Genotyping & Sequencing Technologies in Cancer Studies

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Core Equipment

- Roche GS FLX (454) Next Gen Sequencer
- 3730 capillary sequencers
- Taqman 7900 Real-time PCR instrument
- CEQ 8000 sequencers
- Roche LightCycler 480
- PSQ96 Pyrosequencer
- Liquid handling robots
- PCR Machines

Human Genetics Department

- Affymetrix
- Illumina
- Solexa
- SOLiD
Genetic Assays

- SNP Genotyping
- DNA Methylation Analysis
- Gene Expression
- LOH (loss of heterozygosity)
- siRNA/RNAi, microRNA
- In/Del Analysis
- Microsatellite Genotyping
- Large Fragment Sizing
- AFLP
- RFLP

- BAC Fingerprinting
- SAGE
- HLA Typing
- Conformation Analysis
- Allele Quantification
- Sequencing
- Resequencing
- Comparative Sequencing
Maximize DNA Quality

All genotyping methods are sensitive to DNA...

- **Quantity** – Do you have sufficient DNA for your assay? Should you consider Whole Genome Amplification (WGA)? Advantages of WGA can be quantity and consistency. Disadvantages – amplification bias, expense.

- **Quality** – What is the quality of your DNA? High concentrations of poor quality DNA will not help you.

- **Consistency** – Even if the quantity and quality are adequate, if they vary widely from sample to sample, you are liable to get poor results.

Is your technology more robust to DNA quality or quantity? What is the dynamic range of detection?
Sample Collection

• Where will you get your DNA?
  • Blood or Buccal Swab / Saliva?
    • Quality
    • Consistency
    • Compliance
DNA / RNA Collection

1. Spit your saliva into the Oragene container.
2. Keep spitting until the amount of liquid saliva (not counting foam) reaches the top of the white label.
3. Tighten the cap very firmly.
4. Gently mix your saliva.

Figure 1. Agarose gel electrophoresis of DNA extracted from Oragene/saliva samples. A Lambda-Hind III digest was used as the marker in Lane 1.
Genetic Marker Maps

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Genetic Markers

• Variable Number Tandem Repeats (VNTRs)
• Short Tandem Repeats (STRs)
• Dinucleotide Repeats (and tri- and tetra-)
• CA Repeats
• Microsatellites
• Single Nucleotide Polymorphisms (SNPs)
Genotyping Gel Autoradiograph
Fluorescent Genotyping Gel
Capillary Sequencing

- Background
- Methods
- Applications

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SNPs

• Single Nucleotide Polymorphism
• Responsible for 90% of all human genetic variation
• A SNP occurs every 100-300 base pairs
• Currently almost 12 million SNPs in the NCBI SNP database
• May be within genes (coding SNP, cSNP) or outside gene (non-coding, the majority)
• May cause amino acid changes or not. If it causes an amino acid change it is called non-synonymous (nsSNP)
• Most SNPs are not responsible for a disease.
• Like microsatellites, they are used as markers for pinpointing a disease on the genome map. SNPs make particularly good markers because
  - They occur frequently throughout the genome.
  - They are older and more stable genetically.
SNP Platforms

Study Size

• **Pyrosequencing** – few SNPs, few samples, low-throughput, labor intensive

• **Taqman** – fewer SNPs, fewer samples, moderate-throughput

• **SNPlex** – moderate numbers of SNPs and samples, high-throughput (hundreds of thousands per day)

• **Sequenom** – moderate numbers of SNPs and samples, high-throughput (hundreds of thousands per day)

• **Affymetrix** – many SNPs, many samples, ultra-high-throughput (millions per day)

• **Illumina** – many SNPs, many samples, ultra-high-throughput (millions per day)

• **Next Generation Sequencing** – massively parallel, ultra-ultra-high-throughput
SNP Platforms
Technology

- **Pyrosequencing** – Sequencing by synthesis. Addition of dNTPs one at a time. Luciferase generates light when nucleotides incorporated. Gives you the neighboring sequence. Labor intensive.

- **Taqman** – Allelic discrimination. Fluorescent probe for each SNP variant. Simple, robust chemistry.

- **SNPlex** – Multiplexed, fluorescently labeled oligonucleotides on capillary electrophoresis.

- **Sequenom** – Mass Spectrometry. Highly accurate and reproducible.

- **Affymetrix** – Microarray of probe DNA spots printed on a glass or plastic chip.

- **Illumina** – Microarray of tiny beads with bound oligonucleotides. Sample DNA binds to the bead oligo, and is detected by an optical fiber.
Instruments

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**Pyrosequencing**

*Figure 1* | The principle of Pyrosequencing and the output Pyrogram™. Double peak heights indicate incorporations of two nucleotides in a row.
Allelic Discrimination
Encoding
Generation of genotype (GT) specific products through multiplex oligonucleotide ligation reaction (OLA)

Amplification
Multiplex PCR with universal primers

Decoding
Hybridization of universal ZipChute probes to amplicons and identification of eluted ZipChutes by CE
Ligation

Assemble OLA reaction

SNPlex Ligase
SNPlex Ligation Buffer (10X)

GeneAmp® PCR System 9700 Thermal Cycler

Thermal-cycle the OLA reaction

Legend
- Green: Universal PCR primer sequence
- Red: Universal reverse PCR priming site
- Blue: Genome equivalent region
- Orange: ZipCode sequence
- Circle: Spacer
- Circle with P: 5' phosphate
- LSO: Locus-specific oligo
- ASO\(_{A1}\): Allele-specific oligo A1
- ASO\(_{A2}\): Allele-specific oligo A2
- ASO\(_{L1}\): ASO linker L1
- ASO\(_{L2}\): ASO linker L2
- gDNA: Genomic DNA

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Enzymatic Purification

Legend
- Green: Universal PCR primer sequence
- Red: Universal reverse PCR priming site
- Blue: Genome equivalent region
- Dotted: ZipCode sequence
- Dashed: Spacer

Purification

\[ \lambda \text{ Exo} \]

\[ G \]

\[ \text{Exo I} \]

\[ C \]

\[ G \]

\[ C \]
PCR

Assemble the PCR reaction

SNPlex Amplification Master Mix (20X)
SNPlex Amplification Primers (2X)

GeneAmp® PCR System 9700 Thermal Cycler

Legend
- Universal PCR primer sequence
- Universal reverse PCR priming site
- Genome equivalent region
- ZipCode sequence
- Universal reverse PCR primer, biotinylated
- Universal forward PCR primer

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SNPlex Raw Data from the Core
Examples

Good

Bad

Good

Bad

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Examples

Bad

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SNP MicroArray
SNP BeadArray
SNP Platforms

Cost

- Affymetrix – $0.0006-??
- Illumina – $0.0002-??
- Sequenom – $0.05-$0.20
- SNPlex – $0.08-$0.14
- Taqman – $0.20-$0.70
- Pyrosequencing – $0.35-$1.60
When Choosing a Technology Platform, Consider:

- **Conversion rate** – How many of the SNPs you chose worked - *in silico, in vitro*?
- **Reproducibility** – Do you get the same result when you repeat the whole process?
- **Error Rate** – Compared to “true” result.
- **Concordance** – Agreement with some other method.
- **Call Rate** – How much missing data?
- **Cost** – What can you afford?
Array Data
Chip-based Genomics

- Each spot represents one genetic marker
- New generation chips hold 2.3 million SNPs
- To find genes for common, complex traits it may require DNA from 2000 individuals

4,600,000,000 SNP dataset

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Using High-Resolution Melting on the Roche LightCycler to Determine CpG-site Methylation status
Bisulfite Conversion

- Methyl-C and C are indistinguishable to DNA polymerase
- Methylation state is lost in PCR amplification
- Bisulfite treatment converts unmethylated cytosine to uracil, which becomes thymine after PCR
- Methylated cytosines are protected from bisulfite and thus unchanged
Principle of High Resolution Melting

- C to T proportion significantly changes the melting temperature of the product
- Degree of DNA methylation gives different melting profiles
- The high resolution dye from Roche intercalates and saturates evenly giving a sharp, precise melting profile

HRM Dye vs. SYBR Green
Example of High Resolution Melting curves on Roche LC480

Temperature

fluorescence

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Analysis using Tm Calling

- Significantly different melting temperatures of the two species allows quantitative analysis using the Tm calling
- By comparing the area of each peak relative to the sum of both peaks a quantitative percentage can be obtained
Analysis using Gene Scan

Normalized and Temp-Shifted Melting Curves

- 0% Methylated
- 0.5% Methylated
- 0.75% Methylated
- 1% Methylated
- 5% Methylated
- 10% Methylated
- 20% Methylated
- 30% Methylated
- 40% Methylated
- 60% Methylated
- 80% Methylated
- 100% Methylated
<table>
<thead>
<tr>
<th>Technique</th>
<th>Quantitative/Qualitative</th>
<th>Pros</th>
<th>Cons</th>
</tr>
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<tbody>
<tr>
<td>Methylation Specific PCR</td>
<td>Qualitative</td>
<td>Low cost.</td>
<td>Extensive PCR optimization</td>
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<td></td>
<td>Variable precision</td>
<td></td>
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<tr>
<td>Bisulfite Sequencing</td>
<td>Qualitative/semi-quantitative</td>
<td>Sequence context. Gives precise information for entire sequence</td>
<td>Labor intensive, high per sample cost.</td>
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<tr>
<td></td>
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<td></td>
<td>Time consuming</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>Quantitative</td>
<td>Sequence context</td>
<td>Labor intensive.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3rd primer necessary for extra specificity</td>
</tr>
<tr>
<td>MethyLight</td>
<td>Quantitative</td>
<td>High accuracy.</td>
<td>High setup cost</td>
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<tr>
<td></td>
<td></td>
<td>Can be scaled up</td>
<td></td>
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<tr>
<td>Methylation Sensitive High Resolution Melting</td>
<td>Quantitative</td>
<td>Accurate, scalable, low cost</td>
<td>Optimization dependent</td>
</tr>
<tr>
<td></td>
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<td>High precision</td>
<td></td>
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<td></td>
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<td>One tube method</td>
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</table>
Sequencing Technologies
A Brief History of Sequencing...

- A adenine
- C cytosine
- T thymine
- G guanine
Sanger Sequencing

Chain Termination

Template: 3' - CCGGTAGCAACT - 5'
Primer: 5' - GG - 3'

- dATP + ddATP
- dCTP + ddCTP
- dGTP + ddGTP
- dTTP + ddTTP

GGCCA
GGC
GGCCATCG
GGCCATCG
GGCCATCG
GGCCATCGT
GGCCATCGT
GGCCATCGTT
GGCCATCGTT

A
C
G
T

Sequence complementary to template DNA

Figure 3.21 Fundamentals of Biochemistry, 2/e
© 2006 John Wiley & Sons

1. Sequencing reactions loaded onto polyacrylamide gel for fragment separation

2. Sequence read (bottom to top) from gel autoradiogram
Sequencing Gel Autoradiograph
Dye Termination

TACGAGCAATCCGTTCGAAATA

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Fluorescent Gel
Capillary Sequencing

- Background
- Methods
- Applications
Human Genome Project

3 Billion Base Pairs

- **1990** - Begin: estimate **15 years, $3 billion**
- **1998** - Craig Venter starts new company *Celera*, “will sequence human genome in 3 years for $300 million”
- **2000** - Working draft
- **2003** - Complete in **13 years, $2.7 billion**
NIH $1000 Genome Initiative

RELEASE DATE:  February 12, 2004:

To develop novel technologies to sequence a mammalian-sized genome for approximately $1000.

“…the realization of the goals of this RFA is a long-range effort that is likely to require as much as ten years to achieve.”

Near-term goal - $100,000 genome.
X PRIZE Foundation
$10 million prize

For the first device to sequence 100 human genomes in 10 days or less, for less than $10,000.
Next Generation Sequencing Technologies

Strategy

• Automation
• Parallelization
• Miniaturization
Commercially Available Now

• 454 (now Roche GS FLX) - 2005
  ❖ ~300 base pair reads. Good for novel genomes
  ❖ 100 million base pairs in 8 hour run

• Solexa - 2006
  ❖ Short reads, ~23-35 bp. Best when there is a reference sequence (“resequencing”) or for analyzing small molecules like RNA.
  ❖ 4 gigabases per 3 day run - the equivalent of one-third of the human genome

• SOLiD - 2007
  ❖ Short reads, ~35 bp
  ❖ 3 gigabase per 3 day run

• Helicos - 2008
  ❖ Short reads
Technology

• 454 (now Roche GS FLX)
  - DNA is fragmented, bound to beads, amplified till each bead has 100,000 copies of the DNA fragment
  - Pyrosequencing - When a base is added to the DNA strand pyrophosphate is released, used as a substrate for luciferase, light is emitted and detected by a camera

• Solexa
  - Sequencing by synthesis with fluorescently labeled bases and DNA fragments bound to a slide.

• SOLiD
  - Sequencing by ligation. Bead-bound DNA molecule is interrogated with each of the 16 possible 2 base pair combinations in a fluorescently labeled oligonucleotide.
Clonal Cluster Technology

- Randomly fragment DNA
- Dilute to a single DNA molecule
- Amplify single molecule to get colonies of identical DNA
- Sequence by synthesis or ligation

Illumina Solexa

Roche 454

Lots of Data!
Raw Data Images
**Nano-knife edge - Reveo**
A single-stranded DNA molecule is stretched between nano-edge probes. Sequence DNA not by its chemistry but by its nanomechanics.

**Nanopore**
A single-stranded DNA molecule is driven through a pore so tiny that it partially blocks the pore as it moves through. Each base blocking the pore has different electrical properties and is read off as it passes through the pore.
Next-Next Sequencing

Helicos
Shipped in 2008
Single molecule sequencing

Complete Genomics
“mid-2009”
$5000 for whole human genome
Miniaturized

Pacific Biosciences
2010
Single molecule
Under an hour for hundreds of $
Miniaturized - zeptoliter
Current Cost

• < $100,000 for research
• $350,000 from Knome personal genomics company
## Cost to Sequence Human Genome

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<tr>
<td>Human Genome Project</td>
<td>$2.7 billion</td>
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<td>Current Capillary Sequencer</td>
<td>$3 million</td>
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<tr>
<td>Roche GS FLX (454)</td>
<td>$100,000</td>
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<tr>
<td>Illumina Solexa</td>
<td>&lt; $100,000</td>
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<tr>
<td>Applied Biosystems SOLiD</td>
<td>$60,000</td>
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<tr>
<td>Complete Genomics</td>
<td>$5000</td>
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<tr>
<td>BioNanomatrix???</td>
<td>$100</td>
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Applications

As the introduction of the personal computer created new applications in computing, whole genome sequencing technology has generated new applications.
Whole Genome Sequencing

De novo sequence of novel genomes

- *Mycobacterium tuberculosis*
- *Vibrio cholerae*
- *Streptococcus pneumoniae*
- *Haemophilus influenzae*
- *Helicobacter pylori*
Genomic Diversity and Metagenomics

Microbial diversity in

• Human microbiome
• Microbes in honey bee colony collapse disorder
• Deep sea microenvironments
• Deep mine microbial ecology
• Environmental sampling
Comparative Genomics, Paleogenomics, Ancient DNA Analysis

- Neanderthal Genome
- Mammoth
- Ancient wolves
- Mitochondria from ancient hair shafts
Chromosome Structure

- Deletions
- Duplications
- Copy number variation
- Insertions
- Inversions
- Translocations
- Methylation Analysis
Transcriptome Analysis

- Use massively parallel sequencing to quantitatively measure gene expression across tens of thousands of samples
- Develop a genomic signature of
  - Cell differentiation state
  - Disease state
  - Therapeutic response
  - Tumor signature
Deep Resequencing

Sequencing against a reference genome

• Identify rare disease-causing variants
• Cancer, HIV mutation detection
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<th>Application</th>
<th>Method</th>
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<td>Whole Genomes of Plants, Yeasts, Fungi, Bacteria, Viruses</td>
<td>Shotgun Sequencing, Paired-End Sequencing, De Novo Assembly</td>
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<td>Resequencing</td>
<td>Whole Genomes of Humans, Plants, Yeasts, Fungi, Bacteria, Viruses</td>
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<td>Genomic rearrangements, Copy number variation</td>
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<td>Transcriptome Analysis</td>
<td>Full-length transcripts</td>
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<td>Multiplex Sequencing of Paired-End Ditags (Singapore MS-PET)</td>
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<td>Serial Analysis of Gene Expression (SAGE)</td>
<td>cDNA Fragment Sequencing</td>
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<td>Gene Regulation Studies</td>
<td>Small non-coding RNAs</td>
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<td>Epigenetic Changes</td>
<td>DNA Methylation Pattern</td>
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<td>Nucleosome Modifications</td>
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<td>Metagenomics &amp; Microbial Diversity</td>
<td>Analysis of Environmental DNA</td>
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<td>16S rRNA</td>
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<tr>
<td>Paleogenomics</td>
<td>Whole Genome Sequencing of Ancient DNA</td>
<td>Shotgun Sequencing</td>
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Diagnostics

• $1000 genome will allow individual sequence as diagnostic. Will it come too soon to be useful?

• In genomic research technology and data have often come before our ability to extract information and knowledge from them.
Gene Expression Technologies

- DNA microarray
- Whole Genome Expression Profiling
- ChIP-Seq - Chromatin ImmunoPrecipitation
  - protein interactions with DNA
Gene Expression Microarray

- Make cDNA reverse transcript
- Label cDNAs with fluorescent dyes
- Hybridization to microarray
- Laser excitation at dye-specific Hz
- Laser emission
- Computer calculates ratio of intensity

Principle of cDNA microarray assay for gene expression (after Gibson & Muse 2002)

Red = "up-regulation"
Green = "down-regulation"
Black = constitutive expression
Results - Raw Data
Expression Array Example

Columns = Brain tissue samples

Rows = Genes
Color bands indicate modules

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Expression Array Example
85 Grade III and IV gliomas with 595 survival related genes

"Gene expression profiling of gliomas strongly predicts survival."
Diagnostics: Carbamazepine

FDA ALERT [12/12/2007]: Dangerous or even fatal skin reactions … that can be caused by carbamazepine therapy, are significantly more common in patients with a particular … [gene form], HLA-B*1502. This [form] occurs almost exclusively in patients with ancestry across broad areas of Asia, including South Asian Indians. Genetic tests for HLA-B*1502 are already available. Patients with ancestry from areas in which HLA-B*1502 is present should be screened for the HLA-B*1502 allele before starting treatment with carbamazepine. If they test positive, carbamazepine should not be started unless the expected benefit clearly outweighs the increased risk of serious skin reactions.

http://www.fda.gov/cder/drug/InfoSheets/HCP/carbamazepineHCP.htm