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

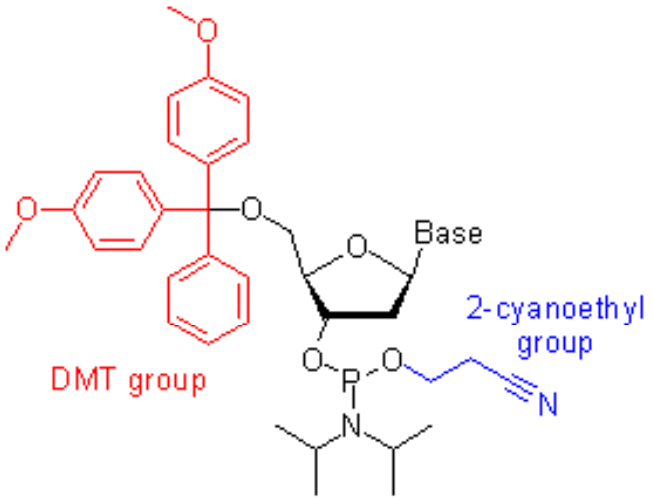
- PCR primers
- Sequencing primers
- Site-directed mutagenesis
- Deletion mutagenesis
- ESMA probes
- Double-stranded linkers
- Interference

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

protecting group - Removed to allow addition of next base



protecting group

Oligonucleotide synthesis

- Synthesis occurs in 3’ – 5’ direction on columns loaded with 3’ end base
- Each base added sequentially
- Efficiency of coupling is not 100% (but close)
- Final product will contain all DNA from each step – failed couplings are one base shorter each time
  - A problem with long (>30) oligonucleotides
- Additional purification needed to obtain only full length product
  - Done by Mobix (affinity column, extra cost)
  - Done by researcher, acrylamide gel purification

Tips for designing oligonucleotides

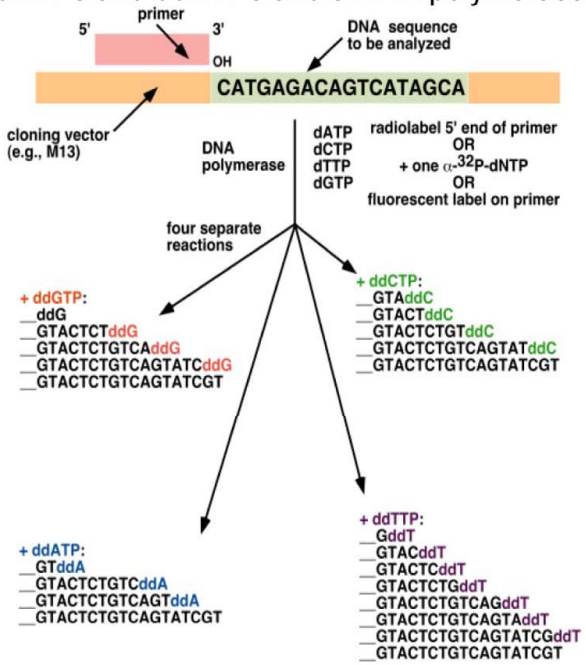
- For sequencing:
  - 18 – 22 bases long, at least 40bp from the region to be sequenced
  - ~50% GC, no long runs of a single base, Tm around 50 – 55^{o}C
  - No hairpin loops, check for self annealing
  - Unique annealing site (BLAST search against organism of origin)
- For PCR:
  - Same as above but 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

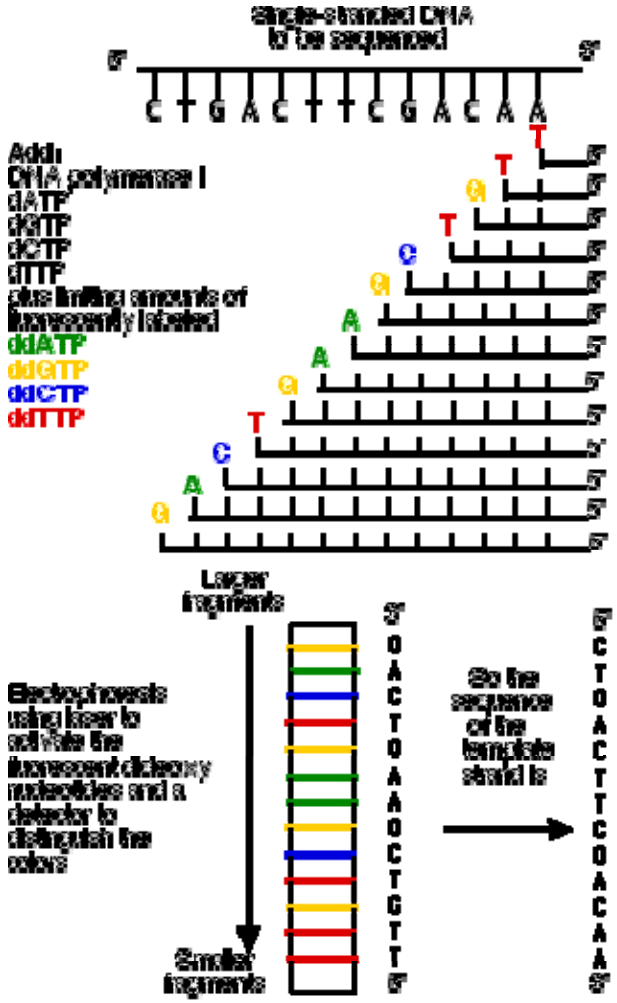
- For site-directed mutagenesis:
  - Target nucleotides must be in the middle of the oligo
  - Approx 20 bases on each side of the mutation site
  - Purified oligos will give best results
- For EMSA probes:
  - Small double stranded DNA fragments
  - Usually 40 - 100bp long (maximum size that can be 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

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.htm

Conditions to prepare DNA for sequencing!

- Mini-prep DNA must be pure – Qiagen kit or similar
- PCR products – primers MUST be removed
- Quantity must be sufficient – 1μl of DNA sample gives a clearly visible band on a gel (100 – 200ng/μl)
- What type of output file:
  - ab1 file – actual run file from sequencer
  - Printout of ab1 file – costs extra but can check quality
  - ab1 file and/or text file edited by Mobix staff – costs extra
  - ab1 file edited by researcher – requires special programs to open
  - Text file of sequence – no indication of quality of sequence, must have it edited by Mobix

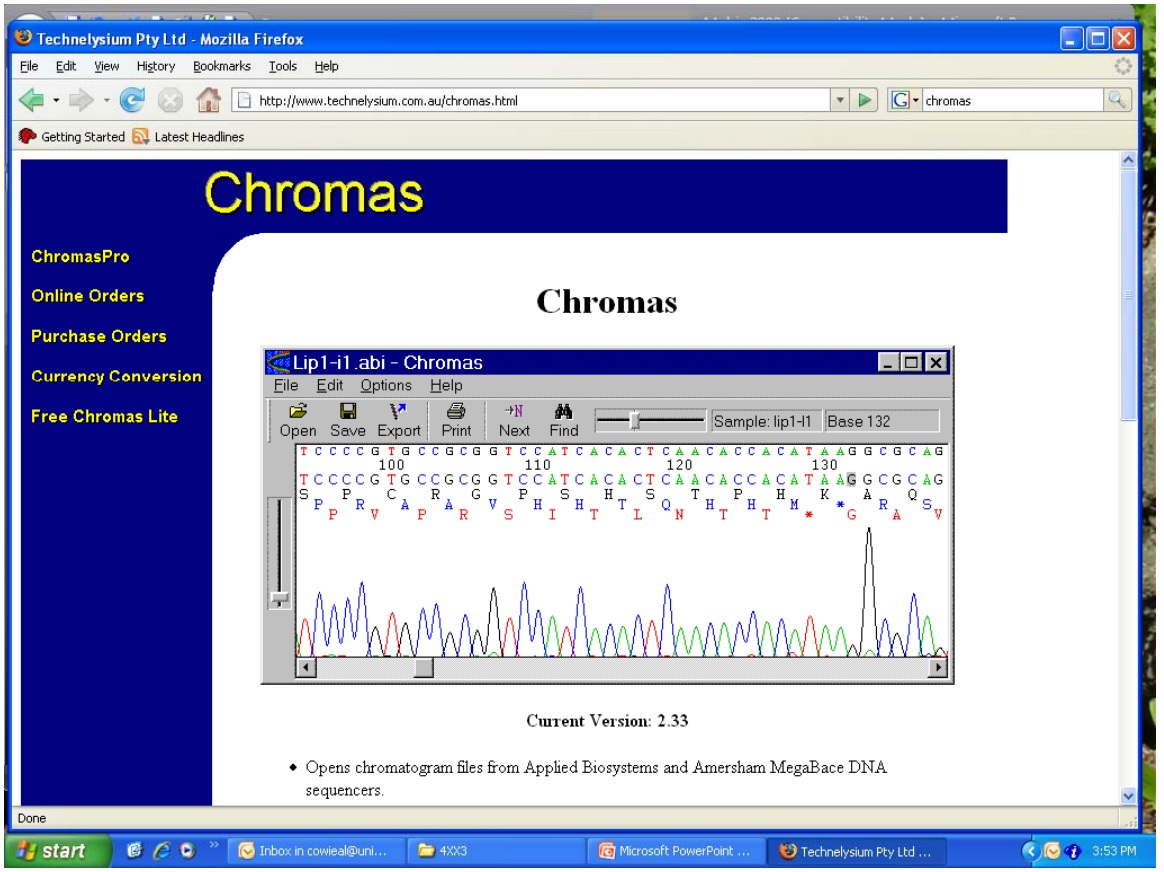
Editing your own sequence data

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/

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 20ml into small tubes then add enzyme (or control)
- PCR cycles:

Denature	95°C	5 min		
Anneal:		60°C	30 sec	30 cycles
Extend:		72°C	2 min	
Denature:		95°C	30 sec	
Final extension:	72°C	5 min		
Stop:	4°C	untimed		

PCR polymerases

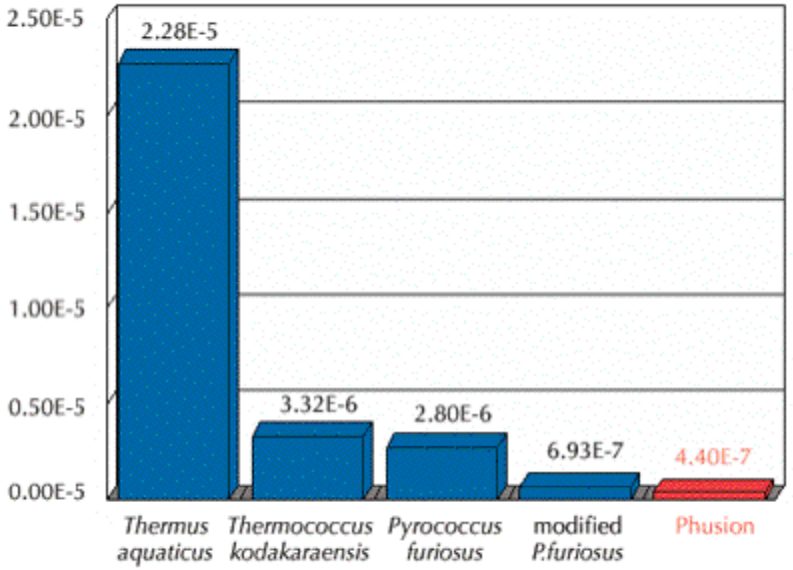
- ***Taq* polymerase:** *Taq* Polymerase contains a polymerization dependent 5'-3' exonuclease activity. It does not have a 3'-5' exonuclease and thus no proof reading function.

  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