Nucleic Acids Methods

(CSI / Law and Order / Forensic Files / House / Crossing Jordan / Quincy, M.E.)

Topics:

1. Brief Review of DNA Double Helix

   Structure of DNA and the double helix

2. DNA Sequencing / Human Genome Project / Genomics

   Chemical sequencing (Maxam / Gilber) / Sanger dedeoxy sequencing

3. Use of DNA Microarrays

4. PCR – Polymerase Chain Reaction

The Nobel Prize in Physiology or Medicine 1962

"for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material"

Francis Harry Compton Crick

1/3 of the prize

United Kingdom

MRC Laboratory of Molecular Biology
Cambridge, United Kingdom

b. 1916
d. 2004

James Dewey Watson

1/3 of the prize

USA

Harvard University
Cambridge, MA, USA

b. 1928

Maurice Hugh Frederick Wilkins

1/3 of the prize

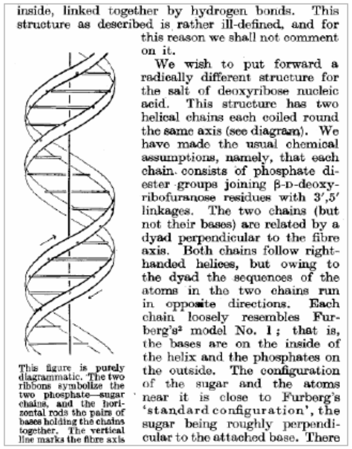
United Kingdom and New Zealand

London University
London, United Kingdom

b. 1916
(in Pongaroa, New Zealand)
d. 2004

.eft to right: Maurice Wilkins, John Steinbeck, John Kendrew, Max Perutz, Francis Crick and Jim Watson after the Nobel Ceremony in Stockholm in December 1962.

The Birth of Molecular Biology: DNA Structure



inside, linked together by hydrogen bonds. This structure as described is rather ill-defined, and for this reason we shall not comment on it.

We wish to put forward a radically different structure for the salt of deoxyribose nucleic acid. This structure has two helical chains each coiled round the same axis (see diagram). We have made the usual chemical assumptions, namely, that each chain consists of phosphate diester groups joining β-D-deoxyribofuranose residues with 3′,5′ linkages. The two chains (but not their bases) are related by a dyad perpendicular to the fibre axis. Both chains follow right-handed helices, but owing to the dyad the sequences of the atoms in the two chains run in opposite directions. Each chain loosely resembles Furberg's[2] model No. 1; that is, the bases are on the inside of the helix and the phosphates on the outside. The configuration of the sugar and the atoms near it is close to Furberg's 'standard configuration', the sugar being roughly perpendicular to the attached base. There

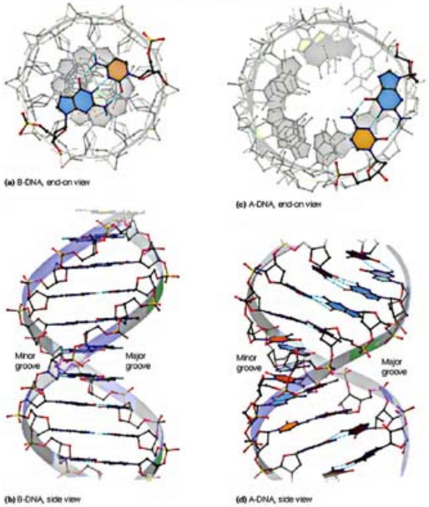
This figure is purely diagrammatic. The two ribbons symbolize the two phosphate—sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the fibre axis

Nature – 1953

15 February 2001
nature
www.nature.com
the human genome
Nuclear fission
Five-dimensional energy landscapes
Seafloor spreading
The view from under the Arctic ice
Career prospects
Sequence creates new opportunities

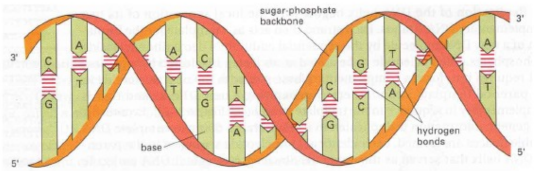
Nature – 2001

A and B Double Helices

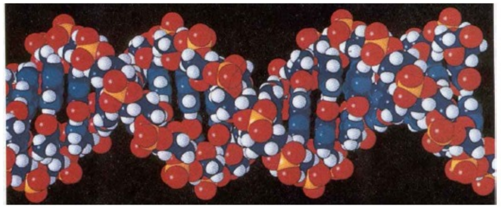


Fall 2007

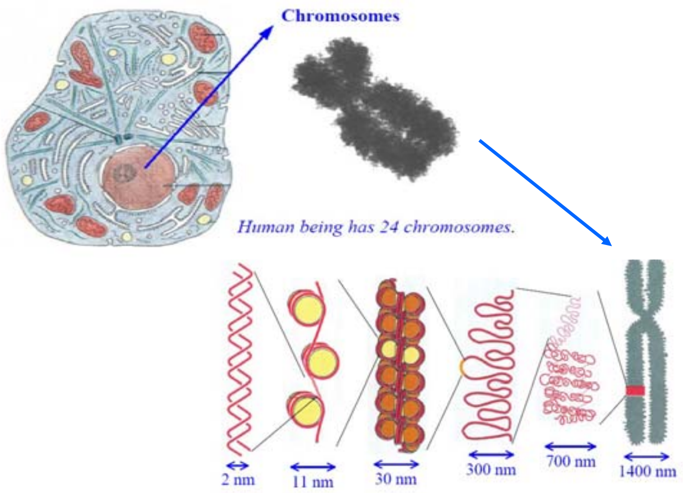
Double stranded DNA



- *Two single stranded DNA paired by Hydrogen bonds.*
- *Helical structure*

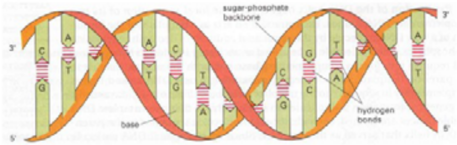


Chromosome



Human being has 24 chromosomes.

Genetic information



... G T A C T G A A C G C A G G T...

Genetic code

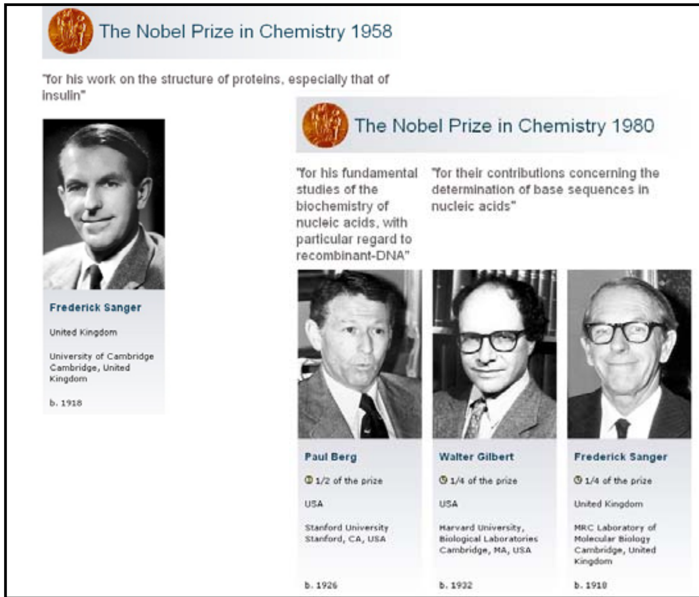
Human being: ~ 3,000,000,000 base-pairs
~ 30,000 – 40,000 Genes

(Public Human Genome Project and Celera Genomics)

Sequencing DNA

Prior to the mid-1970’s no method existed by which DNA could be directly sequenced. Knowledge about gene and genome organization was based upon studies of prokaryotic organisms and the primary means of obtaining DNA sequence was so-called reverse genetics in which the amino acid sequence of the gene product of interest is back-translated into a nucleotide sequence based upon the appropriate codons.

- **Maxam-Gilbert DNA Sequencing**
- **Sanger (didexoy) DNA Sequencing**



The Nobel Prize in Chemistry 1958

"for his work on the structure of proteins, especially that of insulin"

Frederick Sanger

United Kingdom

University of Cambridge Cambridge, United Kingdom

b. 1918

The Nobel Prize in Chemistry 1980

"for his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant-DNA"

"for their contributions concerning the determination of base sequences in nucleic acids"

Paul Berg	Walter Gilbert	Frederick Sanger
1/2 of the prize	1/4 of the prize	1/4 of the prize
USA	USA	United Kingdom
Stanford University Stanford, CA, USA	Harvard University, Biological Laboratories Cambridge, MA, USA	MRC Laboratory of Molecular Biology Cambridge, United Kingdom
b. 1926	b. 1932	b. 1918

Maxam-Gilbert DNA Sequencing

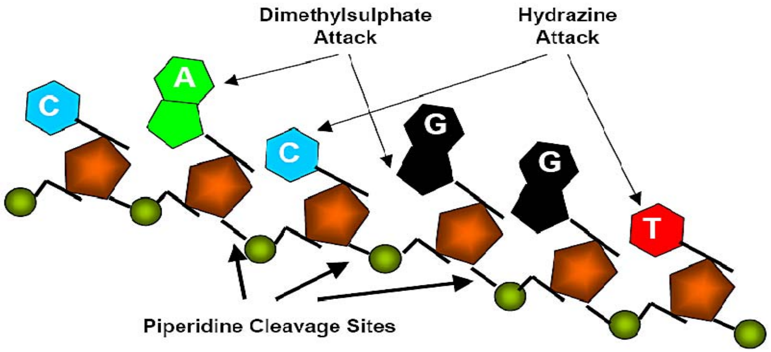


Figure 1. Chemical targets in the Maxam-Gilbert DNA sequencing strategy. Dimethylsulphate or hydrazine will attack the purine or pyrimidine rings respectively and piperidine will cleave the phosphate bond at the 3' carbon.

http://www.idtdna.com/support/technical/TechnicalBulletinPDF/DNA_Sequencing.pdf

IDTutorial: DNA Sequencing

Allan Maxam / Walter Gilbert DNA Sequencing

Sequencing single-stranded DNA

Two-step catalytic process:

1) Break glycoside bond between the ribose sugar and the base / displace base

Purines react with dimethyl sulfate

Pyrimidines react with hydrazine

2) Piperidine catalyzes phosphodiester bond cleavage where base displaced

"G"	- dimethyl sulfate and piperidine
"A + G"	- dimethyl sulfate and piperidine in formic acid
"C"	- hydrazine and piperidine in 1.5M NaCl
"C + T"	- hydrazine and piperidine

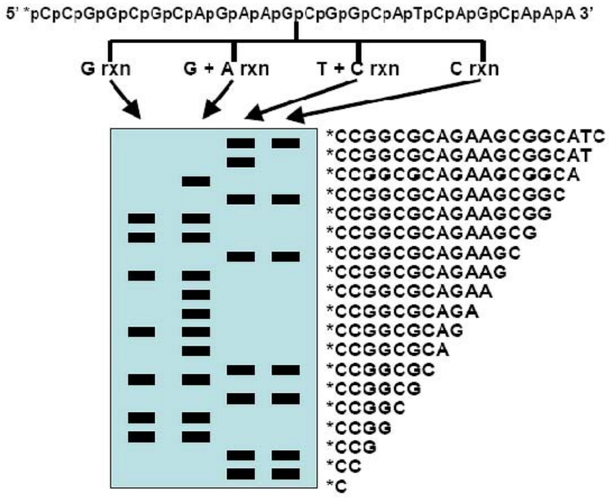


Figure 2. The Maxam-Gilbert manual sequencing scheme. The target DNA is radiolabeled and then split into the four chemical cleavage reactions. Each reaction is loaded onto a polyacrylamide gel and run. Finally, the gel is autoradiographed and base calling proceeds from bottom to top.

Maxam-Gilbert DNA Sequencing

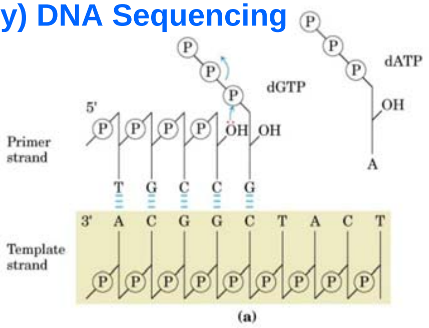
- 200-300 bases of DNA sequence every few days
- Use large amounts of radioactive material, 35S or 32P
- Constantly pouring large, thin polyacrylamide gels
- Hydrazine is a neurotoxin

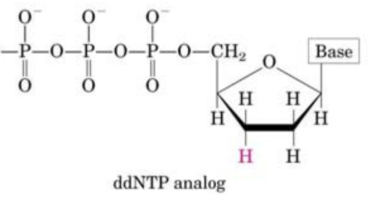
Early Benefits -

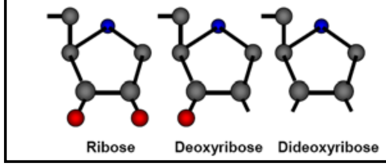
Discovery that the gene for ovalbumin in chicken and the gene encoding β-globin in rabbit contained non-coding gaps in the coding regions. These gaps< were flanked by the same dinucleotides in the two genes; GT on the 5' end of the gaps and AG on the 3' end of the gaps. Soon, the terms intron and exon were added to the genetic lexicon to describe the coding and non-coding regions of eukaryotic genes (1977).

Fred Sanger (dideoxy) DNA Sequencing

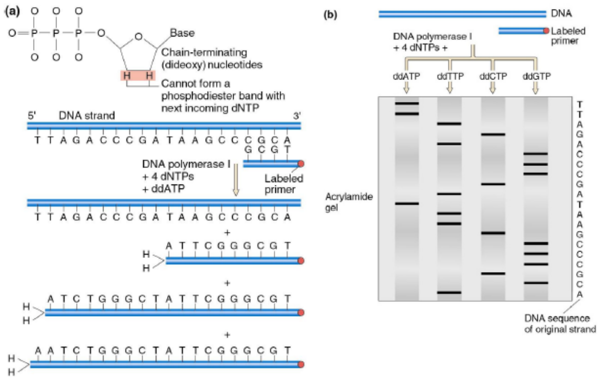
Sanger knew that, whenever a dideoxynucleotide was incorporated into a polynucleotide, the chain would irreversibly stop, or terminate. Thus, the incorporation of specific dideoxynucleotides in vitro would result in selective chain termination.

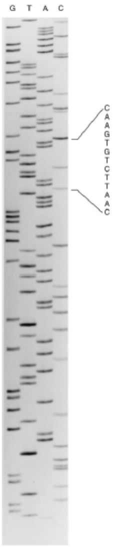






Sanger (dideoxy) DNA Sequencing


(a)
O=P—P—P—O
Base
Chain-terminating (dideoxy) nucleotides
H H
Cannot form a phosphodiester band with next incoming dNTP
5' DNA strand 3'
T T A G A C C C G A T A A G C C C G C A
G C G T
DNA polymerase I
+ 4 dNTPs
+ ddATP
Labeled primer
T T A G A C C C G A T A A G C C C G C A
+
A T T C G G G C G T
+
A T C T G G G C T A T T C G G G C G T
+
A A T C T G G G C T A T T C G G G C G T
(b)
DNA
Labeled primer
DNA polymerase I + 4 dNTPs +
ddATP
ddTTP
ddCTP
ddGTP
Acrylamide gel
T T A G A C C C G A T A A G C C C G C A
DNA sequence of original strand



Advantages of dideoxy DNA Sequencing

- Elimination of dangerous chemicals (hydrazine)
- Greater efficiency (>3x)

Taq polymerase makes DNA strands off of a template at rate of about 500 bases per minute

Chemical synthesis of a 25-mer oligonucleotide takes more than two hours.

→ High Throughput Methods (Human Genome Project)

Automated Fluorescence Sequencing

In 1986, Leroy Hood and colleagues reported on a DNA sequencing method in which the radioactive labels, autoradiography, and manual base calling were all replaced by fluorescent labels, laser induced fluorescence detection, and computerized base calling.

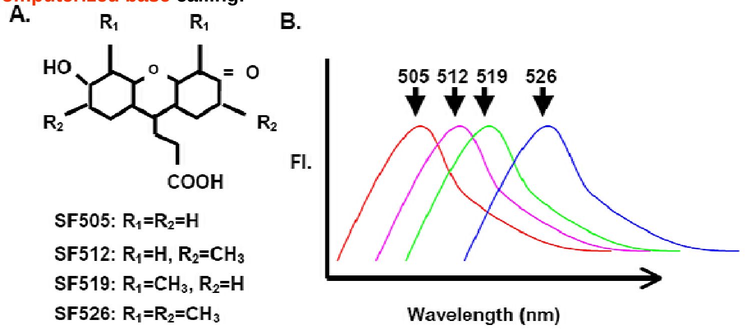
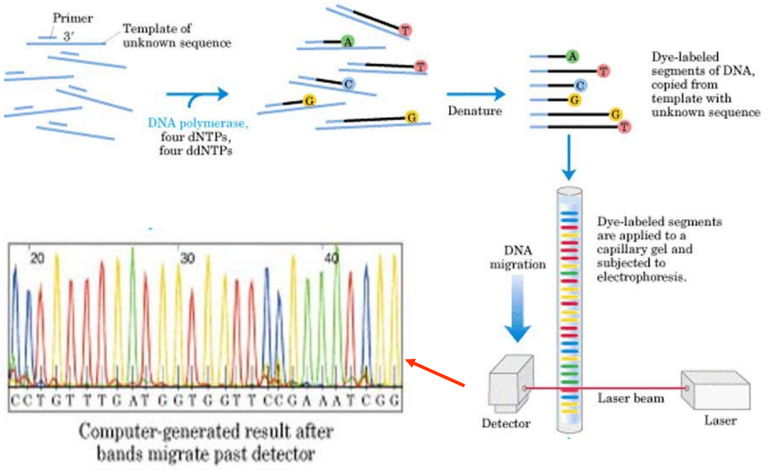


Figure 5. A. Chemical structure of the four succinylfluorescein dyes developed at DuPont. B. Normalized fluorescence emission spectra for each of the four dyes following excitation at 488nm. Shifts in the spectra were achieved by changing the side groups R_1 and R_2.

Automated DNA sequencing



Primer
3′
Template of unknown sequence
DNA polymerase, four dNTPs, four ddNTPs
T
A
T
C
G
G
Denature
A
T
C
G
G
T
Dye-labeled segments of DNA, copied from template with unknown sequence
Dye-labeled segments are applied to a capillary gel and subjected to electrophoresis.
DNA migration
20
30
40
CCTGTTTGATGGTGGTTCCGAAATCGG
Laser beam
Laser
Detector
Computer-generated result after bands migrate past detector

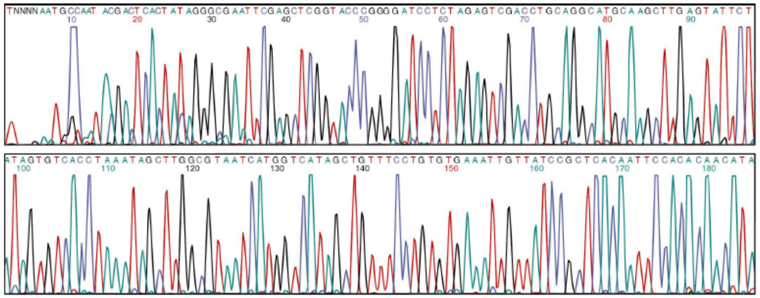
Automated dye-terminator sequencing

4-fluorescently labelled dideoxy dye terminators

ddATP
ddGTP
ddCTP
ddTTP

} pool and load in a single well or capillary

- scan with laser + detector specific for each dye
- automated base calling
- very long reads (~ 1000 bases)/run



Human Genome Project

Begun formally in 1990, the U.S. Human Genome Project was a 13-year effort coordinated by the U.S. Department of Energy and the National Institutes of Health. The project originally was planned to last 15 years, but rapid technological advances accelerated the completion date to 2003.

Project goals:

- *identify* all the approximately 20,000-25,000 genes in human DNA,
- *determine* the sequences of ~3 billion chemical base pairs of human DNA,
- *store* this information in databases,
- *improve* tools for data analysis,
- *transfer* related technologies to the private sector, and
- *address* the ethical, legal, and social issues (ELSI) from the project.
- *sequence 500 Mb/year at < $0.25 per finished base*

  (Sequenced >1,400 Mb/year at <$0.09 per finished base)
- *complete genome sequences of E. coli, S. cerevisiae, C. elegans, D. melanogaster*
- *develop genomic-scale technologies (oligo syn, DNA microarrays, 2-hybrid sys)*

HGP Hero - Jim Kent (research scientist at UC Santa Cruz)

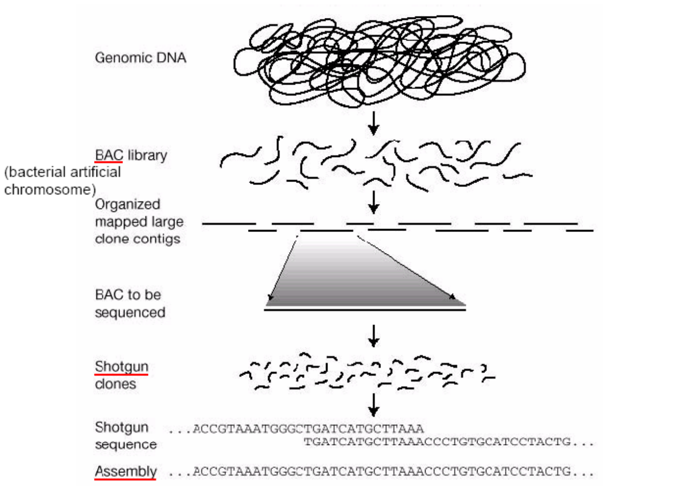
The human genome project was ultimately a race between **Celera Genomics** and the **public** effort, with the final push being a bioinformatics problem to put all of the sequence reads together into a draft genome sequence. **Jim Kent was a grad student at UCSC**, who worked for weeks developing the algorithm to put all of this together, **beating Celera by 3 days** to an assembled human genome sequence.

His efforts ensured that the human genome data remained in the public domain and were not patented into private intellectual property.

Kent built a grid of cheap, commodity PC's running the Linux operating system and other Freeware to beat Celera's, what was thought of then as the, world's most powerful civilian computer. In **June 2000**, thanks to the work done by Kent and several others, the **Human Genome Project** was able to publish its data in the Public Domain just hours ahead of Celera.

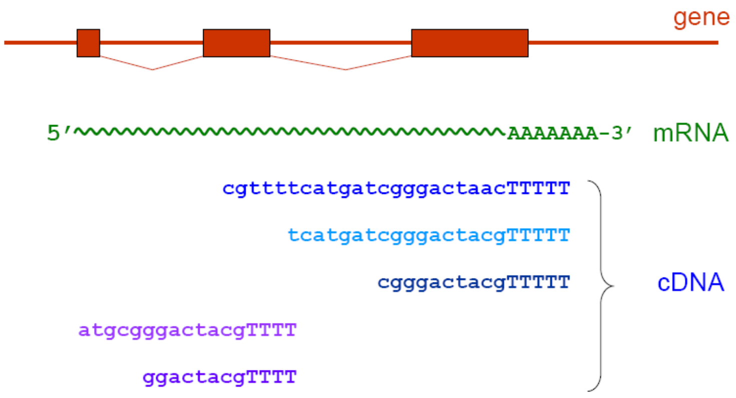
Kent went on to write BLAT and the UCSC Human Genome Browser to help analyze important genome data, receiving his PhD in biology in 2002. He remained at UCSC to work primarily on web tools to help understand the human genome. He helped maintain and upgrade the browser, and worked on projects such as comparative genomics and Parasol.

Physical mapping and sequencing of the human genome


Genomic DNA
BAC library
(bacterial artificial chromosome)
Organized mapped large clone contigs
BAC to be sequenced
Shotgun clones
Shotgun sequence
...ACCGTAAATGGGCTGATCATGCTTAAA
TGATCATGCTTAAACCCTGTGCATCCTACTG...
Assembly
...ACCGTAAATGGGCTGATCATGCTTAAACCCTGTGCATCCTACTG...

Nature (2001) **409** p. 860-921

Genes can also be identified by sequencing cDNAs at random. The sequenced cDNAs are called ESTs (expressed sequence tags)



Finding genes in genomes

- compare to EST or cDNA sequence
- look for open reading frames
- similarity to other genes and proteins
- Gene prediction algorithms (identifying splice sites, coding sequence bias, etc.)

The BIG QUESTION:

Why do we have so few genes?

Species	Genome size	Number of genes
Human (*Homo sapiens*)	2.9 billion base pairs	25,000 - 30,000
Fruit fly (*Drosophila melanogaster*)	120 million base pairs	13,600
Worm (*Caenorhabditis elegans*)	97 million base pairs	19,000
Budding yeast (*Saccharomyces cerevisiae*)	12 million base pairs	6,000
E. coli	4.1 million base pairs	4,800

Genomics vs. Proteomics

With the completion of a rough draft of the human genome in the Spring of 2003, many researchers began looking at how genes and proteins interact to form other proteins. A surprising finding of the Human Genome Project is that there are far fewer protein-coding genes in the human genome than proteins in the human proteome (20,000 to 25,000 genes vs. about 1,000,000 proteins). The human body may contain more than 2 million proteins, each having different functions. The protein diversity is thought to be due to alternative splicing and post-translational modification of proteins. The discrepancy implies that *protein diversity cannot be fully characterized by gene expression analysis*, thus proteomics is needed for characterizing cells and tissues.

Functional genomics and proteomics

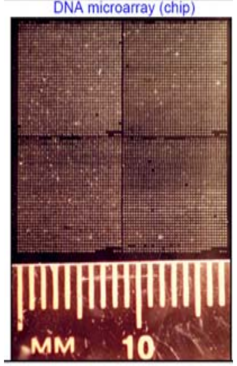
- Identify genes and proteins encoded in the genome (Gene finding)
- Measure gene expression on a genome-wide scale (microarrays)
- Identify protein function

  30-50% of the genes in a genome are of unknown function
- Identify protein interactions, biochemical pathways, gene interaction networks inside cells

Methods of making microarrays

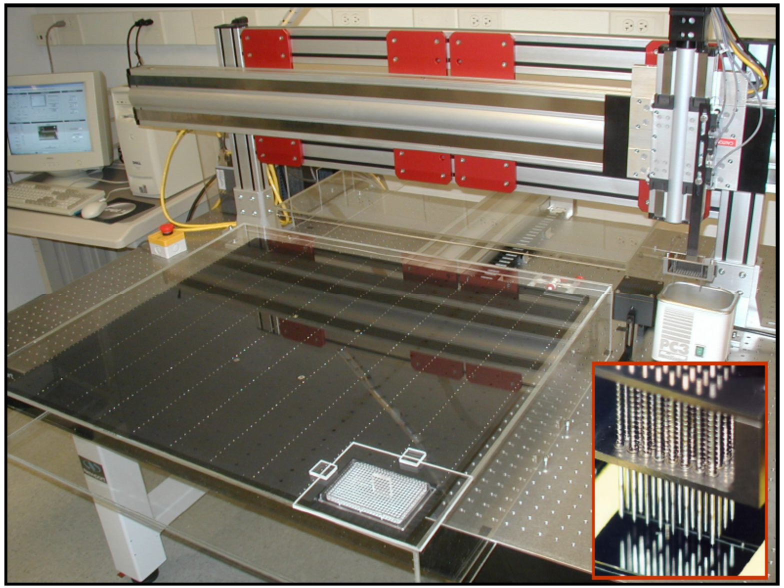
- Robotic spotting
  - using a printing tip
  - using inkjets
- Synthesis of oligonucleotides
  - photolithography (Affymetrix)
  - using inkjets
  - Digital Light Processor (DLP) or Digital Micromirror Device (DMD)

DNA microarray (chip)

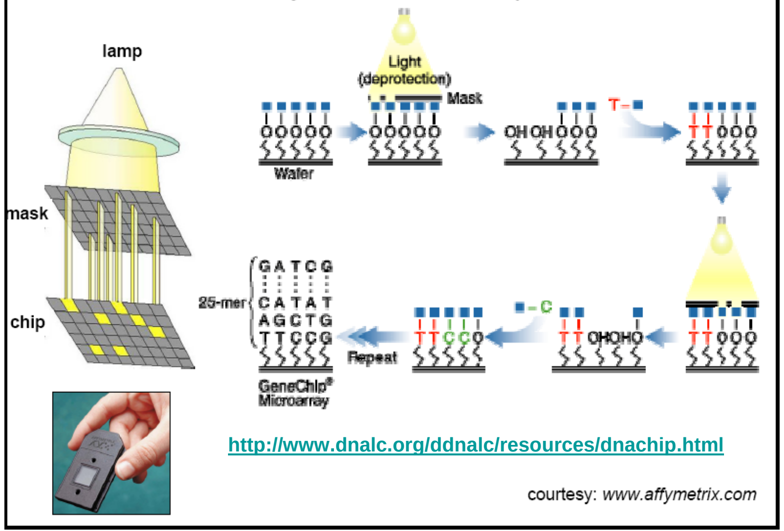


Microarrays can be used to study gene expression, DNA-protein interactions, mutations, protein-protein interactions, etc., all on a genome-wide scale

Note: Thanks to Prof. Vishy Iyer for many of these slides on microarrrays.

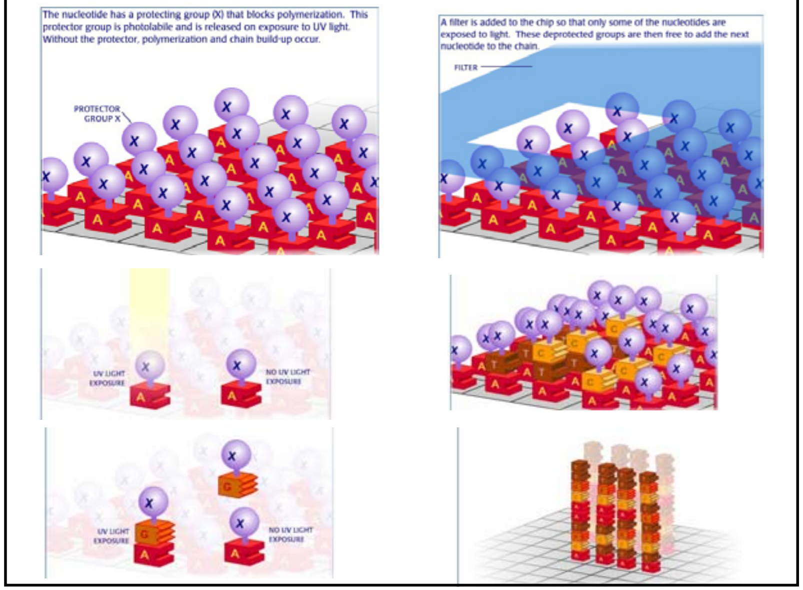


Affymetrix GeneChip



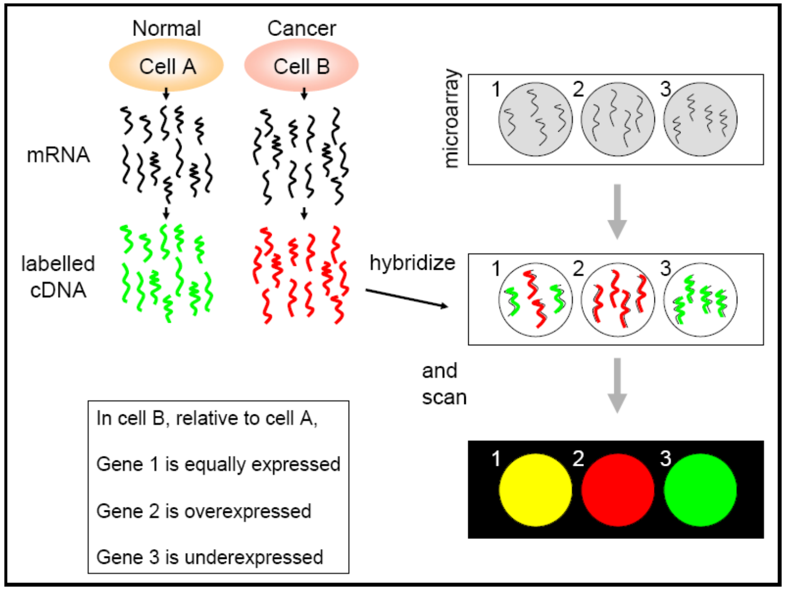
http://www.dnalc.org/ddnalc/resources/dnachip.html

courtesy: *www.affymetrix.com*



The nucleotide has a protecting group (X) that blocks polymerization. This protector group is photolabile and is released on exposure to UV light. Without the protector, polymerization and chain build-up occur.

A filter is added to the chip so that only some of the nucleotides are exposed to light. These deprotected groups are then free to add the next nucleotide to the chain.



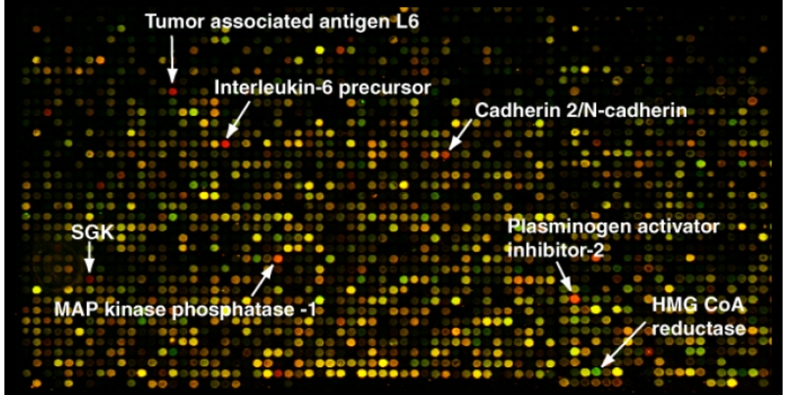
In cell B, relative to cell A,

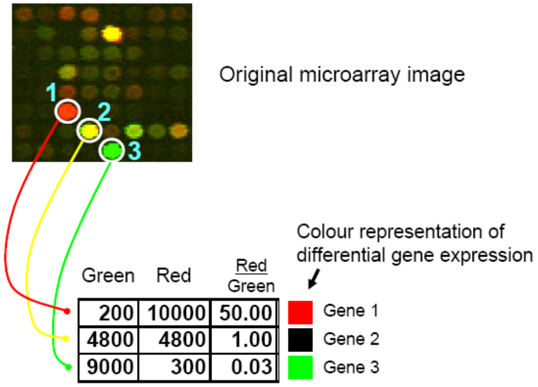
Gene 1 is equally expressed

Gene 2 is overexpressed

Gene 3 is underexpressed

DNA microarray after hybridization of fluorescent probes


Tumor associated antigen L6
Interleukin-6 precursor
Cadherin 2/N-cadherin
SGK
Plasminogen activator inhibitor-2
MAP kinase phosphatase -1
HMG CoA reductase



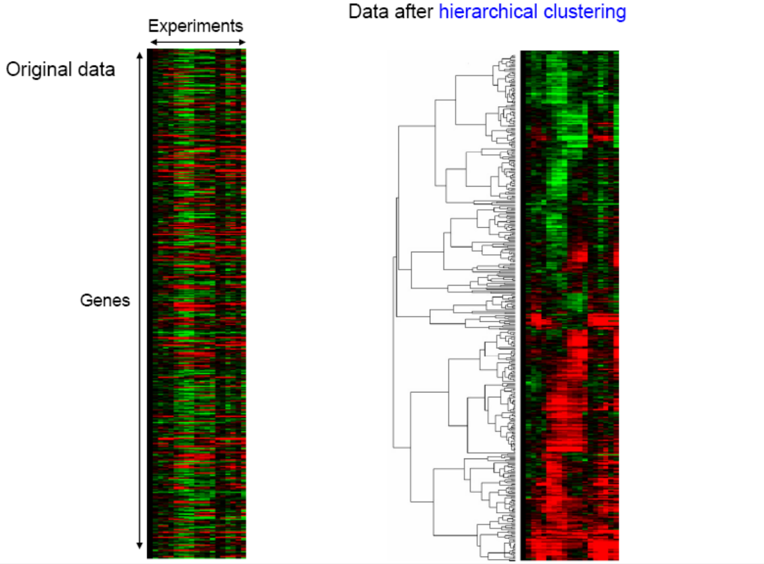
Original microarray image

Colour representation of differential gene expression

Green	Red	Red / Green		
200	10000	50.00	🟥	Gene 1
4800	4800	1.00	⬛	Gene 2
9000	300	0.03	🟩	Gene 3

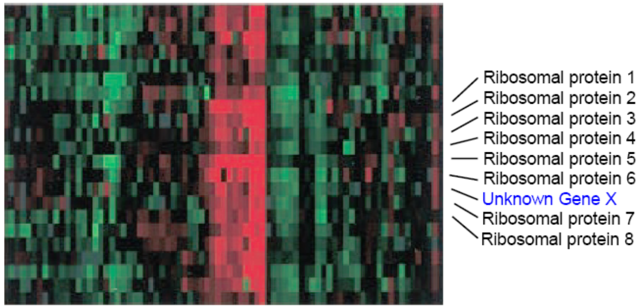
- Large amounts of data can be displayed in this manner
- Gene expression data can be computationally analyzed and organized to reveal patterns

Data after hierarchical clustering



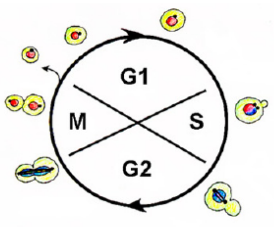
Functionally related genes are often co-expressed

A cluster of co-expressed genes

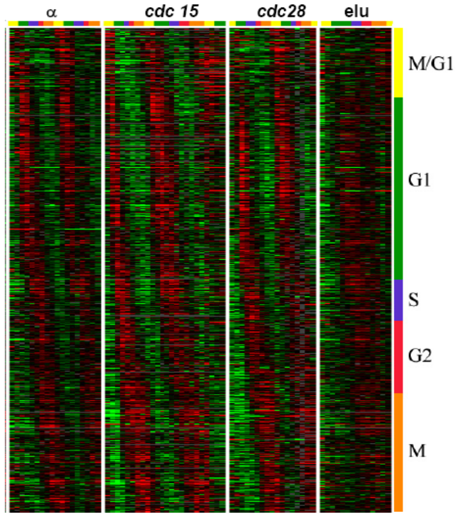


Thus, unknown Gene X may also be a ribosomal protein

S. cerevisiae mitotic cell-cycle



Brenda Andrews lab, University of Toronto



Spellman *et al*, (1998) *Mol. Biol. Cell*

Some Examples / Applications

- DLBCL
- P4 - Medicine

Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling

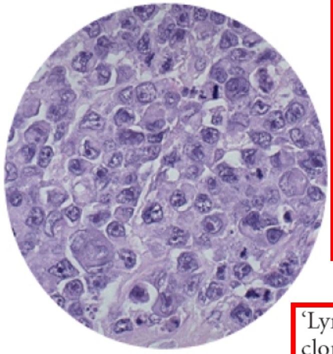
Ash A. Alizadeh[1,2], Michael B. Eisen[2,3,4], R. Eric Davis[5], Chi Ma[5], Izidore S. Lossos[6], Andreas Rosenwald[5], Jennifer C. Boldrick[1], Hajeer Sabet[5], Truc Tran[5], Xin Yu[5], John I. Powell[7], Liming Yang[7], Gerald E. Marti[8], Troy Moore[9], James Hudson Jr[9], Lisheng Lu[10], David B. Lewis[10], Robert Tibshirani[11], Gavin Sherlock[4], Wing C. Chan[12], Timothy C. Greiner[12], Dennis D. Weisenburger[12], James O. Armitage[13], Roger Warnke[14], Ronald Levy[6], Wyndham Wilson[15], Michael R. Grever[16], John C. Byrd[17], David Botstein[4], Patrick O. Brown[1,18] & Louis M. Staudt[5]

NATURE | VOL 403 | 3 FEBRUARY 2000 | www.nature.com

Diffuse large B-cell lymphoma (DLBCL), the most common subtype of non-Hodgkin's lymphoma, is clinically heterogeneous: 40% of patients respond well to current therapy and have prolonged survival, whereas the remainder succumb to the disease. We proposed that this variability in natural history reflects unrecognized molecular heterogeneity in the tumours. Using DNA microarrays, we have conducted a systematic characterization of gene expression in B-cell malignancies. Here we show that there is diversity in gene expression among the tumours of DLBCL patients, apparently reflecting the variation in tumour proliferation rate, host response and differentiation state of the tumour. We identified two molecularly distinct forms of DLBCL which had gene expression patterns indicative of different stages of B-cell differentiation. One type expressed genes characteristic of germinal centre B cells ('germinal centre B-like DLBCL'); the second type expressed genes normally induced during *in vitro* activation of peripheral blood B cells ('activated B-like DLBCL'). Patients with germinal centre B-like DLBCL had a significantly better overall survival than those with activated B-like DLBCL. The molecular classification of tumours on the basis of gene expression can thus identify previously undetected and clinically significant subtypes of cancer.

Despite the variety of clinical, morphological and molecular parameters used to classify human malignancies today, patients receiving the same diagnosis can have markedly different clinical courses and treatment responses. The history of cancer diagnosis has been punctuated by reassortments and subdivisions of diagnostic categories. There is little doubt that our current taxonomy of cancer still lumps together molecularly distinct diseases with distinct clinical phenotypes. Molecular heterogeneity within individual cancer diagnostic categories is already evident in the variable presence of chromosomal translocations, deletions of tumour suppressor genes and numerical chromosomal abnormalities. The classification of human cancer is likely to become increasingly more informative and clinically useful as more detailed molecular analyses of the tumours are conducted.

The challenge of cancer diagnosis



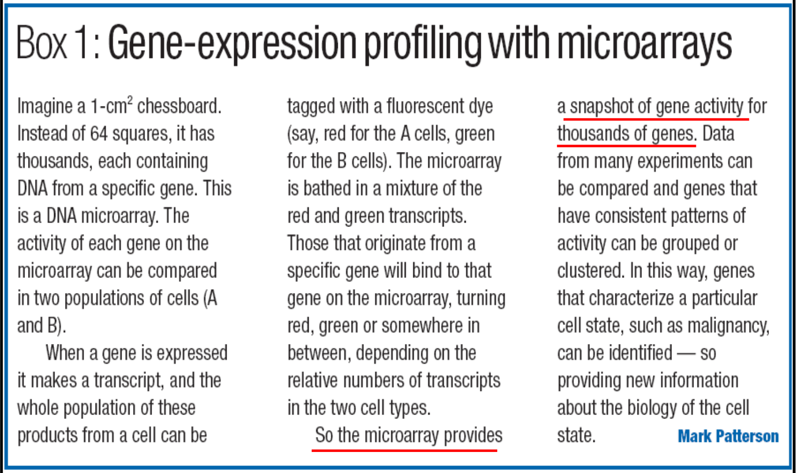
Diffuse large B-cell lymphoma is the most common subtype of non-Hodgkin's lymphoma. With current treatments, long-term survival can be achieved in only 40% of patients. There are no reliable indicators — morphological, clinical, immunohistochemical or genetic — that can be used to recognize subclasses of DLBCL and point to a differential therapeutic approach to patients.

'Lymphochip', a microarray carrying 18,000 clones of complementary DNA designed to monitor genes involved in normal and abnormal lymphocyte development.

What type of cancer?

What is the underlying molecular basis?

What is the optimal treatment?



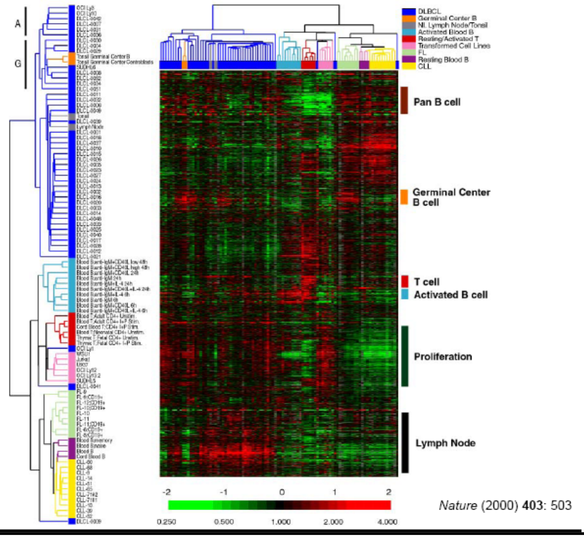
Box 1: Gene-expression profiling with microarrays

Imagine a 1-cm^2 chessboard. Instead of 64 squares, it has thousands, each containing DNA from a specific gene. This is a DNA microarray. The activity of each gene on the microarray can be compared in two populations of cells (A and B).

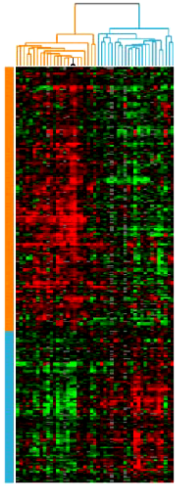
When a gene is expressed it makes a transcript, and the whole population of these products from a cell can be tagged with a fluorescent dye (say, red for the A cells, green for the B cells). The microarray is bathed in a mixture of the red and green transcripts. Those that originate from a specific gene will bind to that gene on the microarray, turning red, green or somewhere in between, depending on the relative numbers of transcripts in the two cell types.

So the microarray provides a snapshot of gene activity for thousands of genes. Data from many experiments can be compared and genes that have consistent patterns of activity can be grouped or clustered. In this way, genes that characterize a particular cell state, such as malignancy, can be identified — so providing new information about the biology of the cell state. **Mark Patterson**

Hierarchical clustering of gene expression data (as ratios).

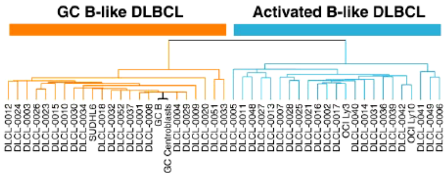


Nature (2000) **403**: 503

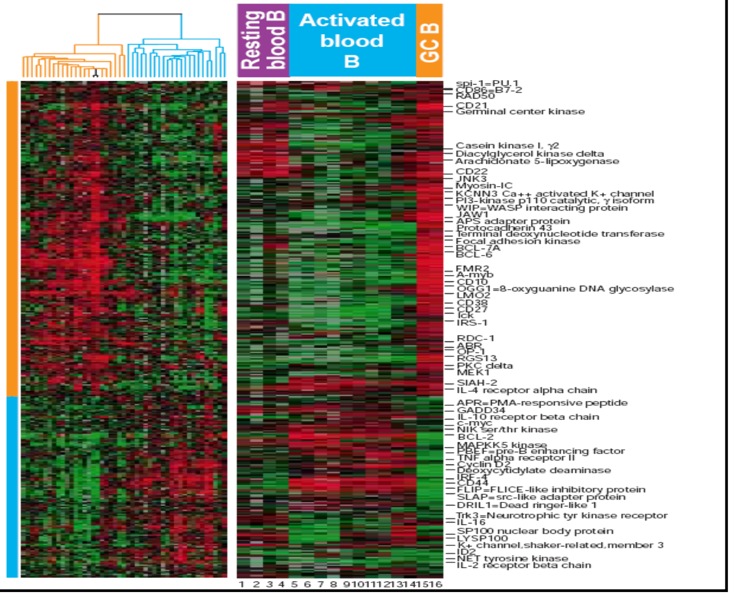


Clustering of tumour samples from cancer patients can be used for molecular classification of cancers. This may be useful for diagnosis and treatment

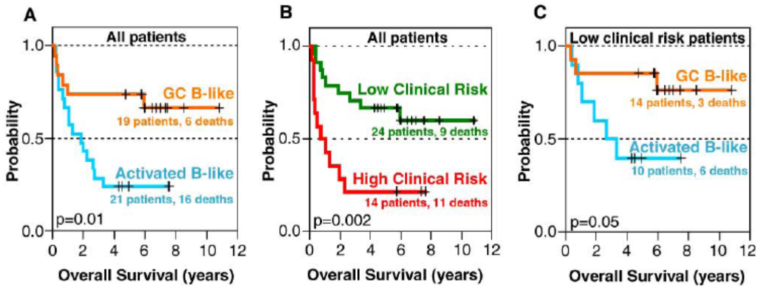
Subtypes of Diffuse Large B-Cell Lymphoma (DLBCL)



Nature (2000) **403**: 503



Using “clustering analysis,” Alizadeh *et al.* could separate DLBCL into two categories, which had marked differences in overall survival of the patients concerned. The gene expression signatures of these subgroups corresponded to distinct stages in the differentiation of B cells, the type of lymphocyte that makes antibodies.



The Nobel Prize in Chemistry 1989

"for their discovery of catalytic properties of RNA"

Sidney Altman

1/2 of the prize

Canada and USA

Yale University
New Haven, CT, USA

b. 1939

Thomas R. Cech

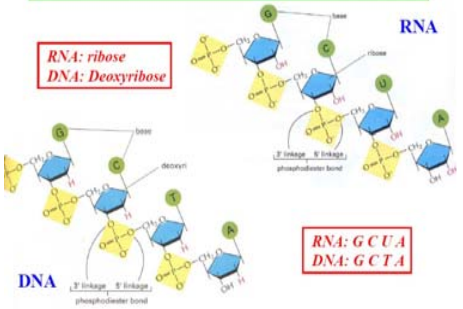
1/2 of the prize

USA

University of Colorado
Boulder, CO, USA

b. 1947

DNA vs. RNA

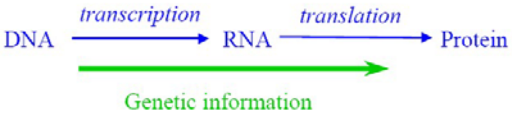


RNA: ribose
DNA: Deoxyribose

RNA: G C U A
DNA: G C T A

Functions of RNA

- **Messenger RNA**



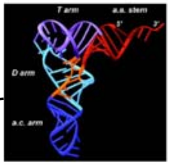
DNA *transcription* → RNA *translation* → Protein

Genetic information

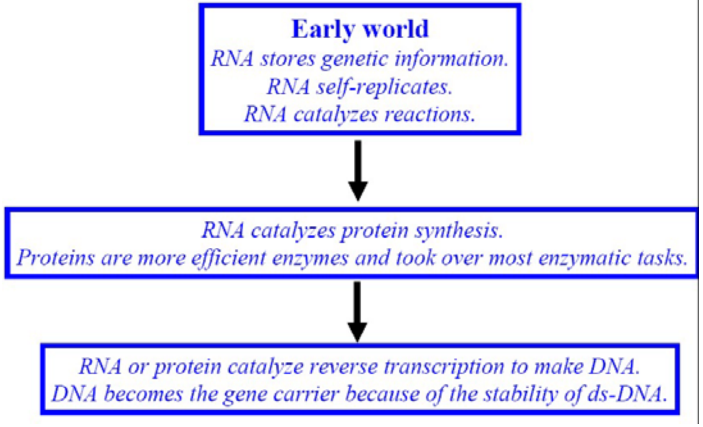
- **Functional RNA**

Transfer RNA

Enzymatic RNA



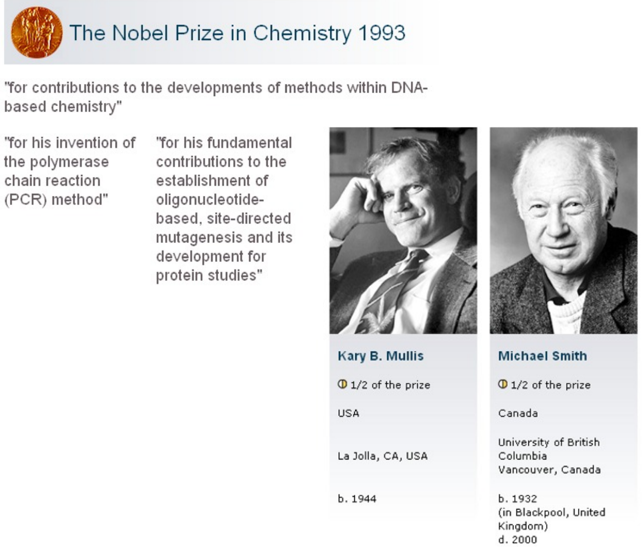
RNA world hypothesis



Early world
RNA stores genetic information.
RNA self-replicates.
RNA catalyzes reactions.

RNA catalyzes protein synthesis.
Proteins are more efficient enzymes and took over most enzymatic tasks.

RNA or protein catalyze reverse transcription to make DNA.
DNA becomes the gene carrier because of the stability of ds-DNA.



The Nobel Prize in Chemistry 1993

"for contributions to the developments of methods within DNA-based chemistry"

"for his invention of the polymerase chain reaction (PCR) method"

"for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies"

Kary B. Mullis

1/2 of the prize

USA

La Jolla, CA, USA

b. 1944

Michael Smith

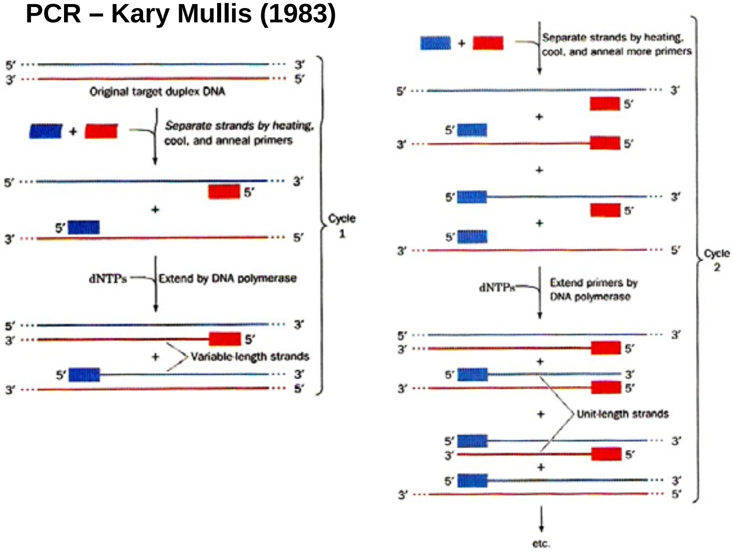
1/2 of the prize

Canada

University of British Columbia
Vancouver, Canada

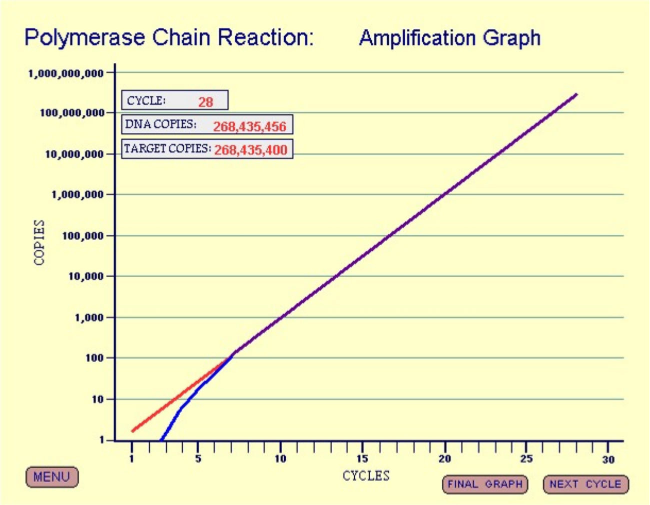
b. 1932
(in Blackpool, United Kingdom)
d. 2000

PCR – Kary Mullis (1983)



5′ 3′
3′ 5′
Original target duplex DNA
Separate strands by heating, cool, and anneal primers
+
5′ 3′
5′
3′ 5′
Cycle 1
dNTPs
Extend by DNA polymerase
Variable-length strands
Separate strands by heating, cool, and anneal more primers
Extend primers by DNA polymerase
Unit-length strands
Cycle 2
etc.

http://www.dnalc.org/ddnalc/resources/animations.html



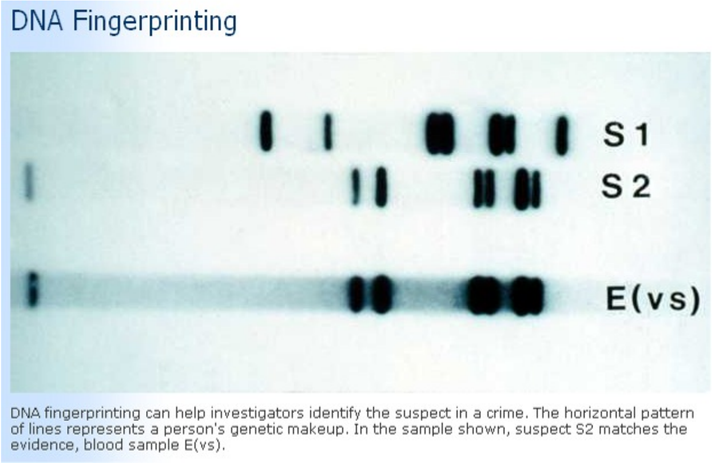
Polymerase Chain Reaction: Amplification Graph

CYCLE: 28

DNA COPIES: 268,435,456

TARGET COPIES: 268,435,400

Sir Alec Jeffreys - 1984



DNA Fingerprinting

DNA fingerprinting can help investigators identify the suspect in a crime. The horizontal pattern of lines represents a person's genetic makeup. In the sample shown, suspect S2 matches the evidence, blood sample E(vs).