Bio(tech) Interlude

3 Nobel Prizes:
PCR: Kary Mullis, 1993
Electrophoresis: A.W.K. Tiselius, 1948
DNA Sequencing: Frederick Sanger, 1980
PCR

1: 25°C

5' A
C
T
G 3'

3' A
G
C
T 5'

2: 95°C

5' A
G
C
T

3: 60°C

5' A
G
C
T

6: 72°C

5' A
G
C
T

4: 72°C

3' A
G
C
T 5'

5: 72°C

5' A
G
C
T 3'

2: 95°C

3' A
G
C
T 5'

4: 72°C

5' A
G
C
T

6: 72°C

3' A
G
C
T 5'
Hot spring, near Great Fountain Geyser, Yellowstone National Park
PCR

Ingredients:
- many copies of deoxy nucleotide triphosphates
- many copies of two primer sequences (~20 nt each)
  - readily synthesized
- many copies of Taq polymerase (*Thermus aquaticus*),
  - readily available commercially
- as little as 1 strand of template DNA
- a programmable “thermal cycler”

Amplification: million to billion fold
Range: up to 2k bp routinely; 50k with other enzymes & care
Why PCR?

PCR is important for all the reasons that filters and amplifiers are important in electronics, e.g., sample size is reduced from grams of tissue to a few cells, can pull out small signal amidst “noisy” background.

Very widely used; forensics, archeology, cloning, sequencing, …
DNA Forensics

E.g. FBI “CODIS” (combined DNA indexing system) data base
As of 1/2013, over 10,142,600 offender profiles
Picked 13 “short tandem repeats”, i.e., variable-length regions of human genome flanked by (essentially) invariant sequences (primer targets), several alleles common at each locus, of which you have 2
Amplify each from, e.g., small spot of dried blood
Measure product lengths (next slides)

http://www.fbi.gov/about-us/lab/biometric-analysis/codis
http://www.dna.gov/solving-crimes/cold-cases/howdatabasesaid/codis/
Gel Electrophoresis

DNA/RNA backbone is negatively charged (they’re acids)
Molecules moves slowly in gels under an electric field
  agarose gels for large molecules
  polyacrylamide gels for smaller ones
Smaller molecules move faster

So, you can separate DNAs & RNAs by size

Nobel Chem prize, 1948 Arne Wilhelm Kaurin Tiselius
DNA Sequencing – Sanger Method

Like one-cycle, one-primer PCR
Suppose 0.1% of A’s:
  are di-deoxy adenosine’s;
  backbone can’t extend
carry a green fluorescent dye
Separate by capillary gel electrophoresis
If frags of length 42, 49, 50, 55 … glow green,
those positions are A’s
Ditto C’s (blue), G’s (yellow), T’s (red)
DNA Sequencing
Sanger with capillary electrophoresis
Sequencing A Genome

Highly automated
Typical Sanger “read” about 600 nt
“Whole Genome Shotgun” approach:
  randomly fragment (many copies of) genome
  sequence many, enough to cover each base 10x or more times
  reassemble by computer

Complications: repeated region, missed regions, sequencing errors, chimeric DNA fragments, …
But overall accuracy $\sim 10^{-4}$, if careful

E.g., human genome project:
$\approx 30$Gbases and
$\approx 3 \times 10^9 / 600 \times 10$
$= 5 \times 10^7$ reads
“Next Generation” Sequencing

Many technical improvements to Sanger approach over many years, culminating in highly automated machines used for the HGP. Since then, many innovative new ideas/products:

- Helicos: single molecule fluorescence tethered to flow cell
- Illumina: colony PCR; reversible dye terminator
- Ion Torrent: semiconductor detection of ions released by polymerase
- Roche 454: emulsion PCR; pyro sequencing
- Oxford Nanopore
- Pacific Biosciences: single tethered polymerases in “zero mode waveguide” nano-wells, circularized DNA, “real time”
- ABI SOLiD: emulsion PCR, sequence by ligation, “color-space”
- Complete Genomics: rolling circle replication/DNA nanoballs

Technology is changing rapidly!
“Next Generation” Sequencing

~1 billion microscopic PCR “colonies” on 1x2” slide
“Read” ~50-150bp of sequence from (1 or 2) ends of each
Ends fluorescently labeled, blocked, chemically cycled
Automated: takes a few days; ~ 100 G bases/day
Costs a few thousand dollars
Generates terabytes of data (mostly images)
I,e., ~ 30x human genome/day (you need 25x-50x to assemble)

Other approaches: long reads, single molecules,…
Technology is changing rapidly!
In contrast with other platforms, therefore, the sequencing by synthesis method must be monitored “live (that is, the camera does not move relative to the template library).” Across multiple cycles (e.g., A-G-C-T-A-G-C-T…), the pattern of detected incorporation events reveals the sequence of template library ends (that is, multi-template PCR, not multiplex PCR, as only a single primer pair is used, corresponding to flanked shotgun library (shown as gold and turquoise adaptors flanking unique inserts) is PCR amplified while simultaneously avoiding overcrowding.

Measurement of the concentration of the template library is critical to maximize the cluster density; each clonal cluster contains ~1,000 copies of a single member of the template library. Accurate clonal amplification of sequencing features. (The Solexa technology relies on bridge PCR –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore)

A major limitation of the 454 technology relates to homopolymers –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore.

Because there is no terminating moiety preventing multiple consecutive incorporations at a given cycle, the length of all homopolymers must be inferred from the signal intensity. This is prone to a greater error rate than the discrimination of incorporation versus nonincorporation. This can lead to shorter and more variable read lengths than what are possible with other technologies, with consequences for the amount of sequence information that can be extracted from a single run. Moreover, it is somewhat unconventional in relying on DNA polymerase activity. Nucleotide incorporations can potentially be made in achieving early proof-of-concept demonstrations –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore. These methods, we are also unlikely to run out of nucleotides to sequence –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore.

Several academic groups and companies are working on different forms (e.g., SOLiD and Solexa) but it may be –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore. Although progress has been made in achieving early proof-of-concept demonstrations –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore.

Fluctuations in DNA conductance through the pore, or, potentially, the detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore (aka ‘cluster PCR’) to amplify

—constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified –constructed adaptor-flanked shotgun library is PCR amplified to distinguishable locations from any single template molecule during the amplification remain immobilized and clus-tered to a single physical location on an array. On the Illumina platform, the bridge PCR method of choice for certain applications, including the potential for longer reads than Sanger sequencing, demonstrated substantial progress toward a working technology, which illumination can be restricted to a zeptoliter-scale volume around a surface-tethered polymerase such that incorporation of phosphate-labeled nucleotides (with fluorescent labels on phosphate groups) can be observed with low background fluctuations in DNA conductance through the pore, or, potentially, detection of interactions of individual bases with the pore, membrane protein such as alpha-hemolysin or a synthetic pore.

Box 3  Sequencing in real time

Modern DNA Sequencing

A table-top box the size of your oven (but costs a bit more … ;-) can generate ~100 billion BP of DNA seq/day; i.e.
= 2008 genbank,
= 30x your genome
Figure 3: Illumina Sequencing Technology Outpaces Moore’s Law for the Price of Whole Human Genome Sequencing
Table 1: HiSeq X Ten Preliminary Performance Parameters*

<table>
<thead>
<tr>
<th></th>
<th>Dual Flow Cell</th>
<th>Single Flow Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Output/Run</td>
<td>1.6–1.8 Tb</td>
<td>800–900 Gb</td>
</tr>
<tr>
<td>Reads Passing Filter†</td>
<td>≤ 6 billion</td>
<td>≤ 3 billion</td>
</tr>
<tr>
<td>Supported Read Length</td>
<td>2 × 150</td>
<td></td>
</tr>
<tr>
<td>Run Time</td>
<td>&lt; 3 days</td>
<td></td>
</tr>
<tr>
<td>Quality</td>
<td>≥ 75% of bases above Q30 at 2 × 150 bp</td>
<td></td>
</tr>
</tbody>
</table>

*Specifications based on Illumina PhiX control library at supported cluster densities (between 1,255–1,412 K clusters/mm²). Supported library preparation kit includes TruSeq Nano DNA HT kit with 350 bp target insert size and HiSeq X HD reagents. HiSeq X was designed and optimized for human whole-genome sequencing; other applications and species are not supported.

†Single-end reads.
Pacific Biosciences

SMRT® Technology

The PacBio® RS II is based on novel Single-Molecule, Real-Time (SMRT) technology which enables the observation of natural DNA synthesis by a DNA polymerase in real time. Sequencing occurs on SMRT Cells, each containing thousands of Zero-Mode Waveguides (ZMWs) in which polymerases are immobilized. The ZMWs provide a window for watching the DNA polymerase as it performs sequencing by synthesis.

Phospholinked nucleotides

Zero-Mode Waveguides

Advantages:
- single molecules
- long reads
- direct CH$_3$ detection

Disadvantages:
- throughput
- error rate; (circularize?)

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**Read Length:**
- Average: 4,606 bp
- 95$^{th}$ Percentile: 11,792 bp
- Maximum: 23,297 bp

**Throughput per SMRT® Cell:**
- 216 Mb
- 47,197 reads

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Based on data from 11 kb plasmid library using a 120 minute movie

http://www.pacificbiosciences.com/img/assets/smrt_sequencing_advantage_readlength_lg.png
Oxford Nanopore

Prerelease claims ≈ 100k read lengths, 150Mb in 6 hrs, $1000
Personal Genomes

2001: ~$2.7 billion (Human Genome Project)
2003: ~$300 million
2007: ~$1 million
2008: ~$60 thousand
2009: ~$4400
2014: ~$1000 (?)  

*bioinformatics not included…*
Summary

PCR allows simple \textit{in vitro} amplification of minute quantities of DNA (having pre-specified boundaries)

Sanger sequencing uses

\begin{itemize}
  \item a PCR-like setup with modified chemistry to generate varying length prefixes of a DNA template with the last nucleotide of each color-coded
  \item gel electrophoresis to separate DNA by size, giving sequence
\end{itemize}

Sequencing random overlapping fragments allows genome sequencing (and many other applications)

“Next Gen” sequencing: many innovations

\begin{itemize}
  \item throughput up, cost down (lots!)
\end{itemize}