Methods

RNA isolation from cell culture using the High Pure RNA Isolation Kit

Culturing HT29 cells and RNA Isolation

HT29 cells were cultivated in McCoy's medium in either T75 cell culture bottles for RNA isolation, or an E-Plate 96 (Roche) for cell growth monitoring, and in three regular microtiter plates for the WST-1 assay. The surface of the bottom of a single well of the E-Plate 96 is approximately 0.2 cm². T75 cell culture bottles have 75 cm². To assure comparable growth conditions within each individual well of the E-Plate 96 and the microtiter plates and cell culture bottles, 4000 cells/well were seeded in the E-Plate 96 and the regular microtiter plates and 7.5 x 10⁵ cells were seeded into each T75 cell culture bottle.

	seeding	area	Culture volume	Cell concentration
E-Plate 96 or regular microtiter plate	4000 cells	0.2 cm ²	100 µl	40 cells/µl
T75 cell culture bottle	1.5 x 10 ⁶ cells	75 cm ²	375 ml	40 cells/µl

After 24 hours incubation at +37°C, paclitaxel was added to a final concentration of 50 nM. As the 2 mM paclitaxel stock was dissolved in DMSO, control cell cultures were treated with DMSO to the final concentration of 0.0025%. In addition, cells treated only with medium were monitored in parallel. All cells were incubated at +37°C. Cell growth was monitored in real time using the RTCA SP Instrument (Roche) for the entire course of the experiment (see Figure 1).

Viability assay

Cells grown in standard microtiter plates were subjected to a cell viability assay using the Cell Proliferation Reagent WST-1 (Roche). One, two and four hours after paclitaxel treatment, 10 µl WST-1 reagent was added to each well, and incubated for one hour before absorption readout at 450 nm using a reference wavelength of 600 nm.

RNA Isolation and cDNA Synthesis

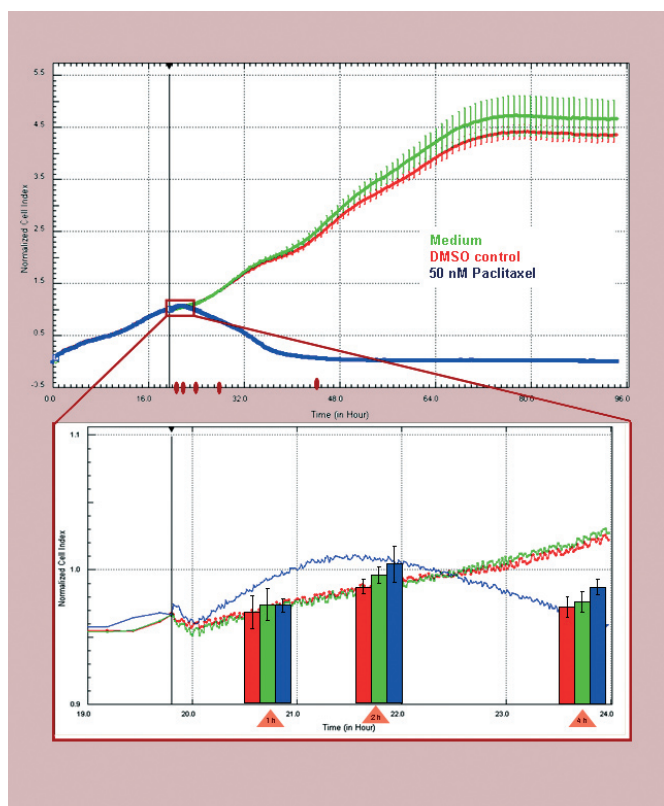
Cells were harvested for RNA isolation after one, two, four and 24 hours. Cell number was determined and fractions of 10⁶ cells were collected for RNA isolation using the High Pure RNA Isolation Kit (Roche) following the manufacturer's instruction. The high quality of the RNA samples was confirmed using the NanoDrop Instrument and the Agilent Bioanalyzer (see Figure 2). From each RNA population, one microgram total RNA was used for cDNA synthesis with the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Real-time qPCR

A single cDNA synthesis reaction using one microgram total RNA was used as template for each RealTime ready Human Apoptosis Panel, 96 (Roche) or each RealTime ready Human Cell Cycle Panel, 96 (Roche). The total PCR reaction volume per well was 20 µl using the LightCycler® 480 Probes Master reagents (Roche). Sample setup and analysis were easy using the provided macro file for each gene panel with the LightCycler® 480 Software, Version 1.5.

3 Results

Growth of the HT29 cells was monitored using the RTCA SP Instrument. The E-Plate 96 was loaded with 4000 cells/well in quadruplicates. As can be seen from the recorded growth curve of impedance derived Cell Index (CI) values produced by HT-29 cells, the confluent state at this initially seeded cell density was reached after approximately 70 hours (see Figure 1). To ensure that untreated cells are within the early logarithmic growth phase at the time point of paclitaxel treatment, cells were treated with 50 nM paclitaxel at approximately one-third of their maximum Cell Index at 20 hours after seeding.



Cells were also analyzed one, two and four hours after paclitaxel treatment using the WST-1 assay carried out in regular microtiter plates. The resulting data was compared to the CI curves recorded using the xCELLigence System. (see Figure 1B).

The WST-1 Reagent is designed to measure cell viability, which is apparently not affected during the first four hours after addition of paclitaxel. Paclitaxel is known to arrest the cell cycle in the G2/M phase by preventing the depolymerization of microtubulin. With the xCELLigence System, any changes in cell morphology –

Using continuous real-time cell monitoring, significant changes in the CI values were recorded immediately after paclitaxel treatment. Interestingly, the CI slightly increased within the first hour after treatment, before dropping down to reach the minimum CI after approximately 24 hours. Based on this data, the first T75 bottle was harvested one hour after paclitaxel treatment for RNA isolation, reverse transcription and qPCR. Additional samples were collected two, four and 24 hours after paclitaxel treatment.

Figure 1: Cell Monitoring using the RTCA SP Instrument.

Cell growth was continuously monitored using the Real-Time Cell Analyzer SP Instrument. Cell Index (CI) values were normalized to the CI at the time point of paclitaxel addition indicated by the black solid line.

A. The CI profiles of paclitaxel treated cells (blue line), cells treated with DMSO as a control (red line) and cells treated with medium only (green line) represent the initial cell attachment, the logarithmic growth phase and the response to the treatments, respectively. The CI values for paclitaxel treated cells are approaching zero approximately 24 hours after treatment, indicating that nearly all of the cells have detached from the bottom of the well, and are no longer contributing to the Cell Index value. The red dots on the x-axis of Panel A indicate time points selected for RNA isolation. Error bars show the standard deviation of the mean of quadruplicates.

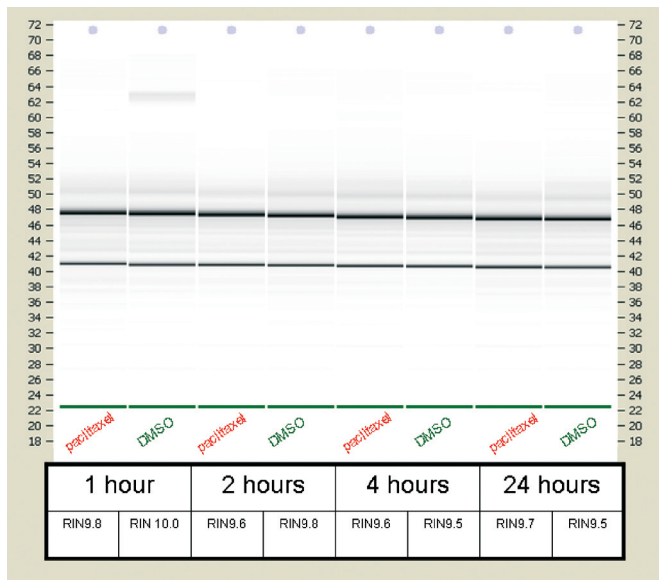
B. Higher resolution of the CI profiles with labeling of the time points selected for RNA Isolation (red triangles). Colored bars represent data using the WST-1 assay. Color coding is the same as in Figure 1A. Please note that the continuous CI data stream recorded by the xCELLigence System in one minute intervals delivers a high density of information, providing a more complete picture than the WST-1 assay alone (see below).

especially in the cytoskeleton are easily detected by the network of microelectrodes in contact with the surfaces of HT29 cells.

The early changes in the CI profile can be assigned to paclitaxel treatment.

This indicated that it could be important to analyze gene expression at the early time points.

Relying only on the WST-1 data, which is an endpoint assay and not a continuous cell monitoring, one may not have isolated RNA at this early time point, and missed the significant changes in RNA expression at this early stage after paclitaxel treatment.



For reliable qRT-PCR analysis, high quality RNA is crucial. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche). The integrity of the RNA preparation was confirmed using the Agilent Bioanalyzer (see Figure 2). All samples showed high RNA Integrity Number (RIN) values between 9.5 and 10 indicating the best possible RNA quality for the subsequent qPCR analysis.

Figure 2: Analysis of total RNA prepared using the High Pure RNA Isolation Kit.

One microliter of each total RNA sample was analyzed using the Agilent Bioanalyzer Gel photograph of each RNA sample. The corresponding RNA Integrity Number (RIN) values were calculated using the Agilent Bioanalyzer Software and are shown below each lane.

Four time points, one, two, four and twenty four hours after paclitaxel treatment, were selected for real-time qPCR analysis using the LightCycler® 480 System with the RealTime ready Human Cell Cycle Panel, 96 (see Figure 3) and the RealTime ready Human Apoptosis Panel, 96 (see Figure 4). The gene names corresponding to the numbers shown in Figures 3 and 4 can be found in the package insert provided with both gene panels (and also on the internet at <https://www.roche-applied-science.com/pack-insert/5392063a.pdf> for RealTime ready Human Apoptosis Panel, 96 and <https://www.roche-applied-science.com/pack-insert/5339359a.pdf> for RealTime ready Human Cell Cycle Panel, 96).

Our findings clearly demonstrate that the most significant change in the expression levels of apoptosis related genes occurs within the first hour after paclitaxel treatment. At two and four hours after paclitaxel treatment, no genes show significant changes in expression compared to DMSO control HT29 cells (see Figure 3).

With the RealTime ready Human Cell Cycle Panel, 96 again most dramatic effects were observed within the first four hours (see Figure 4).

4 Conclusion

The xCELLigence System measures cellular responses in real time without the incorporation of exogenous labels. The cell impedance measurements recorded by the network of microelectrodes found at the bottom of each well of the E-Plates 96, provide quantitative information about the biological status of the cells, including cell number, cell spreading and proliferation and cell morphology. The xCELLigence System thus provides a continuous surveillance in real-time of the cellular “body language” of the cells *in vitro* following a given treatment, such as in this case with paclitaxel.

A standard single endpoint cell viability assay such as the WST-1 assay cannot easily capture all the significant changes in cell morphology and cell adhesion at every stage after paclitaxel treatment. Our present findings show that using our WST-1 data alone, we would have missed the most important changes in gene expression after paclitaxel treatment. Due to continuous record of CI profiles, we performed qRT-PCR assays at much earlier time points.

Most previous studies selected 8 hours after treatment as the earliest time point for qRT-PCR analysis. Monitoring the Cell Index changes using the xCELLigence System is an ideal way to identify the optimal time points for carrying out qRT-PCR. Our findings show an immediate response of HT29 cells to the treatment with paclitaxel visualized by the early changes in the Cell Index values. The corresponding qPCR results demonstrate, that the changes in the Cell Index profile of cellular responses is correlated to significantly increased/decreased expression levels of specific genes regulating both the cell cycle and apoptosis.

The qPCR results obtained using the RealTime ready Human Apoptosis Panel show that the strongest effects on gene expression of apoptosis related genes occur within the first hour after paclitaxel treatment. With the RealTime ready Human Cell Cycle Panel, changes in gene expression are detectable already during the the first four hours. Gene profiling data obtained 24 hours after paclitaxel treatment, may be more of a reflection of DNA degradation and cell death, than a robust cellular mechanism.

RealTime ready Focus Panels using Roche Applied Science’s Universal ProbeLibrary probes are excellent tools for quantifying gene expression of human pathway-specific genes during cell proliferation and apoptosis. The gene content of each panel is designed for the analysis of a specific human cellular pathway, and each panel also has pre-plated controls and reference genes.

Our data using the RTCA SP Instrument and the Cell Proliferation WST-1 Reagent demonstrate that the combination of real time measurement of cellular growth with qRT-PCR at selected time points identified by cell monitoring can provide significant insight into how and when paclitaxel affects cultured human carcinoma cells.

Ordering Information

Product	Cat. No.	Pack Size
High Pure RNA Isolation Kit	11 828 665 001	Up to 50 isolations
Transcriptor First Strand cDNA Synthesis Kit	04 379 012 001	50 reactions
	04 896 866 001	100 reactions
	04 897 030 001	200 reactions
RTCA SP Station	05 229 057 001	1 instrument
RTCA Analyzer	05 228 972 001	1 instrument
RTCA Control Unit	05 229 014 001	1 instrument
Cell Proliferation Reagent WST-1	05 015 944 001	8 ml (800 tests)
LightCycler® 480 Instrument II	05 015 278 001	1 instrument (96-well version)
LightCycler® 480 Probes Master	04 887 301 001	10 x 500 reactions, 20 µl volume
RealTime ready Human Apoptosis Panel, 96	05 392 063 001	2 plates (each containing 96 assays)
RealTime ready Human Cell Cycle Panel, 96	05 339 359 001	2 plates (each containing 96 assays)

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