Clinical Sequencing by Sanger: State of the Art in a Next-Gen World

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Objectives

- Describe assay design considerations for complete coverage of regions to be interrogated
- Discuss validation approaches to establish performance characteristics and ensure test accuracy and robustness
- List challenges in and solutions for complex data analysis and interpretation
- Discuss workflow measures for implementing efficient Sanger sequencing assays into the clinical laboratory
DNA sequencing with chain-terminating inhibitors

(F. Sanger, S. Nicklen, and A. R. Coulson)

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

1977
1986 - ABI Sequencing
(Sanger with Fluorescent Terminator)

**AmpliTaq DNA Polymerase, dNTPs, & DyeDeoxy Terminators**

1. **Primer**
   - DNA Template

2. **Cycle Sequencing:**
   - Annealing of Primer
     - Single Primer
   - Extension
     - Incorporation of Dye Labeled Terminator

3. **Products**
   - Various size fragments with last nucleotide labeled
Clinical Sequencing Assays

• Analytical Validation
  • Familiarity
  • Design
  • Optimization
  • Accuracy
  • Robustness (reproducibility)
  • Interpretation

• Clinical validation
  • Clinical sensitivity
  • Clinical specificity
Familiarization and Planning

- Reference sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>GBK file (analysis)</th>
<th>GBK file (reporting)</th>
<th>Mutation database numbering differences</th>
<th>MLPA exon numbering differences</th>
<th>GVIE - ARUP Wiki</th>
<th>CDS</th>
<th>Inheritance</th>
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<td>NC_000010.10</td>
<td>NM_000314.4</td>
<td>None</td>
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<td>No</td>
<td>No</td>
<td>A.dominant</td>
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</table>

- Alternative transcripts
- Homology checks
  - pseudogenes
- Inheritance
- Databases
  - Locus specific
- Known benign variants
Regions Interrogated

- Targeted exons
  - Example: MEN2
- All coding exons
  - ‘Full gene or full sequence analysis’
- Intron/exon boundaries
  - +20--50
- Known deep intronic mutations
- Regulatory regions
  - 5’ UTR, promoter
  - 3’ UTR
Primer Design

- Often per exon
- Design around pseudogenes
- Avoid known variants
  - Interfere with PCR
- All at same PCR conditions?

### PTEN Amplicon Sizes

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>Exon 1</td>
<td>252</td>
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<tr>
<td>Exon 2</td>
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<td>Exon 3</td>
<td>220</td>
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<td>Exon 3 new</td>
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<td>Exon 4</td>
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<td>Exon 5 short</td>
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<td>Exon 6</td>
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<td>Exon 7</td>
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<td>Exon 8 short</td>
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<tr>
<td>Exon 9</td>
<td>322</td>
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<tr>
<td>Promoter</td>
<td>697</td>
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### PTEN PCR and Sequencing Primers:

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<tr>
<th>Exon</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
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<td>4</td>
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<tr>
<td>5 s</td>
<td>TGTAAGACAGCCGAGCTCTTTTTCATCGAG</td>
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<tr>
<td>5 l</td>
<td>TGTAAGACAGCCGAGCTCTTTTTCATCGAG</td>
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<td>7 new</td>
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<tr>
<td>8 l</td>
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<td>CAGGAAACAGCTATGAGCCAATCAATTTTCACG</td>
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<td>9</td>
<td>TGTAAGACAGCCGAGCTCTTTTTCATCGAG</td>
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<td>promoter</td>
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<td>CAGGAAACAGCTATGAGCCAATCAATTTTCACG</td>
</tr>
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</table>
Sanger Sequencing Alignment Using Mutation Surveyor Software

Reference seq

Forward

Reverse

Reference seq
Difficult Regions

- High GC content
  - Optimization
- Secondary structure
  - Optimization or avoidance
- Benign Insertions/deletions
  - Example: CFTR GATT
- Pseudogenes
  - Example: PMS2
- Repeat motifs
  - Example: CFTR intron 8 TG/T
  - Example: Homopolymers
Primer Design

- Design Long and Short amplicons
- Cover all regions

CFTR intron 8
TG/T region
F and R primers for Long amplicon
Primer Design

CFTR intron 8
TG/T region
F and R primers for Short amplicon
Primer Design

- Loop-out/masking
Analytical Validation

- Performance characteristics
  - *Accuracy*

<table>
<thead>
<tr>
<th>PTEN Exon</th>
<th>Clinically Diagnosed Sample 1</th>
<th>Clinically Diagnosed Sample 2</th>
<th>Accuracy 1 (Cftr35)</th>
<th>Accuracy 2 (M3 DNA)</th>
<th>Accuracy 3 (CF16 11.21.08)</th>
<th>Accuracy 4 (CFTR41 12.3.08)</th>
<th>Accuracy 5 (11F)</th>
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<tr>
<td>3</td>
<td>wt</td>
<td>wt</td>
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<td>wt</td>
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<td>wt</td>
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<td>5</td>
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<td>wt</td>
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<td>8</td>
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<td>wt</td>
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<td>9</td>
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<td>wt</td>
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<td>promoter</td>
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<td>c.1-1085C&gt;CT</td>
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<tr>
<td>Intron</td>
<td>IVS1= c.80-96A&gt;AG IVS8= c.1026+32T&gt;TG</td>
<td>IVS1= c.80-96A&gt;AG IVS4= c.233+1G=GC IVS8= c.1026+32T&gt;TG</td>
<td>wt</td>
<td>wt</td>
<td>IVS1= c.80-96A&gt;AG IVS8= c.1026+32T&gt;TG</td>
<td>wt</td>
<td>wt</td>
</tr>
</tbody>
</table>
Quality checks

- Trace scores: average quality score
- Signal intensity
- Signal to noise ratio
- \( %QV_{20+} \): percentage of bases with quality values \( \geq 20 \)

**Sequencing Results for PTEN**

<table>
<thead>
<tr>
<th>EXON 1</th>
<th>TS</th>
<th>QV20+</th>
<th>AL (bp)</th>
<th>%QV20</th>
<th>S/N (A)</th>
<th>S/N (C)</th>
<th>S/N (G)</th>
<th>S/N (T)</th>
<th>SI (A)</th>
<th>SI (C)</th>
<th>SI (G)</th>
<th>SI (T)</th>
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<tbody>
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<td>227</td>
<td>270</td>
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<td>308</td>
<td>423</td>
<td>275</td>
<td>4031</td>
<td>1997</td>
<td>2396</td>
<td>2284</td>
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<td>sample1_Acc_ex1R</td>
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<td>236</td>
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<td>87.407</td>
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<td>684</td>
<td>9187</td>
<td>4062</td>
<td>7181</td>
<td>5642</td>
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</table>

Average | 43.2 | 228.5 | 270 | 84.63 | 1062.8 | 506.667 | 944.83 | 545 | 7345.5 | 3415.5 | 5646.2 | 4534 |

Standard Deviation | 3.43 | 9.1378 | 0 | 3.3844 | 546.55 | 277.831 | 637.8 | 328.63 | 3477.6 | 1795.3 | 3764.8 | 2619 |
Reproducibility - PCR product

*Intra-run variability

Re-design of exon 3
Reproducibility - PCR products

*Inter-run

All reactions
Workflow

Sample receipt → Extraction → PCR set up

Amplification

PCR clean-up → Sequencing set up → Sequencing

Sequencing clean-up → Detection → Analysis
**Workflow**

- M13 tagged primers
- **Workflow**
  - Low throughput – per sample
  - High throughput – per exon
- Primer plate

---

**PCR Tray Map**

<table>
<thead>
<tr>
<th>Sample #1</th>
<th>Sample #2</th>
<th>Sample #3</th>
<th>Sample #4</th>
<th>Sample #5</th>
<th>Sample #6</th>
<th>Sample #7</th>
<th>Sample #8</th>
<th>Sample #9</th>
<th>Sample #10</th>
<th>Sample #11</th>
<th>Sample #12</th>
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<td>10</td>
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</tbody>
</table>

*Thermocycler method: pcr men*
Sequencing Throughput

- High throughput
  - 96 samples, one exon (amplicon)/plate

- Medium throughput
  - 1 plate – 1-8 samples, 3-48 reactions/sample

- Low throughput
  - Manual is faster
Clinical Parameters

- Clinical sensitivity
  - Percent affected individuals in which mutations can be found in the gene
  - Mutation detection rate
- Clinical specificity
  - Percent of unaffected individuals in which mutations are found in a gene
  - Penetrance
- Reference or reportable range
  - Description of gene regions interrogated
  - Mutations tested
  - Zygosity
Implementation

- Validation summary
  - With refseq, known SNPs, known double mutations, database information
- Standard operating procedure
- Training
- Costs
- Test information
- Reporting
- Internal databases
- Proficiency testing
Reporting

• Result
  • Standard vs Traditional nomenclature
    • Example: Beta globin amino acids are commonly known from the mature protein (-1 amino acid)
  • Nucleic Acid
    • Example: c.2183delAA
  • Amino Acid
    • Example p.G542X
• Reference sequence (version) and numbering scheme
• Interpretation
• Recommendations
ACMG Recommendations

- Report clinical significance

- "... the laboratory must provide the interpretive information and a best estimate of clinical significance for the variants...."

Mutation Categories

- Previously reported
  - Pathogenic
  - Benign
  - But check original reports
- Previously unreported
  - Expected pathogenic
  - Suspected pathogenic
  - Uncertain
  - Suspected benign
- Further classification
  - Severe, moderate, mild, very mild
Interpretation

- Exonic
  - Frameshift (presumed pathogenic)
  - Nonsense (presumed pathogenic, except 3’ end?)
  - In-frame deletion/duplication (may or may not be pathogenic)
  - Missense (may or may not be pathogenic)
Missense Mutation

- Evidences:
  - Reported before?
    - Seen in affected or control individuals?
  - Conserved amino acid?
    - Over gene families or species?
  - Active site in the protein?
  - Affect mRNA levels?
  - Occur in the general population?
  - Co-occurrence with causative mutations
  - Track with disease in the family?
  - Functional studies available?
    - IHC, structural analysis, RNA, biochemical studies
Amino Acid Prediction

- Existing predictions programs
  - PolyPhen 2, SIFT, Pmut, PhD-SNP, nsSNPAnalyzer, AlignGVGD

- Predictions using machine learning classification tools.
  - Gene-specific algorithms outperform generalized tools
  - Developed a standardized metric for evaluation of uncertain gene variants.
  - Visualization models for clinical implementation

- Emerging “authoritative” (clinically curated) gene variant/disease archives
ACADM UNCERTAIN VARIANTS

**ACADM - A170S**

<table>
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<tr>
<th>Predictor</th>
<th>Call</th>
<th>Score</th>
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<tbody>
<tr>
<td>SIFT</td>
<td>tolerated</td>
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<tr>
<td>PolyPhen</td>
<td>benign</td>
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<tr>
<td>PMut</td>
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<td>MutPred</td>
<td>benign</td>
<td>45</td>
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<tr>
<td>PSAAP</td>
<td>benign</td>
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</table>

**Consensus**

![Consensus Diagram](image)

**ACADM - A372D**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Call</th>
<th>Score</th>
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<tbody>
<tr>
<td>SIFT</td>
<td>affects function</td>
<td>98</td>
</tr>
<tr>
<td>PolyPhen</td>
<td>probably damaging</td>
<td>99</td>
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<td>PMut</td>
<td>pathological</td>
<td>82</td>
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<td>MutPred</td>
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<td>88</td>
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<tr>
<td>PSAAP</td>
<td>pathogenic</td>
<td>84</td>
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</tbody>
</table>

**Consensus**

![Consensus Diagram](image)

PREDICTED PATHOGENIC

PREDICTED BENIGN
Intronic Mutation

- Intronic
  - has it been reported before?
  - approximately 20-50 bases
  - potential splice site
    - http://www.fruitfly.org/seq_tools/splice.html
      - consensus sequence GT ..... AG
        - Donor GT (start of intron)
        - Acceptor GA (end of intron)
        - Branch site U (18-40 upstream of 3’ splice site)
Finding Rare Variants

- **CFTR Example**
  - Child with F508del/I1028T
  - Mother also with F508del/I1028T
  - In cis
  - Does not explain symptoms in child

- **Alpha globin Example**
  - Apparent homozygous for p.X143Glu (Hb Seal Rock)
  - Subsequent deletion analysis showed -3.7Kb deletion
  - Compound heterozygous
  - Mild Hb H disease
Genetic Evidence
Family Concordance Studies

- Autosomal dominant/ X-linked/de novo mutations
- Single (affected) individual from a family tested
  - Results: sequence variant of unknown significance
- Test additional family members
  - Affected/Unaffected
  - Greater statistical power with affected distant relatives
- Evaluate pedigree data for evidence of causality
- Test hypothesis: Variant confers specified risk against the hypothesis of complete neutrality
- Determine likelihood ratio for causation
**MECP2 Missense Mutation**

- In silico prediction
  - Polyphen2: unknown
  - SIFT: Tolerated
- Present in ‘normal’ mother
  - Variable phenotype due to X inactivation?
- Present in unaffected brother
Extended Pedigree from Clinical Case

Bayesian Factor = 461:1 in favor of causality

(R479Q)
Likelihood Ratios: In Favor of Causality

<table>
<thead>
<tr>
<th>Pedigree. Gene/Mutation</th>
<th>Bayesian Factor</th>
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<tbody>
<tr>
<td>1. ACVRL1 p.R479Q</td>
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<td>2. ACVRL1 p.G402S</td>
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<td>3. ACVRL1 p.C344R</td>
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<td>5. ENG p.W196R</td>
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<td>31.82</td>
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<td>7. ENG p.R529H</td>
<td>7.98</td>
</tr>
</tbody>
</table>
Variant Annotation Summary

Current manual method:
- Check internal database for variant
- Locus-specific databases
- dbSNP, frequency (not all benign)
- Prediction algorithms (Polyphen-2, Sift, others)
- Literature search
- Google

GALT Database
Automated Pipeline

Input

MySQL database:
Internal database
dbSNP
Biobase
1,000 Genome

Pipeline

Prediction programs

Output

Annotated Variant

Graphical Display

Courtesy of P. Ridge
Revolutionary Approach

- Next-generation sequencing (NGS) 2005
  - Massive Parallel Sequencing in a flow cell (400 Mb to 30Gb)
  - Large scale sequencing/re-sequencing of the chromosome possible
    - Clonally amplified templates
    - Single molecule templates
Next Gen Sequencing

- Gene panels
  - Genes known to cause disease
  - Variant discovery
- Whole exome
  - Gene discovery
- Whole genome
  - Gene discovery
Data Analysis: Variant Filtering

Pinpoint which gene causes HHT4!

Kept affected SNVs (2 shared) ~36%
Remove unaffected SNVs ~25%
Remove 8 HapMap SNVs ~27%
Remove SNVs in dbSNP, pseudogenes, repeat regions ~7%
~5% remaining!

Focus on NS-SNVs in protein coding regions, UTR, splice sites

Lead candidate gene: ADCK2 c.997C>CT, p.Arg333Stp

Courtesy of Drs. P. Bayrak-Toydemir, W Donahue
ADCK2 c.997C>CT, p.Arg333Stop!
Sanger Sequencing Continued Role

- Complex regions difficult to align with NextGen software
- Confirm that variants are “real”
- Confirm that variants are “significant”
  - Family concordance studies
- Familial testing
Conclusions

- Sanger sequencing has allowed clinical testing for numerous diseases
- Proper design and validation of sequencing tests can prevent analytical errors
- Sequence complexity can be addressed by primer design
- Interpretation complexity still a challenge
- Mutation databases with evidences for classification are needed
- Sanger sequencing will remain important as companion to next generation sequencing
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