RNA-seq and Gene Expression Profiling

Alexander Robertson
Scientific Project Coordinator
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Dedicated to Genomic Analysis…

- Genetic Analyzers
  - Gold standard in Sequencing & Fragment Analysis

- SOLiD™ Sequencers
  - Highest quality genome for discovery of novel causative variation

- PostLight™ Semiconductor Sequencers
  - Scalability, simplicity, and speed for every benchtop

- Single Molecule Sequencing
  - Ultra-long sequencing & tunable performance for application flexibility

- Real-Time PCR
  - High performance, fast, Real-Time PCR; variety of chemistry choices

- Invitrogen, Ambion, & Dynal Reagents
  - Breadth & depth of best-in-class reagents and consumables
SOLiD™ System Family tree

SOLiD™ 4 System
- ~7 Gb/day
- 50 base reads
- 99.94% accuracy

5500 SOLiD™ System
- 10-15 Gb/day
- 75 base reads
- 99.99% accuracy
- Configurable slide

Same Chemistry
Same Informatics

5500xl SOLiD™ System
- ~20-30 Gb/day
- 75 base reads
- 99.99% accuracy
- 2 Configurable slides

life technologies™
5500 Series SOLiD™ Sequencer Microfluidic FlowChip

*Flexible, fast and cost-effective sequencing*

- Flexibility to run single or multiple samples
- Fast turn around times: 1 lane = typically 1 day for 35 bp reads
- Cost effective: Pay per use sequencing

Quickest time to result for single lane experiment
Flexible Experiment Configuration

- Targeted resequencing
- Small RNA
- ChIP
- Whole Transcriptome
- Whole Exome
- Whole Genome Sequencing

- Multiple samples in 1 lane
- Multiple experiments on 1 FlowChip
- 1 sample across multiple lanes
“Bulk” reagents are pay-per-use

Microfluidic FlowChip enables running 1 or more independent lanes
Robust – Embedded Sequencing Control

- Quality control at multiple check points in process
- Ability to predict run performance and trouble shoot issues.
- Real time analysis enables detection and disabling of poor samples to save on reagents and time
- Spike In controls
Smart Diagnostics

- It surfaces relevant actions
## Portfolio of Solutions for RNA Analysis

<table>
<thead>
<tr>
<th>Step</th>
<th>Whole Transcriptome</th>
<th>Small RNA</th>
<th>SAGE/dGEx</th>
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<tbody>
<tr>
<td>Design Experiment</td>
<td>TRlizol® Plus RNA Purification System</td>
<td>mirVana™ miRNA Isolation Kit</td>
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<td>Isolate Nucleic Acid</td>
<td>SAGE Analysis Tool</td>
<td>SOLiD™ Total RNA-Seq Kit</td>
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<td>Prepare cDNA Library</td>
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<td>Validate Results</td>
<td>Taqman® Gene Expression Assays</td>
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</table>
Some RNA Publications on SOLiD™ System

Anthrax Transcriptome on SOLiD™ System

Mouse Stem Cell Transcriptome on SOLiD™ System

Stem cell transcriptome profiling via massive-scale mRNA sequencing

Kathleen G. Gowan1,2,6, Alistair R. R. Forrest1,2,6, Gabriel J. R. C. de Oliveira1,2,6, broccoli B. A. Gardiner1,2, Geoffrey J. Fadloun3,4, Melissa K. Brown1,6, Darin E. Taylor1,6, Anita I. Strother1,6, Micayla E. Ward1,6, Graeme Reith1,6, Alan J. Robertson1,6, Andrew C. Perkins1,6, Stephen J. Bruce1,6, Clarence C. Lee6, Neel S. Ramani1,6, Heather E. Pechhurn1,6, Jonathan M. Manning1,6, Kevin J. McKeehan1,6, & Sean M. Grimm1,6

Cancer Transcriptome on SOLiD™ System

Single Cell Expression Analysis on SOLiD™ System

Tumor Transcriptome Sequencing Reveals Allelic Expression Imbalances Associated with Copy Number Alterations

Brian B. Tuchscherger1, Rebecca R. Laborde2, Xing Xu1, Jian Gu1, Christina B. Chung1, Cinna K. Monighetti1, Sarah J. Stanley1, Kerry D. Olsen1, Jan L. Kasperbauer1, Eric J. Moore1, Adam J. Broome1, Ruoying Tan1, Pius M. Brzoska1, Matthew W. Muller1, Asim Siddiqui1, Yan W. Asmann1, Yongming Sun1, Scott Kuersten1, Melissa A. Barker1, Francisco M. De La Vega1, David L. Smith2,3

1 Life Technologies Inc., Foster City, California, United States of America. 2 Division of Experimental Pathology, OHIF, Rochester, Minnesota, United States of America. 3 Life Technologies Inc., Austin, Texas, United States. 4 Duke University, Durham, North Carolina, United States of America. 5 Division of Biomedical Statistics and Informatics, Duke University, Durham, North Carolina, United States of America.

J. Bacteriol. doi:10.1128/JB.00132-12 © 2012, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.

The Structure and Complexity of a Bacterial Transcriptome

Karla D. Pastoreczna, Anjana Varadarajan, Brian D. Ondov, David T. Olcen, Michael E. Zwick, and Nicholas H. Bergman

School of Biology, Georgia Institute of Technology, Atlanta, GA 30332, USA; Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322, USA; Electro-Optical Systems Laboratory, Georgia Tech Research Institute, Atlanta, GA 30332, USA.
Importance of small RNAs or miRNA

MicroRNAs (miRNAs) belong to a class of non-coding, regulatory RNAs that is involved in posttranslational and is known to be involved in the development and progression of cancer. Recent studies have identified miRNAs as potential biomarkers for the early detection and diagnosis of cancer. miRNAs are small, non-coding RNA molecules that regulate gene expression by binding to mRNAs and either promoting their degradation or inhibiting their translation. They are involved in a variety of cellular processes, including cell proliferation, differentiation, and apoptosis. miRNAs are important for maintaining cellular homeostasis and are dysregulated in many diseases, including cancer.

What role do miRNAs play?

Are there still other small non-coding RNAs to be discovered?
Single Cell Transcriptome Analysis

Validated low input protocols conserve precious cancer samples

- Detailed protocol, from single cell collection, library creation, barcoding and SOLiD analysis

  Tang et al, Nature Protocols, 2010

- Demonstrates advantage over arrays in the identification of novel transcripts

  Tang et al, Nature Methods, 2009

- Describes differentiation process from the mouse inner cell mass cells to embryonic stem cells. Measured splice specific differential expression

  Tang et al, Cell Stem Cell, 2010
Combining Sequencing and Transcriptome Data

- Reveals expression levels, allelic imbalance and CNVs all using same platform in an oral cancer
- Demonstrates link between CNVs and gene expression
- Demonstrates that SOLiD™ system can measure allelic imbalance in transcriptomic data

Tuch et al, PLoS ONE, 2010
Composition of the Transcriptome

- 1-5% of RNA in eukaryotic cells is mRNA or polyA RNA that "codes" for proteins
- The remainder is "non-coding" RNA
  - rRNA
  - tRNA
  - miRNA
  - piRNA
  - snoRNA
  - and others....

Many non-coding RNA species have not been characterized because until recently there hasn’t been a way to study them.
The Power of RNA-Seq

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<th>Array</th>
<th>SOLID</th>
<th>TaqMan</th>
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<tr>
<td></td>
<td>2 to 3 logs</td>
<td>5 to 6 logs for all applications</td>
<td>7 to 9 logs</td>
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</table>

**DISCOVERY**

**VALIDATION**

DISCOVERY

VALIDATION
SOLiD™ System enables the detection of:

- Novel transcripts
- Novel exon – exon junctions
- Non coding RNA
- Expressed SNPS
3’ SOLiD™ SAGE™ Workflow

- Optimized 3’ SAGE kit and workflow for SOLiD™ System
- Start with as little as 50 ng poly-A RNA
- The extended 27 bp tag length provided by the EcoP15 digestion improves mapping
- Tag identifies unique full length transcript and is a digital enumeration of the number of times it is expressed
5’ SAGE™ enables genome-wide identification of transcription start sites

- Sensitivity of a single sequence run of the SOLiD platform was 100–1,000 fold greater than that observed from 5’end SAGE™ data generated from the analysis of 70,000 tags obtained by Sanger sequencing.

- The level of expression of these genes ranged from 0.02 to 4,704 transcripts per cell.
“How many samples can I multiplex?”
“How can I decide?”

- For SAGE, expect a million tags to be sufficient*
- For small RNA discovery, expect that 10-40M reads are sufficient
  - AB has detected >100 novel miRNAs with 40M reads
- For small RNA profiling, expect 2-10M reads to be sufficient*
- For Whole Transcriptome Analysis, expect at least 50 reads to be sufficient*
  - Profiling will require less reads
  - Splice junctions and fusion transcripts will require some more reads
  - Allele-specific expression will require more reads, depending on level of depth and allele expression ratios

* “Sufficient” can’t easily be quantified, thus approximate ranges given
ERCC Product

NIST SRM plasmids

*in vitro* transcription

Robotic pooling and formulation

Single-tube sku

Pool A

Pool D

Pool C

Pool B

Two tube sku

ERCC1

- 4X

- 0.5X

- 0.67X

- 1X

OR

ERCC2
What Can We do With 92 RNA Controls and SOLiD Sequencing?

*Provide Quality Control for RNA library preparation and sequencing*

• **Sensitivity**
  • Define the limit of detection and dynamic range for an experiment

• **Accuracy**
  • Determine how close the results are to expectation

• **Reproducibility**
  • Evaluate the variability between samples
Sensitivity
Lower Limit of Detection for SOLiD RNA-Seq

We can detect as few as 0.24 transcript copies per cell with ~19 million uniquely mapped reads or 0.08 copies per cell with ~54 million reads.

Set detection at 1X coverage.
RNA Isolation and enrichment or depletion strategies

TRIzol® Plus RNA Purification System
Poly(A)Purist™ mRNA Purification Kits
RiboMinus™ Eukaryote Kit for RNA Seq
Gene Expression Decision Tree

Total RNA → TRIzol® Plus RNA Purification System

- rRNA Depleted (including Non-coding RNA + Poly (A) RNA)
- Poly (A) RNA

Spike in ERCC

RNA Fragments 50-150 bp

SOLiD™ Whole Transcriptome Analysis Kit

P1  BC  P2
Why is Maintaining RNA Strand Specificity Important?

- RNA is transcribed from both DNA strands
- The SOLiD™ Total RNA-Seq Kit conserves strand information by directional addition of primer/adapters to the RNA
- Mapping sequence back to a specific strand of DNA is crucial to understand transcript structure
**Strand Information Conserved**

- Reads map accurately to sense or antisense strand
- The data shows that both red and blue transcripts reads extend beyond the 3’ end in the genome annotation
- Genome annotation can be updated
Identification of Alternate Transcripts
Number of Reads critical to achieve exon coverage

Long alternate 3’end of transcript **expressed**
Short alternate 3’end of transcript **not expressed**
5’end of transcript
BioScope - Whole Transcriptome Workflow

• Multi-step mapping process to identify sequencing artifacts and exon junctions

- Colorspace Reads
- Filter Mapping
- Junction Mapping
- Genomic Mapping
- Merge
- Mapping Results (.bam)
- Fusion Detection

• Count files contain RPKM* values for detecting differential expression
• Built in tool for identifying fusion transcripts and novel splice variants
• Data visualization via Integrative Genomics Viewer or UCSC Genome Browser

*RPKM reference: http://www.nature.com/nmeth/journal/v5/n7/abs/nmeth.1226.html
- Simplified output for identifying known and putative splice variants
- Confidence score provided for each junction (JCV)
- Allows researcher to quickly identify which junctions to validate

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Data Visualization

• Use .bam file to visualize coverage in the Integrative Genomics Viewer and load annotation tracks

• Use .wig files to visualize coverage in UCSC’s Genome Browser
Tertiary Analysis- 3\textsuperscript{rd} Party Software

- Import BAM file into Partek Genomics Suite
- Detect novel start sites / exons and comparative analysis
• BioScope can detect fusion transcripts using paired-end reads

Red: Inter Chromosome Fusion
Black: Intra Chromosome Fusion
Blue: Inverted Intra Chromosome Fusion
Finding Fusions or Alternative Splicing Events with Short Reads

Transcript

Exon-X

Exon-Y

Putative junction

Single Read Evidence

Paired Read Evidence*

Definition for calling as ‘Present’:

PE+SR – regular junction = 1 unique PE + 1 unique SR
PE+SR – fusion = 2 unique PE + 2 unique SR
SR – junction or fusion = 2 unique SR *

* Insert size matters

Expect: 75 to 125
Single Reads + Paired End Reads Enable More Efficient Detection of Junctions than Single Reads Only

UHR - 150 Known vs Putative Total Junctions Detected

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<th>Run Name (B1 = Barcode1 / B2 = Barcode 2)</th>
<th># of junctions</th>
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<tr>
<td>B2 SR</td>
<td>120,000</td>
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<td>B2 SR+PE</td>
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<td>B1&amp;2 SR+PE</td>
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known
putative
Detection of Putative Fusion Transcripts in UHR RNA Libraries

Library #1-16 M uniquely mappable reads

PE+SR: 9
SR: 58

Library #2-17 M uniquely mappable reads

PE+SR: 10
SR: 67

Library 1+2 - 33 M uniquely mappable reads

PE+SR: 16
SR: 169
Fusions identified by BioScope Software and Validated with TaqMan® Assays

~90% of putative fusions validate with Taqman® assays
TaqlMan® Gene Expression Assays

- Gold Standard in real-time PCR chemistry
  - Wide dynamic range: at least 6 orders of magnitude
  - Reproducible results
  - Sensitivity: detects 1 copy in 10-100 cells
  - >90% coverage of all RefSeq transcripts

- Simple-Fast-Reliable
  - Single tube or Low Density Arrays
  - Universal Thermal Conditions
  - No optimization
TaqMan® MicroRNA Assays

Step 1: Stem-loop RT
1. RT primer
2. Forward primer
3. Reverse primer
4. TaqMan probe

Step 2: Real-time PCR

Four oligos per miRNA

Two enzymes required
1. Reverse transcriptase
2. AmpliTaq Gold® DNA Polymerase

All components are from AB!
Simplify Cell Pathway Research

GeneAssist™ Pathway Atlas

- Learn how your protein is involved in different cellular pathways
- Find the interrelationships between proteins in a pathway
- Select a protein in a pathway to view gene, disease, and product information
- Quickly and easily order gene-specific Silencer® siRNAs and the corresponding TaqMan® Gene Expression Assays

From Proteins to Products in 4 Easy Steps

1. Search/browse by pathway, gene name, ID, symbol or NCBI transcript

   [Search/Browse the Atlas]

For additional guidance in the use of this new tool, please View Instructional Webinar describing the use of the GeneAssist Pathway Atlas in the context of an siRNA library experiment.
Mitotic Roles of Polo-Like Kinase Overview

Cell division is characterized by orchestrated events of chromosome segregation, distribution of cellular organelles, and the eventual partitioning and separation of the two daughter cells. Mitosis is a highly regulated process that ensures the proper allotment of genetic material between each pair of daughter cells. It proceeds through successive stages of well-defined and coordinated sub-processes. Entry into mitosis is regulated by the **CDCA5** (Cell Division Cycle 5)/Cdc5-heterodimer. CDCA5/Cdc5 is an as nuclear breakdown, chromosome condensation and spindle formation by phosphorylating subunit of the heterodimer) is required to drive the events of early mitosis, protein kinases (Kinesin) also make important contributions to cell cycle progression. The **PLKs** (Polo-Like Kinase emerging family of essential cell-cycle regulators (Ref.1).

PLKs are present in fungi and higher organisms and are characterized by the presence of a non-catalytic domain, termed the Polo-box (amino acids 410-439 in PLK), which is critical for the subcellular localization and function of the kinase. Members of this subfamily include mammalian **PLK1** (human Polo-Like Kinase-1), **PLK2** (Proliferating cell nuclear antigen, PCNA)-related kinase, **PLK3** (Proliferation-related kinase), and S. cerevisiae **CDC5**. Polo Kinases regulate various stages of mitotic/M posture to Anaphase transition via its interaction with the APC (Anaphase Promoting Complex) and play important roles in the regulation of cytokinesis (Ref.3).

At the onset of mitosis, the cytoplasmic cells undergo profound structural rearrangements to accommodate the nuclear envelope breakdown. The prominence among the kinases responsible for regulating entry into mitosis is the **CDCA5** (Cell Division Cycle 5)/Cdc5-heterodimer, which regulates the transition between mitosis and interphase. CDCA5/Cdc5 is an as nuclear breakdown, chromosome condensation and spindle formation by phosphorylating subunit of the heterodimer) is required to drive the events of early mitosis, protein kinases (Kinesin) also make important contributions to cell cycle progression. The **PLKs** (Polo-Like Kinase emerging family of essential cell-cycle regulators (Ref.1).

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**References:**

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Disease Implications - CDC2

Warts Tumor Suppressor:
CDC2/cyclin B forms a complex with a fraction of WARTS in the centrosome and phosphorylates the Ser613 site of WARTS during mitosis. S613-phosphorylated WARTS appears in the spindle poles at prometaphase and disappears at telophase. CDC2/cyclin B regulates functions of WARTS on the mitotic apparatus (Ref.1).

Breast Cancer:
Taxol activates CDC2 kinase in MDA-MB-435 breast cancer cells, leading to cell cycle arrest at the G2/M phase and, subsequently, apoptosis (Ref.2). Functional Cyclin B1-CDC2 protein complex is detected in G1 phase of BT-549 and T-47D cells. In a subset of transformed breast cancer cells altered cyclin B1 promoter activity may contribute to the misexpression of Cyclin B protein (Ref.3).

Alzheimer's Disease:
CDC2 might be involved in the abnormal hyperphosphorylation of tau and consequently aggregation of tau into PHF at an early stage and that increased CDC2 activity is not consequent to the deposition of Beta-amyloid in AD brain (Ref.4).

Apoptosis:
The activation of CDC2 participates also in HIV-1 induced apoptosis, upstream of the p53-dependent mitochondrial permeabilization step. An unscheduled CDC2 activation may contribute to neuronal apoptosis occurring in neurodegenerative diseases. The premature activation of CDC2 can lead to mitotic catastrophe for instance after irradiation-induced DNA damage. A cell type-specific modulation of CDC2 might be taken advantage of for the therapeutic correction of pathogenic imbalances in apoptosis control (Ref.5).

References:
THANK YOU-QUESTIONS ???