Tumor heterogeneity
mechanisms, clinical implications & analysis
Agenda

- What is ‘Tumor Heterogeneity’
- Mechanisms
- Impact of tumor heterogeneity
- How can you analyze tumor heterogeneity
- Biology focused approach
  - Genotyping
    - Somatic mutation
    - Copy number variation
    - NGS
  - Gene expression analysis
    - RT² Profiler PCR arrays
- Summary and Questions
What is tumor heterogeneity

- Existence of subpopulations of cells
  - Distinct genotypes and phenotypes
  - Divergent biological behaviours

- Types of tumor heterogeneity: Intertumor & Intratumor
Tumor heterogeneity

Inter-tumor heterogeneity

- Variation between two tumors
- Different individuals, tissues or cells
- Different tissues and cells have different mutational frequencies and response to therapy
- Clinically intertumor variation is handled by classifying tumors into subgroups based on mutations, copy number changes & RNA expression profiles
Tumor heterogeneity

**Intratumor heterogeneity**

- Variation within the primary tumor and its metastases
- Variation type
  - Morphological variation
  - Nuclear pleomorphism
    - breast cancer grading based on size & shape of the nucleus
  - Phenotypic heterogeneity
    - altered chromatin regulation, G1-S transition
- Investigating genomic heterogeneity
  - G-band karyotyping & FISH – demonstrating discrete banding patterns of chromosomal rearrangements and copy number alterations

**Copy number variation**
Tumor heterogeneity

Models of tumor progression & heterogeneity

- Cancer stem-cell model
- Clonal evolutilional model
Tumor heterogeneity

Models of tumor progression & heterogeneity

- **Cancer stem-cell model**
  - Cancer cells organized into a hierarchy of subpopulations of tumorigenic and non-tumorigenic cancer stem cells
  - Tumor growth is driven by a minority population of tumorigenic cells and that most other cancer cells have little or no capacity to contribute to tumor growth
  - Challenges associated with cancer stem-cell model
    - Replicating solid-cancer stem-cell markers
    - Variability from patient to patient
    - Variation in xenograft models
Models of tumor progression & heterogeneity

- **Clonal evolution model**
  - Proposed by Nowell et al., 1976
  - Based on Darwinian model of natural selection
  
  Genetically unstable cells accumulate genetic variations that gives it a selective advantage favouring its growth and survival

- **Eg.** hematological cancers, breast, brain, pancreas
Tumor heterogeneity

Intratumor heterogeneity - clonal evolution

- Heterogeneity as tumor evolves
  - Linear tumor evolution
  - Branched tumor evolution
    - Seen in ALL, CLL, pancreatic, breast cancer
    - Spatial separation of tumor subclones
      eg. clear-cell renal cell carcinoma

- Treatment
Impact of tumor heterogeneity

Targeted therapy

- Based on tumors dependence on a critical proliferation or survival pathway

- Works on solid tumors, but not in majority of advanced disease cases because of tumor heterogeneity

- Understanding tumor heterogeneity and developing rapid tests to comprehensively identify the genetic alterations is critical for its success
Tumor heterogeneity

Overall

- Tumor heterogeneity leads to
  - Highly variable cancers
  - Differential response to treatment
    - Targeted
    - Non-targeted
  - Needs to be monitored over time especially during treatment
  - Low frequency events are as important as the high frequency events
  - Challenge is to identify these low frequency events
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Tumor heterogeneity

Studying tumor heterogeneity

- qPCR
- RNAseq
- microarray
- ISH
- Genotyping
- aCGH
- NGS
- qPCR SOM CNV
- FISH
- MS
- Genotyping
- Differential Gene expression
- qPCR
- RNAseq
- microarray
- MS
Tumor heterogeneity

Studying tumor heterogeneity

- Somatic mutation arrays & assays
- Copy number variations / alterations arrays & assays
- Genotyping
  - NGS
  - GeneRead Gene Panels
- RT² Profiler PCR Arrays
- Differential Gene expression
Steps in Genotyping

- Step 1: Collection and stabilization
- Step 2: gDNA purification
- Step 3: WGA
- Step 4: PCR-based analysis
- Step 5: Detection
**Circulating Nucleic Acid** from human plasma or serum  
QIAamp Circulating Nucleic Acid Kit

**FFPE (Formalin-fixed Paraffin-embedded) samples**  
GeneRead DNA FFPE Kit

**Blood, tissues, & cells**  
DNeasy Blood & Tissue Kit  
Blood & Cell Culture DNA Kit (Mini, Midi, Maxi)

**Whole genome amplification from single cell**  
Repli-g WTA Single Cell Kit

**Quantification & qualification of amplifiable DNA prior to NGS**  
GeneRead DNA QuantiMIZE Kits

**Automated sample prep**  
QIAcube
Challenges in genotyping

- Sample quantity and quality
  - Limited biopsy specimens
  - Degraded nucleic acids – collection, storage, process

- Purity (genetic heterogeneity)
  - Cancerous cells may be a minor fraction of total sample
  - Multiple sub-clones of cancer may be present in one tumor sample
  - Genomic alterations in cancer found at low-frequency

- Data analysis
  - Biologically interpretable data

- Fragmented workflow
REPLI-g Single Cell Kit

**Problem:**
- Isolation of genotyping compatible DNA quantity from single to few cells

**Solution:**
- REPLI-g Single Cell Kit
  - Unbiased amplification of ultra-low DNA amount
How REPLI-g Single Cell Kit work

Simple three step protocol

Cell sample in PBS-sc or media

Add denaturation solution mix

10 min at 65°C

Add stop solution mix

Purified gDNA

Add denaturation solution mix

3 min at 15–25°C

Add neutralization solution mix

Add REPLI-g sc Master Mix

8 hr at 30°C
Stop at 65°C, 3 min

Amplified DNA

(Yield: 20-40 µg)
REPLI-g Single Cell technology provide high genome coverage & high fidelity
Studying tumor heterogeneity

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Genotyping

Next Generation Sequencing

Pre-Screen

Discovery
- Array CGH
- SNP Chips
- NGS

Validation
- FISH
- qPCR

Diagnostic Test
Why NGS?

- High throughput
  - Test many genes at once

- Cost effective
  - Drastic decrease in cost of sequencing

- Systematic and unbiased
  - Detection of all mutation types

- Quantitative
  - Easy to quantify mutation frequency
Clinical utility requires targeted analysis

Linking genetic variants with biology

- Common Variants
- No obvious effect on protein
- Iterative Biological interpretation
- Biologically relevant and actionable causal variants

Use known relationships or associations with:
- Diseases
- Signaling pathways
- Cellular phenotypes
- Clinical phenotypes/symbols
- Disease Genes/subnetworks
- Biomarkers
- Causal networks
- Copy number variations
- Regulatory sites
- Cellular processes
QIAGEN solution

1. Accessing single cell genomes for next generation sequencing
2. Target enrichment enabling rare mutation detection and QC
3. Streamlined one-tube library preparation
4. Raw reads to completely annotated variants
Shrink the Genome

Focus on Genes of interest relevant to research topic

<table>
<thead>
<tr>
<th>Sample</th>
<th>Information</th>
<th>Analysis Time</th>
<th>Coverage Depth</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome</td>
<td>$3 \times 10^9$ bps</td>
<td>7 days</td>
<td>10X</td>
<td>1 ug</td>
</tr>
<tr>
<td>20 Genes</td>
<td>$6 \times 10^4$ bps</td>
<td>8 hours</td>
<td>1000X</td>
<td>10 ng</td>
</tr>
</tbody>
</table>

Diagram: Chromosome with Genes 1 and 2 connected to DNA.
Which is most suitable target enrichment technology?

**Hybridization**
- Microarray
- Genomic DNA
- Biotin-UTP transcription
- Solution hybridization
- Bead capture
- Elution
- PCR
- Catch
- PCR
- Sequencing

**Hybridization, Ligation + PCR**
1. Digest genomic DNA
2. Hybridize the HaloPlex probe library in presence of the Barcode Primer Cassette: Hybridization results in gDNA fragment circuarization and incorporation of barcodes and Ion torrent sequencing motifs.
4. PCR amplify targeted fragments to produce a sequencing-ready target enriched sample.

**Multiplex PCR**
- Gene of interest
## Hybridization

- **Workflow:**
  - Library construction (4 hrs)
  - Hybridization with probes (24-48 hrs)
  - PCR & Indexing (2 hrs)

- **DNA input:**
  - 1-3 ug

- **Time from DNA sample to NGS library:**
  - 2-3 days

## Hybridization, Ligation + PCR

- **Workflow:**
  - Hybridization with probe (16 hrs)
  - Ligation (1 hr)
  - PCR & Indexing (2 hrs)

- **DNA input:**
  - 200-400 ng

- **Time from DNA sample to NGS library:**
  - 2 days

## Multiplex PCR

- **Workflow:** (SIMPLE)
  - PCR amplification (3 hrs)
  - Library construction (4 hrs)

- **DNA input:** (LOW)
  - <100 ng

- **Time from DNA sample to NGS library:**
  - 1 day (RAPID TAT)
GeneRead DNAseq Gene Panel

- Multiplex PCR technology based targeted enrichment for DNA sequencing
- Cover all human exons (coding region + UTR)
- Division of gene primers sets into 4 tubes; up to 1500 plex in each tube
**GeneRead DNAseq Panels V2**

Wet-bench validated panels; require minimal DNA input

- **Actionable Mutations**
- **Clinically-relevant**
- **Disease-specific**
- **Comprehensive**

- Breast Cancer
- Colorectal Cancer
- Myeloid Neoplasms
- Liver Cancer
- Lung Cancer
- Ovarian Cancer
- Prostate Cancer
- Gastric Cancer
- Cardiomyopathy

- Custom & Mix-n-Match

**Only 40 ng DNA needed per panel**
(except Actionable Mutations panel which needs a total of 20 ng)

Largest collection of pre-designed panels suitable for a wide range of applications

*Only 40 ng DNA needed per panel (except Actionable Mutations panel which needs a total of 20 ng)*
3 minutes with QIAGEN
New short 3 minute videos answering specific questions

Featured question of the month
When should one use targeted exome sequencing?
Type ‘targeted exome’ on YouTube to see the video
Studying tumor heterogeneity

- qBiomarker Somatic mutation arrays & assays
- qBiomarker CNV arrays & assays
- RT² Profiler PCR Arrays
- Genotyping
  - NGS
  - GeneRead Gene Panels
- Differential Gene expression
Genotyping

Analyzing somatic mutations

Pre-Screen

Discovery
• SNP Chips
• NGS

Validation
• qPCR
• Sequencing
• Mass Spectroscopy

Diagnostic
## Analysis Methods for DNA Mutations

<table>
<thead>
<tr>
<th>Method</th>
<th>Benefits</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point Mutation Analysis</td>
<td>Exact</td>
<td>Time Consuming</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>Exact</td>
<td>Time Consuming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need Instrument/Training</td>
</tr>
<tr>
<td>High Resolution Melt</td>
<td>Inexpensive</td>
<td>Base change identity not obvious</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simple</td>
<td></td>
</tr>
<tr>
<td>Microarray</td>
<td>Large Coverage</td>
<td>Sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need Instrument/Training</td>
</tr>
<tr>
<td>Next-Generation Sequencing</td>
<td>Exact</td>
<td>Time Consuming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need Instrument/Training</td>
</tr>
<tr>
<td>Quantify DNA Targets</td>
<td>Fast</td>
<td></td>
</tr>
<tr>
<td>➔ Real-time PCR</td>
<td>Reliable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High-throughput</td>
<td></td>
</tr>
</tbody>
</table>
## Comparison of different technologies

<table>
<thead>
<tr>
<th>Technology</th>
<th>qPCR</th>
<th>Sequenome OncoCarta *</th>
<th>Sanger Sequencing</th>
<th>NGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage</td>
<td>Focused content - Expanding coverage - Relevant mutations - Customization - Profile</td>
<td>~300 mutations in 19 genes -include some very rare mutations -not so easy to customize</td>
<td>Homebrew assay, low gene coverage, Some levels of mutation discovery</td>
<td>Genome wide, however this also depends on sequencing depth. -Detecting 1% mutation = 1000X sequencing depth</td>
</tr>
<tr>
<td>Samples</td>
<td>5<del>10ng fresh/frozen 200</del>500ng FFPE</td>
<td>500ng fresh or FFPE</td>
<td>Fresh/frozen, FFPE</td>
<td>Several ug of DNA</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>&lt; 1%</td>
<td>10%</td>
<td>&gt;20%</td>
<td>Depends on sequencing depth</td>
</tr>
<tr>
<td>Throughput</td>
<td>High-Throughput based off of content</td>
<td>&gt; 96/d</td>
<td>High sample/low content throughput</td>
<td>Depends, long waiting time</td>
</tr>
<tr>
<td>Instrument</td>
<td>Real-time PCR cycler -wide accessibility</td>
<td>Sequenome Mass spectrometer -limited access</td>
<td>CE sequencer</td>
<td>NGS sequencer -limited access</td>
</tr>
<tr>
<td>Data analysis</td>
<td>Simple</td>
<td>Complicated</td>
<td>Simple</td>
<td>Complicated</td>
</tr>
</tbody>
</table>
Profile disease or pathway-focused mutation profiling

1. Isolate genomic DNA from fresh, frozen or FFPE samples using QIAamp or DNeasy kits recommended in the handbook.

2. Add qBiomarker Probe mastermix to genomic DNA.
   - 200 – 500 ng fresh/frozen DNA
   - Less requires WGA
   - 500 – 3000 ng FFPE DNA

3. 1 sample goes on 1 plate

4. Standard 40 cycle PCR on most real-time Thermocyclers.

5. Upload C_T values to Data Analysis Webportal
Greek Symbol = Gene
# = Mutation

- For Normalization
- Assays detect non-variable region (not ARMS-based design)
- Designed to control differences in sample input and quality
## Tumor heterogeneity

### Focused arrays for somatic mutation screening

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>FGFR</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>RTK - Panel I</td>
<td>Hematopoietic neoplasms</td>
</tr>
<tr>
<td>RTK - Panel II</td>
<td>Lung cancer</td>
</tr>
<tr>
<td>Ras-Raf</td>
<td>Skin cancer</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Brain cancer</td>
</tr>
<tr>
<td>KIT</td>
<td>Lymphoid neoplasms</td>
</tr>
<tr>
<td>APC / CTTNNB1</td>
<td>Thyroid cancer</td>
</tr>
<tr>
<td>FLT3</td>
<td>Melanoma</td>
</tr>
<tr>
<td>c-MET</td>
<td>Liver cancer</td>
</tr>
<tr>
<td>p53 / Rb</td>
<td>Ovarian cancer</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Gastric cancer</td>
</tr>
<tr>
<td>PI3K-AKT signaling pathway</td>
<td>Esophageal cancer</td>
</tr>
<tr>
<td>DNA - QC</td>
<td>Head &amp; Neck cancer</td>
</tr>
<tr>
<td></td>
<td>AML, MDS</td>
</tr>
</tbody>
</table>
Tumor heterogeneity

Studying tumor heterogeneity

qBiomarker Somatic mutation arrays & assays

qBiomarker CNV arrays & assays

RT² Profiler PCR Arrays

Genotyping

NGS

GeneRead Gene Panels

Differential Gene expression
Genotyping

Copy number variation

- Definition: DNA segment with 1 kb or larger variation in comparison to reference genome
- Copy Number Variation or Copy Number Polymorphism
- Frequent and occur semi-randomly throughout the genome
- Occurs in wide range of organisms
  - Humans, Mice, Chimpanzees, Rhesus macaques, Cows, Chickens, Arabidopsis thaliana, Fruit flies, C.elegans, Saccharomyces cerevisiae
Current methods of copy number analysis

Different Methods for Different Experimental Questions

Pre-Screen

Discovery
• Array CGH
• SNP Chips
• NGS

Validation
• FISH
• qPCR

Diagnostic Test
qPCR

1. Determine the gene and reference genome
2. Design the primers, amplify genes by PCR, determine Ct value
3. As copy number increases Ct value decreases

Sample 1

Sample 2

Sample 3
Multicopy reference assay (MRef)

The ideal reference assay should fulfill the following criteria:

- Not be affected by a local change in the genome
  - Copy Number or SNP
- Copy number:
  - >20 copies in a diploid genome
- Location distribution:
  - Located on different chromosomes
  - ≤ 10% copies concentrated on a single chromosome
  - For copies on the same chromosome, preferably on different arms
- Sequence:
  - Sequence stable in human genome

Benefit: Superior Assay For Input Normalization
Multicopy reference assay yields increased accuracy

CNA Experimental Setup

Genomic DNA Samples
Liposarcoma samples

Gene Of Interest (GOI)

Reference Genome
Multicopy Reference Assay
Thirty (30) liposarcoma samples were tested by aCGH for copy number events.

Results from one of those samples (T50) is shown.

- Analysis with Partek software
- Deletions on Chromosome 11
- Amplifications on Chromosome 12

Initial screen yielded a list of 23 genes with copy number changes.

All samples were re-tested using Custom Copy Number PCR Array.

Data courtesy of Kara Pascarelli and Dominique Broccoli, Memorial University Medical Center, Savannah, GA, USA; and Lesley Ann Hawthorne, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA, USA)
Copy number assay in liposarcoma

Copy Number PCR Array Data for Sample T50

Data courtesy of Kara Pascarelli and Dominique Broccoli, Memorial University Medical Center, Savannah, GA, USA; and Lesley Ann Hawthorne, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA, USA)
Tumor heterogeneity

**Studying tumor heterogeneity**

- Genome instability
- Differential gene expression analysis

- qBiomarker Somatic mutation arrays & assays
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- NGS
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- Genotyping
- Differential Gene expression
Q: How to assess the expression of different mRNAs in a sample involved in a process and compare it across multiple conditions?

A:
Principles of qRT-PCR: overview

• **Real-Time PCR**
  - Amplify and simultaneously quantify target DNA

• **RT² PCR (Reverse transcription real-time PCR)**
  - Amplify and simultaneously quantify mRNA

• **Ct Values: Threshold Cycle**
How RT² Profiler PCR Arrays work

- Treat cells, for example non-cancer or cancer cells

control  sample

- Isolate RNA (Rneasy Kits)  RNase-Free DNase Treatment

- Genomic DNA Removal step  Reverse Transcription step  5mins + 20 mins

- Convert Total RNA to cDNA

- RT-PCR  + SYBR Green Master Mix  Real-time PCR Detection  2hrs

- Data Analysis
  Upload raw data (Ct) and analyze data  15 mins
Anatomy of a RT² PCR Array

- 84 Pathway-Specific Genes of Interest
- 5 Housekeeping Genes
- Genomic DNA Contamination Control
- Reverse Transcription Controls (RTC) n=3
- Positive PCR Controls (PPC) n=3

B2M, HPRT, RPL13A, GAPDH, HGDC
FREE complete & easy analysis with web/excel-based software

- Gene content for each pathway is pre-loaded
  - Custom Arrays: Gene List is all you need

- From raw $C_t$ to fold change results in multiple analysis formats

  Volcano Plot  Scatter Plot  Clustergram  3-D Histogram
Tumor heterogeneity

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- Differential Gene expression
Where will you find these information?

qPCR for Genotyping Portal

Detection of mutations or changes in gene copy number in cell lines or research samples is critical for toxicological, drug development, and cancer studies. qBiomarker Somatic Mutation PCR Arrays are panels of mutation-specific, real-time PCR assays for simultaneously profiling mutations within a disease- or pathway-focused set of genes. qBiomarker Copy Number PCR Arrays and Assays are designed for highly sensitive detection and profiling of copy number alterations and copy number variations.

- qBiomarker Copy Number PCR Arrays
  - For profiling copy number variations and alterations
  - Show details

- qBiomarker Copy Number PCR Assays
  - For focus-specific analysis of copy number variations and alterations
  - Show details

- qBiomarker Somatic Mutation PCR Arrays
  - For rapid and accurate profiling of the somatic DNA mutation status of gene panels
  - Show details

- qBiomarker Somatic Mutation PCR Assays
  - For detecting the presence of specific DNA sequence mutations in cancer and oncogenesis
  - Show details

Add Selection to WishList
Questions

Contact Technical Support
9 AM – 6 PM Eastern M – F

Telephone: 800-362-7737

Email: brcsupport@qiagen.com

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