The Institute for Molecular Biology and Biotechnology
MOBIX

http://www.science.mcmaster.ca/mobix/

Antimicrobial Research Centre (ARC)
Centre for Gene Therapeutics
Centre for Evolutionary and Genomics Biotechnology (CEGeBio)
Functional Genomics Centre

LSB/B123
Oligo Synthesis
DNA-Sequencing
Fragment Analysis

http://www.science.mcmaster.ca/mobixlab/index.html

Mobixlab Services

- **Automated DNA sequencing: 3730 DNA Analyzer processes 96 samples in 5 hours, average read of 700 - 900 bases on good quality DNA**
- **Oligonucleotide synthesis: in-house or through IDT (Integrated DNA Technologies**
- **Microsatellite and SNP analysis**
- **Phosphorimager – LSB314**
- **Alpha Imager – LSB 412**
- **Kodak Image station – LSB B123**
- **Bio-informatics**
- **Biobar**

Common uses for oligonucleotides

- PCR primers
- Sequencing primers
- Site-directed mutagenesis
- Deletion mutagenesis
- EMSA probes
- Double-stranded linkers
- Interference

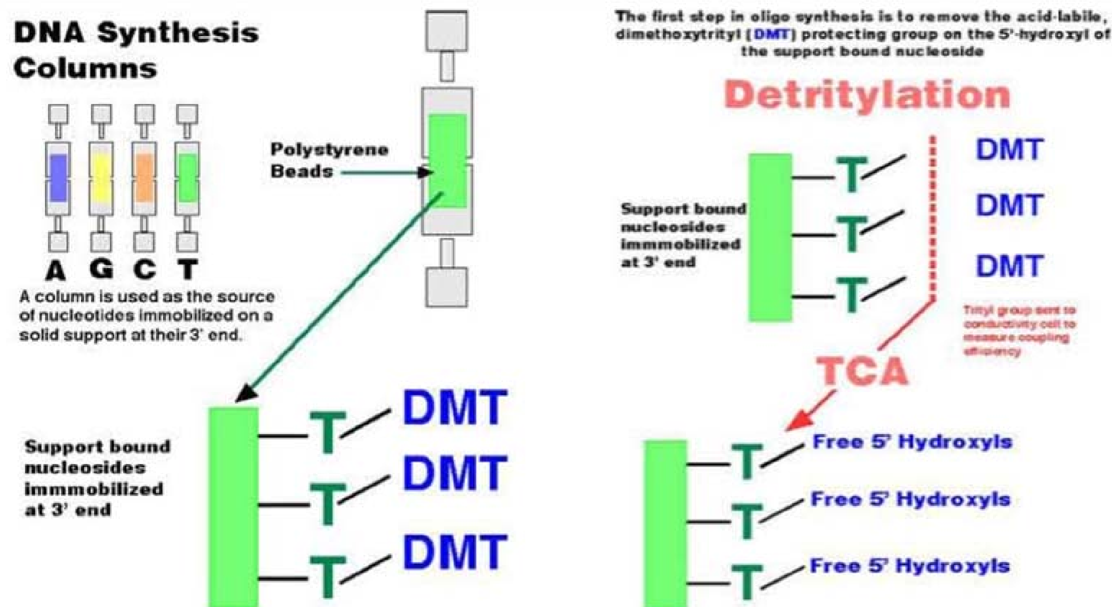
http://www.idtdna.com/home/home.aspx
http://www.invitrogen.com/site/us/en/home/Products-and-Services/Product-Types.html
http://www.sigmaaldrich.com/sigma-aldrich/the-americas/canada-english/sigma-genosys-canada.html

Oligonucleotide synthesis - history

- First published by Todd et al, 1955, 1957
- Alternate method by Khorana et al, 1958
- Modifications by Letsinger and Reese, 1969, 1978
- Improvements lead to use of phosphoramidites and solid phase synthesis and automation
- Synthesis occurs in 3' – 5' direction on columns loaded with 3' end base
- Each base added sequentially

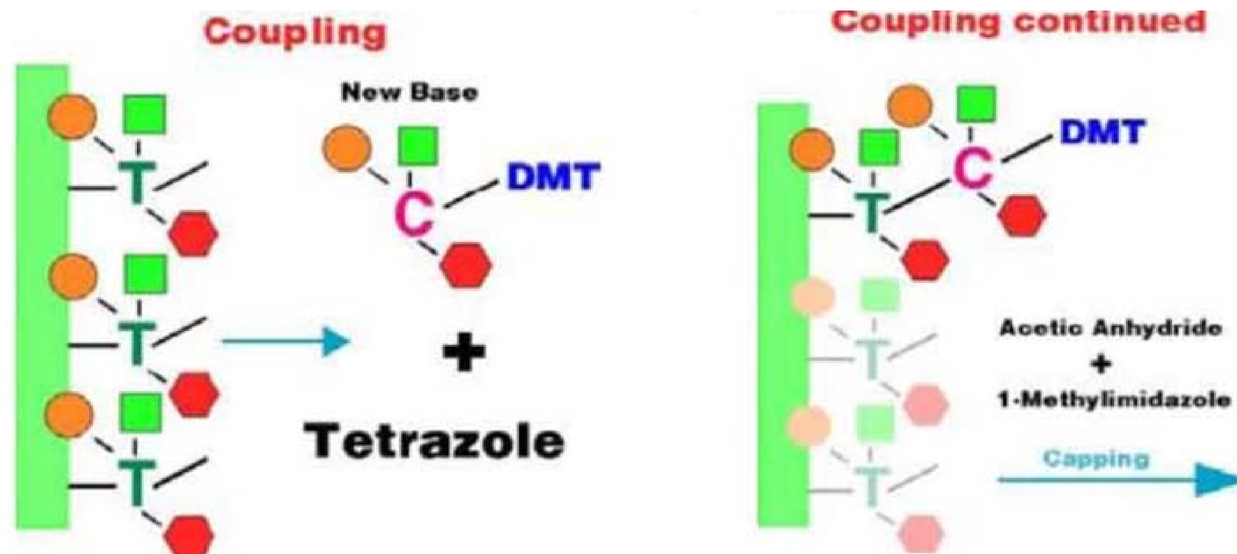
How to synthesize an oligonucleotide 1

- Synthesis starts with a column with the 3’ base attached. The reactive OH group is —protected by dimethoxytrityl group (DMT or trityl). First the trityl protecting group must be removed to generate a free 5’ hydroxyl.



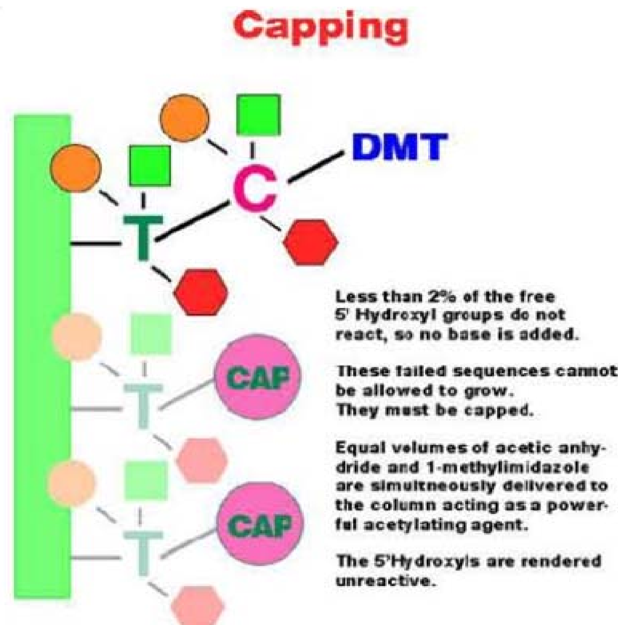
How to synthesize an oligonucleotide 2

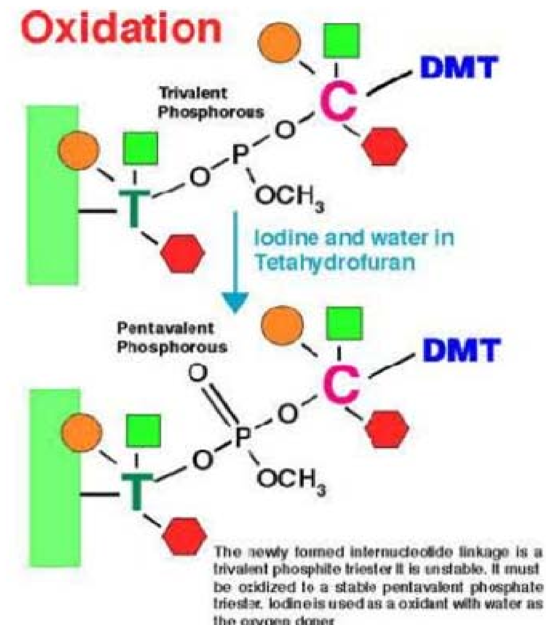
- The next base is added together with the coupling reagent. Some bases may not have coupled (<1%) so any unreacted bases are —capped to block any further reaction.



How to synthesize an oligonucleotide 3

- Coupling produces a trivalent phosphite bond which must be oxidized to the phosphate.Using a sulphurizing agent at this step will generate a phosphorothioate backbone, often required if the oligos are to be used *in vivo*. Cycles are repeated to add each base of the sequence

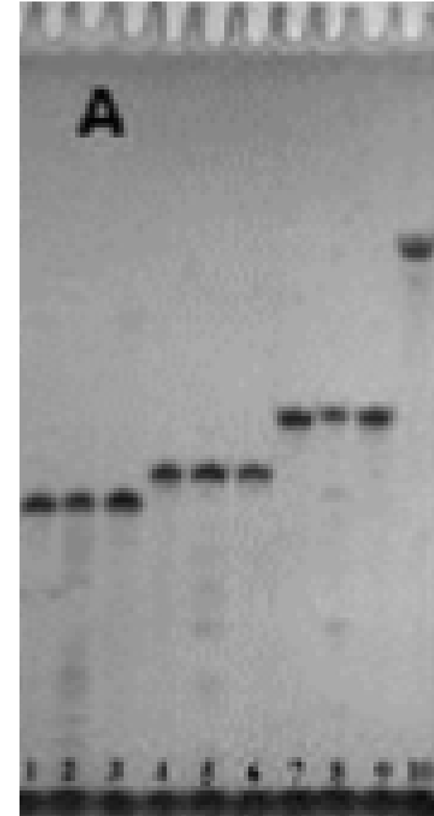




How to synthesize an oligonucleotide 4

- Remove all protecting groups
- Cleave from the solid support
- Elute from the column
- Purify if needed
- Quantify by reading OD_{260}
  - single stranded DNA has different extinction coefficient than dsDNA
  - $OD_{260} = 1 = 33 \mu g/ml$
  - Lots of website calculators for calculating concentration of oligos

Applied Biosystems DNA synthesizer



Because the reaction is not 100% efficient there will be capped products at each step that will not be elongated and result in n-1, n-2 to n-x products

Tips for designing oligonucleotides

- For sequencing:
  - 18 – 22 bases long, at least 40bp from the region to be sequenced
  - ~50% GC, no long runs of a single base, Tm around 50 – 55°C
  - No hairpin loops, check for self annealing
  - Unique annealing site (BLAST search against organism of origin)
- For PCR:
  - Same as above but can be longer (30 – 40 bases)
  - Can include RE sites at 5' end (do not include in Tm calculation)
    - Some enzymes will not cut when site at the end of a DNA fragment – check NEB catalogue or similar
  - Tm is more flexible but both primers should be about the same
  - Check for dimer formation (anneal to each other)

Tips for designing oligonucleotides

- For site-directed mutagenesis:
  - Target nucleotides must be in the middle of the oligo
  - Approx 20 bases on each side of the mutation site
  - Purified oligos will give best results
- For EMSA probes:
  - Small double stranded DNA fragments
  - Usually 40 - 100bp long (maximum size that can be synthesised)
  - Oligos must be further purified for best results
- Double-stranded linkers:
  - Small DNA fragments for cloning specific sequences such as restriction enzyme sites, amino acid tags
  - Purified oligos will give best results

The Story of Synthia

(part I): Craig Venter's plan to build a synthetic life-form

Text: ETC Group. Art: Stig. www.etcgroup.org

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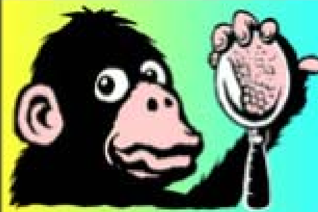
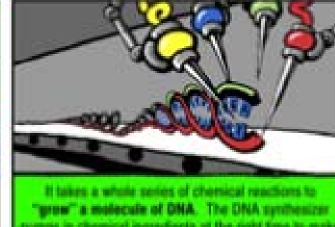


All living things, from microbes to monkeys, are made up of **cells**. Cells contain everything that's needed to transform energy into life. Humans have trillions of cells in their bodies. Simpler organisms, like bacteria, have just one cell. Cells are the basic units of life.

Inside most cells is a molecule known as **DNA**. It looks a bit like a zipper. The "teeth" of the zipper are four different chemical compounds – Adenine, Guanine, Cytosine and Thymine – known as A, G, C and T.

How an organism grows, develops and stays alive depends a lot on its **DNA**. It's like the software program that instructs a computer how to perform a function. The "code" for an organism's software is the order, or sequence, of **A, G, C and T** along the DNA zipper. Every living thing has a unique DNA sequence which is shorter in simple bacteria and longer in complex organisms such as humans.

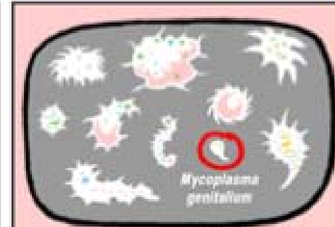
To create a synthetic life-form, you first need to construct its DNA. **Synthetic DNA (sDNA)** is manufactured in a machine called a DNA synthesizer. The synthesizer has a computer in it that can be programmed to create any DNA sequence.



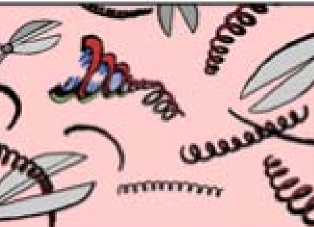
It takes a whole series of chemical reactions to **"grow" a molecule of DNA**. The DNA synthesizer pumps in chemical ingredients at the right time to make the right chemical reactions happen in the right order. Chemists have been tweaking the "recipe" for DNA for almost a half-century. Making the DNA for even a single-celled bacterium requires a long DNA zipper. In a human cell, A, G, C and T appear 3 billion times!

So most scientists interested in building synthetic life take a short-cut – they go on the Internet and order their sDNA from **'foundries'** – companies that crank out lengths of synthetic DNA like a factory makes car parts. An organism's complete zipper of DNA is called its **genome**. To make a synthetic organism, you need an entire genome.

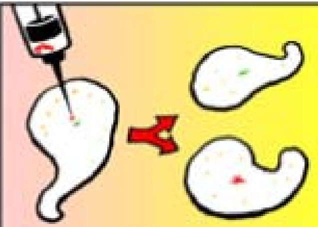
Craig Venter, CEO and founder of **Synthetic Genomics**, Inc. claims that his company is constructing the first-ever, living organism whose genome is not like any known species and is made entirely from synthetic DNA. We call it '**Synthia**.'



The scientists aren't constructing the whole cell, just the DNA inside the cell. To create Synthia, the team started with a bacterium that already exists. It is called ***Mycoplasma genitalium*** and lives in the human genitals. It has a really small genome.



The scientists deleted some pieces of *Mycoplasma genitalium's* DNA, but not enough to kill it... at least not right away. They call this stripped-down DNA a **'minimal' genome**. Then they re-build it out of synthetic DNA.

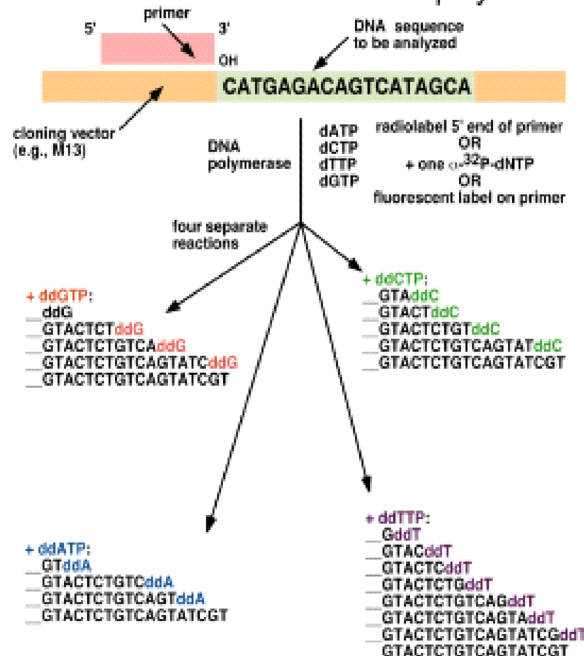


No genome can function by itself – it needs all the other parts of the working cell. So, **the human-made genome**, stitched together from sDNA, **is injected into a natural bacterial cell**. The cell divides, and one of the new cells ends up with the synthetic genome.

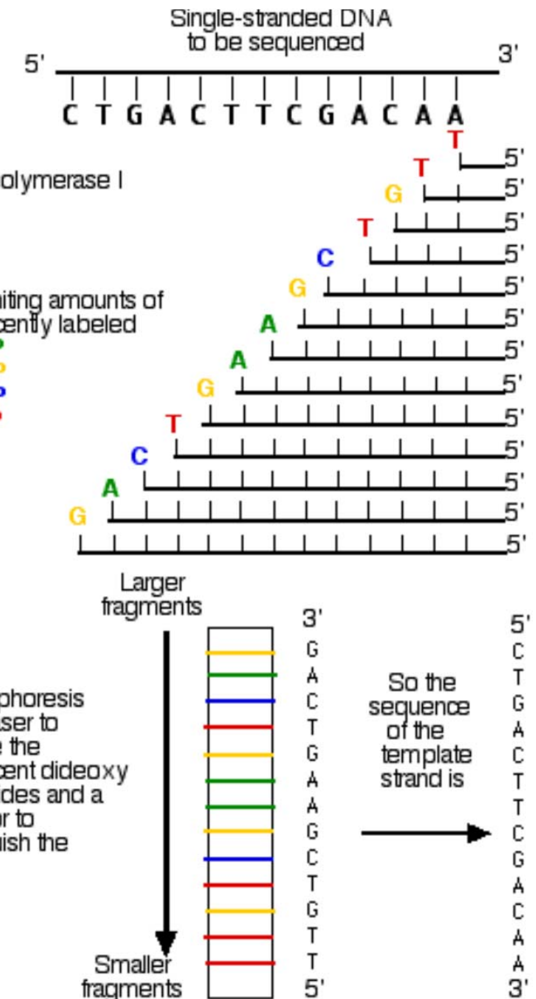
Presto! **Synthia is born!** Scientists pieced together a new species of bacterium with entirely human-made DNA. Like all life-forms, Venter's synthetic organism should be **able to reproduce and mutate... and that's where the trouble could begin...**

Automated DNA Sequencing

Automated sequencing is based on the Sanger dideoxy sequencing method which utilizes the fact that DNA polymerase will stop after a dideoxy-nucleotide (ddNTP) is incorporated. Sequencing requires a small primer close to the region to be sequenced, dNTPs and ddNTPs and a DNA polymerase.

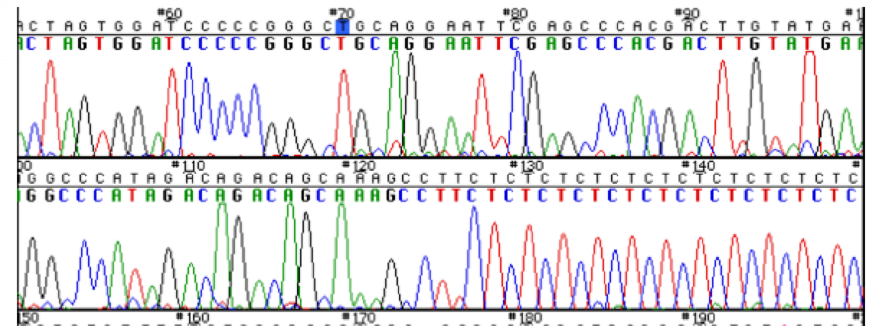


Modifications for automation include the use of fluorescently labeled ddNTPs so that the reaction is carried out in one tube and the use of Taq DNA polymerase to allow repeated cycles of the reaction.



http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/D/DNAsequencing.html

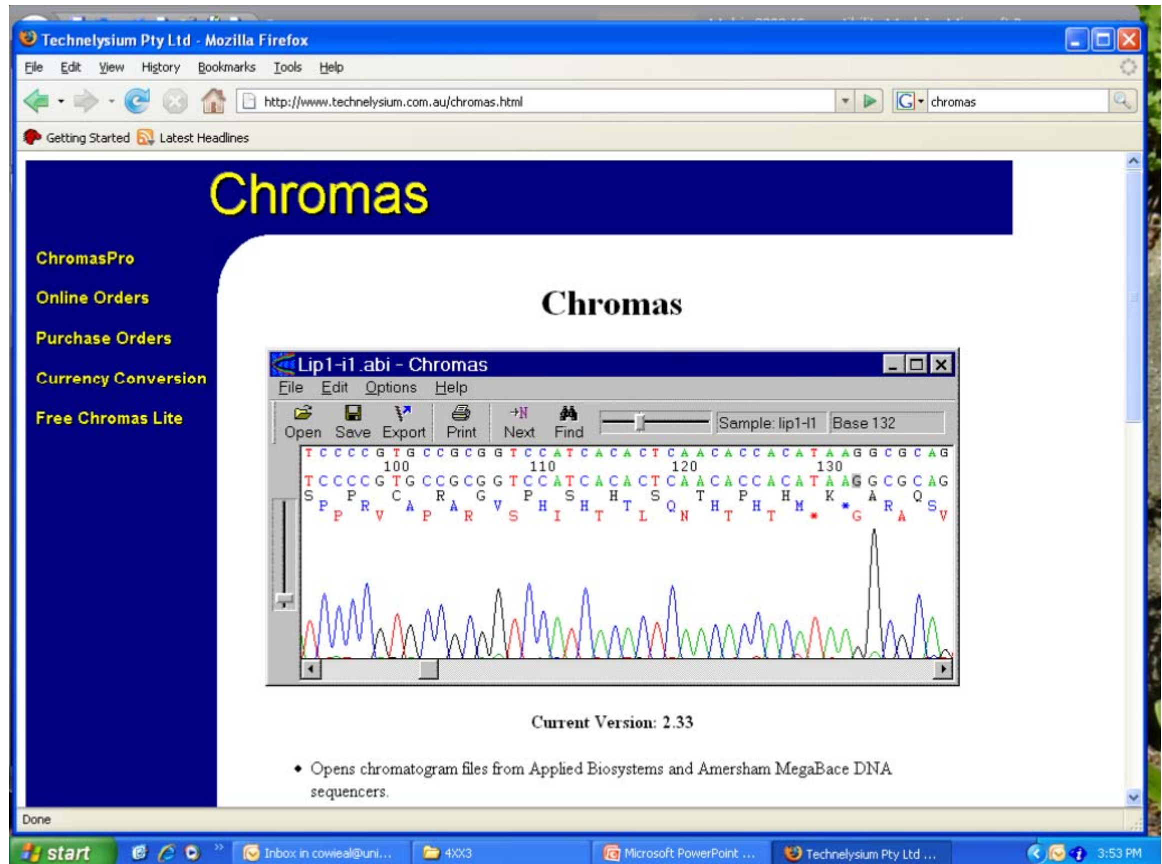
ABI Automated DNA Sequencer



Editing your own sequence data

Software to read the .ab1 files

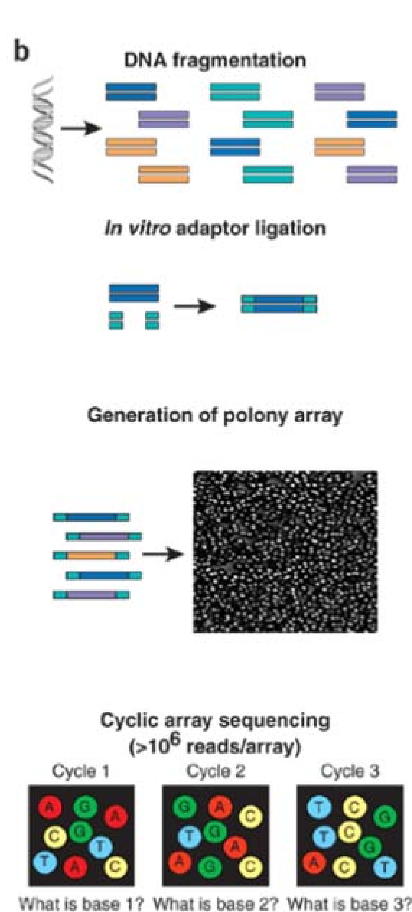
1. Chromas
2. Sequence Scanner Software from ABI
3. FinchTV

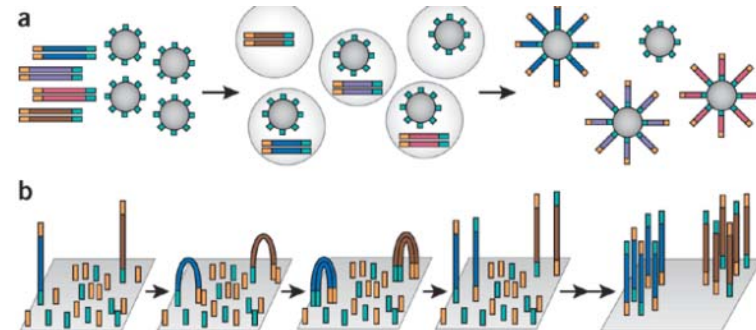


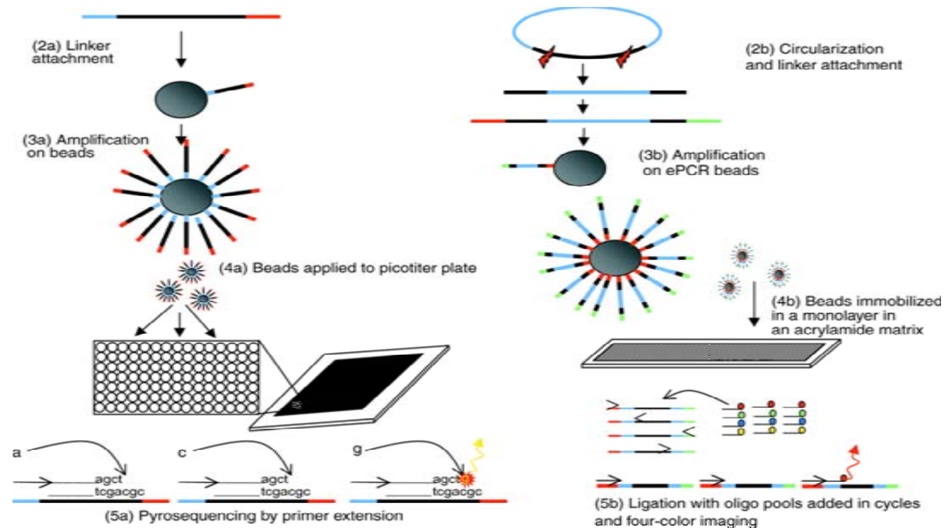
1. http://www.technelysium.com.au/chromas.html
2. http://www.appliedbiosystems.com/support/software/
3. http://www.geospiza.com/

New Generation of Sequencing

Roche 454, Polonator, SOLiD, Solexa



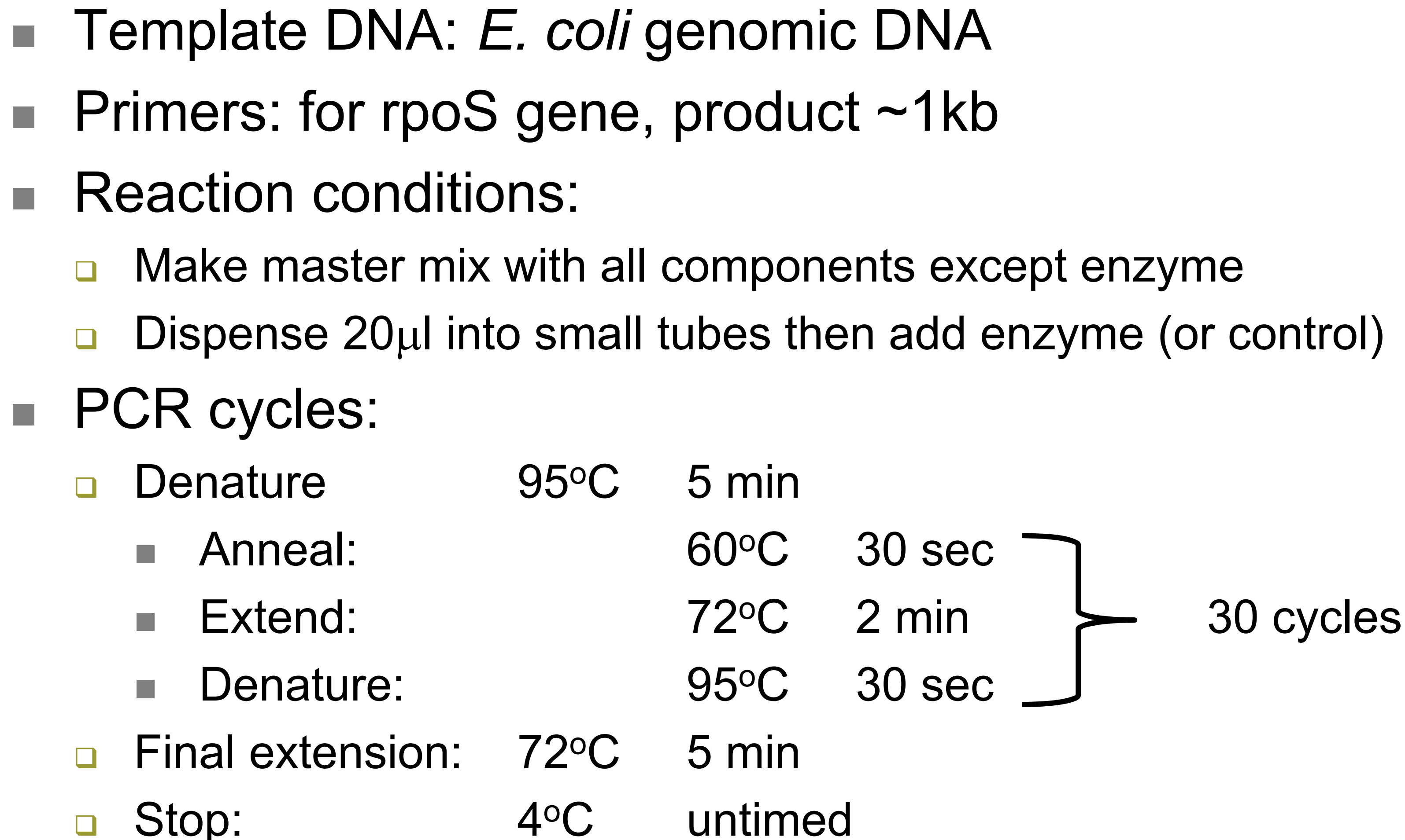




PCR Experiment

- To determine the activity of your purified Taq polymerase prep each group should run 7 reactions:
  - commercial Taq polymerase (undiluted)
  - lysate C (undiluted)
  - final product F (undiluted, 1:10, 1:100, 1:1000, 1:10,000, dilutions made in dialysis buffer)
- Optimization of PCR
  - annealing temperature (gradient)
  - Mg^{2+} concentration
  - annealing time
  - solvents such as DMSO
  - use of PCR for mutagenesis, proofreading enzymes

PCR experiment - details



- Template DNA: *E. coli* genomic DNA
- Primers: for rpoS gene, product ~1kb
- Reaction conditions:
  - Make master mix with all components except enzyme
  - Dispense 20μl into small tubes then add enzyme (or control)
- PCR cycles:
  - Denature 95°C 5 min
    - Anneal: 60°C 30 sec
    - Extend: 72°C 2 min
    - Denature: 95°C 30 sec

    (Anneal, Extend, Denature: 30 cycles)
  - Final extension: 72°C 5 min
  - Stop: 4°C untimed

PCR polymerases

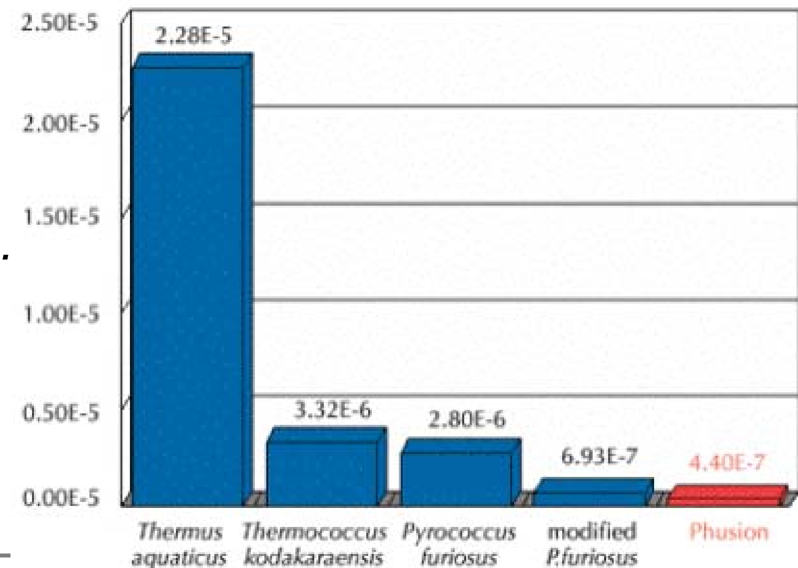
- ***Taq* polymerase:** *Taq* Polymerase contains a polymerization dependent 5'-3' exonuclease activity. It does not have a 3'-5' exonuclease and thus no proof reading function.

  Error rate $1x10^{-4}$ to $8x10^{-5}$
- **Hi Fidelity *Taq* polymerase:** Error rate 6 to 9x better than regular *Taq* pol. Includes 3'-5' exonuclease for proof-reading.
- ***Pfx* polymerase:** from *Thermococcus sp.* Error rate 26x better than Taq pol, has 3'-5' exonuclease
- **Vent *polymerase:** from *Pyrococcus sp.*

  Error rate 5x better than Taq pol,

  no exonuclease activity
- **Phusion* polymerase:** from *Pyrococcus sp.*

  Error rate 50x better than Taq pol ($4x10^{-7}$).



*from NEB, other information from Invitrogen