Gene expression of EdU proliferating cells using Ion Torrent™ sequencing made possible with improved click chemistry

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ABSTRACT

Gene expression profiling of cancer cells has become increasingly common over the past five years. Especially important is identifying changes in gene expression caused by drug treatment leading to side populations with altered pathways. These populations can escape drug treatment and lead to recurrence. Characterizing gene expression of proliferating cells requires a method of identifying proliferation while simultaneously maintaining high quality RNA. Typically, cells are screened in a drug panel in the presence of tritiated thymidine or BrdU to identify the drug resistance in the proliferating population. Proliferation detection with these methods is neither rapid nor does it yield high quality RNA for profiling. A new proliferation method based on click chemistry using an alkyne modified thymidine analog[1-3] (EdU), which can be used to rapidly identify replicating cells appears ideally suited for this application; however, copper used for the catalysis of the click reaction causes undesired degradation of RNA. Recent improvements to the copper based click reaction are presented here whereby the copper is tightly controlled, resulting in higher quality RNA useful for sequencing.

In this study we demonstrate gene expression changes in the proliferating population of a cancer cell line compared to a parental primary cell line. Proliferating cells were identified using an improved click reaction designed to control the amount of copper related unwanted side reactions. Total RNA from these cells was isolated and gene expression was assessed by targeted RNA sequencing using the Ion AmpliSeq™ RNA Apoptosis panel on the Ion PGM™ Sequencer.

Use of an improved copper “safe” click reaction for detection of nascent DNA, allows for detection of incorporated click analog for measuring proliferation and subsequent isolation of higher quality RNA for use in sequence analysis.

INTRODUCTION

- Screen two cell lines (cancer and primary) with drug panel to identify hits from Killer Plate (MicroSource Discovery, Gaylordsville, CT) using CyQUANT® Direct Cell proliferation assay and CellEvent® Caspase 3/7 Green detection reagent (LifeTechnologies, Carlsbad, CA).
- Pulse label ± drug with EdU and detect with a modified Click-iT® EdU kit to identify proliferating cells while protecting RNA quality.
- Isolate RNA from click labeled cells treated ± narciclasine using mirVana™ RNA isolation kit.
- Create Ion AmpliSeq™ Library kit 2.0 with barcode adapters for multiplex sequencing and analysis on Ion AmpliSeq™ RNA Apoptosis panel with Ion PGM™ Sequencer to identify changes in apoptosis relevant genes (Life Technologies, Carlsbad, CA).
- Analyze a selection of apoptosis related gene expression levels isolated from ± click treated cells using real time PCR TaqMan® assay

Figure 1: Workflow

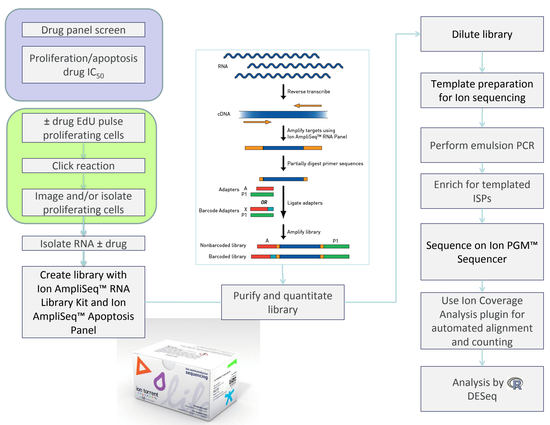


Figure 1: Workflow for making library for sequencing using RNA isolated from EdU pulsed cells labeled with modified click reaction and amplified using Library 2.0. Ion Xpress™ Barcode Adapters 1-16 kit were used to allow for multiplexing of 4 samples on a single chip.

Figure 2: Drug panel screen and IC_{50}

2A: Primary Screen:

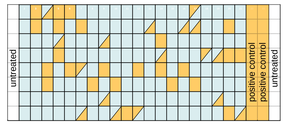


Figure 2A: Apoptosis induction in A549 (◿) and wi-38 (◸) cells was evaluated using Killer Plate drug panel (MicroSource Discovery Systems) for 24 and 48 hours. Caspase 3/7 signal measured using CellEvent® Caspase 3/7 Green detection reagent in drug treated cells shown in orange.

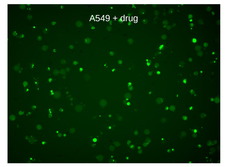


Figure 2B: A549 cells treated with 10 μM narciclasine, a plant alkaloid[4-5], for 24 hours then stained with CellEvent® Caspase 3/7 Green to detect apoptosis.

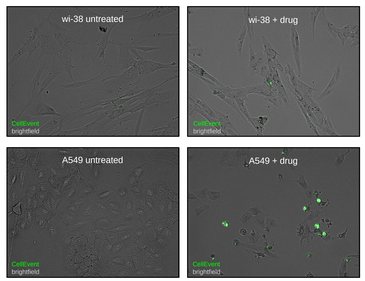


Figure 2C: wi-38 (top) and A549 (bottom) cells treated ±1 μM narciclasine for 48 hours and labeled with CellEvent® Caspase 3/7 Green to detect activated caspase 3 activity, an indicator of apoptosis. Treated A549 cells show more CellEvent™ signal than wi-38.

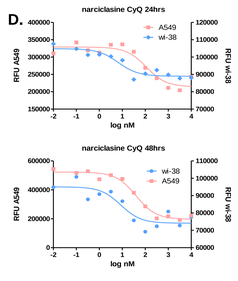


Figure 2D: Narciclasine showing inhibition of cell proliferation at 24 and 48 hr measured with CyQUANT® Direct Cell Proliferation Assay has an apparent IC_{50} at 48 hrs of 39 nM and 8.6 nM for A549 and wi-38 cells (respectively).

Figure 3: Click chemistry reaction with EdU

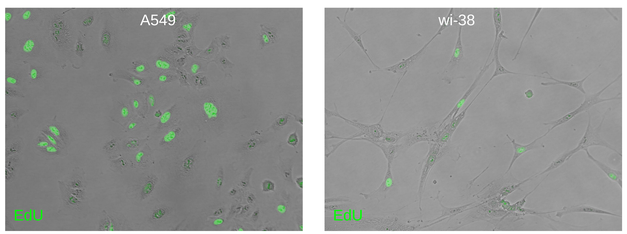
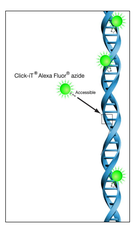


Figure 3: Incorporation of the thymidine analog, EdU, followed by detection with click chemistry labels cells actively replicating DNA (right).

Cells treated ±1 μM narciclasine for 6 hours and pulsed with 10 μM EdU for 2 hours were briefly fixed with 70% ethanol then click labeled (10 minutes) prior to imaging, and isolating total RNA for sequencing. A modified Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies) was used to limit copper mediated degradation of RNA. The non-treated controls show EdU incorporation (green) while narciclasine treated cells showed no EdU incorporation (images not shown).



RESULTS

Figure 5: Volcano Plots: Ion AmpliSeq™ RNA Apoptosis Panel

A. A549 no drug/+drug **B. wi-38 no drug/+drug**

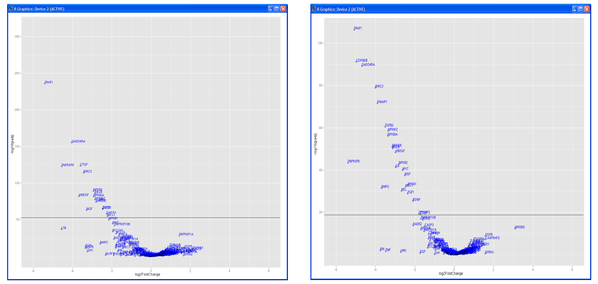


Figure 5: Volcano plot of cancer cell line A549 and primary cell line wi-38 response to apoptosis inducing drug narciclasine. Negative values on x-axis indicate high +drug/-drug ratio. Adjusted P-value >0.05 (dotted line) are considered significant ($-\log_{10}$(p-value) > 1.3)

Figure 6: Fold Change: Ion AmpliSeq™ RNA Apoptosis Panel

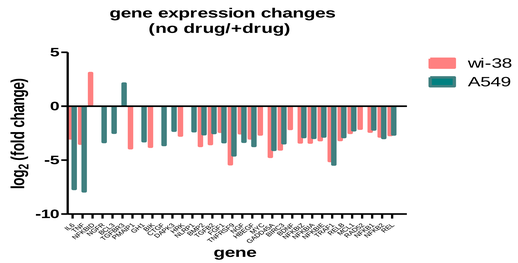


Figure 6: Significant fold changes in expression measured with Ion AmpliSeq™ RNA Apoptosis Panel caused by differences in drug treatment. Chart shows genes with greater than 10 counts per sample having p-values < 0.05, and absolute $\log_2$(fold change) > 2. Negative fold changes reflect an increase in expression after drug treatment.

Figure 7: RNA quality

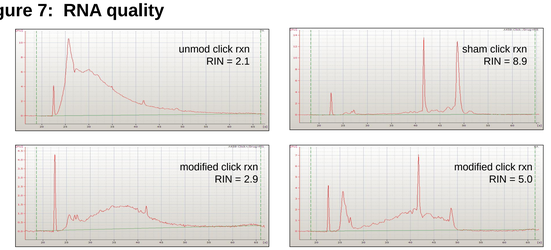
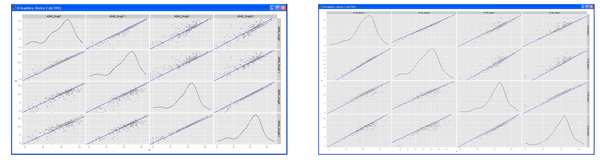


Figure 7: Quality of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Treating cells with unmodified click reaction prior to RNA isolation results in partially degraded RNA (RIN 2.1) compared to a sham click reaction without copper (RIN 8.9). Cells labeled with modified click reaction results in higher quality RNA (RIN 3.0 to 5.0).

Figure 8: Plotmatrix graph of gene expression fold change

A. A549 ± drug **B. wi-38 ± drug**



	A549_drug+ 1	A549_drug+ 2	A549_drug - 1	A549_drug - 2
A549_drug+ 1	1	0.9960023	0.7886056	0.7743991
A549_drug+ 2	0.9960023	1	0.7977393	0.7769184
A549_drug - 1	0.7886056	0.7977393	1	0.9921117
A549_drug - 2	0.7743991	0.7769184	0.9921117	1

	wi-38_drug+1	wi-38_drug+2	wi-38_drug-1	wi-38_drug-2
wi-38_drug+1	1	0.9941396	0.7088489	0.692372
wi-38_drug+2	0.9941396	1	0.7215671	0.7115017
wi-38_drug-1	0.7088489	0.7215671	1	0.9879727
wi-38_drug-2	0.692372	0.7115017	0.9879727	1

Figure 8: Correlation of technical repeats is 0.99 in each case and the distribution of counts is shown where samples intersect with themselves. Plots were made using the ggplot2 library in R.

Figure 9: Effect of click reaction on TaqMan® derived Ct values

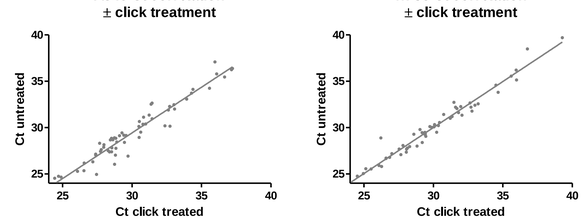


Figure 9: Isolated RNA was reverse transcribed into cDNA using the SuperScript® VILO™ cDNA Synthesis Kit at 2 ng of RNA/μl in the reaction. Real Time PCR was done using Applied Biosystems 7900HT Fast Real-Time PCR System, TaqMan® Gene Expression Master Mix and TaqMan® Assays on Demand with an input of 0.5 μl of the RT reaction in duplicate for each sample. The TaqMan® assays were chosen to match the region interrogated by the Ion AmpliSeq™ RNA Apoptosis Panel. Correlation between ± click reaction shows minimal changes in the profile of expression. A549 cells are more affected than wi-38. Ct values were determined using the SDS software v2.3.

CONCLUSIONS

- RNA isolated from click labeled cells is demonstrated to be of sufficient quality to create libraries for gene analysis using Ion PGM™ Sequencer
- “Copper safe” modifications to the click reaction improves the quality of isolated RNA
- RNA ± click reaction analyzed by real time PCR shows minimal changes to Ct value
- RNA isolated using the mirVana™ Kit allows for efficient capture of partially degraded RNA
- RNA from 6 hour drug treatment reveals early changes in gene expression prior to evidence of activation of the canonical apoptotic pathway of caspase 3
- Ion AmpliSeq™ Apoptosis panel shows the majority significant gene expression changes to be up-regulated in narciclasine treated samples

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