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

Scott T. Clarke1, Laura M. Chapman2, Brian Sanderson3, Janelle Ortiz2, Aleksey Rukavishnikov2, Michelle Yan1, Kyle Gee1, Susan Magdaleno1 1Molecular Probes® Labeling and Detection – Life Technologies™, Eugene, OR, USA; 2Ambion® – LifeTechnologies™, Carlsbad, CA, USA

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 phenotype. 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 stained in a drug panel in the presence of bromo-deoxyuridine (BrdU) to identify the drug resisting phenotype in the proliferating population. Following detection, the proliferating cells are re-isolated and analyzed for differential gene expression using an automated alignment method similar to the method used by the LifeTechnologies® 48-Channel FluorProStar. Using Click-IT EdU click chemistry, a weak cell surface EdU signal is measured using flow cytometry. 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 a click reaction designed to control the amount of copper related unwanted side reactions. Total RNA from these cells was isolated and gene expression was measured 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 masked DNA allows for detection of unmodified cells, making for quantification and subsequent isolation of high-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 a 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 treated a nanoclease using mirTriz™ RNA isolation kit.
• Create Ion AmpliSeq® Library ver 2.0 with barcode adapters for multiplex sequencing and analysis on Ion AmpliSeq™ RNA Apoptosis panel with Ion PGM® Sequencer to screen for changes in apoptotic relevant genes (LifeTechnologies, Carlsbad, CA).
• Use of Click-IT EdU click chemistry with directly labeled EdU fluorescent dye to track cell proliferation.

RESULTS

• Figure 1: Workflow
  - Drug panel screen and IC50

• Figure 2: Drug panel screen and IC50
  - 2A: Primary Screen:

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

• Figure 4: Gene expression changes
  - A: Significant fold changes in expression measured with Ion AmpliSeq™ RNA Apoptosis Panel caused by differences in drug treatment. Chart shows gene names, fold change, and raw P-values. Both drug-treated cell lines show changes in the expression of more than 100 genes per sample having p-values < 0.05, and absolute log2fold change > 2. Negative fold changes reflect an increase in expression after drug treatment.

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

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

• Figure 7: RNA quality

• Figure 8: Correlation of technical repeats is 0.99 in each case and the distribution of counts is shown where samples intersect with plots. Peaks 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 μg of RNA. In the reaction, Real-Time PCR was done using against Bcr-Abl and β-actin using Life Technologies™ 7500HT Fast Real Time PCR System, TaqMan® Gene Expression Master Mix and TaqMan® Assay on Denver with an input of 0.1 μg of the RT reaction in duplicate for each sample. The TaqMan® assay was run for 40 cycles with 38 cycles showing minimal changes to the profile of expression. A549 cells were more affected than with 38_drug+1 cells 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 reNAseq™ Kit allows for efficient capture of partially degraded RNA.
• RNA were 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 major significant gene expression changes to be up-regulated in nanoclease treated samples.

REFERENCES: